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Diagnostic Virology Protocols

Edited by

John R. Stephenson

and Alan Warnes

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Preface

The accurate and reliable diagnosis of transmissible diseases is the most powerful weapon available to ensure their control, and in some cases eradication. The detection of parasites in clinical cases, companion and farm animals, and in the environment is relatively easy since many of them are visible to the naked eye, and those that are not are readily detected by light microscopy. Fungal infections can similarly be determined. Bacteria are somewhat harder to detect. Although their presence can frequently be detected by light microscopy, differential diagnosis, beyond their gross morphology, is almost always impossible. However, most bacterial pathogens can be cultured in the laboratory and can be accurately identified by combinations of a series of simple tests such as morphology, staining, antibiotic sensitivity, biochemical analyses, nutrient dependence, and phage sensitivity.

Viruses, however, have proved much more difficult; their size and absolute dependence of the host cell for propagation have rendered useless the methods traditionally used for other microorganisms. Until the development of tissue culture in the middle of this century, diagnosis was entirely dependent on the skill and experience of the clinician. But this was an unreliable process since many of the common virus infections exhibit similar clinical symptoms, such as coryza, exanthema, vomiting, diarrhea, neuralgia, and lethargy. Indeed many viral infections display clinical signs that are indistinguishable from bacterial or parasitic infections. In a few cases an experienced pathologist could detect viral infections where macroscopic inclusion bodies were present or where infected cells displayed a distinctive morphology (e.g., measles giant cell pneumonia).

With the introduction of electron microscopy in the 1960s, the direct visualization of virus particles in clinical specimens became possible. However, even with the increasing sophistication of electron microscopy, only those viruses with defined and robust structures (e.g., adenoviruses, polioviruses, herpesvirus, and rotaviruses) could be reliably observed. Diagnosis by electron microscopy of diseases caused by other viruses, especially enveloped viruses such as influenza virus, measles virus, and yellow fever virus has not been possible. The only notable exceptions occur in cases where infected cells contain clearly identifiable inclusion bodies or virus-related structures, such

as the distinctive nucleocapsids seen in measles-infected cells. In spite of its limitations, electron microscopy has been the only reliable diagnostic tool for such nonculturable viruses as astroviruses and Norwalk-like agents.

Because direct virus culture from such easily obtainable clinical material as serum, saliva, or urine is nearly always too unreliable for routine use, the majority of viral diagnoses have rested on the detection of a specific immune response. Although serology is in most cases satisfactory, it is nearly always useful only for retrospective analyses and for monitoring virus spread in populations. Specific immune responses can only be detected sometime after the initial virus infection and thus serodiagnosis does not normally benefit the patient. Such assays have traditionally depended on detecting antibodies that inhibit biological functioning in the virus and include plaque reduction neutralization tests (PRNT), hemagglutinin inhibition assays (HI), and complement fixation (CF) assays. These assays can be specific and reliable in the hands of an experienced laboratory worker, but they are expensive, laborious and, in the case of such human pathogens as yellow fever virus and rabies virus, potentially dangerous, requiring sophisticated containment facilities.

Several technological innovations, developed over the last decade or so, are now coming together to revolutionize diagnostic virology. Solid-phase assays such as RIA (radioimmune assay), ELISA (enzyme-linked immunosorbant assay), and latex-agglutination technology, have become increasingly popular since they use less material and can be readily and cheaply adapted to automated laboratory protocols. The explosive development in computer technology and powerful software, combined with the rapid fall in cost, but increasing sophistication and acceptability of robotic analyzers, is radically changing the nature of the hospital pathology laboratory. In addition to developments in analytical technology, the nature of the biological reagents available to the clinical pathologist has improved dramatically. Synthetic peptides can be made that are pure, inexpensive, and highly specific antigens, readily adaptable to automated laboratory systems. For viral antigens that are unsuited to synthetic peptide chemistry, a wide variety of recombinant DNA technologies are available that can make any viral antigen, or fragment thereof, in high yields and to an acceptable standard or purity. These two technologies, taken together, can remove one of the major inhibitions on viral diagnosis: that is, the difficulty of providing reliable batches of high-quality diagnostic reagents. Moreover, there is no need now to grow large amounts of dangerous pathogens for the production of analytical reagents. Not only has the design and production of viral antigens been dramatic, but the advent of monoclonal antibodies has also markedly improved the sensitivity and reliability of detection systems employed by ELISA assays and similar technologies. Thus it is now possible to analyze

the immune response in an individual to give details of each viral protein the immune response is raised against and also provide information on the about the nature of the antibodies involved. This analysis can be done in hours, and not days, and can be automated and performed in a laboratory requiring only low levels of biological containment.

In the past virus diagnosis has been less valuable than similar tests for bacterial and parasitic infections since the technology has been too slow to directly affect the treatment of the patient from whom the sample was taken. The invention and rapid evolution of PCR technology has for the first time enabled pathologists to consider viral diagnosis to affect treatment. PCR is so sensitive that, with the appropriate carefully designed controls, it can detect and identify viral genomes in the early stages of infection. Furthermore, the product of the PCR reaction can have its entire nucleotide sequence determined at later date for the unequivocal confirmation of the diagnosis. Viral antigens can also be detected using specific monoclonal antibodies bound to a variety of conjugates, although this technology is significantly less sensitive than PCR. Such accurate information, early in a viral infection, can determine whether one of the increasing number of antiviral drugs should be used, and the period of treatment minimized to avoid unnecesary side effects. In addition the unnecessary use of antibiotics can be avoided if a viral infection can be reliably identified. The inappropriate use of antibiotics is a matter of increasing concern since overprescription has been implicated in the rise of antibiotic-resistant bacteria.

Viral diagnosis is therefore entering a new and exciting era in which many new technologies are combining to enable both the researcher and the clinician to use rapid, accurate, sensitive, and robust analyses. In *Diagnostic Virology Protocols* we have brought together well-tried diagnostic protocols that use at least one of the modern technologies now available. Chapters covering all the major groups of human viral pathogens have been included, as well as those introducing and assessing the utility a number of modern technologies. Viruses causing diseases of veterinary importance have not been included, but in many cases protocols for similar viruses causing human diseases should be easily adaptable. We hope the information provided in *Diagnostic Virology Protocols* will be of equal value to researchers and clinician alike and be usable by both experienced workers and those just entering the field.

John R. Stephenson
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1

Rapid Detection of Adenovirus from Fecal Specimens

Tanvir Tabish, Alan Warnes, and Stuart Clark

1. Introduction

In 1953, the first adenovirus was isolated from a human and, subsequently, 47 types have been shown to exist. Adenoviruses are now classified into six subgroups (A–F), which are based on their hemagglutination properties *(1)*. They have a icosahedron structure that contains double-stranded linear DNA of 45,000 basepairs. Although, adenoviruses can cause a range of infections in humans, including conjunctivitis, pharyngitis, and gastroenteritis, this chapter focuses on gastroenteric adenoviruses and their rapid detection in fecal samples.

The incidence of adenoviral infections causing gastroenteritis is well-documented, accounting for 5–15% of all viral infections, which occur throughout the year with no particular seasonal peak *(2)*. The incubation period is dose-dependent, but usually takes 5–8 d to the onset of clinical symptoms. The ability of adenoviruses to infect the intestinal tract is due to their ability to survive low pH levels where high titers of virus particles can be produced and subsequently excreted.

Unlike respiratory adenoviruses, those causing gastroenteritis are difficult to grow in culture, therefore, cross-reacting group proteins are commonly used as target antigens for in vitro diagnostic assays. Although there is considerable genetic variation between the different types of adenoviruses, the hexon gene contains highly conserved regions which when translated produce a cross-reacting antigen *(3)*. Thus, assays that will detect adenoviruses in fecal samples have been designed using the hexon protein. Alternatively, owing to advances in genetic engineering, it is now possible to produce recombinant proteins from

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eukaryotic systems, and this technology is described in further detail in Chapter 24.

Recent work has indicated that monoclonal antibodies (MAbs) specific for types 40 and 41 can detect those adenovirus types causing gastroenteritis, but not those causing respiratory or ocular infections *(4)*. Subsequently, enzyme immunoassays (EIAs) based on the use of these MAbs have been developed to detect the types 40 and 41 directly from fecal samples. However, there are reports that a number of other adenoviruses are also responsible for gastroenteritis, and consequently an assay that detects adenovirus group antigen may be better suited for screening purposes *(5–7)*.

Although there are a number of EIAs available for the detection of adenoviruses, most require sophisticated laboratory equipment, which is not always available to all laboratories We, therefore, describe a latex agglutination assay for the rapid detection of adenoviruses in stool specimens that can be as sensitive as electron microscopy *(8)*, which is still used as the confirmatory test for positive fecal samples. Latex agglutination can be performed rapidly taking only 2 min, with 3 min for sample preparation; the result is easy to visualize, with no requirement for support equipment. Upon mixing the extracted sample, with polyclonal antibody-coated latex particles, cross-linking occurs between the antibodies coated to the latex and the virus particles, resulting in the production of visible aggregates.

Owing to the widespread use of adenoviral vectors as delivery systems in gene therapy, there is now the opportunity to utilize the rapid latex assay as an on-line monitoring system to estimate viral load in cell culture. This has the benefits of being able to maximize viral yield for the production of stocks, using an assay that takes only 2 min to perform.

2. Materials

1. Beckman J2-21 centrifuge (Fullerton, CA).
2. Dialysis tubing BDH Size 5 Diam 24/32–19.0 mm (BDH, Poole Dorset, UK)
3. Nalgene glass fiber pre-filters: Sybron International (Rochester, NY).
4 Amicon 76mm Ultracentrifugation cell (Amicon Ltd, Stonehouse, Gloucestershire, UK)
5. Ultrafiltration membrane (PM30 43mm) (Amicon).
6. Roller mix.
7. Sonicator MSE Soniprep 150 (MSE Scientific Instruments, Crawley, UK)
8. Sephacryl S-400-HR (Sigma S-400-HR) (Sigma Chemical Company Ltd, Poole, Dorset, UK).
9 Glycine-buffered saline (GBS)· 100 m*M* glycine, 171 m*M* sodium chloride, 15 m*M* sodium azide, pH to 8.2 with 10 *M* sodium hydroxide solution (BDH).
10. Latex; Prolabo K080: 0.8 μm (Estapor, Manchester, UK).
11 Glacial acetic acid (BDH).

12. n-Octanoic acid (BDH)
13. Phosphate-buffered saline (PBS): 150 m*M* sodium chloride, 4 m*M* potassium dihydrogen phosphate, 11 m*M* disodium hydrogen phosphate dihydrate (BDH).
14. Ethylenediamine tetra-acetic acid (EDTA): 10 m*M* stock solution
15 Acetate buffer, pH 4.0· 100 m*M* sodium acetate, 5 75 m*M* glacial acetic acid
16 Acetate buffer, pH 4.8: 100 m*M* sodium acetate, 5 75 m*M* glacial acetic acid
17. 0.1% Bovine serum albumin (BSA) solution (Bayer Diagnostics, Nankakee, IL) in GBS
18 Normal rabbit serum (NRS).
19. Cell growth media (EMEM): 10% fetal bovine serum, 1X nonessential amino acids, 10 m*M* HEPES, 2 m*M* L-glutamine.
20. Beta-propiolactone.
21. Extraction buffer: available from Microgen Bioproducts Ltd (Camberley, Surrey).
22 Mixing cards (Microgen Bioproducts).
23. Filtration units (Microgen Bioproducts).

3. Methods

The following list of protocols describes the procedures involved in purifying hexon proteins required as immunogen to raise polyclonal anti-adenovirus hexon antisera.

3.1. Production of Adenovirus

1 Grow a Hep2 cell monolayer to 80% confluency in 175 cm^2 tissue-culture flasks in cell-growth media sparged with 95% air/5% CO_2 at 37°C.
2. Infect cells with a multiplicity of infection of 0.01 of adenovirus type 5 and incubate for 1 h at 37°C.
3. Remove the virus and wash the cells with prewarmed PBS.
4. Add 60 mL of fresh prewarmed cell-growth media, gas with 95% air/5% CO_2 and incubate at 37°C
5. Examine the cell monolayer daily and harvest, when the cell monolayer shows 100% cytopathic effect.
6. Freeze thaw the culture four times and then inactivate the virus with 0.4% beta-propiolactone.
7. Store crude virus stock preparations at –20°C.

3.2. Purification of Adenovirus Hexon Protein and Production of Polyvalent Sera

A key stage is the purification of hexon protein and the subsequent production of polyvalent sera; although the precise method cannot be mentioned here, purification processes are now well-advanced and detailed elsewhere *(9)*. The use of genetic engineering should also not be forgotten because these methods now offer the production and purification of proteins as diagnostic antigens with relative ease (*see* Chapter 24). The purified adenovirus hexon protein can

be evaluated for purity using polyacrylamide gel electrophoresis (PAGE) or immunoblotting if polyclonal antibodies are available (*see* **Note 1**).

Polyclonal anti-hexon sera can then be obtained by immunization of animals, and the serum stored at –40°C.

3.3. Fractionation of IgG from Rabbit Antiserum Using n-Octanoic Acid

1. Pour the rabbit antiserum into appropriate plastic containers; add twice the volume of acetate buffer, pH 4.0.
2. Stir each pot vigorously at room temperature with the aid of a magnetic stirrer
3. For every 10 mL of rabbit serum, add 0 75 mL n-octanoic acid in a dropwise manner.
4. Leave containers to stir at room temperature for 30 min
5. Centrifuge in Beckman J2-21, for 20 min, at 15,300*g* and 10°C
6. Decant and retain the supernatant.
7. Resuspend the precipitate in 10 mL of acetate buffer pH 4 8 per 10 mL of original serum volume.
8. Centrifuge at 15,300*g* for 20 min at 10°C
9. Pool the supernatant with that retained in **Subheading 3.3.6.**
10. Boil the dialysis tubing in 10 m*M* EDTA for 2 min in a glass beaker, allow to cool, and wash thoroughly with distilled water
11. Filter treated serum through a Nalgene glass-fiber pre-filter to remove coarse precipitate and pipet into the dialysis tubing
12. Dialyze against GBS, initially for 4 h at 4°C, followed by a further 16 h at the same temperature.
13. Check pH of the dialyzed material, if pH is 8 0–8 2, then proceed, if not, then dialyze for a further period until the desired pH has been achieved
14. Store the purified anti-adenovirus IgG at –70°C

3.4. Manufacture of Test Antibody-Coated Latex

The method for the production of antibody-coated latex particles has already been published in detail *(10)*, as described here; a modification of this method is currently used at Microgen Bioproducts Ltd. This provides hexon-specific polyvalent antibody-coated latex particles produced in **Subheading 3.2.**

1. Wash the latex particles three times with PBS by centrifugation at 8000*g* for 20 min at 4°C
2. Resuspend at a concentration of 0.4% in PBS.
3. Add an equal volume of anti-adenovirus antibody at 16 mg/mL
4. Shake at room temperature for 2 h
5. Wash coated latex particles three times with PBS by centrifugation at 8000*g* for 20 min at 4°C
6. Resuspend to a final concentration of 0.7% in PBS and store at 4°C.

Table 1
Comparison of Adenovirus Latex Against EM

		Adeno latex	
		+	–
EM results	+	53	3[a]
	–	1[b]	79

[a]This sample was retested with other commercial assays and was shown to be positive

[b]On retesting with other commercial assays 2 of the 3 samples were also shown to be negative

3.5. Manufacture of Control Antibody-Coated Latex

As part of the assay, it is essential that a control latex is always used so that the test can be correctly evaluated and nonspecific reactions detected. The control rabbit IgG is derived from normal rabbit serum (NRS) as described in **Subheading 3.3.** and processed as described in **Subheading 3.4.**

3.6. Test Procedure

1. Prepare an approximate 10% suspension of the fecal sample by transferring 0.1 g (0 1 mL) of sample into 1 mL of extraction buffer in a stoppered tube (supplied as a component of the filter pack). Mix the contents well
2. Allow the reagents to reach room temperature for 1–2 min before processing.
3. Remove the stopper and fit integral filter/dropper unit
4. Holding the whole assembly vertically, dispense 1 drop of clear filtrate onto each of 2 wells on the test slide.
5. Add 1 drop of well-mixed test latex reagent to one well and 1 drop of control latex reagent to the other
6. Mix the contents of each well using a separate mixing stick for each sample, covering the entire area of the well
7. Gently rock the slide and observe for agglutination for up to 2 min
8. A positive result is indicated by agglutination of the test latex reagent with no agglutination of the control latex reagent.
9. The result is negative if no agglutination of either the test latex reagent or the control latex reagent is observed within the 2-min test period
10. Agglutination both in the test and control latex indicates a nonspecific result and the sample should be retested.

3.7. Expected Results

To contrast the efficacy of the assay the following results were obtained when a panel of fecal samples was independently assessed by electron microscopy (EM) (*see* **Table 1**).

These results produced a sensitivity of 95% and a specificity of 99% compared to the "Gold standard" EM. Other commercial kits are available from other companies (*see* **Note 2**).

4. Notes

1 If polyclonal or MAbs are available, the purified antigen should be characterized using both PAGE and immunoblotting. If fusion proteins are produced via genetic engineering techniques, then most protein end products can be visualized used supplied antibodies against the tagging protein

2 Commercial assays based on latex agglutination are available from Microgen Bioproducts (Adenoscreen); Orion, Finland (Adenolex); Biokit, Spain (Adenogen)

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2

Alphaviruses

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1. Introduction

Alphaviruses are enveloped, positive-stranded RNA viruses that are the etiologic agents of severe encephalitis and polyarthritis. These viruses can be divided into six or seven serocomplexes *(1)*. Four of these serocomplexes—represented by eastern equine encephalitis (EEE), western equine encephalitis (WEE), Venezuelan equine encephalitis (VEE), and Semliki Forest viruses—comprise the most medically important alphaviruses. The VEE serocomplex can be further divided into at least six subtypes (1 to 6), with subtype 1 having at least five different varieties (1AB, 1C, 1D, 1E, and 1F). The importance of VEE virus subtyping is that varieties 1AB and 1C viruses cause epidemic/epizootic VEE infection, whereas disease caused by other VEE viruses is endemic/enzootic. Ross River, Chikungunya, Mayaro, and Getah viruses are members of the Semliki Forest serocomplex. Sindbis and Ockelbo viruses are members of the WEE virus serocomplex. A newly emerging alphavirus, Barmah Forest, may represent a new serocomplex of alphaviruses.

Laboratory diagnosis of human alphavirus infections has changed greatly over the last few years. In the past, identification of alphavirus antibody relied on four tests: hemagglutination-inhibition, complement fixation, plaque reduction neutralization test, and the indirect fluorescent antibody (IFA) test. Positive identification using these immunoglobulin M- (IgM-) and IgG-based assays required a fourfold increase in titer between acute and convalescent serum samples. A number of very good procedural reviews contain the specifics of these older assays *(2)*.

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With the advent of solid-phase antibody-binding assays, such as enzyme-linked immunosorbent assay (ELISA), the diagnostic algorithm for identification of viral activity has changed. Rapid serologic assays such as IgM-capture ELISA (MAC-ELISA) are now employed early in infection *(3)*. In many cases, a positive MAC-ELISA with an acute serum sample precludes the need for testing of a convalescent serum sample. Early in infection, IgM antibody is more serocomplex specific, while later in infection, IgG antibody is more serocomplex crossreactive. Inclusion of monoclonal antibodies (MAbs) with defined virus specificities in these solid-phase assays has allowed for a level of standardization that was not previously possible. All tests described in this chapter are equally applicable to all alphaviruses.

Virus isolation and identification have also been useful in defining viral agents in serum, cerebrospinal fluid (CSF), or mosquito vectors. Although virus isolation still depends upon growth of an unknown virus in cell culture or neonatal mice, virus identification has also been greatly facilitated by the availability of virus-specific MAbs for use in IFA assays. Similarly, MAbs with avidities sufficiently high to allow for specific binding to virus antigens in a complex protein mixture (e.g., mosquito pool suspensions) have enhanced our ability to rapidly identify virus agents *in situ*. Although polymerase chain reaction has been developed to identify a number of viral agents, such tests have not yet been developed for routine rapid identification of alphaviruses in the clinical setting.

2. Materials

2.1. General ELISA Materials List

The following materials list is employed in all subsequent ELISA procedures:

1. 96-Well Immulon 2 microtiter plates (Dynatech Industries, Inc., Chantilly, VA).
2. Carbonate-bicarbonate (pH 9.6) coating buffer: 1.59 g of Na_2CO_3, 2.93 g $NaHCO_3$ in 1 L of distilled water *(4)*.
3. Phosphate-buffered saline (PBS): BBL FTA buffered saline (9.23 g/L, Becton Dickinson, Cockeysville, MD)
4. Blocking buffer: 5% skim milk, 0.5% Tween-20 in PBS.
5. Rinse buffer: 0.05% Tween-20 in PBS.
6. ELISA plate-reader.
7. Refrigerator.
8. Humid incubator, 37°C.

2.2. Antigen Detection ELISA in Virus-Infected Mosquitoes

1. Grinding apparatus (Ten Broeck homogenizers or mortars and pestles).
2. Microcentrifuge with accompanying 1.5-mL microcentrifuge tubes
3. Probe sonicator.

4 BA-1 diluent. 1X cell culture medium M199, 0.05 *M* Tris-HCl, 1% bovine serum albumin, 0.35 g/L $NaHCO_3$, final pH 7.6. Filter sterilize
5. Lysis buffer: 5% Tween-20 in PBS
6. Substrate: 3-3',5-5'-tetra-methyl benzidine (TMB) Commercial source: TMB-ELISA reagent (Gibco-BRL, Gaithersburg, MD)
7. Stopping reagent: 1 *N* H_2SO_4.
8 Positive control antigen (suckling mouse brain [SMB] antigen of either EEE virus strain NJ-60, or WEE virus strain Fleming) Procedures for preparation of SMB antigens have been previously described *(2)*
9. Capture antibody· Murine MAb, 1A4B-6, for EEE virus or 2A3D-5, for WEE virus *(5,6)*.
10. Detector antibody: Murine MAb, 1B5C-3, conjugated to horseradish peroxidase (HRP) for EEE virus, or 2B1C-6, conjugated to HRP for WEE virus *(5,6)*.
11 Polyclonal control antibodies: Procedures for producing murine polyclonal antivirus antibodies for use in the inhibition assay have been previously described *(2)*.

2.3. IgM-Capture ELISA (MAC-ELISA)

1 Previously titered goat antihuman IgM capture antibody (Cappel Labs, Organon Teknika, Durham, NC)
2 Previously titered virus and control SMB antigens *(2)*.
3. Previously titered HRP-conjugated MAb detector, 2A2C-3 *(5)*
4 Known-positive human serum or CSF samples reactive with test viruses to serve as positive controls.
5 Known-negative human serum or CSF samples to serve as negative controls

2.4. IgG ELISA

1 Capture antibody· Murine MAb, EEE 1A4B-6 *(5)*
2. Previously titered virus and control SMB antigens *(2)*.
3. Detecting antibody: Goat antihuman IgG (Fc-specific)-alkaline phosphatase (AP) conjugate (Jackson Immunochemicals, West Grove, PA).
4. Known-positive human serum samples reactive with test viruses to serve as positive controls.
5. Known-negative serum samples to serve as negative controls
6. Substrate: 3 mg/mL Sigma 104 in 1 *M* Tris-HCl, pH 8.0 (Sigma, St. Louis, MO).
7. 3 *M* NaOH (120 g in 1 L water) to stop reaction.

2.5. IFA Assay

1. Unconjugated MAbs of various specificity (**Table 1**).
2. Fluoresceinated antimouse antibody (Jackson Immunochemicals).
3 Sodium azide (as preservative).
4. Penicillin-streptomycin.
5. PBS.
6. Counterstain: Trypan blue diluted 1:4000 in PBS.

Table 1
Monoclonal Antibodies Useful in Alphaviruses Serology

MAb	Virus	Specificity	Serologic reactivity	Ref.
1A2B-10	VEE peptide	E2	All VEE except TC-83 and subtype 6	*7*
5B4D-6	VEE TC-83	E2	TC-83 specific	*8*
3B4C-4	VEE TC-83	E2	VEE 1AB, 1C, 1D, 2	*8*
1A3A-9	VEE TC-83	E2	VEE1AB,1C,1D,1E,1F	*9*
1A1B-9	VEE Mena 2	E2	VEE 1D,1E,1F,3	*10*
1A3B-7	VEE TC-83	E2	VEE complex	*10*
2B1C-6	WEE McMillan	E1	WEE specific	*6*
2A6C-7	WEE McMillan	E1	WEE complex	*6*
2A3D-5	WEE McMillan	E1	WEE complex	*6*
2D4-1	HJ Original	E2	HJ specific	*11*
49	Sindbis Ar339	E2	Sindbis specific	*12*
1B5C-3	EEE NJ-60	E1	North American EEE	*5*
1B1C-4	EEE BeAn5122	E1	EEE complex	*5*
UM5 1	Semliki Forest	E2	Semliki Forest specific	*13*
2A2C-3	WEE McMillan	E1	All alphaviruses	*6*
1A4B-6	EEE NJ60	E1	All alphaviruses	*5*

7. Mounting solution: Aqua-mount (Lerner Labs, Pittsburgh, PA)
8. High-quality fluorescence microscope with epifluorescence interference filters and a tungsten light source
9. Twelve-spot IFA slides (Erie Scientific Co., Portsmouth, NH)
10. Coverslips (Corning Co , Corning, NY).

3. Methods

3.1. Antigen Detection ELISA in Virus-Infected Mosquitoes

Monitoring levels of virus in vector mosquitoes allows for rapid assessment of disease threat. The antigen-capture ELISA does not require use of expensive isolation techniques such as cell culture or animal inoculation. The test is not as sensitive as virus isolation in cell culture; however, if mosquito pools are kept below 25 individuals, the test has appropriate sensitivity to detect virus at levels necessary for alphavirus transmission ($\geq$5.0 $\log_{10}$ PFU).

3.1.1. Mosquito Pool Preparation

1. Using sterile procedures, triturate pools of suspect mosquitoes (25 mosquitoes/pool or less) in 1.5 mL BA-1 buffer by standard protocol. We have used two methods. Method one employs homogenization in Ten Broeck tissue homog-

enizers. Method two employs homogenization in mortars and pestles *(14)*. The method of trituration seems to be less important than the pool size. Preliminary data indicate that as pool size increases from 25 to 50 or 100, the ELISA signal is diminished. The diminution of ELISA signal with larger pools is probably associated with the larger concentrations of irrelevant material in the larger pools

2. Following trituration, centrifuge the suspension in a microfuge at 15,000*g* for 2 min. At this point, a small amount of the sterile supernatant can be removed for subsequent virus isolation in plaque assay in Vero cells. Split the remaining volume into two aliquots. Reserve one aliquot for confirmation of testing. The second aliquot serves as antigen for the antigen-capture ELISA.
3. Also prepare at least six independent pools of normal, noninfected mosquitoes to serve as ELISA-negative antigens.
4. Resuspend one 0.25-mL aliquot (including the pellet) Sonicate each sample in a biosafety cabinet using a microprobe at 100 W for 10 s. Centrifuge in a microfuge at 15,000*g* for 2 min. Transfer supernatant to new vial. Immediately before ELISA testing, add 10 µL lysis buffer per 100 mL mosquito pool sample. Addition of the lysis buffer frees virus antigens from larger particles. Incubate 15 min at room temperature (RT). After incubation, centrifuge in a microfuge at 15,000*g* for 2 min The supernatant from this centrifugation will be the mosquito pool antigen used in the antigen-capture ELISA (*see* **Subheading 3.1.2.**)

3.1.2. Antigen-Capture ELISA

1. Dilute capture antibody (1:20,000 of MAb 1A4B-6 for EEE, virus or 1:5000 of MAb 2A3D-5 for WEE virus *[5,6]*) in coating buffer. Coat wells of a 96-well Immulon 2 microtiter plate with 100 mL capture antibody per well. Incubate coated plates overnight at 4°C.
2. Rinse plates 5 times with ELISA rinse buffer
3. Block plates with 300 µL per well blocking buffer for 1 h at 37°C. Repeat the rinse step.
4. Add 100 mL per well detergent-treated mosquito pool antigen. Test mosquito pools in triplicate. Incubate plates overnight at 37°C. Include space for six normal uninfected mosquito pool homogenates. These normal homogenates will be used to calculate test background. Also include space for positive control antigen diluted 1:1000 and treated with lysis buffer (**step 4, Subheading 3.1.1.**) Use positive control antigen at 100 mL per well. Repeat the rinse step.
5. Add 100 µL per well detector antibody 1B5C-3-HRP-conjugate, diluted 1·1000 for EEE virus detection or 2B1C-6-HRP conjugate diluted 1:5,000 in ELISA rinse buffer for WEE virus. Incubate 1 h at 37°C
6. Rinse 10 times with ELISA rinse buffer.
7. Add 100 mL per well substrate (TMB-ELISA) Incubate 30 min at RT and stop the reaction with 50 mL per well 1 *N* H_2SO_4. Measure the absorbance at 450 nm ($A_{450\ nm}$) in a microplate reader.

3.1.3. Inhibition Assay

All mosquito pools presumed to be positive for viral antigen should be tested in the inhibition assay.

1. Dilute EEE, or WEE, and St. Louis encephalitis virus polyclonal antibodies 1:20 in PBS
2. Mix 100 µL mosquito supernatant with 20 µL of either EEE virus or WEE virus polyclonal antibody. Also mix 100 µL mosquito supernatant with 20 µL St Louis encephalitis virus polyclonal antibody, incubate at 37°C for 1 h If there is enough mosquito supernatant, do the procedure in duplicate.
3. Add mixture to ELISA plate (*see* **step 4** in **Subheading 3.1.2.**) Incubate overnight at 4°C. Perform ELISA as in **Subheading 3.1.2.**
4. If the mean absorbance value of the pool is reduced by 50% or more when it is preincubated with the polyclonal antialphavirus antibody, sample is considered specific for alphavirus antibodies.

3.1.4. Data Analysis

1. Derive the mean $A_{450\ nm}$ for each duplicate or triplicate and also the mean of the six normal mosquito pool samples The negative cutoff will be twice the $A_{450\ nm}$ of the mean of the six normal mosquito pools. Any experimental pool with a mean $A_{450\ nm}$ greater than twice the mean of the $A_{450\ nm}$ of the six negative control pools should be considered presumptive for the presence of EEE or WEE virus antigen These pools should be tested in the inhibition assay (*see* **Notes 1–4**).
2 The experimental sensitivity of this assay is 3.5–4 0 $\log_{10}$ PFU per 0.1 mL. Pools with titers lower than this cutoff will give negative or variable results.

3.2. IgM-Capture ELISA (MAC-ELISA)

Assays that detect virus-specific IgM are advantageous because they detect antibodies produced within days of infection, obviating the need for convalescent-phase specimens in many cases. The MAC-ELISA is the optimum approach to detect IgM because capturing the antiviral IgM antibody negates the competitive effects seen with antiviral IgG in the more standard indirect ELISA format. The MAC-ELISA is simple, sensitive, and applicable to serum and CSF samples. False-positive reactions owing to rheumatoid factor are minimized.

3.2.1. ELISA

1. Coat 96-well Immulon 2 plates with 75 µL per well of goat antihuman IgM in coating buffer, pH 9.6. Coat enough wells to test each sample against both positive and negative antigens in triplicate. Do not use the outer wells on the plate. Incubate overnight at 4°C. Wash plates in microplate washer five times with rinse buffer.
2. Block plates with 300 µL per well blocking buffer. Incubate covered plates at RT for 30 min. Repeat wash step.

3. Add 50 µL per well of the patient's serum diluted 1:400 in rinse buffer or patient's CSF undiluted to six wells. Incubate for 1 h at 37°C Also test appropriately diluted positive control human serum and a normal human serum. Repeat wash step
4. Dilute virus-infected SMB antigen in rinse buffer according to previous titration. Add 50 µL per well to three wells of each test sample. To the other three wells add 50 µL per well of normal SMB antigen diluted in the same manner. Incubate overnight at 4°C. Repeat wash step.
5. Add 50 µL per well of HRP-conjugated MAb, 2A2C-3, diluted as per the previous titration in the blocking buffer. Incubate 1 h at 37°C
6 Repeat wash step twice
7. Add 75 µL per well of TMB substrate. Incubate at RT for 10 min.
8. Add 50 mL per well of 1 *M* H_2SO_4 to stop the reaction Allow to sit at RT for 1 min Read plates in microtiter plate-reader using 450 nm.

3.2.2. Data Analysis

Calculate the positive/noise (*P/N*) values as follows:

[Average 450 nm reading of patient's serum plus antigen (P)]/
[Average 450 nm reading of normal human serum plus antigen (N)]

The *P/N* ratio must be at least 2.0. The positive human serum control *P/N* ratio should be at least 2.0 and the normal human serum control *P/N* ratio should be less than 2.0. If any OD readings or control serum P/N values fall outside these threshold values, the test must be repeated. All patient *P/N* values greater than or equal to 2.0 should be reported as positive with the understanding that *P/N* values between 2.0 and 2.5 could represent false positive reactions (*see* **Notes 5–8**).

3.3. IgG ELISA

Rapid testing for IgG antibody in a solid-phase assay precludes the necessity for other IgG measuring tests such as hemagglutination-inhibition, complement fixation, and plaque reduction neutralization tests. Serologic crossreactivity in the IgG response to the alphaviruses makes the IgG ELISA less specific than the MAC-ELISA. The ELISA assay design of this IgG test allows for concurrent application with the MAC-ELISA. The use of a MAb capturing antibody allows for easy standardization of antigen quantities between laboratories.

3.3.1. ELISA

1. Dilute MAb 1A4B-6 1.10,000 in coating buffer and coat wells of a 96-well microtiter plates with 75 µL overnight at 4°C. Coat enough wells to test each sample against both positive and negative antigens in triplicate. Wash plates in microplate washer 5 times with rinse buffer.

2 Block plates with 300 µL blocking buffer per well for 30 min at RT. Repeat wash step.
3. Add 50 mL per well of appropriate SMB virus or control antigen (*see* **Subheading 3.2.1., step 4**) diluted in rinse buffer and incubate overnight at 4°C. Rinse plate five times with rinse buffer.
4 Add 50 µL per well unknown sera diluted 1:400 in rinse buffer and incubate 1 h at 37°C. Rinse plates 5 times with rinse buffer.
5. Add 50 µL goat antihuman IgG (Fc-specific)-AP conjugate diluted 1·1000 in rinse buffer per well and incubate 1 h at 37°C.
6 Rinse plates 10 times with rinse buffer.
7. Add 75 mL per well of substrate (Sigma 104) and incubate 30 min at RT. Stop color development, if necessary, by adding 25 µL 3M NaOH per well, and read absorbance at 405 nm.

3.3.2. Data Analysis

P/N ratios are determined as in **Subheading 3.2.2.** Ratios greater than or equal to 2.0 are considered positive with the understanding that *P/N* values between 2.0 and 2.5 could represent false positive reactions (*see* **Notes 9–11**).

3.4. IFA Assay

Immunofluorescence tests provide a useful means of identifying viral antigen directly in clinical specimens and of providing specific immunologic identification of isolates in the laboratory *(15–17)*. If the antigen is known, the presence of specific antibodies in a test serum may also be documented. After incubation of antiserum and antigen, the presence of a reaction is detected by observation of fluorescence in a microscope that is equipped with a source of ultraviolet light. A sequence of filters is used to generate excitation light of optimal wavelength and to block light of harmful wavelengths before viewing.

3.4.1. Infecting Cells and Preparing Spot Slides

1. Select a cell culture type appropriate for the virus to be used. Inoculate a monolayer culture less than 1 wk old with the virus seed stock
2. Incubate at 37°C and observe daily for virus cytopathic effects When it involves at least 25% of the cell sheet, harvest the cells, saving the media for virus seed (if necessary).
3. Dilute the harvested cells so that sufficient cells are added to each spot on the slide Add about 10 µL of diluted cells to each well on the slide.
4. Allow slides to air dry at least 2 h Fix slides in cold acetone for 15 min, dry, and store at –70°C

3.4.2. IFA Assay

1. Remove antigen slides from –70°C and allow to air dry or optionally, refix in cold acetone for 10–15 min.

2 Dilute all antibodies in PBS with 0.1% sodium azide and 2% penicillin/streptomycin Dilute MAb ascites to appropriate concentrations Add 10–12 mL of diluted antibody to one well on the spot slide Run all necessary control slides (*see* **Subheading 3.4.3.**)
3 Incubate in a moist chamber for 1 h at 37°C
4 Wash slides for 15 min in PBS Allow to air dry.
5 Add 10–12 mL of pretiterated antimouse antibody (*see* **Subheading 3.4.3.1.**) conjugated to fluorescein isothiocyanate made up in 1:4000 trypan blue with 0.1% sodium azide to each well. Incubate for 1 h at 37°C in a moist chamber. Repeat wash step
6. Add mounting solution and coverslips. Examine slides by fluorescence microscopy no later than 24 h after completing procedure. Store slides at 4°C. A positive reaction appears as apple-green fluorescence against a background of red counterstained cells

3.4.3. Test Controls

3.4.3.1. Titrating MAbs and Conjugates

1 Each new lot of MAb and commercial conjugate must be titrated before use This is best done in a box titration Choose several antiviral antibodies for which you have homologous antigen slides.
2 Serially dilute MAb in PBS with 0 1% sodium azide and 2% penicillin-streptomycin, starting at 1:100 in twofold dilutions to 1.10,240
3. Refix antigen slides as for indirect assay protocol.
4 Add 10–15 mL of each detecting antibody in the dilution series to one spot on the antigen slide Incubate at 37°C for 1 h. Wash slides as in **Subheading 3.4.2.**
5. Prepare a dilution series for the fluorescein-isothiocyanate-conjugate in 1:4000 trypan blue with 0.1% sodium azide.
6. Add 10–15 µL of each dilution in the series to one set of the dilutions of detecting antibody. Incubate at 37°C for 1 h Wash slides as in **step 4**
7. Affix coverslips as for the indirect assay protocol
8. Read slides with a fluorescence microscope. There must be at least 100 cells per well to assess accurately the extent of the antibody-antigen reaction Optimal conjugate dilution is that dilution yielding 4+ fluorescence at the highest antibody dilution. This dilution is used in all subsequent tests performed with this conjugate lot.

3.4.3.2. Normal Tissue Culture Cell Slide

A slide of uninfected cells prepared in the same manner as for infected cells must be run in the same manner as the unknowns in all tests. This slide indicates any nonspecific reaction between the antibody and normal tissue culture cells of the type used.

3.4.3.3. Serum and MAbs

1. Use normal sera from the species in which the antibody was produced to show the level of nonspecific fluorescence between the species and the tissue culture cell type used.

2. Use a homologous antigen slide and an unrelated antigen slide for each MAb used in the test. Use the homologous MAb and another unrelated antibody on each slide. This will demonstrate that the MAb is specific for the antigen and shows no crossreactivity. Use a normal MAb that is an ascite produced from mice inoculated with parental Sp2/0-Ag14 myeloma cells. Alternatively, any MAb specific for an antigen other than alphaviruses (e.g., flaviviruses) can be used as a negative control. This preparation will show if the procedure for producing the MAb causes any nonspecific reactions between the antibody and the tissue culture cells used.

3.4.4. Data Analysis

When the slides are read, each well is ranked upon the following scale: 4+ (positive cells fluoresce intensely); 3+ (positive cells fluoresce brightly); 2+ (positive cells fluoresce to some degree, less than brightly); 1+ (cells fluoresce dully); ± (varying degrees of fluorescence that may or may not be specific); – (no fluorescence of the cells; cells appear red from the counterstain). Positive wells must be ranked 2+ or higher. Results are reported as a simple positive or negative by IFA (*see* **Notes 12** and **13**).

4. Notes

4.1. Antigen Capture ELISA

1. If you are unsure about the results because absorbance values are close to the negative cutoff, retest the pool, or use a backup test such as plaque assay in Vero cells.
2. The serologic reactivities of the MAbs used in these assays are shown in **Table 1**.
3 Occasionally, high backgrounds with uninfected control mosquitoes may be observed. In this case the test should be repeated.
4 For this and all other ELISA assays, MAb reagents are in the form of mouse-ascitic fluids. MAb-enzyme conjugates are commercial preparations using ascitic fluids supplied by our laboratory (Jackson Immunochemicals). Reagent potency may vary depending upon preparations and should be independently determined before use.

4.2. IgM-Capture ELISA

5. Store all diagnostic specimens at –20°C prior to and after testing. Avoid repeated freeze-thaw cycles, which tend to inactivate IgM.
6 This test is used if serum or CSF samples have been drawn within 45 d of onset
7 In the event that a very early CSF or serum is negative by this test, a convalescent serum specimen must be requested and tested before that patient is reported as negative for serological evidence of recent viral infection. Without testing of a convalescent specimen, a negative result may reflect testing of an acute-phase specimen obtained before antibody response.

8. Occasionally the test serum will be highly positive when tested with the normal SMB antigen. The reason for this is unknown. If this happens, the test should be repeated. If high backgrounds persist, another test must be used.

4.3. IgG ELISA

9. We have tried a number of detector antibodies in this test The IgG (Fc-specific)-AP conjugate gives us the best results with the lowest backgrounds.
10 Using the 1A4B-6 MAb as capture antibody for all alphaviruses allows for easy antigen standardization.
11. Remember that antialphavirus IgG is in general more crossreactive than IgM; therefore, the specificity of this test is less than that of MAC-ELISA

4.4. IFA Assay

12. If any of the controls do not perform within the expected reaction range, the test must be repeated.
13 Unlike normal polyclonal antiviral antibodies, MAb reagents are of extremely high potency. Be sure to dilute them out appropriately. Using MAb reagents at low dilutions results in false-positive staining This high activity is why it is imperative to quantitate MAb dilution by endpoint box titration prior to use.

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3

Detection of Human Caliciviruses and Astroviruses in Stools by RT-PCR

Xi Jiang and David O. Matson

1. Introduction

Human caliciviruses (HuCVs) and astroviruses are single-stranded RNA viruses that cause acute gastroenteritis in humans. HuCVs include several prototypes of small, round-structured viruses (SRSVs) as well as morphologically typical caliciviruses. Recent genetic characterization of HuCVs has divided them into three genogroups: Norwalk virus (NV), Snow Mountain agent (SMA), and Sapporo (Sapp) viruses *(1–3)*. Astroviruses include at least seven antigenic types *(4)*. Complete genomic sequences of astrovirus types 1 and 2 and partial sequences of other strains have been reported *(5–7)*. In both families, differences in antigenic types appear to correspond to differences in genetic groups.

HuCVs and astroviruses share some morphologic and genomic features. Both are small (28–40 nm) and round, and have distinct structural features when visualized by electron microscopy (EM). Astroviruses have 5- or 6-pointed stars on the surface of the virion and a smooth particle edge. HuCVs have two morphologic appearances. Typical caliciviruses have a "Star of David" appearance on the particle surface, 32 surface hollows, and 10 spikes, depending upon the orientation *(8,9)*. Detailed structural studies have revealed that the surface hollows are formed by 90 arches protruding from the surfaces. However, many HuCVs do not have this typical appearance and these strains are called SRSVs. In SRSVs, the surface arches are blunted.

Both astroviruses and HuCVs contain a single-stranded, positive-sense RNA genome (**Fig. 1**); *(5–7,10,11)*. The RNAs contain a poly-A tail to give a final length of about 7.7 kb for HuCVs and 7.0 kb for astroviruses. Astroviruses and HuCVs share a similar genomic organization in which nonstructural genes lie

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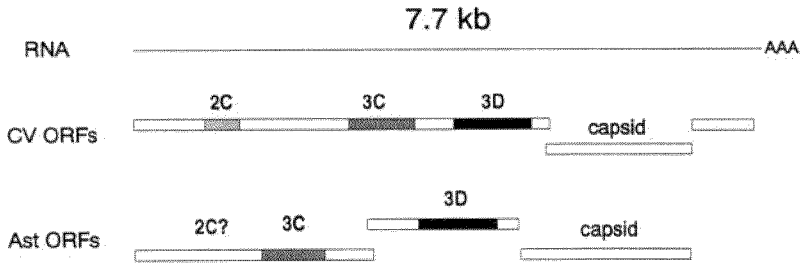

Fig. 1. Genomic organization of HuCV and astrovirus. The top line represents the single stranded caliciviral and astroviral genomic RNAs. The genomic organizations of calicivirus (CV) and astrovirus (Ast) are shown below. The 2C, 3C, and 3D motifs are conserved in most single-stranded RNA viruses, including picornaviruses, caliciviruses, and astroviruses. A frame-shift mechanism is responsible for the expression of the second open reading frame (ORF) of astroviral genome.

5' to the capsid gene on the genome. In astroviruses, a frameshift mechanism is required for expression of the entire nonstructural genes ***(5,7)***. Amino acid sequence motifs (2C, 3C, and 3D) commonly found in the other single-stranded RNA virus families, such as picornaviruses, also are found in the genomes of astroviruses and HuCVs, but the 2C region in the astroviral genome is not clear ***(5–7,10,11)***. The nucleotide sequences encoding these motifs are highly conserved within a family but vary significantly among the families.

This chapter describes a method for detecting astroviruses and HuCVs in stool specimens using the reverse transcription-polymerase chain reaction (RT-PCR). Similar methods likely can be applied to other RNA viruses in stool specimens, if the appropriate nucleotide primers are used. The methods are divided into two parts: extraction of viral RNA from stool specimens and RT-PCR amplification and detection of viral RNA. We emphasize the extraction of viral RNA from stool specimens, because the quality of the viral RNA is the most important element for success of the method. Human stool specimens contain a number of uncharacterized inhibitors of reverse transcriptase and *Taq* polymerase. Removal of these inhibitors without loss of the viral RNA is essential. In addition, viral RNA is easily degraded if stool specimens contain RNase. The cetyltrimethylammonium bromide (CTAB) method described here efficiently removes inhibitors of the enzymes from stool and generates high quality viral RNA for RT-PCR. This method was developed originally for the detection of Norwalk virus ***(12)***. In the last several years, it has been adapted by many laboratories for detection and genetic analysis of other caliciviruses, astroviruses, and picornaviruses ***(1,3,13–16)***. This chapter describes recent modifications of the method based on these studies.

2. Materials

2.1. Reagents and Equipment Used for Extraction of Viral RNA from Stools

1 Freon (1,1,2-trichloro-1,2,2-trifluoroethane); also called Genetron (Dupont, Wilmington, DE). store at room temperature.
2. 2X PEG solution. prepare 16% polyethylene glycol-6000 or 8000, 0 8 *M* NaCl in sterile distilled water. Aliquot and store at room temperature.
3. 2X Proteinase K digestion buffer: 0.2 *M* Tris-HCl, pH 7 5, 25 m*M* ethylenediamine tetraacetic acid (EDTA), 0 3 *M* NaCl, 2% (w/v) sodium dodecyl sulfate (SDS). Aliquot and store at room temperature.
4 Proteinase K stock solution make 10 mg/mL of proteinase K in 1X proteinase K digestion buffer. Aliquot and store at –20°C.
5 10% CTAB solution. prepare 10% CTAB (also called hexadecyl, trimethylammonium bromide, Sigma #H5882, Sigma, St. Louis, MO) in distilled water. Store at room temperature This solution may crystallize at room temperature Warm at 55°C to dissolve the crystals prior to use.
6. 4 *M* NaCl solution· prepare 4 *M* NaCl solution with distilled water and store at room temperature
7. Water-saturated phenol: any commercially available phenol, phenol/chloroform, or phenol/chloroform/isoamyl alcohol that is saturated with distilled water, biotech research grade, peroxide-free. Store at 4°C.
8 Chloroform: molecular biology grade, peroxide-free Store at room temperature.
9. 4 *M* Sodium acetate. prepare 4 *M* sodium acetate solution in distilled water and store at room temperature
10 Ethanol solutions· prepare 100 and 70% solutions in distilled water and store at –20°C
11 Microcentrifuge and Eppendorf 1 5-mL reaction tubes

2.2. Reagents and Equipment Used in RT-PCR

1. 10X PCR buffer. 100 m*M* Tris-HCl, pH 8.3, 15 m*M* $MgCl_2$, 500 m*M* KCl Aliquot and store at –20°C.
2. RNasin: 40,000 U/mL (Promega, Madison, WI). Store at –20°C
3. Deoxynucleoside triphosphate mixture (dNTPs): combine 10 m*M* dATP, dGTP, dCTP, and dTTP (Promega). Dilute to 0.5 m*M* each and store at –20°C.
4. AMV-RT. avian myeloblastosis virus reverse transcriptase, 20 U/μL (Life Sciences, Inc. 007-5, St Petersburg, FL). Store at –20°C.
5. Ampli *Taq:* recombinant *Taq* DNA polymerase, 5 U/μL (Perkin-Elmer/Cetus N801-0060, Foster City, CA) Store at –20°C.
6. Primers for type-specific and type-common detection of caliciviruses.
7. Primers for type-specific and type-common detection of astroviruses
8 Mineral oil.
9. Programmable thermocycler.
10 Eppendorf 0.5-mL reaction tubes

11 6X Sample buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 40% (w/v) sucrose in distilled water. Store at 4°C
12. 5X TBE buffer: add 54 g Tris base, 27.5 g boric acid and 20 mL of 0.5*M* EDTA (pH 8 0) into distilled water to make 1 L final vol. Store at room temperature.
13 Ethidium bromide: prepare a stock solution of 10 mg/mL in water Store at room temperature and keep away from light
14 SeaKem agarose: make 0 5%–1% SeaKem agarose powder (FMC BioProducts, Rockland, ME) in 1X TBE buffer Boil for 3–4 min and pour the gel
15 Equipment for agarose gel electrophoresis. submarine gel electrophoresis apparatus and power supply.
16. UV light illuminator

3. Methods

3.1. Storage of Stool Specimens

Stool specimens can be kept at 4°C for weeks after collection. For long-term storage, –20°C or –70°C is recommended. Avoid multiple freezing and thawing of stool samples and exposure to high pH because viral particles tend to degrade under these conditions.

3.2. Extraction of Viral RNA from Stools

Extraction of viral RNA from stool specimens is the critical step of the method. The viral RNA is particularly susceptible to RNase after samples are treated with proteinase K, which removes the viral capsid and exposes the viral RNA. Process the samples as quickly as possible according to the following protocol. To prevent cross-contamination and contamination by carryover PCR products, the location for extracting viral RNA should be separated from that for amplification, detection, and cloning of PCR products (*see* **Note 6**).

1. Place 300 mL of stool suspension (10–50% in water) in an Eppendorf tube Extract once with an equal volume of freon by vortexing for 30 s followed by centrifugation for 5 min in a microcentrifuge Remove the supernatant (do not disturb the interface) and transfer to a new Eppendorf tube (*see* **Note 1**).
2. Add 300 mL 2X PEG buffer to the supernatant at a final concentration of 8% PEG and 0.4 *M* NaCl. Incubate the sample for 30 min at 4°C and then centrifuge in a microcentrifuge for 15 min at 4°C.
3. Remove the supernatant by aspiration with a micropipet tip. Resuspend the pellet in 150 µL of water (vortex the tube vigorously until the pellet is complete dissolved) and add 150 µL of 2X proteinase K digestion buffer and 12 µL of stock proteinase K (10 mg/mL) to obtain a final concentration of 400 µg/mL. Incubate the sample for 30 min at 37°C.
4. Add 50 mL 10% CTAB solution and 50 mL 4 *M* NaCl solution, vortex for 10 s, and incubate the sample for 30 min at 56°C.

5. Extract once with an equal volume of water-saturated phenol or phenol/chloroform Spin the tube in a microcentrifuge for 5 min at room temperature. Carefully remove the aqueous (upper) phase and transfer it to a new tube. Re-extract the sample once with chloroform by vortexing for 1 min followed by centrifugation in a microcentrifuge for 5 min at room temperature. Transfer the aqueous phase to a new tube.
6. Add 2.5 volume ethanol and 20 mL of 4 *M* sodium acetate to the tube containing the aqueous solution to give a final concentration of 0.2 *M* sodium acetate Precipitate the viral RNA for at least 30 min at –20°C Pellet the RNA by centrifugation for 15 min at 4°C in a microcentrifuge Viral RNA can be stored in the ethanol solution for several months at –20°C or –70°C without loss of the RNA.
7. Pour off the supernatant first and then remove the residual ethanol solution using a micropipet tip. Wash the pellet once with 70% ethanol Be careful not to lose the pellet. Centrifuge the tube in the same orientation as **step 6** for 1 min and remove the ethanol again using a micropipet tip. Remove as much residual ethanol as possible without disturbing the pellet. Let the pellet dry for 2–3 min at room temperature. The pellet should be very small and sometimes is barely seen by eye. Resuspend the pellet in 20 mL of water Use 1–5 mL for each RT-PCR reaction This amount of viral RNA in the extracts is sufficient to give a positive result (*see* **Note 7**) Greater amount of extract may introduce sufficient inhibitors to inhibit the reverse transcriptase and *Taq* polymerases. If multiple bands or smears occur, RT-PCR can be attempted with less extract. Store the remainder of the viral RNA at –70°C.

3.3. RT-PCR Detection of Viral RNA

Following is a method typically used for detection of HuCVs to produce reproducible results. Slight modifications, such as changing the volume of the RT and PCR reactions, the primer concentration, and the thermocycle program may be necessary to detect a specific target. The application of RT-PCR to clinical, epidemiological, and molecular biological studies of astroviruses and HuCV is addressed in **Note 8**.

1. RT reaction: For the RT reaction, a 50-mL reaction mixture is made. It contains 1X PCR buffer, 40 m*M* deoxynucleoside triphosphates, 1.0 m*M* negative-strand primers (*see* **Note 2**), 10 U RNasin, 10 U reverse transcriptase, and 1–5 mL purified viral RNA. Vortex the reaction mixture for 5 s and centrifuge briefly. The RT reaction is carried out for 1 h at 42°C. A master RT reaction mix usually is prepared and aliquoted to provide 1 reaction per tube. Each batch of aliquots is stable for several months at –70°C (see Note 5)
2. PCR For PCR, 50 mL of 1X PCR buffer containing the positive-strand primers (1 0 m*M*) and *Taq* polymerase (5 U) are added to the RT mixture, overlaid with mineral oil, and placed into the thermocycler. The amplification cycle program includes denaturation for 1 min at 94°C, 30–40 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min 30 s at 55°C (*see* **Notes 3** and **4**),

and primer extension for 1 min at 72°C A final extension then is performed for 15 min at 72°C

3. Detection of amplified DNA products by agarose gel electrophoresis. One-tenth of the RT-PCR reaction mixture is mixed with 2 μL of 6X sample buffer and loaded onto an agarose gel The gel is electrophoresed in 0 5X TBE buffer containing 0 5 μg/mL of ethidium bromide for 1 5 h at 150 volts The DNA bands are visualized with illumination by a UV light

4. Notes

1 Removal of inhibitors from stools RT-PCR is an enzymatic method; therefore, removing inhibitors of the enzymes is critical. EM examination of stool specimens has shown that the concentration of HuCVs and astroviruses in human stools is usually low. In the method described above, steps have been included to concentrate viruses and remove inhibitors. The first step is to extract the stool suspension with freon followed by precipitation with PEG This step removes significant amounts of organic and soluble materials. Following the PEG precipitation, the samples are usually less colored than samples not treated by PEG The second step is to extract RNA from the samples by phenol/chloroform in the presence of CTAB. This step was a modification from our previous method used to extract viral RNA from environmental samples and shellfish *(17)* By monitoring radiolabeled viral RNA with variable concentrations of CTAB and NaCl, the effect of salt in the CTAB solution on the yield of viral RNA was determined At high salt concentrations, viral RNA tended to remain in solution, but at low salt concentrations, the viral RNA was precipitated. We also observed that treatment of the samples with phenol/chloroform in the presence of CTAB selectively precipitated large amounts of fecal debris, leaving the viral RNA in the aqueous phase. The nucleic acid pellet resulting from the CTAB treatment usually was colorless and small. Stool samples treated with CTAB consistently produced positive results by RT-PCR whereas untreated samples did not

2. Principle of designing primers: As described in the Introduction, astroviruses and HuCVs each contain several genetic groups and antigenic types Highly conserved regions in the viral genomes within each family have been identified in the area of the 2C, 3C, and 3D motifs and in the capsid regions (**Fig. 1**) In astroviruses, the 3' end of the viral genomes is highly conserved among different antigenic types These conserved regions have been utilized to design group-specific primers for broad detection of viruses in each family Many primers reported in the literature show variable ability to detect astroviruses and HuCVs. Parameters that we believe are important for designing a primer pair include:
   a. High GC content (30–50%) within the primer sequence;
   b. Primer length 18–25 nucleotides;
   c. High conservation at the 3' end of the primer (if mismatches between the primer and the target must be included, they should be at the 5' end); and
   d. Product size between 200 and 800 bases.

3. Type-common and type-specific detection of HuCVs and astroviruses. The advantages of RT-PCR for detection of HuCVs over other methods such as immune EM and enzyme-linked immunosorbent assay (ELISA) include not only high sensitivity, but also high specificity. The specificity of RT-PCR is flexible depending on the primers used. As mentioned above, primers in the highly conserved region are broadly reactive and primers derived from unique regions of the viral genome usually are highly specific for strains or types. HuCVs are highly diverse genetically and it is difficult to use one primer pair for all members of the family. Astroviruses seem to be less diverse than HuCVs in the conserved regions. Primers for type-common and type-specific detection of astroviruses have been reported but further sequencing of the family is important to select more efficient primers. Continued sequencing of HuCVs also is important to design group- and type-specific primers for detection of different portions of the family. An alternative technique that improves broad detection of astroviruses and HuCVs is to use low stringency in the RT-PCR reaction. This means lowering the primer annealing temperature in the PCR cycles to allow mismatches. In our experience, the lowest temperature used was 37°C, which permitted detection of many HuCV strains quite different: from the NV on which the primers were based
4. Nonspecific reactions. Multiple bands will be noted, particularly when low annealing temperatures are used and occasionally when highly stringent conditions are used. Usually these nonspecific bands can be easily differentiated from the intended products by including appropriate size markers in the agarose gel electrophoresis. Confirmation of the RT-PCR products usually is performed by analyzing products with restriction enzyme digestion (this needs to be considered when designing the primers), hybridization (using an internal probe), and sequencing the amplified products.
5 Techniques for handling large numbers of specimens. RT-PCR procedures involve multiple pipeting steps, each of a small volume. Therefore, pipeting accuracy is critical for reproducible results We prepare the reaction mixtures in large volumes and aliquot them into reaction tubes. This practice also decreases the opportunity for cross-contamination between test samples. The RT and PCR reaction mixture aliquots can be stored at –70°C for several months without loss of sensitivity. This method is particularly useful for large clinical studies.
6. Techniques to prevent cross-contamination. The most common sources of contamination are carryover of amplified PCR products and plasmids in the laboratory environment into test samples during extraction of viral RNA We have monitored plasmid DNAs deliberately applied to surfaces in the laboratory environment and recovered them up to 2 mo later, although the surfaces were dry and exposed to air. To minimize the possibility of contamination, room and equipment for the preparation of the reagents and samples should be separated from rooms and equipment used to clone or otherwise manipulate RT PCR products. Strict cleaning procedures, such as changing of bench top covers, should be routine.
7. Sensitivity of RT-PCR compared with immunological methods and hybridization. Direct comparison of RT-PCR with ELISA and hybridization demonstrated

that RT-PCR was as sensitive as ELISA, but more sensitive than hybridization, for detection of HuCVs in stool specimens. The ELISA in this instance used hyper-immune antisera to highly purified viral antigen, a situation not usually achieved for production of antisera The similar sensitivity of RT-PCR and ELISA may be due to a higher concentration of soluble viral capsid antigen than of viral RNA in the stool. RT-PCR is significantly more sensitive than currently available ELISAs for detection of astroviruses *(15)* Because of the extremely low concentration of HuCVs and astroviruses usually present in human stools, it is not possible to count viral particles or quantitate viral RNA Using viral cDNA and synthetic viral RNA, RT-PCR detected 100–1000 copies of the viral genome, which is 1000–10,000 times more sensitive than hybridization *(12)*.

8 Application of RT-PCR to clinical, epidemiological, and molecular biological studies of astroviruses and HuCVs The above sections have described the RT-PCR method to detect astrovirus and HuCVs in human stools. This method should have broad application in basic and applied research on these viral families. Because of the common chemical properties of the viral genome of many single stranded RNA viruses, this method should also apply to those viruses. RT-PCR should be useful in monitoring attempts at HuCV replication in cell culture Previous attempts to cultivate HuCVs may have been hampered by a lack of sensitive methods to monitor even abortive viral replication RT-PCR also can be used to localize viral replication in the gastrointestinal tract and to monitor the duration and patterns of viral excretion in stool or other clinical samples. The RT-PCR technique also is applicable to environmental samples that may be contaminated by HuCVs and result in unsafe food, water, and shellfish These methods may promote public health safety and protect the shellfish industry

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4

Enteroviruses and Rhinoviruses

Peter Muir

1. Introduction

1.1. Human Picornaviruses

The enteroviruses and rhinoviruses are two large groups of viruses belonging to the *Picornaviridae* that regularly cause infections in humans. Like all picornaviruses they are small nonenveloped viruses with a single-stranded, positive sense RNA genome that contains a single open reading frame encoding a polyprotein that undergoes autocatalytic cleavage to give virus structural proteins and replication enzymes. The human enteroviruses consist of 66 serotypes *(1)* that include the polioviruses, group A and B coxsackieviruses, and echoviruses. More recently identified serotypes have been numerically classified as enterovirus serotypes 68–71 (some serotypes have been reclassified, hence the missing serotype numbers), while the rhinoviruses consist of over 100 distinct serotypes *(2)*.

1.2. Enterovirus Infections

1.2.1. Enterovirus-Related Disease

The majority of enterovirus infections are usually mild or asymptomatic, however some may produce a wide range of clinical manifestations of varying morbidity and mortality. This can involve cardiac and skeletal muscle, neuronal tissue, pancreas, skin, and mucous membranes. Enteroviruses are the most commonly implicated viral agents of acute myocarditis and aseptic meningitis. Although controlled in many parts of the world by vaccination, paralytic poliomyelitis resulting from poliovirus infection is still endemic in some countries.

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1.2.2. Diagnosis of Enterovirus Infections

Due to the diversity and nonspecificity of enterovirus-induced disease manifestations, diagnosis cannot be made on clinical grounds alone. Diagnosis is important to distinguish between enterovirus-induced and other causes of disease, and to allow monitoring of enterovirus outbreaks. Traditionally enterovirus infections have been diagnosed by isolation of the virus from target organs (e.g., myocardial biopsy or autopsy tissue, cerebrospinal fluid), blood, throat swabs, stool, or urine, followed by typing via neutralization of infectivity. However, some enteroviruses, notably many group A coxsackieviruses, can only be isolated in cell culture with difficulty (they were originally identified by inoculation into suckling mice). In addition, enteroviruses are rarely isolated from patients with acute myocarditis, since symptoms often appear after the peak of virus replication. When virus isolation is unsuccessful or when suitable specimens are not available, demonstration of seroconversion, with a fourfold or greater rise in type-specific virus-neutralizing antibody titer, or virus-specific IgM antibody in serum and/or cerebrospinal fluid (CSF) *(1,3)* may provide evidence of infection. However, serological diagnosis is complicated by the large number of serotypes, the occurrence of anamnestic, heterotypic antibody responses, and the absence of antibody responses directed toward crossreactive enterovirus group antigens in many patients. The requirement for appropriately timed paired sera to demonstrate a diagnostically significant rise in antibody titer further limits the utility of enterovirus serology

1.2.3. Enterovirus PCR

Recently, PCR has been explored as a means of enterovirus detection. Comparison of published enterovirus genome sequences has allowed highly conserved sequences to be identified and used as primer recognition sequences for reverse transcriptase PCR (RT-PCR) assays, capable of detecting most or all enteroviruses, including those which cannot be propagated in cell culture *(4–7)*. Many groups have found that RT-PCR is at least as sensitive as cell culture systems for detection of enteroviruses in clinical specimens *(8–19)* and sewage or environmental water *(20–25)*. It is also possible to detect enterovirus RNA by PCR in specimens from which infectious virus is never or only rarely recovered by culture, such as myocardial tissue from patients with acute myocarditis or dilated cardiomyopathy *(26–29)* and in formalin-fixed paraffin-embedded tissue *(30–35)*. These advantages will therefore ensure PCR a major role in the laboratory diagnosis of enterovirus infections. An enterovirus PCR test kit for the diagnosis of enteroviral meningitis, now commercially available from Roche Molecular Systems, will assist the transition to PCR-based diagnosis *(36,37)*. This test is based on a single step reverse transcription and

amplification of enterovirus RNA, and incorporates enzymatic elimination of amplicon contamination.

1.3. Rhinovirus Infections

1.3.1. Rhinovirus-Related Disease

Despite the large number of serotypes, the clinical manifestations of rhinovirus infections are far less diverse than those of enterovirus infections. The major presenting illness is the common cold, although approximately one-third of infections are asymptomatic *(2)*. However, rhinoviruses may be detected in children and infants with lower respiratory tract infections, and in patients with exacerbation of chronic bronchitis or asthma, although evidence of a causal role in these conditions is not yet conclusive *(38)*. In these situations, a specific diagnosis may prove useful.

1.3.2. Diagnosis of Rhinovirus Infections

Numerous respiratory and enteroviruses may cause symptoms indistinguishable from those caused by rhinoviruses, thus making laboratory diagnosis necessary for identification of the causative agent. Rhinoviruses are also traditionally detected by virus isolation from nasal washings or throat swabs in cell culture, followed by demonstration of acid lability, which distinguishes rhinoviruses from enteroviruses. Serological assays have been described, but not widely used, again due to the serological complexity of this virus group.

1.3.3. Rhinovirus PCR

Here again RT-PCR methods have the potential to simplify and improve diagnostic procedures *(39,40)*. Owing to the genetic similarity between enteroviruses and rhinoviruses, several groups have employed PCR methods that are capable of detecting both virus groups with a single set of primers, with distinction between enteroviruses and rhinoviruses based on the difference in size or internal sequence of the amplification target *(5,13,14,17,41,42)*.

1.4. Principles of Methodology

This chapter describes in detail a RT-PCR method employing nested primers for the detection of enterovirus RNA *(16)* which has been extensively evaluated in our laboratory and used by several other groups. The assay is capable of detecting 0.01–0.001 50% tissue culture infective doses ($TCID_{50}$) of enterovirus, and the principles described could readily be applied to other PCR systems for the detection of enteroviruses and/or rhinoviruses. A convenient and sensitive RNA extraction method is described, which is suitable for recovering RNA from a wide range of biological specimens prior to RT-PCR amplifica-

tion. Methods for treating cryopreserved or formalin-fixed paraffin-embedded solid tissue specimens prior to RNA extraction are also described. This is followed by a description of reverse transcription, PCR amplification, and PCR product analysis procedures.

2. Materials (*see* Note 1)

2.1. RNA Extraction

2.1.1. Disposable plastics

1 Filtered 2–20-, 20–200-, and 100–1000-µL pipet tips
2 Sterile, nuclease/protease free 0.65-µL and 1.7-µL Eppendorf tubes
3 Sterile disposable fine nozzle transfer pipets.

2.1.2. Reagents

1 Sterile molecular biology grade water (Sigma W-4502, Sigma, Poole, UK).
2 Acrylamide (Sigma A-9099).
3 1 *M* Tris-HCl, pH 8 0 (Sigma T-3038).
4 TEMED (Sigma T-7024)
5 150 mg Ammonium persulfate capsules (Sigma A-3426).
6. Sodium acetate (Sigma S-2889).
7. 0 5 *M* Ethylenediaminetetraacetic acid (EDTA) (Sigma E-7889)
8. Absolute ethanol
9 25 mg/mL Linear acrylamide solution prepared as follows

Concentration required	Volume required for 20 mL
5% Acrylamide	1 g Acrylamide
40 m*M* Tris-HCl, pH 8.0	800 µL 1 *M* Tris-HCl, pH 8 0
20 m*M* Sodium acetate	400 µL 1 *M* sodium acetate

Dissolve in a final volume of 20 mL sterile water, then divide solution into four 5-mL amounts in sterile universal bottles, and add 50 µL 10% ammonium persulfate (one capsule dissolved in 1.5 mL sterile water) and 5 µL TEMED to each bottle. Allow polymerization to take place for 30 min, then add 12.5 mL absolute ethanol to each bottle to precipitate the acrylamide Hold at –20°C for 10 min, then centrifuge at 6000*g* in an MSE centrifuge. Aspirate supernatant and dissolve each pellet in 10 mL sterile water by mixing overnight at room temperature. Store at 4°C This preparation is a based on the method of Gaillard and Strauss *(43)*.

10 RNAzol B (Cinna Biotecx; cat. no CS-105, Friendswood, TX).
11. Chloroform.
12. Isopropanol
13. 70% Ethanol/30% sterile molecular biology grade water

2.2. Homogenization of Cryopreserved Tissue

1. Sterile disposable scalpels
2. Sterile disposable fine nozzle transfer pipets
3. Porcelain mortars and pestles These should be wrapped in foil and sterilized by baking for 8 h at 200°C. After cooling they should be prechilled to –70°C for at least 1 h before use. After use they should be cleaned by immersing in 10% Domestos for at least 1 h, rinsed and soaked in 1 *M* hydrochloric acid for 1 h, then rinsed and dried at 80°C before wrapping in foil for resterilization.
4. Glass 0.1-mL Minihomogenizers. These should also be wrapped in foil (wrapping plungers separately) and baked as above They should be chilled to 4°C for at least 1 h before use. After use residual tissue should be removed mechanically using a transfer pipet They can then be cleaned in the same way as mortars and pestles.
5. Filtered 100–1000-µL pipet tips.
6. Sterile, nuclease/protease free 1.7-mL Eppendorf tubes.
7. Liquid nitrogen
8. Crushed dry ice.
9. Crushed wet ice.
10. RNAzol B.

2.3. Preparation of Paraffin-Embedded Tissue

1. Microtome employing disposable knife blades.
2. Sterile, disposable plastic forceps.
3. Filtered 2–20-, 20–200-, and 100–1000-µL pipet tips.
4. Sterile, nuclease/protease free 0 65-mL Eppendorf tubes
5. Programmable thermal cycler.
6. *n*-Octane (Sigma O-2001)
7. Absolute ethanol.
8. 1 *M* Tris-HCl, pH 8.0
9. 0 5 *M* EDTA
10. Tween-20 (Sigma P-9416).
11. Proteinase K (Sigma P-4914).
12. Sterile molecular biology grade water.
13. Proteinase K digestion buffer, prepared as follows

Concentration required	Stock solution	Volume required for 1 mL
50 m*M* Tris-HCl, pH 8.0	1 *M* Tris-HCl, pH 8.0	50 µL
1 m*M* EDTA	0 5 *M* EDTA	2 µL
0.5% Tween-20	Tween-20	5 µL
	Sterile water	923 µL
Total volume		980 µL

2.4. Reverse Transcription-PCR

1. Programmable thermal cycler
2. Filtered 2–20, 20–200, and 100–1000-µL pipet tips.
3. Sterile, nuclease/protease free 0.65-mL and 1 7-µL Eppendorf tubes.
4. Sterile molecular biology grade water
5. Moloney murine leukemia virus (MMLV)-reverse transcriptase (RT) (Life Technologies 28025-021, Gaithersburg, MD) (*see* **Note 33**)
6. 5X First-strand synthesis buffer (Life Technologies Y00146, supplied with MMLV-RT).
7. Deoxynucleotide triphosphates (dNTPs) (Promega U1240, Madison, WI) Add 400 µL of each 100 m*M* dNTP solution to 14.4 mL sterile water to give a 10 m*M* total dNTP stock solution. Dispense into aliquots and store at –20°C.
8. PRIME RNase Inhibitor 5 Prime—3 Prime Inc 9-901109; Boulder CO (*see* **Note 34**)
9. Primers EVD1, EVU1, EVD2, EVU2 (**Table 1**), adjusted to 15 µ*M*
10. Mineral oil (Sigma M-5904).
11. 25 m*M* $MgCl_2$ (Promega A351B, supplied with *Taq* polymerase).
12. 10X PCR buffer without $MgCl_2$ (Promega M190A; supplied with *Taq* polymerase) (*see* **Note 38**).
13. *Taq* polymerase (Promega M186B).
14. 1 *M* NaCl, prepared by diluting 5 *M* NaCl (Sigma S-5150) 1 5 in sterile water

2.5. Agarose Gel Electrophoresis (AGE)

1. Electrophoresis tank, gel mold, gel combs, and power pack.
2. 0.65-mL Eppendorf tubes.
3. 200-µL pipet tips.
4. Ultraviolet transilluminator.
5. Polaroid camera or image analyzer
6. Deionized or reverse-osmosis purified water.
7. 50X Tris-acetate-EDTA (TAE) buffer, prepared according to Sambrook et al. *(45)* as follows:
   a. 242 g Trizma.
   b 57.1 mL Glacial acetic acid.
   c. 100 mL 0.5M EDTA pH 8 0.
   d. Deionized water added to a final volume of 1000 mL.
   50X TAE should be sterilized by autoclaving.
8. Agarose (Sigma A9311)
9. 50X Gel loading buffer, prepared as follows:
   a 2 mL 50X TAE buffer.
   b 2.5 g Ficoll (Sigma F-2637)
   c. 0 04 g Bromophenol blue (Sigma B-8026).
   Add approx 10 mL deionized water, and make up to a final volume of 20 mL once Ficoll has dissolved. This is a modification of a gel loading buffer described by Sambrook et al. *(45)*.

Table 1
Nucleotide Sequences and Positions of Annealing Sites of Primers for Enterovirus Nested PCR

Primer designation	Primer sequence (5' to 3')[a]	Nucleotide positions of primer[b]
EVU1	GGTGYGAAGAGYCTAYTGAG	417-436
EVD1	CACYGGRTGGCYAATCCA	645-628
EVU2	CCCCTGAATGCGGCTAAT	456-473
EVD2	ATTGTCACCATAAGCAGCCA	602-583

[a]Mixed bases, indicated by the letters Y (C or T), and R (A or G), are incorporated to accomodate variations in the sequence of primer annealing sites among different enterovirus genomes at these positions

[b]Nucleotide positions are based on the published sequence of coxsackievirus B3 *(44)*

10. 10 mg/mL ethidium bromide solution (Sigma E 1510).
11. 1X TAE buffer, prepared by adding 20 mL 50X TAE to 980 mL deionized water.
12. DNA size markers, e g., Ready-Load kb DNA ladder (Life Technologies 10381-010)

3. Methods

3.1. RNAzol Extraction of RNA (see Note 2)

1. Add 400 mL RNAzol B to 100 mL of sample (e.g., tissue culture supernatant, cerebrospinal fluid, throat swab transport medium, urine, stool filtrate, or proteinase K-digested tissue section [see Subheading 3.3]) See Note 3 for specimen storage. Include a positive control and extraction blanks (RNAzol B plus sterile water) (see Note 4).
2. Add 80 µL chloroform to each sample.
3. Mix and hold on ice for 5 min
4. Centrifuge tubes in microfuge at top speed for 15 min
5. Add 2 µL of 25 mg/mL linear acrylamide solution to clean large Eppendorf tubes, one for each sample (*see* **Note 5**)
6. Transfer upper, aqueous phase of each sample to a tube containing linear acrylamide solution and mix. Discard lower organic phase.
7. Add an equal volume (typically 360 µL) isopropanol to each sample (*see* **Note 6**).
8. Close tubes, mix well, and store at –20°C for at least 1 h (*see* **Note 7**).
9. Centrifuge tubes in microfuge at top speed for 5 min.
10. Aspirate liquid phase, taking care to leave precipitate in tube.
11. Wash precipitate with 100 µL 70% ethanol (*see* **Note 8**)
12. Centrifuge tubes briefly and completely aspirate liquid.
13. Allow precipitates to air dry for 5 min (*see* **Note 9**)
14. Proceed with reverse transcription and PCR as described in **Subheading 3.4.**

3.2. RNA Extraction from Cryopreserved Solid Tissue (see Note 10)

1. Remove a portion of tissue up to approx 100 mg in weight using a sterile scalpel prechilled in dry ice (*see* **Note 11**)
2. Transfer tissue to a sterile mortar and pestle prechilled to –70°C
3. Pour 10–20 mL liquid nitrogen into mortar, and allow to evaporate (*see* **Note 12**)
4. Grind tissue to a fine powder
5. Add 400 µL RNAzol B to a minihomogenizer and store on wet ice (*see* **Note 13**)
6. Collect ground tissue and transfer to minihomogenizer containing RNAzol B using cold scalpel blade.
7. Homogenize ground tissue in RNAzol B with ten strokes of the plunger (*see* **Notes 13** and **14**
8. Transfer homogenate to a sterile 1 7-mL Eppendorf tube using a fine nozzle disposable transfer pipet (*see* **Note 15**).
9. Continue with RNA extraction from step 2 as described above in **Subheading 3.1.**

3.3. RNA Extraction from Paraffin-Embedded Tissue (see Notes 16–22)

3.3.1. Paraffin Removal

1. Transfer a single 5–10 µm thick tissue section using disposable forceps to a sterile 0 65-mL Eppendorf tube (*see* **Notes 23–25**)
2. Add 0 5 mL octane (see **Note 25**)
3. Mix for at least 30 min at room temperature.
4. Aspirate octane (*see* **Note 26**).
5. Repeat **steps 2–4**.
6. Add 0 5 mL absolute ethanol.
7. Mix for at least 5 min
8. Centrifuge in a microfuge for 1 min at 17,000*g*.
9. Aspirate ethanol
10. Repeat **steps 6–9**.
11. Air dry sections overnight; leave tubes open but covered to protect from dust.

3.3.2. Proteinase K digestion (see **Note 27**)

1. Prepare a 10 mg/mL stock solution of proteinase K. Dispense into conveniently sized aliquots (e.g., 20 µL) and store at –70°C Use each aliquot once only, discarding any remaining solution
2. Prepare proteinase K digestion buffer per sample as described in **Subheading 2.3., item 13** (*see* **Note 28**). Immediately before use, add 20 µL of 10 mg/mL proteinase K stock solution to each 980 µL volume of buffer.
3. Add 100 µL proteinase K digestion buffer to each deparaffinized section. Process empty tubes in parallel as proteinase K digestion blanks, and if available, a positive control tissue section (*see* **Note 29**).

4. Incubate at 55°C for 3 h, then at 99°C for 5 min using thermal cycler.
5. Centrifuge tubes briefly using microfuge (*see* **Note 30**).
6. Proceed immediately with RNA extraction as described in **Subheading 3.1**

3.4. RT-PCR

3.4.1. Reagent Preparation

1. Determine the number of samples to be analyzed (including RNAzol extraction blanks and proteinase K digestion blanks) (*see* **Notes 31** and **32**). Prepare reverse transcription master mix as follows (*see* **Notes 33** and **34**)

Reagent	Volume for 1 tube (mL)
First strand synthesis buffer	4
Sterile water	3.3
dNTPs	8
1.0 U/μL Inhibit-ACE	0 7
MMLV-RT	1
Primer EVD1	1
Total volume per tube	18

2. Dispense 18 μL RT mix into 0.65-mL Eppendorf tubes, and overlay with 3 drops of mineral oil (see **Note 35**).
3 Prepare first PCR master mix in bulk, by multiplying the following reagent volumes as required (*see* **Notes 32** and **38**).

Reagent	Volume for 1 tube (mL)
25 m*M* $MgCl_2$	5.6
10X PRC Buffer	8
Sterile water	64.9
Primer EVU1	1
Taq polymerase	0.5
Total volume per tube	80

4. Prepare nested PCR master mix in bulk, by multiplying the following reagent volumes as required (*see* **Note 32**).

Reagent	Volume for 1 tube (mL)
25 m*M* $MgCl_2$	3.6
10X PCR Buffer	5
Sterile water	36 15
dNTPs	4
Primer EVD2	0.5
Primer EVU2	0.5
Taq polymerase	0.25
Total volume per tube	50

5. Dispense 50 µL nested PCR mix into 0.65-mL Eppendorf tubes, and overlay with 3 drops of mineral oil (*see* **Note 36**)

3.4.2. Reverse Transcription

1. Reconstitute RNA pellets prepared in **Subheading 3.1.** by adding 20 µL sterile water Close tubes.
2. Denature extracted RNA samples at 80°C for 5 min using a thermal cycler, chill on ice, and centrifuge briefly Ensure that pellet has dissolved (*see* **Note 36**).
3. Add 2 µL of each RNA sample to a tube containing RT mix as prepared in **Subheading 3.4.1.** Add sample below the mineral oil layer
4. Incubate at 37°C for 90 min, followed by 99°C for 5 min using thermal cycler
5. Reprecipitate remainder of RNA samples for future use by adding 10 µL 1 *M* NaCl, then 75 µL absolute ethanol to each tube Store RNA samples at –70°C To reconstitute, follow **steps 9–14 in Subheading 3.1.2.** (*see* **Note 37**)

3.4.3. First PCR

1. After reverse transcription is complete, centrifuge tubes briefly and add 80 µL of first PCR mix prepared as described in **Subheading 3.4.1.** to each tube
2. Perform DNA amplification in a Hybaid Omnigene (Teddington, Middlesex, UK) thermal cycler programmed with the following cycling parameters.
   94°C; 5 min, 52°C; 15 s, 72°C; 15 s (1 cycle).
   94°C; 15 min, 52°C; 15 s, 72°C; 15 s (29 cycles).
   72°C; 5 min (1 cycle) (*see* **Note 39**)

3.4.4. Nested PCR

1. Centrifuge first PCR reaction tubes briefly.
2. Transfer 1 µL of first PCR reaction to tubes containing nested PCR reaction mix prepared as described in **Subheading 3.4.1, steps 4** and **5** Use filtered pipet tips, adding sample directly to nested PCR mix below the mineral oil layer.
3. Perform nested PCR amplification in a Hybaid Omnigene thermal cycler programmed with the following cycling parameters:
   94°C, 15 s, 50°C; 15 s, 72°C; 15 s (25 cycles).
   72°C; 5 min (1 cycle) (*see* **Note 39**).

3.5. Agarose Gel Electrophoresis

3.5.1. Gel Preparation

1. Prepare a 2% agarose gel in TAE buffer (*see* **Note 40**) Dissolve agarose in buffer by heating in a microwave oven for 2 min. Add 1.25 µL of 10 mg/mL ethidium bromide solution to each 40 µL of molten agarose solution, and mix well Pour into gel mold while still molten and insert gel combs.
2. When gel has solidified and cooled, transfer to electrophoresis tank, cover with 1X TAE buffer, and remove gel combs

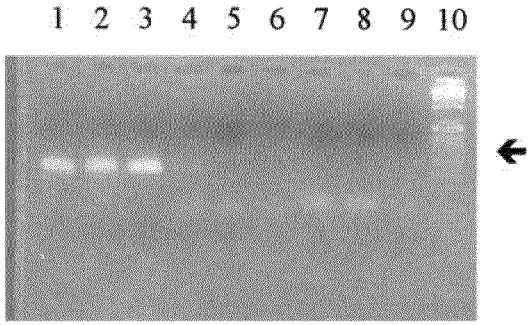

Fig. 1. Electrophoretic analysis of PCR reactions: Lane 1, CVB3 positive control; Lanes 2 and 3, enterovirus-positive CSF samples from two patients with aseptic meningitis; Lane 4, throat swab sample from patient with Bornholm Disease (weakly positive), Lanes 5–9, negative control RNA extractions; Lane 10, electrophoretic size standards (kb ladder; Life Technologies: the arrow indicates the position of a 142/154 bp doublet used to size enterovirus nested PCR products).

3.5.2. Electrophoresis

1. Add 16 µL of each nested PCR reaction to 4 µL 5X gel loading buffer in 0.65-mL Eppendorf tubes.
2. Add 10 µL of each PCR product in gel loading buffer to one lane of the gel.
3. Include DNA size markers on one lane of each row of lanes.
4. Electrophorese samples for approx 15–20 min at 125 volts.
5. Visualize using transilluminator. A positive result is indicated by the presence of a band corresponding to approximately 148 bp (**Fig. 1**) (*see* **Note 41**).

4. Notes

4.1. PCR-Grade Reagents and Disposables

1. Molecular biology grade plastic disposables and reagents that are sterile and certified free of nucleases and proteases should be used for RNA extraction and RT-PCR procedures. This avoids the necessity of treating and sterilizing plastics and reagents on site, thus minimizing the risk of accidental contamination.

4.2. RNA Extraction

2. Extraction of RNA prior to RT-PCR is necessary to inactivate ribonucleases present either in the sample or from possible environmental contamination (ribonucleases are present in dust and perspiration) and to separate RNA from enzymatic inhibitors. Single-stranded RNA is particularly susceptible to ribonuclease activity, which is difficult to inactivate. Most RNA extraction procedures employ an initial lysis stage containing the chaotropic agent guanidine thiocyanate, which rapidly denatures proteins, releasing viral RNA from virions and infected cells,

eliminating any ribonuclease activity, and inactivating most enzymatic inhibitors. Although toxic, the number of manipulations involving guanidine thiocyanate is minimal. In the original procedure described by Chomczynski and Sacchi *(46)*, phenol and chloroform are added after the initial lysis step. A phase separation thus occurs, in which denatured proteins and most of the DNA partition into the denser, organic phase, and total RNA is recovered from the aqueous phase after centrifugation by isopropanol precipitation. The method described in this chapter is based on this method, and uses a commercially available lysis buffer containing guanidine thiocyanate and phenol. Other RNA extraction methods described in the literature employ a guanidine thiocyanate-based lysis buffer, with RNA being recovered from the lysis buffer by isopropanol precipitation without a phenol-chloroform phase separation *(10,36)*, by adsorption to silica particles or diatoms *(47)*, or by hybridization to oligo d(T) or sequence-specific oligonucleotide capture probes bound to paramagnetic particles *(16,48,49)*. Solid phase-bound RNA is then washed and eluted in a small volume There are several commercial RNA extraction kits based on these RNA extraction principles

3 Specimens for RT-PCR analysis should be processed as quickly as possible after collection to minimize sample deterioration due to endogenous nucleases. Tissue samples should be snap frozen in liquid nitrogen and stored on dry ice or in a –70°C freezer, or fixed as soon as possible in chemical fixative (*see* **Note 17**) Other samples should also be stored at –70°C if there is a delay in processing. Although viral RNA has been successfully amplified from serum or plasma samples stored at –20°C, in general samples should not be stored at this temperature It appears that degradation of nucleic acid following disruption of cellular lysosomes is not completely inhibited at –20°C. If samples cannot be stored immediately at –70°C, short-term storage at 4°C is preferable to storage at –20°C. Short-term storage at 4°C is also preferable to repeated thawing and freezing of specimens If samples are required for multiple laboratory procedures, portions or aliquots should be reserved for PCR analysis so that inappropriate storage and accidental contamination can be avoided.

4. Blank RNA extractions, in which the test sample is replaced with an equal volume of sterile water, should be processed in parallel with each batch of samples to demonstrate the absence of PCR contamination. The number of extraction blanks should be similar to the number of test samples. A positive virus control should also be processed in parallel to validate the RNA extraction procedure. An appropriate positive control would be an enterovirus-infected cell culture supernatant diluted to a titer of 1–10 $TCID_{50}$ per mL

5. Precipitation of nucleic acids is inefficient when the total nucleic acid concentration is low. This is likely to be the case with many clinical specimens, especially cell-free specimens. Recovery is greatly increased by inclusion of a carrier during the precipitation step—a substance which coprecipitates with nucleic acids Commonly used carrier molecules are transfer RNA, salmon sperm DNA, or glycogen. This protocol employs linear polyacrylamide, a highly efficient coprecipitant *(43)* that results in the formation of a discrete, firm pellet following centrifugation in **step 9** of **Subheading 3.1.** This allows complete aspiration of the

supernatant in **steps 10** and **12** of **Subheading 3.1.**; although the pellet sometimes dislodges from the base of the Eppendorf tube, it does not disintegrate and is too large to be aspirated accidentally when using fine nozzle transfer pipets or micropipet tips It is essential to ensure that the polyacrylamide solution is evenly dispersed in the RNA-containing aqueous phase at **Subheading 3.1., step 6.** If not, the polyacrylamide will precipitate immediately on contact with isopropanol in **step 7,** of **Subheading 3.1.,** without coprecipitating the RNA present in the sample. Dispersion of polyacrylamide is most easily achieved by adding polyacrylamide solution to the tube before adding the sample, as suggested

6 Addition of isopropanol: The volume of aqueous phase recovered at **step 7,** of **Subheading 3.1.** is typically around 360 µL when the initial volume of the specimen used is 100 µL If the initial sample volume is less than 100 µL, the volume of aqueous phase will be less, and the volume of isopropanol added should be reduced accordingly. This is likely to be the case, for example, when processing small cryopreserved tissue specimens. Addition of excess isopropanol results in poor pellet formation after centrifugation in **Subheading 3.1., step 9.**

7. Interruption of RNA extraction procedure: If desired the procedure can be interrupted following addition of isopropanol, with samples being held at –20°C for up to 1 wk

8 The precipitate is washed in 70% ethanol to remove residual salt carried over from the lysis buffer. This is important, since sodium ions inhibit downstream enzymatic reactions. Precipitated nucleic acids will not resolubilize at this ethanol concentration Only a brief (pulse) centrifugation is required at **step 12 (Subheading 3.1.)** to collect the wash solution at the bottom of the tube This facilitates complete aspiration of the liquid, thus reducing the drying time required.

9. Provided the 70% ethanol wash has been completely aspirated, leaving tube lids open for 5 min is sufficient to allow evaporation of residual ethanol prior to RT-PCR. Use of a vacuum desiccator is not required, and may actually result in dislodging and loss of pellets when releasing the vacuum.

4.3. RNA Extraction from Cryopreserved Solid Tissue

10. Automation of homogenization: Homogenization of cryopreserved tissue is the most messy procedure described in this chapter. An automated mechanical homogenization system is available (Ribolyzer, Hybaid Ltd) that simplifies this procedure. The manual procedure is described here for those who do not have access to this equipment. Owing to the nature of the procedure it is recommended that the work surface be covered with bench paper, on to which crushed dry ice is spread to keep the tissue frozen while a portion is removed for analysis. After each specimen has been processed, bench paper, dry ice, latex gloves, and any other material that might have come into contact with the tissue should be replaced. When processing multiple samples, it is usually convenient to conduct **steps 1–8 (Subheading 3.2.)** individually for each specimen. Homogenized specimens can then be stored in the ice bath until all samples are homogenized, at which point all samples can be processed simultaneously according to **Subheading 3.1.**

11. Tissue grinding: To minimize endogenous RNase activity, it is important to keep tissue frozen during the grinding procedure Prechilling scalpel blades and mortars and pestles to −70°C is essential for this.
12 Chilling the tissue portion in liquid nitrogen facilitates grinding by making the tissue brittle
13. Although 0.1-mL minihomogenizers are used to minimize sample loss, homogenization is more effective when the full 400 µL of RNAzol B is added to the homogenizer. Homogenization in smaller volumes of RNAzol B results in excessive foaming. However, ensure that tissue fragments enter the narrow stem during homogenization.
14 Do not use more than the recommended 10 strokes of the plunger; this results in increased shearing of RNA
15. Use fine nozzle transfer pipets to remove homogenate to Eppendorf tubes. Standard micropipet tips do not reach the bottom of a 0.1-mL minihomogenizer.

4.4. RNA Extraction from Paraffin-Embedded Tissue

16 RNA integrity in paraffin-embedded tissue· Histological procedures designed to preserve tissue architecture and antigens also result in satisfactory preservation of RNA, which can be recovered by RNA extraction following deparaffinization and protease digestion of thin tissue sections. However the efficiency of RNA amplification may be less than from comparable cryopreserved tissue owing to loss of RNA *(50–53)* or presence of enzymatic inhibitors *(54)*. The quality of RNA may depend on a number of factors, as discussed in **Notes 17–20.**
17 Tissue autolysis: Long delays between sample collection and fixation may result in loss of RNA through autolysis. This is a particular issue when processing post mortem tissue which may have been collected some time after death.
18. Tissue fixative: The chemical fixative used may influence the quality of RNA recovered The most commonly used histological fixative for light microscopy is neutral-buffered formalin However some investigators have found that RT-PCR detection of RNA was more successful using tissue fixed with ethanol, Omnifix, or Carnoy's fixative, while Bouin's fixative, Zenker's fixative, B-5, or unbuffered formalin has produced poor results *(55–58)*.
19 Fixation time. The length of fixation prior to tissue dehydration and paraffin embedding may also affect RNA integrity. Bresters et al. *(51)* and Ben-Ezra et al. *(55)* found that fixation times in neutral buffered formalin greater than 24 h resulted in reduced RNA yield.
20 Storage time: Some investigators have reported loss of detectable RNA with increasing storage time *(58)*.
21 Assessment of RNA integrity When negative results are obtained from RT-PCR analysis of archival tissue the possibility of RNA degradation during tissue collection and storage should always be considered. Many groups have attempted to assess RNA integrity in such specimens by RT-PCR amplification of ubiquitously expressed cellular mRNA species. However, it should be remembered that such RNA species may be present in significantly higher copy numbers than viral

RNA species. In addition, the mRNA specificity of such assays should always be confirmed by performing parallel amplifications in which reverse transcriptase is omitted. Although mRNA primers are usually designed to amplify target sequences containing splice junctions so that PCR products derived from mRNA can be distinguished from those derived from genomic DNA or unprocessed RNA, the existence of pseudogenes, the DNA sequence of which corresponds to that of spliced mRNA, may give rise to misleading results *(59)*.

22. Validity of test results: Despite the limitations of RT-PCR analysis of paraffin-embedded tissue outlined above, several groups have successfully recovered and amplified viral RNA from old archival tissue samples that had not been processed with a view to preservation of nucleic acids However because of the possibility of false-negative results, RT-PCR analysis of archival tissue samples should not be considered an accurate means of conducting retrospective epidemiological study of viral infection Nonetheless, the ability to study archival material may allow disease associations and pathogenetic mechanisms to be investigated that could not be rapidly achieved by prospective study *(31,60,61)* Sensitivity of RT-PCR may be optimized by amplifying short (<300 bases) target sequences *(54)* using nested primers *(58,62)*. When performing RT-PCR analysis of paraffin-embedded tissue as a means of viral diagnosis, clinicians and pathologists should be advised of appropriate tissue collection and fixation procedures
23. Tissue sectioning: The use of a separate disposable knife blade for each paraffin block minimizes the risk of cross-contamination of samples Where this facility is not available, the knife should be cleaned thoroughly between blocks by wiping the blade with xylene **Caution:** xylene is a carcinogen
24. Collecting and dispensing tissue sections· Ten to twenty tissue sections from each block should be collected in a sterile universal container Thin tissue sections are frequently difficult to handle both prior to and after deparaffinization because of electrostatic charges that cause sections to become airborne and to adhere to plastic utensils and latex gloves This problem can be reduced by allowing sections to roll up during tissue sectioning. With experience, sterile micropipet tips can be used instead of disposable forceps to transfer individual tissue sections to Eppendorf tubes. Multiple (3–5) sections from each block should be analyzed in parallel to minimize false-negative results owing to small sample size or focal distribution of viral RNA within the tissue block.
25. Solubilization of paraffin: Paraffin wax may also be dissolved by two extractions with xylene. However, since xylene also dissolves some plastics, Eppendorf tubes and transfer pipets should first be tested for xylene resistance. Small (0.65-mL) Eppendorf tubes are recommended because this obviates the need to transfer tissue to a tube that can be accommodated in a thermal cycle prior to proteinase K digestion The initial stages of the subsequent RNAzol extraction procedure can also be performed in the same 0.65-mL tube.
26. Tissue recovery: Tissue sections frequently disintegrate during paraffin removal. This inevitably results in some tissue loss, since tissue fragments in octane cannot be pelleted by centrifugation. This can be offset by adding multiple sections

to each tube when sections are small, for example, bioptome biopsy samples Alternatively, performing octane washes for longer times but without mixing may reduce tissue fragmentation. In contrast, tissue fragments in 70% ethanol can be pelleted by centrifugation This serves to deposit tissue at the base of the tube prior to the final aspiration and drying.

27 Determination of optimal digestion conditions· The method for deparaffinization and protease digestion described here was originally described for DNA extraction by Wright and Manos *(63)*. Several protease digestion protocols have been described by others *(30–35,64)*, differing in buffer constitution, protease concentration, incubation temperature and length of incubation (3 h to 5 d) In a recent comparison of several of these methods, we found the protocol presented here resulted in maximal yield of RNA as measured by PCR product yield (unpublished observation)

28 Inhibition of RNase activity: In adapting the method of Wright and Manos *(63)* for RNA extraction, we originally incorporated vanadyl ribonucleoside complexes into the protease digestion buffer to inactivate ribonuclease activity *(15)* However this has since been found unnecessary when using short protease digestion times; the protease activity effectively performs this function. Since vanadyl ribonucleoside complexes have been reported to inhibit reverse transcriptase activity *(65)* we no longer use this reagent

29. Negative and positive controls: Proteinase K digestion blanks, in which empty tubes without tissue sections are processed through the deparaffinization and proteinase K digestion procedures, should be included in each batch. The number of such blanks should be similar to the number of sections processed. Proteinase K digestion blanks can replace the RNAzol extraction blanks described in **Note 4,** although it is wise to include some additional blanks during RNA extraction so that the source of any observed PCR contamination can be readily identified. Identifying a suitable positive control tissue, in which viral RNA is consistently detected in all sections, is problematic If available, experimentally infected murine tissue may be used.

30. Tissue digestion: Fragments of tissue often remain at the completion of proteinase K digestion. However they usually disperse following addition of RNAzol B Even when tissue is not completely digested, RNA yield is adequate.

4.5. RT-PCR Amplification

31. Negative and positive controls· A positive control RNA extract, prepared from infected cell culture supernatant containing 1–10 $TCID_{50}$ per mL, should be included in each batch. A number of RT-PCR reagent blanks, in which 2 µL sterile water is added in place of test sample, should also be included to demonstrate absence of PCR reagent contamination.

32. Master mix preparation: Having determined the number of samples to be analyzed, including all positive and negative controls, master mixes should be prepared in bulk and the appropriate volume dispensed to individual reaction tubes When calculating reagent volumes for master mixes, sufficient reagents for one

additional reaction tube should be prepared for every 15–20 reactions to be performed to allow for pipeting inaccuracies.

33. MMLV-RT: The reverse transcriptase enzyme used in this protocol is efficient yet relatively inexpensive, even though the number of units required per reaction is more than reported for other reverse transcriptase enzymes. This reverse transcriptase is suitable for generating cDNA fragments up to 300–400 nt. For generation of longer cDNA molecules, a genetically modified RNase H minus enzyme (Superscript II; Life Technologies 18064-071) is more efficient.
34. Inhibit-ACE: This RNase inhibitor was found to result in improved PCR product yield when compared to placental RNasin *(15)*. Unlike RNasin, its activity is not dependent on the presence of dithiothreitol, and dithiothreitol should not be added to the reverse transcription buffer.
35. Mineral oil overlay: Overlaying reagents with mineral oil reduces evaporation and formation of condensation on the lid of the reaction tube during subsequent thermal cycling, which would change the concentration of reactants. Many of the recent thermal cyclers overcome this problem by fitting the thermal block with a heated lid, and using specially designed thin-walled tubes with rounded lids, which prevents condensation forming on the tube lid. In this situation the mineral oil overlay can be omitted.
36 Delaying the reconstitution of dried RNA pellets until reverse transcription reagents have been prepared minimizes any RNA degradation that may occur while RNA is in aqueous solution. Heating samples to 80°C aids solubilization of pellets and denatures any RNA secondary structure that might interfere with reverse transcription.
37. RNA is more stable in ethanol than in water at –70°C. Addition of salt is necessary for precipitation. Samples can then be quickly reconstituted for additional testing
38. PCR buffer. The first PCR amplification of this protocol was found to proceed more efficiently using the 10X reaction buffer provided with Promega *Taq* polymerase than with other reaction buffers, possibly because of its higher pH (9.0) compared to many other PCR buffers.
39 Thermal cycling: The cycling protocols described in this protocol are suitable for use with a thermal cycler employing an in-tube thermistor control, or when using thin-walled tubes. Otherwise, 15-s temperature holds should be extended to 1 min. The initial 5-min denaturation at 94°C in the first PCR amplification is required to ensure that long RNA/cDNA hybrids are fully denatured. A long initial denaturation is not required in the nested PCR, since the template DNA generated by the first PCR amplification is much shorter in length.

4.6. Agarose Gel Electrophoresis

40. The volume of molten agarose required to prepare a gel depends on the size of the gel mold. Commonly used gel sizes are "minigels" for analyzing up to 20 samples, which require approx 40 mL agarose, and "midigels" for analyzing up to 40 samples, prepared from approx 80 mL agarose

41 Interpretation of results. Following amplification of enterovirus RNA, the nested PCR protocol described here usually gives rise to a single band of approx 148 bp that comigrates with the 142/154 doublet band of Life Technologies' kb DNA ladder, although a smaller band corresponding to primer dimers is usually present. Occasionally larger bands are also seen, which may be due to PCR product carried over from the first PCR cycle, or seminested PCR products We do not consider Southern blot hybridization to be necessary to confirm positive PCR results After continuous use of this assay for 3 yr in this laboratory, no sample has ever given rise to a PCR product of correct size that was not subsequently confirmed to be of enterovirus origin by nucleotide sequence analysis or reamplification using alternative primers The specificity of nested PCR amplification, which is dependent on four specific primer/target interactions, can thus be assumed to be equal to or greater than that of single round PCR followed by Southern blot hybridization, which is dependent on only three specific primer/target or probe/target interactions

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5

Rapid Detection and Identification of Dengue Viruses by Reverse Transcriptase/Polymerase Chain Reaction

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1. Introduction

Introduction of the polymerase chain reaction (PCR) technique as a rapid and sensitive method for amplification of DNA has resulted in development of new specific nucleic acid-based techniques for clinical diagnosis of infections *(1)*. Improvements in nucleic acid amplification by PCR, and development of sensitive nonisotopic technologies for detection of amplified viral genes have facilitated the introduction of molecular methods into the diagnostic laboratory. The availability of simple and well-characterized protocols, commercial reagents of high quality, improved equipment for performing PCR reactions, and nonisotopic methods for detection of amplicons has stimulated development of PCR-based tests for detection of both DNA and RNA viral nucleic acids in clinical samples *(1)*.

The flaviviridae family of viruses includes the etiologic agents of dengue (DEN), West Nile encephalitis, Japanese encephalitis (JE), yellow fever (YF), and St. Louis encephalitis, which are transmitted to humans by infected vector mosquitos *(2)*. The flavivirus genome is a positive-sense single-stranded RNA molecule of approx 11 kb *(2)*. Flavivirus infections give rise to a wide spectrum of disease manifestations ranging from a mild, self-limiting febrile illness to more severe vascular and hemostatitic abnormalities and encephalitis. Routine serological diagnosis involves the identification of antibodies against the virus by hemagglutination inhibition (HI), IgM capture enzyme-linked immunosorbent assay (ELISA), or virus

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neutralization *(3–5)*. Only the virus neutralization test can identify the infecting virus to species or serotype because of antigenic crossreactivity between the viruses which share group reactive and complex reactive epitopes *(6,7)*. Because of the low-titer viremia which occurs during the early acute phase of neurotropic flavivirus infection it is impractical to attempt cultivation of these viruses except in fatal cases when central nervous system (CNS) autopsy tissues are available. Infectious virus can be isolated from serum during the acute phase of DEN *(8)* and YF virus infections. Flaviviruses can be isolated from infected mosquitoes, blood/serum or autopsy samples by inoculation of mosquito or mammalian cell cultures, suckling mice, or mosquitoes *(2)*.

Amplification of the flavivirus RNA genome by PCR is a two-step process which first requires conversion of the genome target to DNA by the enzyme reverse transcriptase and amplification of the target by repetitive cycles of oligonucleotide primer (amplimer)-directed DNA replication *(9–15)*. Repetitive amplification cycles involving DNA denaturation, amplimer annealing, and extension of the annealed amplimer by the thermal stable DNA polymerase results in exponential accumulation of the amplified DNA fragment (amplicon) flanked by two amplimers. In this chapter, we report development and application of PCR technology for detection and typing of DEN viruses. Oligonucleotide consensus primers were designed to anneal to a common sequence in the genome of each of the four DEN viruses and amplify a 511-bp product which is identified to serotype by a second round of PCR using nested serotype-specific amplimers which produce products of unique sizes specific for each DEN virus serotype *(9)*.

2. Materials

1. Extraction buffer

	Amount	Final conc.
Guanidine isothiocynate	18 9 g	4 *M*
Na citrate, pH 7 0	1.0 mL of 1 *M* solution	25 m*M*
Sarkosyl	0 2 g	0 5%
Mercaptoethanol	313 µL	100 m*M*
Transfer RNA	4 mg	10 µg/100 µL
Water	qs 40 mL	

Note· transfer RNA can be left out of the mixture if cells or tissues are to be extracted. Too much RNA in the reaction can inhibit the synthesis.

2. Acetate Buffer, 2 *M*, pH 4.0 and 3 *M*, pH 5.2 Na acetate.
3 Phenol, buffer saturated, pH 4.7 (Amresco, Solon, OH, cat. no. 0981)

Table 1
Oligonucleotide Primers Used to Amplify and Type DEN Viruses[a]

Primer	Nucleotide sequence	Genome position[b]	Size, of amplicon[c]
D1	5'-TCAATATGGTGAAACGCGCGAGAAACCG-3'	134–161	511
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	616–644	511
TS1	5'-CGTCTCAGTGATCCGGGGG-3'	568-586	482
TS2	5'-CGCCACAAGGGCCATGAACAG-3'	232–252	119
TS3	5'-TAACATCATCATGAGACAGAGG-3'	400–421	290
TS4	5'-CTCTGTTGTCTTAAACAAGAGA-3'	506–527	392

[a]Used with permission of the American Society for Microbiology

[b]The genome positions of D1 and D2 are given according to the DEN type 2 virus published ***(16)*** sequence, and map of the positions of the dengue virus type-specific primers (TS1,TS2,TS3, and TS4) are given according to their respective published sequences ***(16–19)***

[c]The size of the amplified product obtained after each of the type-specific primers (TS1) to (TS-4) was determined from the priming positions of the D1 primer within each respective genome The priming position of D1 on the genome of each DEN virus serotype is type 1, 105, type 2, 134, type 3, 132, and type 4, 136 ***(9)***

4 PCR reaction buffer· Final concentrations of reagents 50 m*M* KCl; 10 m*M*, pH 8 5 Tris-HCl, 1 5 m*M* $MgCl_2$; 0.01% gelatin. Prepare a 10X stock solution and aliquot. Store at –20°C

5 Nucleotides· Prepare of buy 10 m*M* solutions of each deoxynucleotide triphosphate (dNTP) Final concentration in the reaction is 200 μ*M* Store at –20°C

6 Primers: Primers used for the detection of DEN virus by the reverse transcriptase and PCR technique are listed in **Table 1** Primer concentrations are adjusted to 100 μ*M* (100 p*M*/μL). Final concentration in the reaction is 50 p*M* per 50 μL reaction. Store at –20°C

7. Dithiothreotol (DTT) 0 1 *M* DTT is prepared by dissolving 0.154 g of DTT in 10 mL of nuclease-free water Store at –20°C

8 RNase inhibitor: May be obtained from US Biochemical (Indianapolis, IN), Amersham Life Sciences (Arlington Heights, IL), or Boehringer Mannheim (Indianapolis, IN The final concentration in the reaction is 0.5 U/μL

9 Reverse transcriptase (cat no E2610Y Amersham Life Sciences, Arlington Heights, IL Final concentration in the reaction is 2 25 U/reaction

10 Amplitaq polymerase (Perkin Elmer Cetus, Norwalk, CT): Final concentration in the reactions is 2 5 U/reaction

11 Nuclease free water (cat. no E47 Amresco)

12. Tris borate buffer (TBE), pH 8 3

13. Phosphate-buffered saline (PBS), pH 7 5

14 Chloroform.
15 Isopropanol
16 Agarose (NuSieve, FMC BioProducts, Rockland, MA)
17 DNA molecular weight size standards
18. Sample loading buffer (10X): 20% Ficol 400, 0.1 *M* Na_2 ethylenediamine tetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), 0 25% bromphenol blue
19 QIAampHCV kit (QIAGEN, Chatsworth, CA).

3. Methods

Viral RNA is extracted from virus infected cells, culture fluids, or serum by a modification of the procedure described by Chomczynski and Sacchi *(20)* or the QIAGEN QIAmpHCV kit *(21)*. All procedures are performed in DNase and RNase free 1.5 mL presiliconized microcentrifuge tubes.

3.1. Extraction of DEN Virus RNA from Serum or Supernatant from Virus-Infected Cell Cultures with Guanidine Isothiocyanate

1 Add 200 µL of 1X lysis buffer to 200 µL of serum or supernatant fluid from virus-infected cell cells and vortex
2 Add 40 µL of 3 *M* Na acetate, pH 4 2 and vortex
3 Add 400 µL of buffer-saturated phenol, pH 4.5 and vortex
4. Add 80 µL of chloroform and vortex.
5. Chill on ice for 15 min and centrifuge at 4°C for 15 min
6. Remove aqueous phase, place in a clean 1 5-microfuge tube, add an equal volume of isopropanol, mix by inversion several times, and place at –70°C for 15 min
7 Microfuge at 4°C for 15 min, pour off the supernatant and add 200 µL of ice cold 70% ethanol to wash the pellet and centrifuge at 4°C for 15 min. Discard the ethanol, centrifuge briefly, remove the ethanol with a pipet, and air-dry the pellet
8. Resuspend the pellet in 50 µL of sterile RNase-free water. Freeze at –70°C until ready for use

3.2. Extraction of Viral RNA from Plasma, Serum, or Infected Cell Culture Fluids Using the QIAamp HCV Kit

Viral RNA is isolated from plasma or serum using QIAmpHCV Kit according to the handbook published by QIAGEN *(21)*.

1. Pipet 140 µL of serum or cell culture fluid into a 1.5-mL centrifuge tube.
2. Add 560 µL of buffer AVL and mix by vortexing (5 s).
3 Incubate at room temperature for 10 min
4. Add 560 µL of ethanol to the sample and vortex (5 s).

5. Place a QIAmp spin column into a 2.0-mL collection tube
6. Pipet 630-μL of the sample solution (sample/AVL/ ethanol) into the center of the spin column.
7 Centrifuge for 1 min at 6000*g* (8000 rpm in an Eppendorf microfuge).
8. Remove the spin column and place into a clean 2-mL collection tube and discard the tube containing the filtrate.
9. Add the remainder of the sample solution (approx 630 μL) to the QIAmp spin column and centrifuge as previously described; discard the supernatant fluid from the spin column.
10 Add 500 mL of wash buffer AW to the spin column and centrifuge as before; discard the filtrate
11 Repeat the wash with 500 μL of wash buffer AW; centrifuge at 20,000*g* (14,000 rpm in an Eppendorf microfuge).
12. Place the QIAmp spin column in an nuclease free 1.5-mL centrifuge tube and allow 10 min at room temperature or 5 min in a 60°C heating block for the residual ethanol in the column to evaporate.
13 Elute the RNA by pipeting 50 μL of RNase-free water into the QIAmp column followed by centrifugation at 6000*g* (8000 rpm in an Eppendorf microfuge) for 1 min.
14 The aqueous RNA solution should be stored at –20°C or on ice if it is to be used within a 2-h period

3.3. Extraction of RNA from Viral-Infected Cells

1. Virus-infected cells growing as a confluent monolayer in a 150-cm^2 flask are washed with PBS and then scraped and resuspended in 5.0 mL of PBS.
2 200 μL of the cell suspension are added to 200 μL of 1X lysis buffer and the RNA extracted as previously described using the guanidine isothiocynate procedure *(20)*.

4. Reverse Transcriptase-DNA Polymerase Amplification of DEN Virus RNA

Dengue viral RNA is converted to cDNA using the enzyme reverse transcriptase primed with the consensus downstream primer (D2) which is complementary to the genomic RNA of all four DEN serotype viruses (**Table 1**). Subsequent *Tac polymerase* amplification is performed on the resulting cDNA using the consensus upstream primer (D1). A second amplification of the cDNA has been developed using type-specific primers TS1, TS2, TS3, and TS4 (**Table 1**). The position in the cDNA at which priming with these primers occurs is different, therefore, the size of each amplimer is unique facilitating virus typing by gel electrophoresis of the amplimer (**Fig. 1, Table 1**).

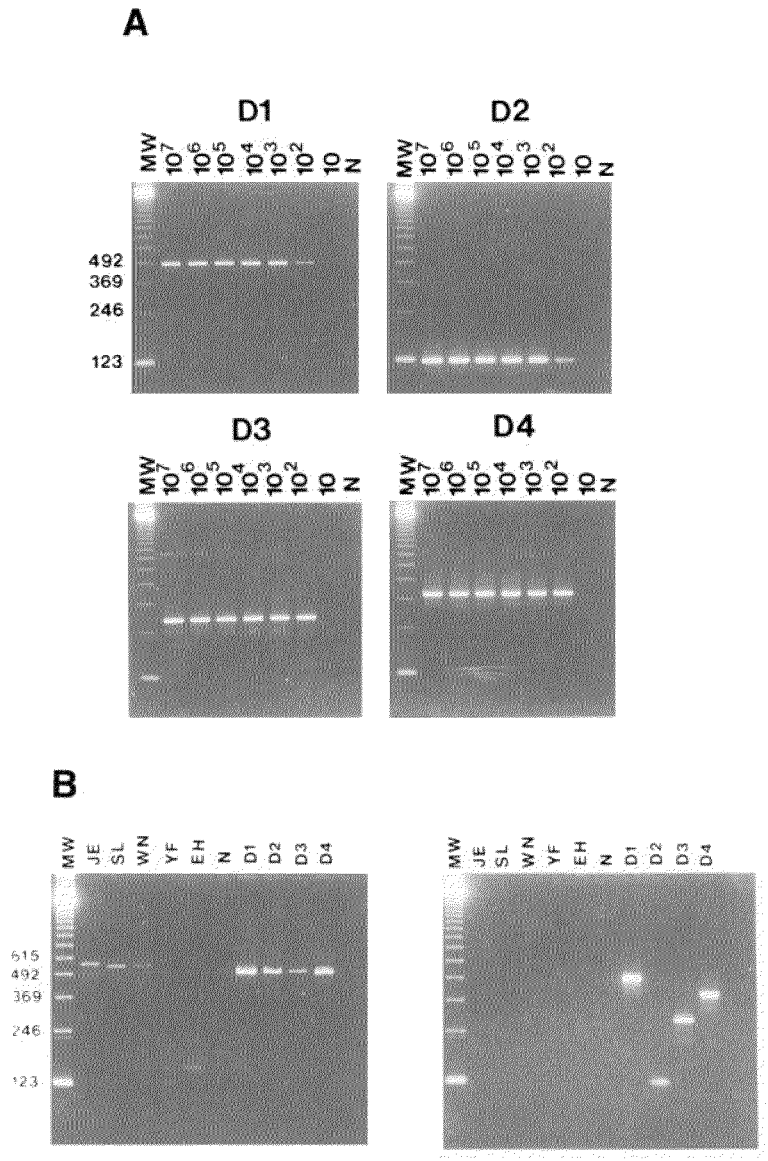

Fig. 1. Agarose gel electrophoresis of DNA products from RT-PCR amplification of RNA samples isolated from DEN viruses and related flaviviruses. **(A)** Amplification with consensus priners D1 and D2. **(B)** After second-round amplification with type-specific primers TS1, TS2, TS3, and TS4. Molecular weight markers are shown on the left; DNA sizes are given in basepairs. Lanes show amplification of RNA from the following viruses: JE, Japanese encephalitis; SL, St. Louis encephalitis; WN, West Nile encephalitis; YF, yellow fever; EH, Edge Hill; N, Western equine encephalitis; D1, DEN type 1; D2, DEN type 2; D3, DEN type 3; D4, DEN type 4. (Used by permission of the American Society for Microbiology.)

4.1. Reverse Transcriptase and DNA Polymerase Amplification of DEN Virus RNA (First Round of Amplification)

Component	Volume/reaction
RNase-free water	75.0 µL
10X PCR buffer	10 .0 µL
dNTP stock (10 m*M*)	2.0 µL
0 1 *M* dithiothreitol (DTT)	5 0 µL
Consensus primer 1 (D1)	1 0 µL
Consensus primer 2 (D2)	1 0 µL
Rnasin (40 U/µL)	0.2 µL
RAV-2 (15 U/µL)	0.12 µL
Taq polymerase (5 U/µL)	0 15 µL

Prepare a reagent "master mix" according to the number of reactions to be tested. Combine the reagents in the above order in a 1.5 ml microfuge tube on ice. Add 95 µL of the master mix to each 0.5 mL PCR reaction tube. Next, add 5 µL of the RNA sample to be tested to each tube and mix thoroughly. If necessary overlay the reaction mixture with 50 µL of mineral oil and place the samples in the thermocycler.

Cycling conditions for first amplification:

1. 1 Cycle. 42°C for 60 min and 96°C for 3 min.
2. 35 Cycles: 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min
3. 1 Cycle: 72°C for 10 min, hold at 4°C.

4.2. Second Amplification with Nested Primers for DEN Serotype-Specific Identification of Virus cDNA

After 35 cycles of PCR amplification, a 4 µL portion of the reaction mixture from the first amplification is removed and diluted to 400 µL with water. Extreme caution is necessary to avoid carryover contamination of the first amplification products at this stage. A 5-µL sample of the diluted first amplification cycle mixture is used as a template for amplication in a second cycle using the D1 consensus primer and type-specific primers TS1 through TS4.

Component	Volume/reaction
Water	78 µL
10X PCR buffer	10 µL
dNTP stock (10 m*M*)	2 µL
Primer D1 (100 µ*M*)	1 µL
Type specific primer TS1 (100 µ*M*)	1 µL

Type specific primer TS2 (100 μ*M*)	1 μL
Type specific primer TS3 (100 μ*M*)	1 μL
Type specific primer TS4 (100 μ*M*)	1 μL
Taq polymerase	0 5 μL

Each amplification reaction contains 95 μL of the PCR "master mix" reaction cocktail plus 5 μL of diluted cDNA from the first amplification cycle.

Cycling conditions for second amplification:

1. 20 Cycles· 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min
2. 1 Cycle: 72°C for 10 min, hold at 4°C

5. Agarose Gel Electrophoresis of PCR Products

Each amplified sample is electrophoresed in a 3% agarose gel in TBE, pH 8.3 containing 0.5 μg/mL of ethidium bromide.

1. Powdered agarose is added to the desired final volume of 1X TBE buffer to give a final concentration of 3% The agarose is dissolved completely by heating. Care should be taken to ensure the solution is homogeneous.
2. The solution is allowed to cool to approx 50–60°C before pouring and ethidium bromide is added to a final concentration of 0.5 μg/mL
3. Pour gel into the mold. The precise nature of this operation will depend on the design of the apparatus The comb is placed in the mold insuring that the bottom of the comb does not touch the plate underneath the gel
4. After setting the gel should look uniformly opalescent, any tape is removed, the gel submerged in the buffer, and the comb gently removed. The apparatus is topped up with buffer until the gel surface is just submerged
5. The capacity of a standard well (2 mm deep × 5 mm wide) is about 20 μL/lane. For analysis, 10 μL of the sample is mixed with 2 μL of dye mix and loaded into the well Electrophoresis is usually done at 100 V, at room temperature until the bromphenol blue dye is approximately half way down the gel.
6. The gel is removed from the tray and examined by UV radiation and DNA band pattern photographed. A typical agarose gel pattern showing DEN 1, 2, 3, and 4 DNA PCR products is shown in **Fig. 1** The unique molecular weights of the DNA PCR products for each DEN serotype is evident and distinguishes the individual viruses (**Table 1**)

6. Notes

1. When working with RNA, it is essential that gloves are worn at all times to prevent RNase contamination of the sample Open and close tubes quickly and avoid touching the inside of the tube or cap Use RNase-free plastic disposable tubes and pipet tips. Use RNase-free water to prepare rea gents and prepare all reagents on ice. Have a dedicated RNA work area that can be used for RNA extraction and RT-PCR set up

2. When setting up the second reaction, prepare the mix and reaction tubes in the clean area. Than take them to another area to make the dilutions and finish the set up. Do not return to the clean area wearing the same gloves, gown, equipment, and so on
3. Contamination with reaction products from previous PCR amplifications can be a major problem. Multiple negative controls, including reagents controls should be included with each experiment. If consistent false positives are experienced, it is preferable to discard all reagents currently in use and prepare new reagents. It is highly recommended that the rooms in which the PCR reactions are assembled, PCR reactions done, and analysis of the PCR products be separate and that their is no exchange of equipment or reagents from one room to another.
4. Ethidium bromide is a known carcinogen which is widely used to stain agarose gels to detect PCR products. Because of the volumes used, this chemical may easily contaminate the laboratory if precautions are not taken. Work areas can be surveyed using a hand-held short wavelength UV light Alternatively, SYBR green dye (Molecular Probes, Eugene, OR) may be used which is less toxic and is used in smaller amounts for post electrophoresis staining. It should be noted that SYBR green requires a different wave length UV fluorescence (254 nm) and a yellow filter for Polaroid photography
5. Oligonucleotide primers are stable at –20°C and have a 4 wk shelf-life at 4°C. When positive controls in the test fail, it is preferable to discard the entire set of primers currently being used in the reactions.
6. High concentrations of extracted nucleic acids in the PCR reaction mixture can inhibit the amplification process. This may result from extraction of tissue-culture cells, tissues, or excessive carrier RNA in the precipitation process. For critical samples, it is advisable to amplify the extracted nucleic acid mixture at concentrations of undiluted and diluted 1:5 and 1·25 to insure proper analysis

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6

Hepatitis Viruses

Girish J. Kotwal

1. Introduction

Acute and chronic liver disease is most commonly caused by hepatitis viruses. Liver diseases resulting from hepatitis viruses share the common characteristic of causing inflammation of the liver. Hepatitis viruses affect a significant population in the world and is a serious public health concern requiring considerable effort to ensure that the blood, water, and food supply remains free of these viruses. The early discovery of hepatitis viruses began in the mid-1960s with the discovery of hepatitis B virus (HBV) and continued in the 1970s with the discovery of hepatitis A (HAV) and D (∂) (HDV) viruses. Two other major causative agents of viral hepatitis were recognized and referred to as non-A, non-B hepatitis viruses (NANBH) for almost two decades. Advances in molecular techniques in the late 1980s, especially the polymerase chain reaction (PCR), have ushered in the discovery of two new viruses, hepatitis C (HCV) and Hepatitis E (HEV) viruses earlier referred to as NANBH. Thus, the most common causes of viral hepatitis are hepatitis A, B, C, D, and E *(1)*. Recently, the discovery of three other hepatitis viruses, two of which are referred to as GB-A and GB-B *(2)* and a third as GB-C, has been reported. All are closely related to the flavivirus family. It remains to be seen how many more will be discovered; there is a general belief at this time that Genelabs, Inc. (Redwood City, CA) has isolated a new hepatitis virus called hepatitis G virus, but there is uncertainty as to the significance of the recently discovered and less prevalent viruses. The focus of this chapter therefore is the more common hepatitis viruses. It needs to be emphasized that there are three other pathogenic viruses, Epstein-Barr virus (EBV), cytomegalovirus (CMV), and enterovirus, that are capable of causing inflammation of the liver, but as they are not primarily hepatotropic, diagnostic protocols for these viruses are discussed

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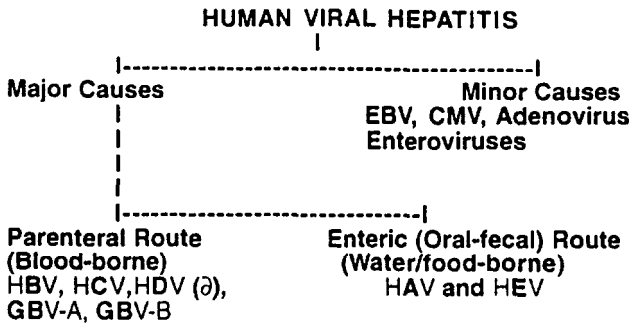


Fig. 1 Schematic of the major and minor causes of viral hepatits and the primary routes of transmission of the major hepatitis viruses

in detail elsewhere in this book. A schematic of the major and minor causes of viral hepatitis and the primary routes of transmission of the major hepatitis viruses is illustrated in **Fig. 1**.

Since the diagnosis of hepatitis virus infection is also a commercial business, I feel compelled to add the following qualifier Several protocols are discussed in this chapter, some of which require the use of commercial kits, and some of which can be performed by purchasing individual items. Every effort has been made to provide the information that was published as well as submitted by companies, and to include all the available options for the diagnosis and monitoring of viral hepatitis infection without endorsing or recommending any particular product. Also, the exclusion of any procedure or product/kit should not be taken as an indication of not being suitable for substitution of the ones that are described here.

1.1. Major Hepatitis Viruses A, B, C, D, and E

Before proceeding to performing the diagnostic protocol, it is imperative to possess a certain basic understanding of the major hepatitis viruses. These viruses are one of the most serious biohazardous occupational risks, and any handling of specimens should be done in places of restricted admittance by persons trained in the proper handling and disposal of biohazardous material. The route of transmission, the period of incubation, the vaccine administered in the person being tested, and the treatment the person is undergoing have an important bearing on the appearance of the molecule being tested in the material being tested Therefore, information summarizing the classification, mode of transmission, period of incubation, preventive intervention, prophylaxis, and treatment of major hepatitis viruses is given in **Table 1**.

Table 1
Characterization of Major Hepatitis Viruses

Virus characteristics	HAV	HBV	HCV	HDV	HEV
Virion size	27 nm	42 mn	30–50 nm	35 nm	27–34 nm
Nucleic acid (genome)	ssRNA	dsDNA	ssRNA	RNA	RNA
Family	Picornaviridae	Hepadnaviridae	Flaviviridae	unclassified	Caliciviridae
Envelope	NoYes (HBsAg)	Yes (E1/E2)	Yes (HBsAg)	No	
Stability	Heat & acid stable	Acid sensitive	Ether sensitive	Acid sensitive	Stability (?)
Clinical and Epidemiologic features					
Incubation period	15–45 d	45–150 d	15–160 d	21–90 d	20–40 d
Onset	Abrupt	Abrupt	Insidious	Insidious	Abrupt
Transmission	Fecal-oral, water, food, parenteral (rare)	Parenteral, sexual, perinatal	Parenteral (common) sexual and perinatal (possible)	Parenteral and sexual (common) perinatal (possible)	Fecal-oral, water, and food
Symptoms					
Jaundice	Children 10% Adults 70–80%	25%	25%	Varies	Varies
Asymptomatic	Most children	Most children Adults 50%	About 75%	Rare	Rare
Presence of virus	Feces, blood, urine	Feces, body fluids	Body fluids	Body fluids	Feces, blood, urine
Severity/fatality	Mild (0 6% fatality)	Severe (1 4%)	Moderate (1–2%)	Often severe (30%)	0 5–3%, 10–20% in pregnant women
Prognosis	Generally good	Worse with age	Moderate	Worse with age	Good
Progression	Complete recovery	Cirrhosis, HCC	Cirrhosis, HCC	Unknown	Complete recovery
Chronic state	None	Adults 6–10% Children 25–50% Infants. 70–90%	50%	10–15%	None
Diagnosis	EIA, RIA of IgM, RT-PCR	HBsAg, anti-HBe/c/s, bDNA, PCR	EIA, RIBA II, bDNA, RT-PCR	RT-PCR, Delta antigen/Ab	EIA IEM, RT-PCR
Immunization	Inactivated, IgG	rHBsAg, HBIG		rHBsAg	None
Therapy		rIFN-α	rIFN–α		

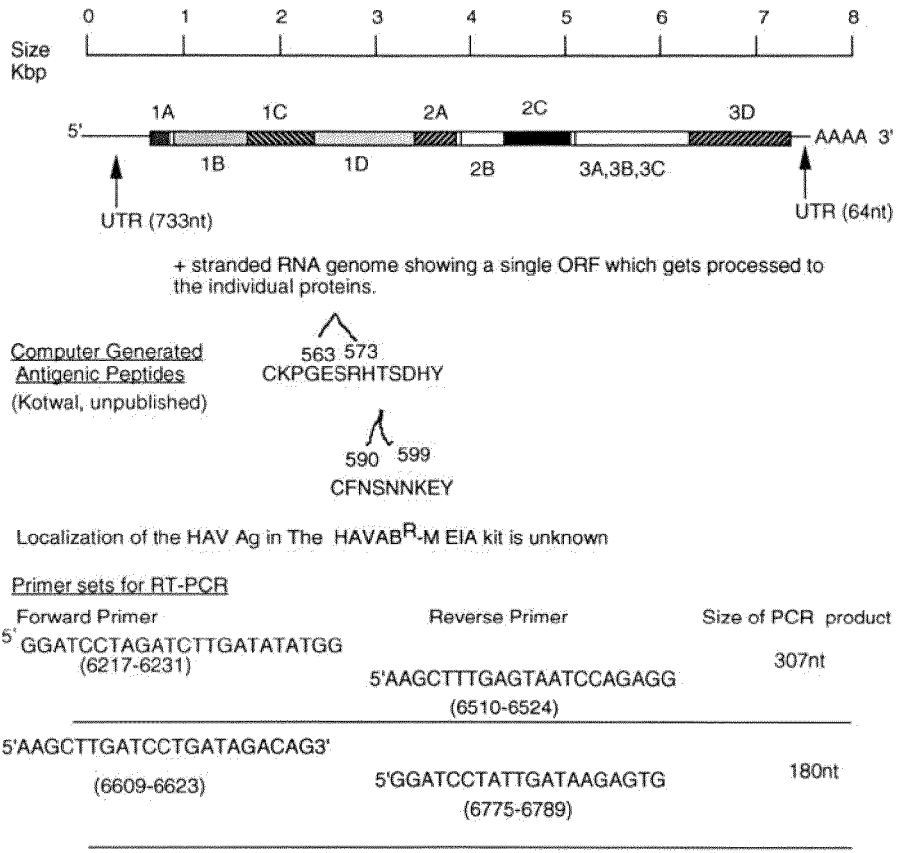


Fig. 2. Hepatitis A virus (HAV).Genome organization, location of individual proteins, antigenic regions, and primers for RT-PCR.

The HAV genomic organization, and primers for reverse transcription-PCR (RT-PCR) ***(3–5)***, and antigens for enzyme-linked immunoabsorbent assay (ELISA) ***(6)*** are shown in **Fig. 2**. HAV-specific IgM antibodies are present in serum by the time symptoms of acute hepatitis appear ***(6)***. In cases in which symptoms appear a few days prior to the appearance of IgM, a second sample should be drawn after 1–2 wk. HAV-RNA appears as early as 2 d postinfection ***(7)*** and the amount of RNA as an indication of viremia is directly proportional to the IgM titers. Serum IgG is indicative of exposure and persists for long periods. Kits to assay the presence of antibodies against HAV can be obtained from Abbott Laboratories (North Chicago, IL). Both kits use inactivated virus as the antigen, making it difficult to prepare antigens. The sequence of the most antigenic region of the HAV capsid, derived by computer analysis using the MacVector

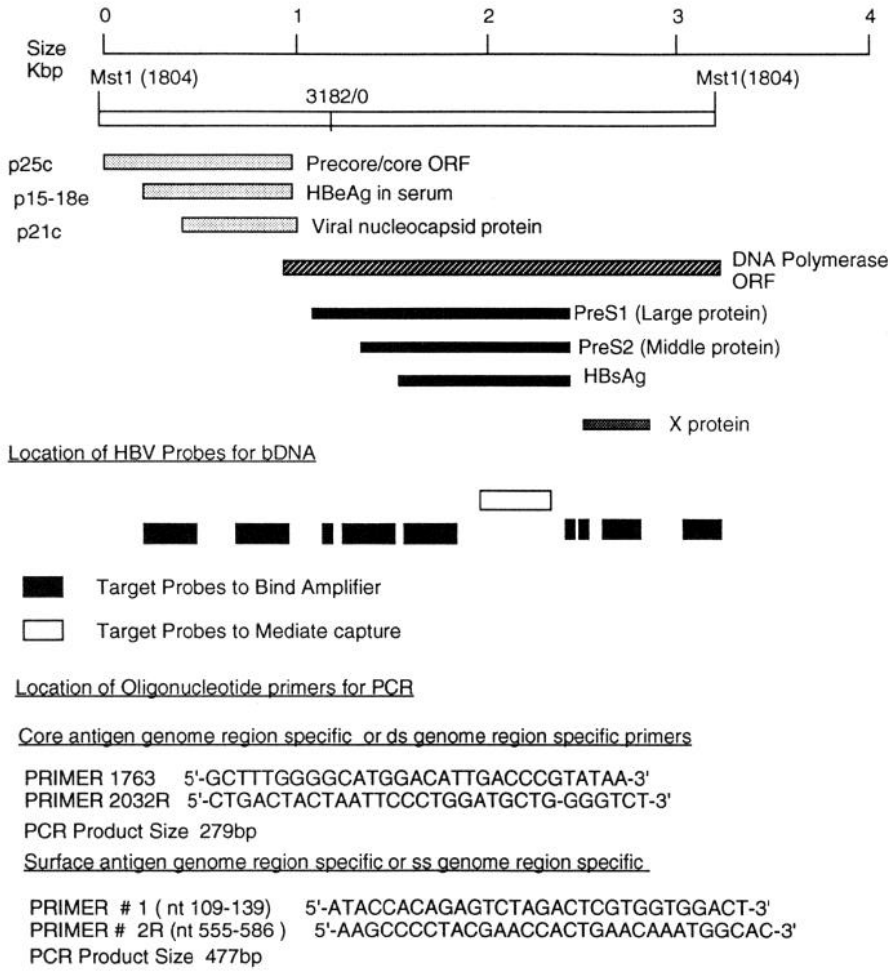


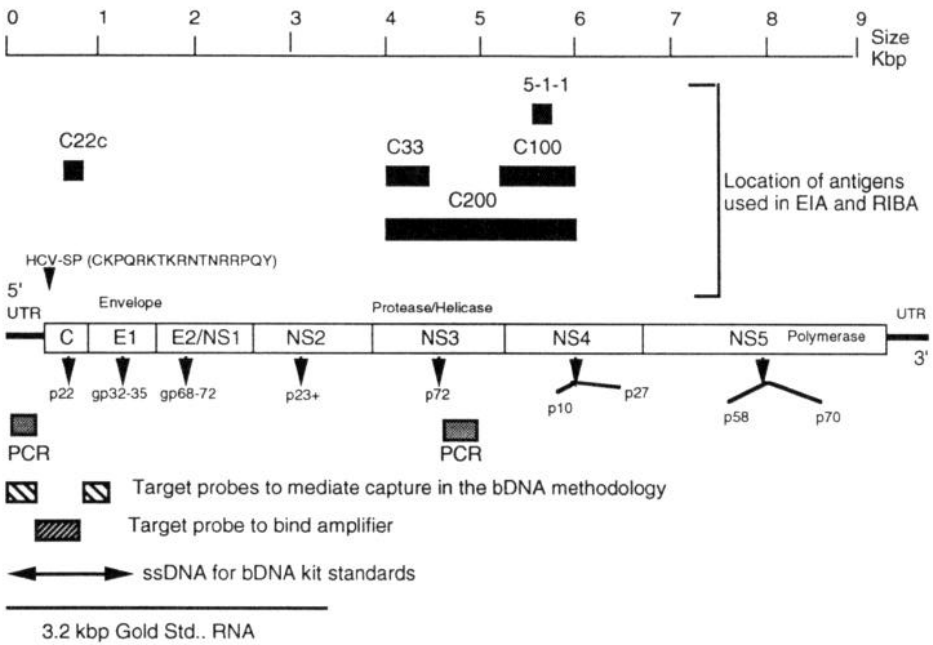
Fig. 3. Hepatitis B virus (HBV). Genome organization, location of individual proteins, probes for bDNA, and primers for PCR.

program (Oxford Molecular Group, Campbell, CA), is provided for those willing to attempt to set up their own ELISA. Use of RT-PCR can allow the detection of RNA isolated from stools and can confirm diagnosis. Methods for quantitation of HAV by PCR have been described recently ***(4)***.

The genomic organization of HBV and the primers used for detection of the HBV-DNA in serum or plasma are shown in **Fig. 3**. Also shown are the open reading frames for the different proteins of HBV. The presence of HBV surface antigen (HBsAg) and "e" antigen (HBeAg) is indicative of active viral

replication during the acute phase in most patients and it usually suggests severe liver damage. An accurate and sensitive reflection of viral replication in the liver is the presence of HBV-DNA in serum *(8)*. Absence of HBV-DNA in serum, but presence in liver tissue could be an indication of integration in the genome HBV-DNA is present in serum in 50% of patients with fulminant atypical HCV infection. The presence of antibodies to HBVSAg is indicative of recovery and immunity or a successful immune response to vaccination High IgM antibody titers against the core protein are indicative of active infection, while low titers are indicative of chronic persistent infection. The response to interferon treatment results in lowering of serum HBV-DNA, and it is highly recommended that those patients undergoing interferon treatment be monitored by a quantitative HBV-DNA procedure to determine the response.

The genomic organization of HCV and the primers used for detection of the HCV-RNA in serum or plasma are shown in **Fig. 4**. Also shown are the open reading frames for the different proteins of HCV and the antigenic regions used to detect the antibody. Characterization of nucleotide sequences of HCV isolates from around the globe indicate a sequence conservation within the 5'-noncoding region and in and around the region encoding the putative RNA-binding nucleocapsid protein *(9–15)*. These conserved regions have resulted in the development of new diagnostic assays for detecting HCV-specific antibodies *(17–19)* and for the detection of HCV-RNA by RT-PCR *(20,21)*. The new assays have overcome the high false-positive rate and have greatly enhanced the chances of detecting HCV antibodies within the acute phase of infection *(22,23)*. Additionally, direct detection of viral presence has become possible by setting up an ELISA to detect HCV-specific antigens *(24,25)*. Most of the current assays measure only the IgG class, although one assay measures both IgG and IgM *(17)*. This is achieved by the application of a mixture of the two secondary antibodies. Recent studies have shown that a transient IgM response can be detected concurrent with or earlier than IgG *(26)*. A four-antigen recombinant immunoblot assay (RIBA) serves as a confirmatory test. The use of RT-PCR in the diagnosis of HCV infection or its confirmation is becoming common. The detection of HBV-DNA and HCV-RNA in the same sample has now become possible for those labs interested in minimizing costs and procedures *(27)*. The need for quantitative estimation of RNA levels to monitor patients' response to treatment either with interferon or other agents has resulted in the introduction of two new commercial assays, the branched DNA procedure *(28)* and the quantitative PCR method *(29)*. In addition, the ribonuclease protection assay can be set up from available individual reagents *(30)*. Genotyping of the HCV causing hepatitis, may become increasingly essential to perform, because of the predictive value of the genotype of the HCV found in patients and the severity of infection as well as the



Sequence of Oligonucleotide primers for PCR

Primers derived from the conserved 5'-untranslated region (UTR) of the HCV genome

Antisense (-) primer 5'-TCG CAA GCG CCC TAT CAG GCA G-3'
Sense (+) primer (-314 to -291) 5'-GGC GAC ACT CCA CCA TAG ATC-3'
PCR product 292bp
Internal (+) probe (-290 to -269) 5'-GGA ACT ACT GTC TTC ACG CAG A-3'

Primers derived from the NS3 region of the HCV genome

Antisense (-) primer 5'-GACATGCATGTCATGATGAT-3'
Sense (+) primer 5'-GGCTATACCGGCGACTTCGA-3'
PCR product 623bp
Internal (+) probe 5'- GGCTGTGCTTGGTATGAGCTCACGCCCGC-3'

Fig. 4. Hepatitis C virus (HCV). Genome organization, location of individual proteins, probes for bDNA, and primers for PCR.

probability of favorable response to treatment with a given antiviral agent. There are to date 14 recognized genotypes, 6 of which are major genetic groups, based on the sequence analysis of the core gene from several isolates ***(31)***.

HDV is a defective virus requiring HBsAg to form infectious virion particles and cause hepatitis ***(32)***. Therefore, it can only cause severe hepatitis if it

is acquired as a coinfection with HBV or as a superinfection in persons with chronic HBV. The genome of HDV is a single-stranded RNA that forms a structure similar to plant viroids owing to several base pairs that span the length of the RNA *(32)*. The antigenomic strand encodes a single antigenic nucleocapsid protein referred to as the HDV-specific antigen (HDAg), which functions in RNA packaging and replication. The HDAg consists of 2 forms, HDAg-L, a 27 kDa large form (215 amino acids), and HDAg-S, a 24 kDa small form (195 amino acids) *(32)*. The use of an immunoblot to ascertain the presence of HDAg is a very reliable and sensitive assay for active viral infection *(33)*. The IgG antibodies to the HDAg can be determined by ELISA, but the appearance of the IgG antibodies normally occurs after the loss of HDAg. Presence of the IgM antibodies to the HDAg may have to be determined by ELISA, since they appear prior to the IgG and may overlap with the period when HDAg is still present. The detection of HDV-RNA by RT-PCR greatly facilitates the sensitive detection of the genomic RNA *(34)*. The antisense primer for such an RT-PCR would be AD2 (5'-ATGAGCCGGTCCGAGTCGAGGAAG-3') and the sense primer would be SD1 (5' TCACTGGGGTCGACAACTCTGGGG-AGA-3'). An internal primer D3 (5' CGAACGGACCAGATGGAGGTAGAC-TCCGGACCTAGGAAGAG-3') can be end-labeled and used as a probe to confirm the PCR product.

HEV genome organization and location of the antigenic regions *(35)* are shown in **Fig. 5**. The HEV-RNA can be isolated from stools and with the primers described above for HDV-RNA, an RT-PCR can be performed to determine the presence of the virus during the acute phase of the disease *(36)*. The antibody response to the virus that is elicited by the patients can be measured using an ELISA *(37)* or western blot *(38)*. It should be noted that by the time the antibodies appear, the patient will have recovered from the acute phase and therefore attempts must be made to perform the RT-PCR. If RT-PCR is unavailable, the testing for the antibody must be repeated within 2 wk if the earlier sample was negative. Because of the high mortality rate (20–30%) resulting from HEV acquired during pregnancy, it is critical that RT-PCR be performed as early as possible after the first symptoms appear or if exposure is suspected.

The protocols for RNA isolation are based on the assumption that at the molecular level, RNAs from different viruses have similar properties to one another and that one isolation procedure can result in the extraction of RNA of similar purity from any virus. With respect to RT-PCR, although the conditions differ (but not significantly), optimization of conditions should always be done. Optimal conditions for each of the four RNA viruses are provided, but they should be standardized by the individual using the procedure.

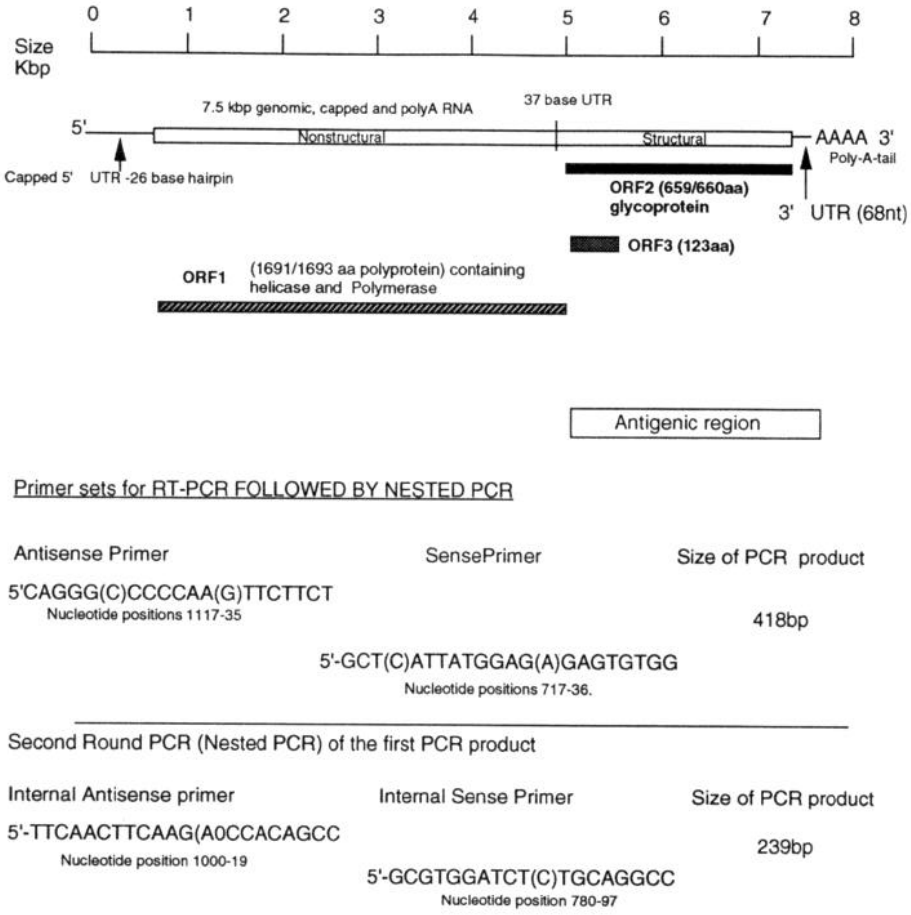


Fig. 5. Genome orgaization, location of individual proteins, antigenic regions, and primers for RT-PCR.

1.2. Collection, Storage, and Processing of Specimen

The success and reproducibility of the protocols described is heavily dependent on the quality of the specimen, which in turn is dependent on the collection procedure, processing of the specimen and its storage.

The most commonly used specimen for diagnostic virology is serum. Most procedures for detection of antibody are performed using serum. In addition, the Quantiplex bDNA assay (Chiros Corp. Emeryville, CA) is performed using serum. Serum is the cell-free and fibrin-free portion of the blood and is obtained by allowing blood to clot. Plasma is the cell-free portion of blood obtained by centrifuging containing anticoagulant blood and

siphoning off the supernatant. It has fibrin in it and is the preferred specimen for PCR and RT-PCR.

1.2.1. Serum Collection

Blood is drawn into a test tube without any anticoagulant, and the blood is allowed to clot at room temperature for no more than 1 h and either processed immediately as described or placed at 4°C or in wet ice for 2–4 h. Longer periods of waiting may cause degradation of the RNA. The serum is removed and placed in a sterile capped microfuge tube and the tube is spun at 12,000*g* for 5 min at 4°C The serum is then removed without disturbing the pellet and aliquoted as desired; the aliquots are then placed in an ultralow temperature freezer at –80°C or lower. Repeated freezing and thawing may affect the quality of the specimen and should be avoided Any transportation of serum samples should be done on dry ice. Serum that is to be used exclusively for antibody detection in an ELISA can be treated with 0.5% Nonidet P-40 (NP40) to give a final concentration of 0.1% NP40 and centrifuged at 10,000*g* for 5 min. The clear liquid between the pellet and the cloudy lipid layer on top is carefully removed and used in protocols for detection of antibody.

1.2.2. Plasma Collection

Blood is drawn into a test tube containing ethylenediaminetetraacetic acid (EDTA), mixed by inverting the tube 3–4 times, and placed immediately in a refrigerator at 4°C or on wet ice. Within 1–2 h, the blood is transferred to a centrifuge tube and centrifuged at 2000*g* for 10 min. The supernatant, or plasma, is carefully removed using a sterile plastic pipet, and transferred to several tubes, and stored at ultralow temperature (–80°C). **Caution:** Blood that has been frozen prior to separation cannot be used for obtaining plasma or serum, since the blood cells lyse, with only one cycle of freezing and thawing, and release their contents, such as, nucleases and proteases, which will affect the outcome of the experiment.

1.2.3. Water Collection

Since contaminated water is the most likely cause of transmission of HAV and HEV, it may be necessary from time to time to test the drinking water supply for the presence of these agents. In all likelihood even though the water used may be a source of the viral spread, it is likely to contain only a few virus particles in a fairly large amount of water. Therefore, the first step is to concentrate the water at least 100-fold before proceeding with any of the procedures described here. Concentration can be easily achieved by using ultrafilters such as the Amicon (Beverly, MA) stirred cell filters with 100,000 cutoff membranes under 70 psi N_2 in a cold room adjusted to 4°C. An ideal volume of

preclarified water (achieved by centrifugation at 3000*g* for 10 min) to start with would be 500 mL and concentrated down to 5 mL. The concentrate can then be divided into aliquot and stored at –90°C.

1.2.4. Stool/Feces

One gram of stool sample is vigorously suspended (if the stool is hard this may require a homogenizer) in 10 mL of phosphate-buffered saline (PBS) or physiological saline in a sterile conical polypropylene or polystyrene tube and centrifuged at 3000*g* for 15 min at 4°C. The supernatant is carefully removed and passed through a bacteria-proof membrane (Millipore, Bedford, MA) filter (0.45 μm pore size), and the filtrate is concentrated about 25-fold using a centricon-10 filter (Amicon). The retentate is then stored at –90°C or processed for RNA extraction as described below (*see* **Subheading 3.1.1.**).

1.2.5. Liver

Liver removed during transplantation is cut into strips about one-half-inch wide, snap-frozen directly in liquid nitrogen, and stored at –70°C or below. Liver biopsy specimens are similarly frozen and stored.

1.2.6. Other Body Fluids

Body fluids other than plasma and serum may require testing. Such fluids should be placed in empty sterile tubes and quickly frozen and stored at ultra-low temperatures (–90°C).

1.2.7. Perpheral Blood Lymphocytes (PBLs)

Five milliliters of whole blood containing an anticoagulent agent such as, heparin or EDTA, stored at room temperature for no longer than 2 h is carefully overlayed on top of an equal volume of sterile Histopaque 1077 (Sigma, St. Louis, MO) in a sterile centrifuge tube and the tube is spun at 3000*g* for 10 min. This results in all the RBCs settling at the bottom and the plasma sitting on the Histopaque, with the lymphocytes at the interface. Carefully remove the plasma and collect the PBLs; transfer the PBLs to another tube, wash them in physiological saline, and store the pellet at –90°C.

2. Materials

2.1. Isolation of DNA or RNA

1. Trizol LS Reagent (Life Technologies Inc , Gaithersburg, MD) for isolation of nucleic acid from liquid samples.
2. Trizol Reagent (Life Technologies) for isolation of nucleic acid from cells and tissues.
3. Phenol:chloroform: isoamyl alcohol (25:24·1, v/v, Life Technologies)

4 Molecular biology grade (DNase- and RNase-free grade) isopropanol (Sigma)
5 Ethanol.
6. Molecular biology grade sterile distilled water (conductivity 18Ω; Sigma)
7. Cloned ribonuclease inhibitor; 10,000 U/mL (Life Technologies), or RNasin (40,000 U/mL; Promega Corp , Madison, WI)

2.2. Reagents for PCR

1 Recombinant *Taq* DNA polymerase (Life Technologies), includes 10X PCR buffer (200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl).
2 50 m*M* $MgCl_2$.
3 Either 10 m*M* dNTP solution mix (Life Technologies, Pharmacia, Uppsala, Sweden, or Boehringer Mannheim, Indianopolis, IN), or GeneAmp PCR reagent kit (Perkin-Elmer/Cetus, Norwalk, CT) containing AmpliTaq DNA polymerase, all 4 nucleotide solutions (10 m*M* each), 10X reaction buffer, and control template and primers These reagents should be stored at –20°C, preferably in a Labtop cooler (Nalgene), and should be transported for short periods in the cooler.
4 Molecular biology grade mineral oil (protease-, DNase-, and RNase-free; from Sigma) Not required if using the Thermal cycler 9600, or any other cycler in which the top of the tube is also heated.
5 Oligonucleotide primers can be synthesized in-house using standard procedures or custom synthesized and purchased from any of the companies, e.g., Midland Certified Reagent Company, Midland, TX, or Bio-synthesis, Inc., Lewisville, TX Generally, the crude-column-purified grade is adequate for PCR and RT-PCR.

2.3. Materials for RT-PCR (from Body Fluids/Tissues)

1. Superscript II RNase H-reverse transcriptase (Life Technologies), supplied with 5X first-strand buffer (250 m*M* Tris-HCl, pH 8 3, 375 m*M* KCl, 15 m*M* $MgCl_2$), and 100 m*M* dithiothreitol (DTT).
2. All reagents listed under PCR or GeneAmp complete RNA PCR kit (Perkin-Elmer/Cetus)

2.4. In Situ RT-PCR

1. Chambers for thermal cycling (Gene Cone from Gene Tec Corporation, Durham, NC).
2. Slidetemp adaptor kit (Gene Tec Corporation).
3 All reagents required for RT-PCR
4. 11-dUTP digoxigenin.

2.5. Detection of [^{32}P]-Labeled PCR Products on Southern Blots

1. 3MM paper from Whatman or medium thickness paper grade GB002 (Schleicher & Schuell, Keene, NH).
2. Gel blot paper, GB004 (Schleicher & Schuell)
3 0 2 μm Nitrocellulose pore size (Schleicher & Scheull).

4. 20X SSC: Dissolve 175.3 g NaCl and 88.2 g sodium citrate in 800 mL of distilled water and adjust the pH to 7.0 with 10 *N* NaOH; adjust the total volume to 1 L.
5. 50X Denhardt's solution (dissolve 1 g Ficoll, 1 g polyvinylpyrrolidone, and 1 g bovine serum albumin (BSA) in 100 mL distilled water and filter sterilize the solution).
6. 10% (w/v) Sodium dodecyl sulfate (SDS) stock solution.
7. Yeast tRNA solution (50 mg/mL) or denatured salmon sperm DNA (50 mg/mL) (Sigma)
8. [γ^{32}P] ATP, specific activity >3,000 Ci/mmol (Amersham, Arlington Heights, IL)
9. T4 Polynucleotide kinase and 5X polynucleotide kinase buffer (Gibco-BRL, Gaithersburg, MD).

2.6. Nonradioisotopic Detection of PCR Products on Southern Blots

1. 20X SSC and 10% SDS (same as in **Subheading 2.5.**)
2. Buffer 1: 0.15 *M* NaCl (8.77 g/L), 0.1 *M* Tris base (12.1 g/L), adjust to pH 7 5 with concentrated HCl and make up to 1 L.
3. Buffer 2: 0 4 *M* NaCl (23.4 g/L), 0 1 *M* Tris base (12 1 g/L), adjust to pH 7 5 with concentrated HCl and make up to 1 L.
4. 3MM paper from Whatan or medium thickness paper grade GB002 (Schleicher & Schuell)
5. Gel blot paper, GB004 (Schleicher & Schuell).
6. Hybond-N+, positively charged nylon membrane (Amersham), or maximum strength Nytran nylon transfer membrane (Schleicher & Schuell)
7. Enhanced chemiluminescence (ECL) 3'-oligolabeling and detection systems (Amersham), or containing **items 8–15**
8. 50 µL Flourescein-11-dUTP.
9. 100 µL Terminal transferase, 2 U/mL in a buffer solution, pH 7.0.
10. 100 µL Cacodylate buffer, 10X concentrated buffer containing sodium cacaodylate, pH 7 2.
11. Sterile deionized distiller water, RNase- and DNase-free (Sigma).
12. Blocking agent (for use in hybridization buffer and membrane blocking solution).
13. Antifluorescein horseradish peroxidase (HRP) conjugate.
14. Hybridization buffer component
15. Detection reagents 1 and 2 (to be mixed 1:1 prior to use) provided in the detection system (**item 7**)

3. Methods

3.1. Isolation of Nucleic Acids

3.1.1. Isolation of RNA from Body Fluids (e.g. Plasma/Serum)

1. Add 750 µL Trizol LS reagent to 250 µL plasma, serum, or filtered and concentrated extract from stools/feces in a sterile 1.5-mL microfuge tube and mix at room temperature for 5 min.

2 To this mixture add 200 µL phenol chloroform isoamyl alcohol (25·24·1, v/v) is added and mixed.
3 Centrifuge at 12,000*g* for 15 min at 4°C
4 Remove the colorless aqueous phase at the top and place in a new tube with 500 µL of isopropanol
5. Centrifuge at 4°C for 10 min.
6 Gently remove the isopropanol and immerse the pellet in 1 mL 75% ethanol; centrifuge at 12,000*g* for 2 min
7 Gently remove the supernatant and replace with 500 µL 100% ethanol and centrifuge.
8 Discard the supernatant and air dry the pellet by placing the inverted tube at an angle on a thin pencil or a 1-mL pipet. The drying procedure can also be performed in a Speedvac (Savant Instruments, Hicksville, NY) but if not carefully done can result in the loss of the pellet or its contamination with nucleases
9 Suspend the dry pellet in 6 µL sterile RNase- and DNase-free distilled water (18 MΩ) containing RNasin at a concentration of 1 U/10 µL.

3.1.2. Isolation of RNA Using the Qiagen Kit

This procedure eliminates the use of phenol and chloroform but requires the purchase of kits from Qiagen.

1. Mix 100 µL of plasma or serum with a buffer containing guanidium isothiocyanate.
2 Adjust the lysate to optimal binding conditions, and apply it to the spin column provided with the kit by two quick spins in a benchtop microcentrifuge The RNA binds to the special silica-gel-based membrane and contaminants that remain are separated from the adsorbed RNA by two short spin washes.
3. Elute the purified RNA in water, precipitated with ethanol, and dissolve in distilled water containing RNasin

3.1.3. Isolation of DNA from Body Fluids

1 Add to 750 µL of Trizol LS reagent to 250 µL plasma in a sterile 1.5-mL microfuge tube and mix at room temperature for 5 min. To this mixture add 200 µL phenol· chloroform isoamyl alcohol mixture is added and cap the tube securely
2 Vigorously shake the tube by inverting it several times for 15 s, incubate at room temperature for 5–10 min
3. Place the tube in a microfuge and centrifuge at 12,000*g* for 15 min at 4°C. The mixture will separate into a colorless upper phase, which contains the RNA; the interphase, which contains the protein and some DNA, and a red-colored lower phase, which contains the DNA. Carefully remove the aqueous phase overlaying the interphase, and add 0.3 mL 100% ethanol to the interphase and the lower phase; and mix by inversion
4 Leave the samples at room temperature for 2–3 min, then centrifuge at 2000*g* for 5 min at 4°C. Remove the phenol-ethanol supernatant and discard it.

5 Wash the DNA pellet twice with 1 mL of a solution containing 0 1 *M* sodium citrate in 10% ethanol, leaving the washing solution for 30 min at room temperature (with periodic mixing); centrifuge at 2000*g* for 5 min at 4°C
6 After the two washes, suspend the pellet in 1 5 mL 75% ethanol and store for 10–20 min at room temperature (with occasional mixing); centrifuge at 2000*g* for 5 min at 4°C.
7 Gently remove the supernatant and dry the pellet for a brief period by inverting the tube at a very slight angle; dissolve the pellet in about 100 µL of 8 m*M* NaOH by gently pipeting the solution up and down.
8. Add an adequate amount of 8 m*M* NaOH (typically, 0 3–0 6 mL per 50–70 mg of tissue or 1×10^7 cells) to obtain a DNA concentration of 0 2–0 3 µg/mL. If at this stage the preparation contains insoluble gel-like material, centrifuge at 12,000*g* for 10 min and transfer the supernatant to a new tube.

3.1.4. Isolation of RNA from Liver Tissue

1 Homogenize 100 mg of liver tissue, using a microtissue homogenizer (Fisher Scientific Co., Pittsburgh, PA) with a Teflon pestle, in 750 µL of a lysis-buffer consisting of 20 m*M* Tris-HCl (pH 8.0), 20 m*M* EDTA (pH 8 0), 2% SDS, 1 mg/mL proteinase K (Life Technologies or Boehringer Mannheim).
2. Transfer the homogenate to a microfuge tube and incubate at 60°C overnight.
3 Centrifuge at and treat about 250 µL of the resulting supernatant as described under isolation of RNA from body fluids.

3.1.5. Isolation of DNA from Liver Tissue

1. For every 50–100 mg of fresh-frozen liver tissue, add 0 75 mL of Trizol reagent and make a suspension with either a glass-Teflon or power homogenizer (Polytron or Tekmar's tissuemizer). If homogenizers are not available process tissue as described for RNA from liver tissue and then proceed as described under isolation of DNA from body fluids.
2. To extract nucleic acids (DNA or RNA) from formalin-fixed, paraffin-embedded (FFPE) liver tissue, trim the excess paraffin with a single-edged razor blade and extract the remaining paraffin twice with 1 mL xylene for 5 min at 55°C Wash the tissue twice with 100% ethanol and once with 50% ethanol to remove xylene, as described for fresh frozen tissue

3.2. RT-PCR from Isolated RNA (Optimized for HCV-RNA)

1. Prepare a master mix sufficient for several different RNA samples *(n)* for the reverse transcription step as follows:
   a $(n + 1) \times 3$ 5 mL sterile deionized distiller water
   b. $(n + 1) \times 2$ 0 mL 5X buffer (250 m*M* Tris-HCl, pH 8 3, 375 m*M* KCl, 15 m*M* $MgCl_2$ (Gibco-BRL).
   c. $(n + 1) \times 1.0$ mL 0.1 M DTT (Gibco-BRL)
   d. $(n + 1) \times 1.0$ mL dNTP mix (10 m*M* each dATP, dTTP, dCTP, and dGTP, Gibco-BRL)
   e. $(n + 1) \times 0$ 25 mL RNasin (10,000 U/mL [Gibco-BRL])

f. $(n + 1) \times 0.2$ mL (40 U) of Superscript II (Gibco-BRL)
g. $(n + 1) \times 0.1$ mL of the antisense oligonucleotide primer (5–6 mg/mL)

It is assumed here that $n = 10$ or more. If the number of samples is less than 10, then dilute the primers, superscript, and the RNasin 1/10 prior to adding to the master mix and adjust the water volume accordingly.

2. Gently mix the master mix and add 8 µL directly to a PCR tube containing 2 µL RNA. Incubate the tube at 43°C for 1 h, either in a water bath or in the thermal cycler. After 1 h quickly centrifuge the tube and place it in ice
3. To this tube, add 40 µL of a PCR master mix prepared as follows.
   a. $(n + 1) \times 34$ µL of sterile, deionized distilled water
   b. $(n + 1) \times 4$ µL 10X PCR Buffer (200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl [Gibco-BRL])
   c. $(n + 1) \times 2$ µL 50 m*M* $MgCl_2$.
   d. $(n + 1) \times 0.1$ µL of the sense oligonucleotide primer (5–6 mg/mL).
   e. $(n + 1) \times 0.25$ µL *Taq DNA polymerase* (5 U/µL [Gibco-BRL]).

   Mix the contents and overlay with 50 µL mineral oil
4. Place the tubes in a thermal cycler and perform 35 cycles of PCR amplification as follows:
   a. Denature at 94°C for 1 min.
   b. Anneal at 50°C for 1 min.
   c. Extend at 72°C for 1 min.

   Perform 3 autoextentions for 30 s and maintain the reaction at 4°C after completion of the cycles
5. Analyze the amplification products by mixing 16 µL PCR mix (carefully removed from under the mineral oil) and 4 µL loading buffer (0.05% w/v, bromophenol blue, 40% sucrose, 0.1 *M* EDTA, pH 8.0, and 0.5% SDS [Sigma]); load the mixture on a 2% agarose gel with *Hae*III digested phiX DNA as marker. Soak the gel in two vol of 0.5 µg/mL ethidium bromide. Place the gel on a UV light box and photograph. The amplification product should be a sharp band of the expected size (*see* **Note 5**)

3.2.1. PCR from HBV-DNA

1. Prepare the PCR master mix as follows.
   a. $(n + 1) \times 32$ µL sterile, deionized distilled water
   b. $(n + 1) \times 5$ µL 10X PCR Buffer (200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl [Gibco-BRL]).
   c. $(n + 1) \times 1.5$ µL 50 m*M* $MgCl_2$
   d. $(n + 1) \times 5$ µL of 1 m*M* each primer pair.
   e. $(n + 1) \times 1$ µL of 10 m*M* dNTP mix (10 m*M* each of dATP, dTTP, dCTP, and dGTP [Gibco-BRL]) to give a final concentration of 200 m*M* each of the four dNTPs
   f. $(n + 1) \times 0.5$ µL *Taq* DNA polymerase (5 U/µL [GibcoBRL]). Add 45 µL PCR master mix to the sample DNA in a PCR tube in 5 µL of water. Overlay the final reaction mix with 50 µL mineral oil.

3. Place the tubes in a thermal cycler and perform 35 cycles of PCR amplifications as described in **Subheading 3.2.** with one exception: the annealing is done at 60°C for 1 min. At the end of 35 cycles, place the tubes at 4°C and analyze the product as described for RT-PCR.

3.3. Confirmation of PCR Signal

3.3.1. Nested PCR

Nested PCR is the second round of PCR, using the amplification product of the first round as the template and oligonucleotides from internal or inner regions as primers.

1. Transfer a fraction of the first round of PCR product (generally 1 µL) to the second round 50-µL reaction mix prepared so that the final concentration is 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 U recombinant *Taq* DNA polymerase, 200 m*M* of each dNTP, and about 125 ng of each inner primer. Overlay with mineral oil if using the earlier versions of the thermal cycler
2. Perform 25 cycles of PCR amplification as in **Subheading 3.2.**, followed by a 3-s autoextention at 72°C
3. Mix 16 µL of the PCR product with 4 µL of the gel-loading buffer (.25% w/v bromophenol blue, 0.25% w/v xylene cyanole FF, 40% w/v sucrose in water), and analyze by electrophoresis on a 2% agarose gel. Visualize bands by ethidium bromide staining and photograph. Alternatively gel loading solution can be purchased from Sigma (St. Louis, MO, cat. no. G-7654)

3.3.2. Southern Blotting

1. Transfer the gel to a glass baking dish and denature the DNA by soaking the gel in several vol of 1 5 *M* NaCl and 0.5 *M* NaOH for 1 h at room temperature with constant shaking. (The actual transfer could be performed in the same gel box that was used for separating the DNA bands on the gel.)
2. Neutralize the gel by soaking in several vol of a solution of 1 *M* Tris-HCl, pH 8.0, and 1.5 *M* NaCl for 1 h at room temperature with constant shaking.
3. Fill the cathode and the anode sides of the gel box to about half with 10X SSC solution.
4. Cut a 3MM paper with the width equal to that of the gel and about 2 in longer on either side and place on the tray. Fold the long sides equally on both sides so that both ends remain immersed. Wet the entire filter with the 6X SSC and remove all the bubbles between the tray and the filter. Place the gel in an inverted position on the filter paper with the open end of the wells facing downwards and the edges of the gel aligning with those of the tray. Cut the nitrocellulose paper to the size of the gel, float on 2X SSC in a glass dish for a few seconds and then immerse for 2 min Place it on the inverted gel and cover with two 3MM papers immersed in 2X SSC Remove air bubbles.
5. Place a stack of the blotting paper on the two 3MM filter papers, cover the whole setup with Saran wrap or equivalent, and weigh it with a heavy item so that the

weight is distributed evenly over the blotting paper Allow the transfer of DNA to proceed for 12–24 h

6 Remove the wet filters and the 3MM paper above the gel. Mark the positions of the gel slots on the nitrocellulose with a pencil. Soak the nitrocellulose in 6X SSC at room temperature for 5 min

7 Allow the nitrocellulose to dry at room temperature by laying it over a 3MM filter paper Place the dried nitrocellulose between sheets of 3MM paper Bake the nitrocellulose for 2 h at 80°C under vacuum in a vacuum oven If the nitrocellulose is not to be immediately processed, store at room temperature under vacuum between the sheets of the 3MM paper.

8. Immerse the baked nitrocellulose in 6X SSC solution for 2 min

9 Slide the baked filter into a glass tube, add about 25 mL of the pre-hybridization solution (5 mL distilled water, 15 mL 10X SSC, 2 5 mL 50X Denhardt's solution, 2.5 mL 10% SDS, and 50 µL tRNA at 50 mg/mL) Close the tube tightly and incubate in a rotisserie-type hybridization oven at 68°C for 2–4 h. Eliminate any bubble between the nitrocellulose and the side of the tube Add 100 µL [^{32}P]-radiolabeled probe and 0 5*M* EDTA to the prehybridization solution and let the tube incubate at 40°C for 18–24 h The labeled probe is prepared as follows Mix 10 µL 5X buffer, 30 µL [γ^{32}P] ATP, 10 µL oligonucleotide probe (0 1 mg/mL), and 1 µL T4 polynucleotide kinase in a 0 5-mL microtube, and incubate at 37°C for 30 min At the end of 30 min transfer the contents to an ultrafilter with a cutoff mol wt of 1000 containing 1 mL distilled water Centrifuge the ultrafilter for 1 h at 5000*g* Resuspend the concentrate in 1 mL water and concentrate as before by centrifugation The concentrated solution free of the unincorporated label is the probe

10 After the hybridization is complete, discard the liquid from the glass tube into the radioactive liquid waste container or in a sink designated for radioactive waste disposal. Wash the nitrocellulose in a glass dish containing 2X SSC and 0 5% SDS at room temperature for 5 min

11 Discard the radioactive wash solution as before and replace with a solution of 2X SSC and 0 1% SDS, wash at room temperature with shaking for 15 min Replace the solution with 0 1X SSC and 0 5% SDS and incubate in the oven at 40°C for 2 h Discard the final wash and wrap the wet filter in Saran wrap. Expose the filter to an X-ray film for sufficient time to see a signal at –80°C and develop in a film developer

3.3. Nonisotopic Detection of Signal

Follow **steps 1–3** in **Subheading 3.3.2.**, and continue with **step 4**

4. Cut a 3MM paper with the width equal to that of the gel and about 2 in longer on either side and place on the tray Fold the long sides equally on both sides so that both ends remain immersed. Wet the entire filter with the 6X SSC and remove all the bubbles between the tray and the filter. Place the gel in an inverted position on the filter paper with the open end of the wells facing downwards and the edges of the gel aligning with those of the tray. Cut the nylon membrane to the size of

the gel, float on 2X SSC in a glass dish for a few seconds and then immerse for a couple of min Place it on the inverted gel and cover with two 3MM papers immersed in 2X SSC Remove air bubbles.

5. Place a stack of the blotting paper on the two 3MM filter papers, cover the whole setup with Saran wrap or equivalent, and weigh it with a heavy item so that the weight is distributed evenly over the blotting paper Allow the transfer of DNA to proceed for 12–24 h
6. Remove the wet filters and the 3MM paper above the gel Mark the positions of the gel slots on the nylon membrane with a pencil Soak the nylon membrane in 6X SSC at room temperature for 5 min
7. Allow the nylon membrane to dry at room temperature by laying it over a 3MM filter paper. Place the dried nylon membrane between sheets of 3MM paper Bake the nylon membrane for 2 h at 80°C under vacuum in a vacuum oven
8. Perform the 3'-end labeling of the oligonucleotide as follows
   Mix 100 pmol oligonucleotide, 10 µL fluorescein-dUTP, 1 6 µL cacodylate, and 16 µL terminal transferase in a 0.5-mL tube and bring the total vol up to 160 µL Incubate the tube for 60 min at 37°C The labeled probe may be stored at –20°C in a nonfrost-free freezer.
9. Float the filter in 6X SSC for 2 min and prehybridize the blot by placing it in a glass tube, adding to the tube about 50 mL of prehybridization solution (5X SSC, 0.1% (w/v) hybridization buffer component, 0.02% (w/v SDS, and 0 5% blocking agent). Alternatively, the prehybridization solution from **Subheading 3.3.2.** may be used Prehybridize in a rotisserie oven at 42°C at least 30 min, but this may go overnight
10. Hybridize by adding 10 ng/mL probe to the prehybridization solution in the tube, place in the rotisserie oven at 42°C for a minimum of 1–2 h.
11. At the end of the hybridization period remove the solution and replace it with 5X SSC and 0 1% SDS. Wash the blot twice at room temperature for 5 min, agitating on a belly dancer shaker. Perform a stringency wash at 42°C twice for 15 min each time with 0.1–1X SSC and 0.1% SDS with agitation Rinse the blot in buffer 1 for 1 min and then incubate in the block buffer for 30 min Rinse in buffer 1 again, and place in a solution containing the antifluorescein antibody at a 1 1000 dilution in 0.5% BSA in buffer 2 and incubate for 30 min. Wash the blot four times in buffer 2 for 5 min each time. Detection of the signal is performed by incubating the blot in the solution (0.125 mL/cm_2) containing equal vol of the two detection reagents for 1 min. Drain the blot against the sides of the tray or tube and cover with Saran wrap Immediately expose to X-ray film Generally the amount of time required to see a signal is <1 h

3.4. Quantitation of Nucleic Acids

3.4.1. Branched DNA/RNA Assay

The following brief protocol was prepared from information provided by Chiron Corp. (Emeryville, CA). It requires the use of Quantiplex HBV-DNA

assay (bDNA) kit for determining the direct quantitation of HBV load in serum or HCV-RNA (bDNA) kit for determining the direct quantitation of HCV load in serum. Chiron also requires any new user to take a course on the assay's use and a detailed protocol is provided with the kit. This assay is highly specific, and reliable, and requires only about 10 μL of the serum specimen.

1. To perform the HBV-DNA assay, mix 10 μL specimen with 10 μL extraction buffer for 30 min at 63°C To this mixture add 10 μL denaturation buffer and HBV probes and mix once more at 63°C for 30 min
2. Add 10 μL of neutralization buffer to the mixture and place in wells
3. Incubate at 63°C for 16–18 h and wash the plate
4. Add 50 μL amplifier (bDNA) to the washed wells and incubate at 53°C for 30 min, then wash.
5. Add 50 μL labeled probe at 53°C for 15 min and wash.
6. Add 50 μL chemiluminescent substrate to the mixture and incubate at 37°C for 25 min. Measure the light emission and determine the HBV-DNA equivalents/mL in the specimens using a standard curve.

3.4.2 Colorimetric Assay

The following protocol was prepared using information provided by Roche Diagnostic System, Inc. (Branchburg, NJ) and it requires the use of Amplicor HCV Monitor kit. Roche also requires any new user to take a course on use of the assay. It is currently suggested to be used only as a research tool for monitoring treatment. The principle is as follows: RNA is prepared using standard procedures described above, but is suspended in a buffer containing manganese, necessary in the PCR procedure. The RNA is then placed in a combined reverse transcription-PCR reaction, referred to as the tRT-PCR reaction, that uses a single set of primers, a single enzyme (DNA polymerase *Thermus thermophilus* rTth1 instead of reverse transcriptase and *Taq* polymerase), and one set of optimized buffer conditions. A 244-p amplification product termed the amplicon is obtained. The amplicon is denatured and transferred to a special microwell detection plate coated with a unique probe sequence specific for capturing the amplicon but not overlapping with the primers. Horseradish peroxidase conjugated to avidin binds to the amplicon when added, and the color is produced when the substrate tetra methylbenzidine (TMH) is added. The color produced is measured at 450 nm and is proportional to the amount of DNA.

3.5. Serological Assay for Detection of Antibody

Serological assays vary mainly with respect to the antigen used; once the antigen is bound to a 96-well plate (ELISA) or beads or nitrocellulose (RIBA), the subsequent procedure is more or less identical. Two different substrate

solutions can be used depending on whether the secondary antibody is coupled to horseradish peroxidase or phosphatase. If the secondary antibody is conjugated to peroxidase, the substrate solution of H_2O_2 as the substrate and 1,2 phenylenediamine dehydrochloride as the color-forming compound is used, and the yellow color product is measured at 450/490 nm.

3.5.1. ELISA for Hepatitis A Virus

Prior to discussing a specific ELISA/EIA, the various commercially available plates coated with different antigens for the antibody to the different hepatitis viruses is discussed.

Two enzyme immunoassay kits are available from the Abbott Laboratories Diagnostic Division (Chicago, IL) with a detailed protocol. A sandwich assay specific for IgM antibody (indicative of the acute phase) to HAV allows its qualitative detection in human serum or plasma as follows. Beads coated with goat antihuman antibody specific for human IgM (μ-chain) are incubated with either the controls or diluted specimens at 40°C ± 1°C for about 1 h. The IgM from the sample binds to the antibody on the beads. All unbound material is washed. HAV antigen is mixed with the beads and it binds to any anti-HAV IgM present on the beads. Unbound HAV Ag is washed away and Human anti-HAV conjugated with horseradish peroxidase is mixed and incubated with the beads; it will bind to the HAV Ag present on the beads. Unbound conjugate is washed and the beads are incubated with the substrate solution. The color development procedure is described in **Subheading 3.5.3.** The absorbance is directly proportional to the amount of anti-HAV IgM present in the specimen

In a second assay (competition), the qualitative and semi-quantitative detection of total antibody to HAV is achieved. This assay is mainly performed to determine previous exposure to HAV for assessing immune status or for epidemiologic studies. Beads coated with formaldehyde inactivated HAV are mixed with the controls or the specimens and anti-HAV antibody conjugated with horseradish peroxidase. The anti-HAV antibody in the sample competes with the limited number of HAV binding sites present on the beads. All unbound material is washed away and the beads are incubated with the substrate solution as described in **Subheading 3.5.3** in detail. The intensity of the color that develops is inversely proportional to the amount of anti-HAV in the sample.

3.5.2. ELISA for Hepatitis B Virus

Abbott Diagnostics markets ELISA kits accompanied by detailed protocols (essentially the same as those for HAV described in **Section 3.5.1.** or HCV described in **Subheading 3.5.3.** for the following markers: HBsAg, HBeAg, anti-HBc (total antibody to hepatitis B core antigen), anti-HBc IgM (IgM antibody to hepatitis B core antigen), anti-HBs (antibody to hepatitis B surface

antigen). In addition, Organon Technikon (Chicago, IL) also has an ELISA kit for anti-HBs.

3.5.3. HCV-SP ELISA for Detection of HCV-Specific Antibodies

A polypeptide HCV-SP is synthesized. Each well of 96-well ELISA plates is coated with 100 ng HCV-SP by adding 100 µL of a 1 µg/µL stock solution of the peptide followed by washing with 200 µL of PBS:0.1% Tween. Serum from patients or healthy donors is pretreated with 0.5% NP40 to a final concentration of 0.1% NP40 and diluted 1:5 in PBS:Tween and 100 µL diluted serum sample is placed in a well and the plate is covered and incubated on a shaker for at least 4 h at room temperature or 1 h at 37°C. At the end of the incubation the plate is washed at least 5 times with 200 µL of PBS:Tween. A mixture of rabbit antihuman IgG and IgM conjugated to horseradish peroxidase diluted 1:500 is added to the wells and the plate is incubated at room temperature for 1–4 h. At the end of the incubation the antibody solution is discarded and the plate washed at least five times. Absorbance of the orange color formed on addition of 200 µL of soluble peroxidase substrate solution—prepared fresh by dissolving two tablets from Sigma referred to as Sigmafast OPD tablets (one is *o*-phenylenediamine dehydrochloride [OPD] and the other is urea H_2O_2) to make 20 µL of buffered substrate—is read on a microwell plate reader at 492 nm in about 15 min

3.5 4. Antibody to Hepatitis D Virus

This can be measured by an anti-hepatitis δ ELISA kit from Abbott. The protocol is essentially similar to the ones described above

4. Notes

1 Handling of RNA requires extreme care. Always wear gloves when holding samples and reagents. Avoid freezing and thawing of samples and reagents. Never let individual reagents reach room temperature Ensure that the reagents are used by only one or two persons. If there are multiple users, aliquot the reagents. The tubes used for the reactions and any pipet tips should be autoclaved prior to each use. If changes are to be made in the source of a reagent, make the change one at a time and perform a run with the old and new reagent side by side in a given reaction prior to switching to the new reagent

2 PCR and RT-PCR are susceptible to contamination from aerosol resulting in false-positive reactions and it is therefore essential that these procedures be performed in a separate room or an isolated corner designated solely for this purpose After each use the pipetman should be placed in a 50 mL conical tube containing about 5 mL of 1% SDS and 1 m*M* EDTA solution Each time the pipetman is removed from the solution, shake off all the dripping liquid otherwise it will enter the reaction mixture

3. Since there are multiple additions for the PCR and RT-PCR reactions it is very important to make a note (tick mark) each time a reagent is added, so that none of the reagents are missed or added more than once.
4. The enzymes such as Superscript and *Taq* are generally in glycerol solutions, making their solutions very viscous Always make sure that these enzymes are added last and the reaction is mixed by pipeting up and down with the microtip
5. Run several internal and external controls. An internal control would be previously tested known positive and negative RNA plasma samples processed at the same time as the batch of samples and in exactly the same fashion Thus, a problem in the isolation will be detected Also, run a known positive and negative RNA, previously isolated and found to give the required outcome. These will also serve as internal controls of the RT-PCR procedure. In addition to these internal controls run an external control along with the amplified product on the gel This control would be a known RT-PCR positive product of the correct size and a negative control showing no product from a previous amplification of a sample negative for HCV. This will serve both as a size marker as well as an indication of whether the set of reactions from the current lot are comparable to the previous lot In addition to the samples and internal controls, always run a sample including all the reagents, except the RNA or DNA. This should not give rise to any product, but if it does it will mean there is some contamination of the reagents or oligonucleotides with the nucleic acid being tested In this case, it is imperative to determine which reagent is contaminated by the process of elimination and discard that reagent Alternatively, all reagents can be replaced with new ones

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7

Herpesviruses

Mark J. Espy, P. Shawn Mitchell, David H. Persing, and Thomas F. Smith

1. Introduction

1.1. General

Eight viruses are included in the herpesvirus group: herpes simplex virus (HSV) (types 1 and 2), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesviruses 6 (HHV-6), 7 (HHV-7), and 8 (HHV-8). All of the herpesvirus have icosahedral symmetry, are surrounded by a lipid-containing envelope derived from the host cell membrane, and have a double strand of linear DNA ranging from 125 kb (VZV) to 230 kb (CMV). After primary infection, herpesvirus DNA becomes latent in an episomal form in specific target cells; reactivation and active virus infection generally occur with immune deficit or suppression of the host *(1)*. Infections with herpesviruses generally occur early in life. Apparent clinical manifestations in children are recognized with dermal lesions of HSV (stomatitis), VZV (chickenpox), HHV-6, and perhaps HHV-7 (roseola). Reactivated CMV and EBV infections in immunocompromised patients are recognized most often as causes—pneumonia, hepatitis, gastroenteritis, encephalitis, and lymphoproliferative disorders (EBV) in organ transplant recipients and in individuals with AIDS. HHV-8 has been recently detected in affected skin tissue (Kaposi's sarcoma) and from body cavity-based lymphomas from AIDS patients with human immunodeficiency virus (HIV) infection *(1)*.

Laboratory diagnosis of HSV, VZV, and CMV infections is currently obtained by immunologic detection of early antigens of these viruses by the shell vial assay with results available within 16 h postinoculation. EBV does not replicate in cell cultures used in the routine diagnostic laboratory, but serologic tests are helpful for the recognition of acute infections. Nevertheless, current

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cell cultures and serologic methods are inadequate for the diagnosis of central nervous system- and lymphoproliferative diseases caused by these herpesviruses. For example, of 311 viruses recovered from cerebrospinal fluid (CSF) samples over the last 14 yr at the Mayo Clinic, 302 (97%) were enteroviruses. Herpesvirus (HSV, 6; CMV, 2) and an adenovirus isolate were the only other viruses detected from this source *(2)*.

Of the eight herpesviruses, protocols are provided herein for the PCR detection of HSV, CMV, EBV, and VZV, which have been recognized to cause most disease in current clinical practice.

1.2. Herpes Simplex Virus

Herpes simplex virus is estimated to cause 10–20% of acute focal CNS infections; the disease has a mortality rate of nearly 70% without early institution of specific therapy. Over the years, HSV has been the single most common virus recovered in our laboratory. Many isolates are recovered from dermal and genital sites of immunocompromised patients. Collectively, the herpesviruses (HSV, CMV, VZV) comprise between 80 and 90% of the total viruses recovered in cell cultures; however, more than one-half of these strains are HSV isolates *(2)*.

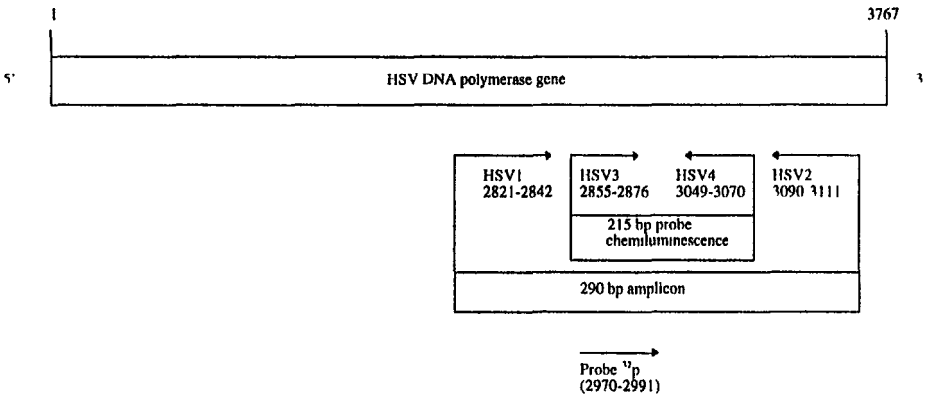
In 1990, Rowley et al. *(3)* were the first to demonstrate nucleic acid sequences of HSV by PCR in the CSF of four patients with culture or histologic evidence of encephalitis caused by this virus. Subsequently, several reports have documented the sensitivity and specificity of PCR for collected HSV sequence initially in stored, but more recently, with prospectively collected specimens *(4–8)*.

The gene target and primer sequence information for detection of HSV DNA is given in **Fig. 1**.

1.3. Cytomegalovirus

Cytomegalovirus has been identified in 20–40% of AIDS patients based on histologic evidence in autopsy brain tissue *(9)*. Specific neurologic syndromes associated with CMV infection include subacute radiculomyelopathy, peripheral neuropathy, and encephalitis. However, because HIV can also infect the CNS, specific involvement by CMV cannot be easily determined by clinical features alone *(10)*.

Several methods have been developed for the sensitive and rapid detection of CMV infections in the laboratory. For example, immunologic demonstration of CMV from urine, blood, and tissue is routinely achieved within 24 h after inoculation of shell vial cell cultures. However, although evidence of disseminated CMV infection in anatomic areas outside the CNS is readily

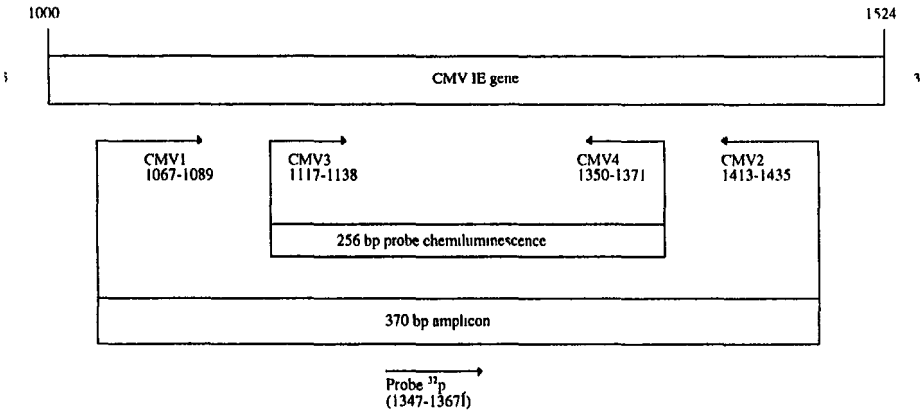


Function	Name	Nucleotide sequence, 5' to 3'
291 bp amplicon	HSV1	TAC ATC GGC GTC ATC TGC GGG G
	HSV2	CAG TTC GGC GGT GAG GAC AAA G
Internal probe sequence 215 bp amplicon	HSV3	TCA AGG GCG TGG ATC TGG TGC G
	HSV4	GGT CGG TGA TGC GCC GAT GGG C
Internal probe sequence ^{32}p	HSV5	AGC CGA GCG CCC CGC GGA GGA G

Fig 1 Map of the HSV-DNA polymerase gene and schematic diagram of the products of PCR detection of HSV.

achieved in the laboratory, recovery of this virus in cell cultures from CSF specimens from patients with neurologic involvement is uncommon *(11)*. Nevertheless, early recognition of CMV involvement of CNS disease in AIDS patients may be useful for implementation of therapy specific for this viral infection *(12)*.

PCR primers to genomic regions coding for immediate early and late products of CMV were used to identify the viral DNA in CSF of AIDS patients with CNS disease. In three separate studies, CMV-specific DNA in CSF was correlated with clinical disease and pathologic findings; in contrast, PCR results were uniformly negative in AIDS patients with (22 patients) or without (34 patients) extra-CNS infection with CMV *(11,13,14)*. For example, Cinque et al. *(13)* demonstrated positive PCR results for CMV in 15 of 19 CSF specimens from AIDS patients with CNS disease, but not in 15 patients (in this study population) who had CMV infection without apparent CNS involvement. In contrast, the PCR test was negative both in 10 AIDS patients without any active evidence of CMV infection and in 10 CMV-seropositive individuals without HIV infection *(13)*. The specificity of this test was also documented by negative PCR results for CMV with CSF specimens from patients with other documented neurologic disease (toxoplasmosis, *6*, *Cryptococcus*, 4; progres-



Function	Name	Nucleotide sequence, 5' to 3'
370 bp amplicon	CMV1	GGG TGC TGT CCT GCT ATG TCT TA
	CMV2	CAT CAC TCT GCT CAC TTT CTT CC
Internal probe sequence	CMV3	CGG CCT CTG ATA ACC AAG CCT G
256 bp amplicon Chemiluminescence	CMV4	AGT CGC GGG TAC AGG GGA CTC T
Internal probe sequence 32p	CMV5	CCA GAG TCC CCT GTA CCC GC

Fig 2. Map of the CMV immediate-early (IE) gene, and schematic diagram of the products of PCR detection of CMV

sive multifocal leukoencephalopathy, 3; lymphoma. 2, tuberculosis, syphilis, HSV encephalitis, discopathy, and medullar ischemia, 1 each) *(13)*.

The PCR test was evaluated for the laboratory diagnosis of congenital CMV infection. Using primer sets designed to amplify DNA coding for the pp65 and viral capsid antigen, CMV infection was detected in 6 of 10 infants with symptomatic infection *(15)*. The PCR test was negative with CSF from 100 control patients with alternative clinical diagnosis.

The gene target and primer sequence information for detection of CMV DNA is given in **Fig. 2**.

1.4. Epstein-Barr Virus

Epstein-Barr virus was the first herpesvirus in which the linear double-strand (172 kb) genome of DNA was completely cloned and sequenced *(16)*. Infection with EBV occurs early in life; by 10 yr, 70–90% of children have been infected. Usually infection in children is asymptomatic or mild and may be associated with minor upper respiratory tract infection, although EBV is the causative agent of infectious mononucleosis, Burkitt's lymphoma, and in southern China, nasopharyngeal carcinoma. In recent years, EBV infection has been associated with lymphoproliferative disorders, non-Hodgkin B-cell and CNS lymphoma, and rare T-cell lymphomas. Encephalitis and hepatitis are recog-

nized as a sequelae of EBV infection, particularly in immunosuppressed populations such as, AIDS and organ-transplant patients *(17–20)*.

Routine laboratory diagnosis of EBV infections is by hematologic (atypical lymphocytes, lymphocytosis) and serologic testing (rapid tests for heterophile antibodies and immunofluorescence/enzyme immunoassays for detection of antibodies to EBV antigens); growth of the virus has been obtained only in research settings using susceptible cord blood cells cultured in vitro. Alternatively, the detection of EBV-DNA in tissues and CSF specimens is a practical approach to serology and culture diagnostic methods for patients with long-term complications of this viral infection *(17,18)*.

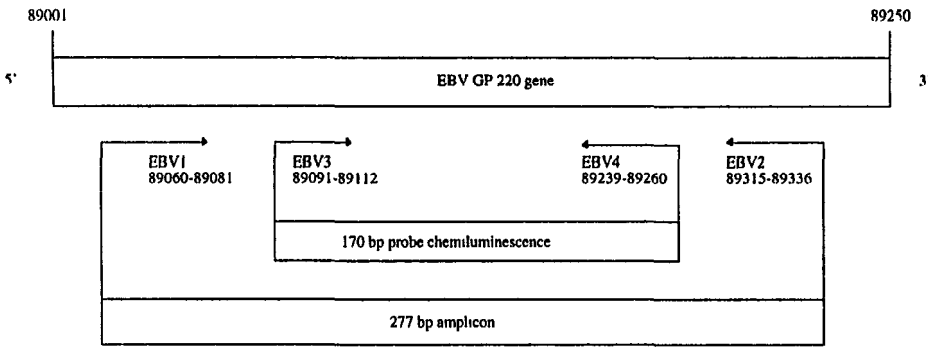
To evaluate PCR for laboratory diagnosis of complicated EBV infections, we used oligonucleotide primers directed to a conserved region of the genome encoding the capsid protein gp 220 (BamHI region) to detect target DNA in tissue specimens from transplant and AIDS patients with lymphoproliferative disorders *(21)*. More recently, EBV-DNA in CSF was evaluated for use as a tumor marker for CNS lymphomas induced by the virus. Of 16 patients with histologically proven EBV-associated lymphomas (*in situ* DNA hybridization), all had EBV-DNA sequences found in CSF samples. In contrast, EBV-DNA was found in CSF from only 1 of 68 HIV-infected patients without histologically detectable lymphoma at necropsy *(22)*.

The gene target and primer sequence information for detection of EBV DNA is given in **Fig. 3**.

1.5. Varicella-Zoster Virus

Varicella-zoster virus, like all members of the *Herpesviridae* family, is a DNA virus of 125 kb surrounded by a lipid envelope. The genome has been completely sequenced *(23)*. VZV infection causes chickenpox (varicella) as a primary infection and shingles (zoster) when reactivated. Both clinical manifestations of VZV infection are generally mild in the immunologically normal host. Conversely, VZV infection can cause severe disseminated and even fatal infections in the patient immunocompromised because of neoplastic disease, organ transplantation, or HIV infection *(24)*. Primary infection is much more severe in adults, and complications such as encephalitis and disseminated fatal disease occur but are uncommon. VZV-associated neurological disorders include meningitis/encephalitis, cerebellar ataxia meningitis, cerebral infarction, and facial palsy.

Vesicular fluid from dermal lesions inoculated into shell vial cell cultures account for almost all VZV strains detected in the laboratory. However, the virus is rarely detected in CSF specimens. Although herpes zoster is a common early manifestation in HIV infection, the diagnosis of encephalitis complications of this virus in this population is generally recognized postmortem. Alter-



Function	Name	Nucleotide sequence, 5' to 3'
277 bp amplicon	EBV1	TGT GCC TCG TCT ACC TCT GTT C
	EBV2	TTG ATT CTC GTG GTC GTG TTC C
Internal probe sequence	EBV3	CAG TGC CTC CGC CTG AGC CGC T
170 bp amplicon Chemiluminescence	EBV4	GGT CAG ATT TTG CAA TAT ATT T
Internal probe sequence 32p	EBV5	CGG GCC GTT TGG CGT CTC AG

Fig 3. Map of the EBV GP220 gene and schematic diagram of the products of PCR detection of EBV.

natively, detection of VZV-DNA in the CSF allows early recognition of CNS involvement of this infection and treatment with acyclovir or ganciclovir.

PCR was initially used in 1991 to detect VZV-DNA in the CSF of patients with neurological complications (postchickenpox cerebellitis and herpes zoster patients with neurological symptoms) of infection with this virus *(25)*. These findings were confirmed by others in more comprehensive studies of patients with acute aseptic meningitis *(26)*.

Our laboratory had detected VZV-DNA from 5 of 38 (13%) CSF specimens and from 14 of 49 (29%) dermal, eye, and liver specimens Conversely, CSF from 20 patients without infectious disease and liver tissue from 20 immunocompromised and 10 immunologically normal individuals were negative.

The gene target and primer sequences information for detection of VZV-DNA is given in **Fig. 4**.

2. Materials

1. IsoQuick Nucleic Acid Extraction Kit (ORCA Research, Inc , Bothell, WA).
2. 20 mg/mL Glycogen.
3. PCR master mix. 200 μM each deoxyribonucleotide triphosphate, 10X buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 2.5 mg/mL bovine

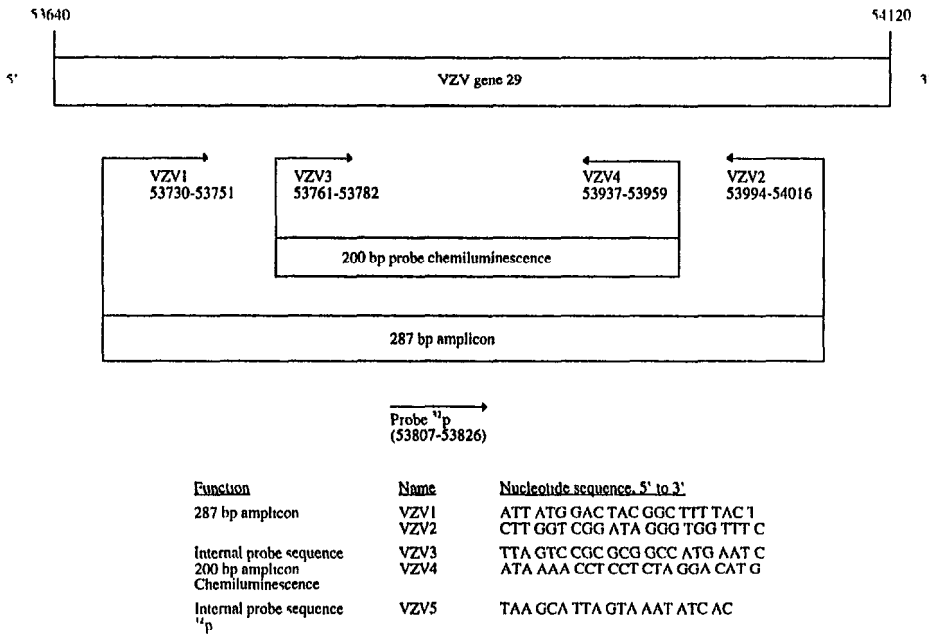


Fig. 4 Map and primer sequence of gene 29 and schematic diagrams of the products of PCR detection of VZV

serum albumin [BSA]; final concentration, 1X), 100 pmol of each PCR primer, 25 µg/mL of isopsoralen (IP-10; HRI Associates, Concord, CA), 10% glycerol, and 1 2 U of *Taq* polymerase (Perkin-Elmer, Norwalk, CT) per 50-µL reaction (*see* **Notes 1, 4,** and **5**)

4 TE buffer 10 m*M* Tris-HCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA) (pH 8.0)

5 Wash buffer 0 1X SSC, 0 1% sodium dodecyl sulfate (SDS) (20X SSC is 3 0 *M* NaCl plus 0 3 *M* sodium citrate)

6 Programmable thermal cycler

7. HRI-300 UV photochemical reaction chamber for post-PCR sterilization

8. Agarose: Nusieve GTG and SeaKem GTG (FMC, Rockland, ME).

9 Ethidium bromide

10. Gel loading buffer (dye with bromphenol blue).

11. Horizontal electrophoresis unit and power supply.

12. UV transilluminator.

13. Polaroid photography equipment.

14. Platform rotator

15 Denaturation solution· 0.5 *M* NaOH, 1.5 *M* NaCl.

16. Neutralization solution. 0.5 *M* NaCl, 0.5 *M* Tris-HCl (pH 7.5).

17 Nylon membrane (NYTRAN, Schleicher and Schuell, Keene, NH)

18 UV crosslinker
19. Water baths
20. Hybridization oven.
21. ECL direct labeling and detection kit (Amersham, Arlington Heights, IL).
22 X-ray film, cassette, and developer

3. Methods

3.1. Sample Collection

1 For CSF, collect by lumbar puncture; the volume received by the laboratory will vary (10 µL–2 0 µL) CSF may be stored at 4°C for up to 2 wk prior to analysis. The extraction technique will determine minimum volume of CSF required.
2 For tissue, fresh or fixed specimens are acceptable. Paraffin-embedded tissue should be cut and placed on glass slides so that the collective thickness of the sections total approx 70-µm thickness.

3.2. Sample Preparation

3 2.1. CSF

Several techniques have been evaluated for extracting nucleic acid from CSF for HSV-DNA detection. Adding CSF directly to the PCR master mix will usually permit detection of HSV-DNA when present in high concentrations. Boiling CSF before adding to the PCR master mix will disrupt the cells and virus particles in the CSF specimen, releasing DNA. In our laboratory, we utilize the chaotropic properties of the IsoQuick Nucleic Acid Extraction Kit to lyse the CSF sample and concentrate the HSV nucleic acid. In addition to the extraction techniques above, microcentrifuge spin columns have been developed that allow centrifugation of a sample through a column allowing the nucleic acid to adhere to a membrane filter. After washing the membrane several times, the nucleic acid is eluted off for analysis.

Extract nucleic acid according to the manufacturer's instructions (IsoQuick, ORCA Research. Inc.), with one modification: the addition of 20 µg of glycogen to each sample as a carrier during isopropanol precipitation.

3.2.2. Tissue

3.2.2.1 Fixed (Paraffin-Embedded)

1 Remove paraffin from tissue section by placing the slides in two containers of xylene for 5 min each.
2. Place the slide in absolute ethanol for 5 min, dry the slide in room air.
3 Moisten the slide with water and scrape the tissue sections into 500 µL of TE buffer Add 25 µL of 10% SDS and 50 µL proteinase K, shake the sample (125 rpm) 16 h at 55°C.
4 Extract the tissue twice with an equal volume phenol chloroform and once with an equal volume chloroform isoamyl alcohol. Discard organic phase. Add 1/10

volume salt (potassium acetate), 2 µL glycogen, and 2 volumes of absolute ethanol to the aqueous phase. Incubate the sample for 10 min at –20°C.

5 Centrifuge the extract at maximum speed in a microcentrifuge for 30 min Decant the alcohol, and add one volume 70% ethanol. Centrifuge the suspension at maximum speed for 5 min

6 Aspirate the 70% alcohol and resuspend the pellet in 60 µL TE buffer

3.2.2.2. Fresh or Frozen (Not Fixed)

Process specimen from **Subheading 3.2.2.1., steps 4–6.**

3.3. PCR Procedure (Perform in a PCR Clean Room)

3.3.1. Sample Addition and Amplification of Product

1 Prepare PCR master mix according to **Subheading 2.3.** and add 45 µL to each reaction tube (*see* **Note 2**) Add 50 µL to the "no-target control" (blank control) reaction tube

2 Add two drops of sterile mineral oil to each tube

3 Add 5 µL prepared target DNA to each tube under the oil layer

4. Place the reaction tubes in a DNA thermal cycler programmed for a three-step protocol: 2 min at 94°C for one cycle, and then a 1-min denaturation at 94°C, a 1-min annealing at 60°C, and a 1-min extension at 72°C for 60 cycles (*see* **Note 3**)

5 After the PCR program is complete, place tubes in an HRI-300 UV photochemical reaction chamber for 15 min at 4°C to inactivate amplified DNA isopsoralen sterilization).

3.3.2. Detection of PCR Products

3 3.2.1 Agarose Gel Electrophoresis

1. Prepare 2% NuSieve: 1% SeaKem agarose gel in 100 mL 1X TBE buffer with 5 µL ethidium bromide.
2. Place agarose gel in electrophoretic box and fill with 1X TBE until gel is submerged (approx 600 mL 1X TBE/30 µL ethidium bromide) Use electrophoresis to separate the amplification products (20 µL/well plus 4 µL gel loading buffer)
3. After the amplified products have migrated sufficiently, visualize DNA bands with UV transillumination and record results with Polaroid photograph.
4. Denature and neutralize the PCR DNA products by soaking the gel in denature and neutralization buffers for 30 min each. Transfer the amplified targets to a nylon membrane by the Southern blot procedure.
5. Expose membrane to UV light (1200 × 100 µJ, energy mode setting) in UV crosslinker box to crosslink DNA to membrane.

3.3.2.2. Chemiluminescence

1. Develop a probe by amplifying a sequence of the original PCR amplicon that is internal to each primer set.

2. Label the internal amplicon for chemiluminescence using the enhanced chemiluminescence kit (ECL) from Amersham.
3 Wet the nylon membrane with 2X SSC and place in a hybridization canister. Add 20 mL of hybridization solution, provided by Amersham, and incubate for 1 h at 42°C.
4 Add the labeled probe to the membrane in the canister and allow it to hybridize for 4 h to overnight
5. Wash the membrane twice with 50 mL of wash buffer 6 *M* urea, 0.4% SDS, 0 5X SSC, at 42°C for 20 min, and twice with 100 mL of 2X SSC at room temperature for 5 min
6 Place the membrane in 30 mL detection reagent provided in the ECL kit for 1 min at room temperature.
7. Wrap the membrane in plastic wrap and expose the membrane to X-ray film for 1 h
8 Develop the film by a Kodak X-Omat X-Ray film processor

3.3 2 3 Radiolabel

1 Wet the membrane with 2X SSC and place in a hybridization canister Add 20 mL of hybridization solution and incubate for 1 h at 42°C
2 Prepare the labeled probe To a 1.5-mL microcentrifuge tube, add 10 µL of H_2O, 1.5 µL T4 kinase buffer, 100 pmol internal probe sequence, 2 µL [γ^{32}P] ATP (6000 Ci/mmol), and 1 µL T4 polynucleotide kinase. Mix and incubate for 30 min at 37°C.
3. Add the solution of labeled probe to the hybridization solution and allow the membrane to incubate at 42°C for 1 h.
4 Wash the membrane twice for 10 min in wash buffer and allow it to air dry
5 Wrap the membrane in plastic wrap, expose it to Kodak X Omat film for 4 and 24 h (*see* **Note 9**).

3.4. Quality Control

1. Negative extraction/amplification control· CSF known to be negative for HSV-DNA is processed and amplified to demonstrate that the extraction and amplification reagents are free of contaminants Assay at least one control CSF specimen for every five patient specimens that are processed
2 Blank amplification control. 50 µL master mix only is amplified (no-target DNA) to demonstrate that the PCR master mix reagents are free of contaminants
3 Positive extraction/amplification control: Extract CSF specimen, seeded with HSV or cell cultures infected with the virus; amplify as protocol for specimens from patients This will indicate that the extraction technically was adequate to yield DNA for PCR. Strong and weak gel-positive (gel ± but Southern blot positive) controls should be used to test the sensitivity of the assay

4. Notes

1 The primers used for the amplification of HSV, CMV, EBV, and VZV are specific for each target, no crossreactions have been detected

2. Sensitivity of PCR detection of HSV-DNA is equivalent by amplifying a single rather than duplicate sample of the specimen extract.
3. Materials, reagents, sample preparation, PCR amplification routine, and detection methods are common for testing specimens for all of the herpesviruses (HSV, CMV, EBV, VZV).
4. Inclusion of 25 μg/mL of isopsoralen does not affect the ability of the internal probe to hybridize to amplified target DNA *(27)*.
5. Controlling the spread of amplified DNA can be accomplished by physical and chemical barriers. Dedicate laboratory rooms and pipets (barrier tips and/or positive displacement pipets) for specific procedures. Limit travel between amplicon-rich and amplicon-free rooms. Bleach and UV can eliminate contaminating nucleic acids. The use of isopsoralen compound 10 and dUTP/UNG (d-uraciltriphosphate/uracil-*N*-glycosylase) as part of the PCR master mix have been used to minimize carryover of PCR products into subsequent reactions.
6 Chemiluminescence (ECL) (i.e., radioisotopic labeling) provides 10–100-fold increase in sensitivity compared with gel electrophoresis.
7 Storage technology of phosphor images is now being offered that rapidly images both radioisotopic and chemiluminescent samples without X-ray film. This technology allows detection of both strong and weak signals with extreme sensitivity and quantitative capabilities. Companies offering this technology include Bio-Rad (Hercules, CA), Molecular Dynamics (Sunnyvale, CA), Fugi (Tokyo, Japan), and Packard (Meriden, CT)
8 Capillary electrophoresis (CE) is being evaluated as a tool for detecting amplified products. This technology is faster and would offer a means for automating a detection system.
9 The primer set for detection of HSV-DNA has sequence homology and equal sensitivity for amplification of both serotypes of the virus
10. Peripheral blood leukocytes (PBL) from unselected, asymptomatic immunocompromised patients are commonly (~30%) positive for CMV- and EBV- DNA sequences. PBLs from immunologically competent subjects normal blood donors rarely contain DNA for any of the herpesviruses *(28)*.

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8

Diagnosis and Direct Automated Sequencing of HIV-1 From Dried Blood Spots (DBS) Collected on Filter Paper

Sharon Cassol, Stanley Read, Bruce G. Weniger, Richard Pilon, Barbara Leung, and Theresa Mo

1. Introduction

Since its discovery in 1981, human immunodeficiency virus type 1 (HIV-1) has rapidly emerged as one of the most devastating infectious pathogens of this century *(1–3)*. The World Health Organization estimates that, as of 1995, there were at least 15 million HIV-1-infected men, women, and children worldwide, with the vast majority of infections occurring in developing countries and isolated rural regions where specimen collection, preparation, and shipment are difficult *(4)*. Simple and improved sampling methods that can be widely applied under difficult field conditions are needed to effectively monitor the changing dynamics of the HIV-1/AIDS pandemic, track the spread of HIV-1 variants among different population groups and ensure that research and interventive activities are directed against biologically important variants of the virus. To date, at least eight major HIV-1 subtypes, designated A through H, have been identified *(5,6)*. More recently, a ninth subtype, I, has been detected *(7)*, as well as several highly divergent, or "outlying" variants of HIV-1 that have been tentatively classified as group O *(8,9)*. This sub-typing is based on a relatively small number of specimens collected from a few geographic areas and the full range and distribution of HIV-1 variants remains to be established.

The collection of whole blood on filter paper provides an innovative and powerful approach for the systematic and unbiased collection of large numbers of field specimens for diagnostic and surveillance purposes *(10–19)*. These specimens, commonly known as Guthrie or dried blood spots (DBS), are collected by carefully applying a few drops of fresh blood, drawn by venipunc-

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ture, or by finger- or heel-prick with a lancet, onto an absorbent filter paper matrix. After the blood has saturated the filter, it is simply air dried, placed in a high-quality bond envelope *(20)*, and shipped to a centralized reference laboratory for analysis. This chapter describes a reliable protocol for obtaining consistent yields of high-quality HIV-1 proviral DNA from small amounts of dried blood and outlines some of the more important applications of this technology for the PCR-based diagnosis and genetic characterization of HIV-1.

2. Materials

2.1. Reagents

1 Centricon 100 columns (Amicon, Beverly, MA, cat. no. 4211).
2 Chelex-100, 100–200 mesh (Bio-Rad, Hercules, CA)
3. Expand long template PCR system, containing 10X concentrated PCR buffer (22.5 m*M* $MgCl_2$, 500 m*M* Tris-HCl, 160 m*M* $[NH_4]_2SO_4$) and 2.6 U expand enzyme consisting of a mix of *Pwo* and *Taq* DNA polymerases, sufficient for 190 reactions (Boehringer Mannheim, Indianapolis, IN, cat no. 500847).
4. NuSieve agarose (FMC BioProducts, Rockland, ME, cat no. 50084)
5. Ultrafree-MC filters (Millipore, Bedford, MA, cat. no UFC3LTK00).
6 Uracil *N*-glycosylase (Perkin-Elmer Corp., Foster City, CA, cat. no. N808-0096)
7. ABI Prism DNA sequencing, chemistry guide (Perkin-Elmer, cat. no 903563, version A, May 1995).
8 Dye terminator cycle sequencing kit with FS DNA polymerase, FS (Perkin-Elmer, cat. no. 401384).
9. 100-bp DNA ladder (Pharmacia, Piscataway, NJ, cat. no. 27-4001-01).
10. dATP, dCTP, dGTP, and dTTP (Pharmacia, Piscataway, NJ, cat. nos. 27-2050, 27-2060, 27-2070, and 27-2080, respectively).
11 Centri-Sep spin column, 100 pack (Princeton Separations, Inc., Adelphia, NJ, cat. no CS-901).
12. QIA quick gel extraction kit, 250 columns (Qiagen, Inc , Chatsworth, CA, cat. no. 28704).
13. Specimen wash bufer from the Amplicor whole blood specimen preparation kit (Roche Diagnostic Systems, Inc., Branchburg, NJ, cat. no. US.87253).
14. Amplicor HIV-1 amplification and detection kits (Roche Diagnostic Systems, cat. nos. US:87260 and US:87271, respectively).
15. #903 Blood collection paper (Schleicher & Schuell, Keene, NH, cat no 58730)
16 Ethidium bromide (Sigma, St. Louis, MO, cat no. E8751)
17. Bromophenol blue/xylene cyanol (Sigma, cat. no. B3269)

2.2. Reagents Not Included in Diagnostic and Sequencing Kits

1 0.5X TBE gel electrophoresis buffer: 44 m*M* Tris-borate; 44.5 m*M* boric acid, 1 m*M* EDTA. One liter of 10X stock is prepared by dissolving in deionized H_2O 54 g of Tris base, 27 5 g of boric acid and 20 mL of 0.5 *M* EDTA
2 5X Ficoll/loading dye: 25% Ficoll, 1% bromophenol blue/xylene cyanol

3. Methods

3.1. Preparation of DBS

Caution: Universal precautions should be followed during the collection and preparation of DBS for shipment. The following protocol provides sufficient DNA for at least 2 diagnostic and 12 sequencing reactions.

1 Collect approx 2 mL of whole blood by venipuncture in an acid-citrate-dextrose Vacutainer tube (Becton Dickinson) (*see* **Notes 1** and **2**).
2 Using a micropipeter and sterile plugged tips, apply 50-µL aliquots of the blood to the back of a standard newborn screening blotter (Schleicher & Schuell, #903) in a dropwise fashion (*see* **Note 3**)
3. Air dry for at least 3 h and place each filter in an individual bond envelope Seal the envelope and enclose in a second, outer bond envelope for storage or shipment (*see* **Notes 4** and **5**)

3.2. Removal of Hemoglobin, Elution, and Concentration of DNA

The following procedure is rapid and efficient. The eluted DNA can be concentrated and amplified directly, obviating the need for phenol/chloroform extraction (*see* **Note 6**).

1. If the DNA is to be used for diagnostic purposes, a single 50-µL blood spot will suffice. For sequencing applications, we routinely process and pool the DNA from three 50-µL spots
2. Excise each circle of dried blood (approx 1 cm^2) with clean, acid depurinated scissors (*see* **Note 7**), cut in half, and place each half in a 1.5-µL sterile screw-cap microfuge tube (*see* **Note 8**). Use a different pair of scissors for each patient and each control specimen.
3. Add 1.0 mL of specimen wash buffer (Amplicor Whole Blood Specimen Preparation Kit; Roche Diagnostic Systems) to each tube.
4 Incubate at 25°C for 30 min on an Eppendorf Thermomixer™ set at 1000 rpm to lyse the red cells and release hemoglobin
5. Aspirate the hemoglobin-containing supernatant, taking particular care to remove as much solution as possible (*see* **Note 9**).
6. Combine the two halves of each filter in the same microfuge tube and add 200 µL of a 5% slurry of Chelex-100 (Bio-Rad) in H_2O.
7 Incubate at 95°C for 1 h on Thermomixer at 1000 rpm to elute the DNA from the filter (*see* **Note 10**).
8. Spin the tubes (14,000*g* for 5 min) to bring down the Chelex resin
9. Remove and pool Chelex supernatants for each patient (i e., from 1 to 3 DBS).
10. Concentrate the pooled supernatant to approximately 40 µL in an Ultrafree-MC centrifugal filtration unit as specified by the manufacturer (Millipore) (*see* **Note 11**).
11. Collect the DNA concentrate from the filter cup and use directly in PCR amplification reactions.

3.3. Diagnosis of HIV-1 Infection

1. For large-scale diagnostic screening using the Roche microwell system, adjust the DNA concentrate to 50 µL with sterile water and add $MgCl_2$ to a final concentration of 7.5 m*M* Amplify and detect the viral DNA exactly as specified in the Amplicor HIV-1 Amplification and Detection kits (*see* **Note 12**)
2. Alternatively, a 40-µL aliquot of the DNA can be amplified in a standard nested PCR reaction using dUTP and uracil *N*-glycoslase (Perkin-Elmer) to prevent carry-over contamination and guard against false-positive results (*see* **Notes 13** and **14**)
3 Check second round reactions for amplification product by loading 8 µL of the PCR product, mixed with 2 µL of 5X-loading dye, on a 1.5% agarose gel in 0 5X TBE buffer. Electrophorese at 100 V for 30–60 min (*see* **Note 14**).
4 Stain the gel with ethidium bromide (0.5 µg/mL in H_2O) and detect the product by UV transillumination. Photograph and record the results

3.4. Sequencing of the HIV-1 Genome

For sequence analysis, we routinely use a two-step, nested PCR protocol and conserved primers, such as the C2V3 *env* primer sets shown in **Table 1** (*see* **Note 15**).

1. For the first amplification, add 40 µL of the DNA concentrate to a thin-walled 0 5 µL GeneAmp reaction tube (on ice) containing.
   a. 500 µ*M* each of dATP, dCTP, dGTP, and dTTP (*see* **Note 16**)
   b 500 n*M* of each outer PCR primer (MK603-F and CD4XBO-R2, **Table 1**)
   c. H_2O (to 50 µL final volume)
2 While still on ice, add 10 µL of cold master mix 2, system 2 (Expand™ Long Template PCR System; Boehringer Mannheim, cat no. 1661-842) (*see* **Note 17**).
3. Place the cold PCR tubes in a 9600 GeneAmp Perkin-Elmer thermocycler set at at a 94°C soak cycle Begin cycling immediately, as follows:
   a. 94°C for 2 min to denature template (once)
   b. 94°C for 10 s, 50°C for 30 s, 68°C for 2 min (repeat 37 times).
   c 68°C for 7 min (once)
   d. 4°C overnight, or until required for amplification 2.
4. For amplification 2, transfer 3–5 µL of the first PCR product into a second GeneAmp tube and amplify for 40 cycles using the inner primer set (MK650iF and V3iR). Use the same reaction components and cycling parameters.
5 Add loading dye to 8 µL of PCR product and electrophorese on a 1.5% NuSieve as described above (*see* **Notes 18** and **19**).
6 Photograph the gel, record the results, and cut out the PCR product band using a UV transilluminator and a clean scalpel blade.
7 Place the gel slice in a microfuge tube, solubilize, and purify the DNA using a QIA Quick Gel Extraction kit (Quiagen) (*see* **Note 20**).
8 Quantify the DNA by reanalyzing a small aliquot on NuSieve gel using 300 ng of a 100-bp ladder (Pharmacia, cat. no. 27-4001-01) as the reference standard (*see* **Note 21**).

Table 1
Frequently Used Oligonucleotide Primers

PCR Primer	Orientation	Sequence (5'–3')	Location
Outer MK603-F	→	CAGAAAAATTGTGGGTCACAGTCTATTATGGGGTACCT	89–126
Outer CD4XBO-R2	←	*gttctcgag*TATAATTCACTTITCCAATTGTCC	1426–1439
Inner MK650ıF	→	AATGTCAGCACAGTACAATGTACAC	715–739
Inner V3ıR	←	AGGTCCCCTCCTGAGGA	

[a]Sequence and map locations of oligonucleotides used as nested primers in PCR reactions Numbered according to the gp120 sequence of HIV-1 $_{LAI}$ (GenBank accession no K02013). The first nine nucleotides of the CD4XBO-R2 primer consists of a *gttctcgag* clamp that is not HIV-1 specific

9 Sequence directly using a Dye Terminator Cycle Sequencing Kit with Ampli*Taq*, or preferably, Ampli*Taq* DNA Polymerase, FS as specified by the manufacturer, Applied Biosystems Inc (ABI)
10 Remove the excess dye on a Centri-sep spin column (Princeton Separations) according to manufacturer's instructions and electrophorese sequencing reaction for 10 h on a 373A Fluorescent DNA Sequencer (ABI)
11 Assemble, align, and edit the data for the forward and reverse strands (*see* **Note 22**)

4. Notes

1 A wide range of clinical specimens can be applied to filter paper and used as a source of material for HIV-1 genetic analysis. Some examples are fresh capillary blood collected by heel-stick or venipuncture, anticoagulated whole blood (citrate, EDTA), peripheral blood mononuclear cells, and cultured cell lines Although most work with DBS has been done on specimens collected from pediatric patients ***(15,16)***, adult DBS specimens have included patients from different risk categories (persons attending sexually-transmitted disease clinics, prostitutes, injecting drug users, homosexual and bisexual men, and spouses of HIV-1-infected individuals) and at various stages of disease (ranging from asymptomatic infection to AIDS) ***(17)*** In the pediatric setting, the sensitivity of the DBS-PCR method is comparable to conventional PCR and better than virus isolation in culture, and approaches 100% in infants older than 16 d of age ***(16)***. As in other diagnostic tests, the sensitivity of DBS-PCR in neonates younger than 15 d is significantly lower, in the range of 26%. To reduce test error in the neonatal period and achieve earliest possible definitive diagnosis, it is recommended that all neonates be tested by repeat DBS-PCR of independent samples collected at birth and at 30 and 60 d of life
2. In addition to clinical specimens, it is recommended that at least one positive and two negative DBS controls be included in each PCR assay As with any laboratory method, additional controls may be useful in start-up protocols to ensure that the procedure is reproducible and achieving its maximum sensitivity. Negative controls can be prepared from uninfected, HIV-1 seronegative blood donors, while positive controls of known HIV-1 copy number can be prepared by progressively diluting cultured 8E5 cells ***(21)***, containing a single integrated copy of HIV-1_{LAV} proviral DNA per cell, with normal seronegative donor blood DNA extracted from DBS containing these dilution mixtures can then be used to construct a standard curve and assess DNA recovery. The HIV-1 copy number in positive controls should be kept at the lowest possible concentration to avoid the risk of PCR product carryover to clinical samples. The 8E5 HIV-1_{LAV} cell line (which can be obtained from the NIH Reference Reagent Program, cat no 95) is also an excellent positive control for sequence analysis of the V3-envelope region since it contains a highly characteristic QR amino acid insert immediately upstream of the hexameric V3-tip sequence. This insert readily distinquishes 8E5 from most clinical variants of the virus and serves as a control for carryover contamination.

3. We have routinely used Schleicher & Schuell #903 paper, originally designed for neonatal metabolic screening, as the absorbent matrix *(13–19)*. This collection device consists of a blotter containing five 1-cm^2 circles for blood application and a sturdy paper overlay that covers the absorbent blotter and the dried blood. The precise method of applying the blood to the blotter is not critical, provided that the blood is fresh and not clotted. In the case of heel-pricks, care should be taken to avoid diluting the sample with extracellular fluid. A standardized method for the collection and preparation of blood spot specimens has been published by the National Committee for Clinical Laboratory Standards *(22)*. In most of our studies, we have simply applied 20–40 drops of whole blood to the back of the filter paper using a syringe or pipeting device.
4. The blood spots must be thoroughly dried at room temperature (in a biocontainment hood, when possible) before covering them with the attached paper overlay and sealing them in a high-quality, sturdy bond envelope, preferably one that is air-permeable and water-resistant *(20)*. When the back of the filter is used, the dried blotter should be double-enveloped to ensure safe shipping. The plastic bags used in some early studies are to be avoided since they release undesirable chemicals and cause heat buildup and moisture accumulation, leading to degradation of the DNA. In regions where the humidity is excessively high, inclusion of a pack of desiccant may be desirable to prevent moisture accumulation and microbial contamination. A recently described modification, designed to ensure that the sample remains "sterile," is to use blotters that have been preimpregnated with 2*M* guanidine thiocyanate *(23)*. Although this matrix has been successfully used for PCR-based studies of human genomic sequences, its applicability to low-abundancy HIV-1 sequences has not yet been demonstrated
5. When double-packaged using either an inner envelope or the filter paper overlay as the inner container, and a high-quality, extra-strong bond envelope as the outer container, as recommended by the Centers for Disease Control in Atlanta *(20)*, DBS specimens can be safely shipped or transported by mail or courier. Upon arrival at the reference or research laboratory, DBS are routinely stored at –20°C to ensure optimal specimen integrity Although not recommended, we were able to obtain high-quality V3-loop sequence data from a series of DBS that were inadvertently left at room temperature for 6 mo *(17)* In other studies, we found that repeated freezing and thawing and storage of DBS for up to 15 wk at room temperature had no adverse effect on the ability to detect HIV-1 DNA in DBS *(14)*
6. To obtain high yields of amplifiable DNA from whole blood, heme and inhibitory substances must be removed from the preparation and the DNA must be effectively released and collected from the nucleated cells. The earliest DBS protocols *(11,13)* were geared toward obtaining highly purified nucleic acid and involved lengthy organic extraction, followed by DNA precipitation, amplification, and detection of the PCR product by solution hybridization with radiolabeled probes. These methods were tedious and labor intensive, and did not lend themselves to large-scale screening and throughput. In addition, the DNA was frequently lost during the precipitation step, leading to false-negative results.

7. To depurinate and prevent cross-contamination between specimens, scissors are routinely washed in 0.25 *N* HCl for 10 min followed by a thorough rinsing in sterile water
8. If screw-cap tubes are not used, the lids will pop open during Chelex treatment
9. If carefully performed, this step removes >95% of the hemoglobin. If not removed, residual heme will act as a potent inhibitor of PCR.
10. The DNA remains bound to the filter and can be efficiently recovered by heating at >95°C for 1 h at pH 10 in a suspension of 5% Chelex-100, a polyvalent resin that chelates metal ions and prevents the breakdown of DNA during subsequent processing *(24)*. Alkaline lysis at high temperature also significantly enhances the PCR amplification signal by ensuring the mononuclear cell membranes are disrupted and that the DNA is completely denatured One of the most common causes of PCR failure is incomplete denaturation of the DNA. Care must be taken to remove all of the Chelex resin, or metal ions will leach back into solution.
11. Although not always necessary, a quick concentration step using a Centricon-100 (Amicon) or Ultrafree-MC filter (Millipore) leads to more reliable detection of low-copy HIV-1 sequences. In both of these systems, the DNA-containing solution is simply poured into a filter cup and concentrated (5- to 10-fold) by centrifugal force. The DNA is retained in the cup, while small-molecular weight substances, such as, salts and PCR inhibitors, pass through the filter and are collected in the filtrate collection tube. These concentration devices are used according to manufacturer's specifications, but some "in-house" experimentation with times and speeds of centrifugation may be required to achieve optimal DNA yields. Recent studies in our laboratory suggest that the Millipore filter may give the most reproducible results (unpublished data), but the system has not been rigorously tested in different population cohorts. Further scale-up of this part of the protocol would be highly beneficial, especially for longer PCR products and large-scale sequencing efforts. In theory, it should be possible to extract and purify large amounts of high-quality DNA from filter supports The challenge is to recover large amounts of DNA in an intact and concentrated form, free of whole-blood inhibitors
12. One of the most promising applications of DBS-PCR has been its adaptation to newborn screening using a rapid microwell plate assay developed by Roche Diagnostic Systems. The use of this standardized, quality-controlled ELISA-type kit broadens the applicability of DBS technology and renders it suitable for large-scale, population-based screening in clinical and public health laboratories In the microwell detection system, an aliquot of the concentrated Chelex supernatant is amplified with biotinylated *gag* or *pol* primers as specified by the manufacturer (Amplicor HIV-1 Amplification and Detection Kit, Roche Diagnostic Systems) After amplification, the PCR product is denatured and hybridized to individual wells of a plate coated with the appropriate HIV-1-specific oligonucleotide probe. The plate is then washed and incubated with avidin-horseradish peroxidase conjugate. Following further washing and incubation with the chromogen, the labeled amplicon is detected colorimetrically by reading the reaction on a microwell plate reader at a wavelength of 450 nm. Details of the procedure, its controls, interpretation, and performance in the perinatal setting have been described pre-

viously ***(16)***. Studies to assess the assay's performance on specimens collected internationally, and in adult populations to screen for new HIV-1 infections in incidence cohorts, are ongoing.

13. For diagnostic applications, all amplifications are performed using uracil-N-glycosylase (UNG) to reduce the risk of false-positive results due to PCR carry-over contamination ***(25)*** In this method, dUTP is substituted for dTTP in the PCR reaction mixture, and all subsequent PCR reactions are pretreated with UNG for 10 min at room temperature to cleave and excise uracil from any dU-containing PCR product that has been inadvertently carried over from a previous reaction This is followed by a heat inactivation step to remove residual UNG and prevent degradation of the desired product. Provided that UNG digestion is carried out to completion, and that the residual UNG enzyme is completely inactivated prior to thermocycling, this method is highly effective in controlling against minute amounts of contamination. It is not, however, a substitute for the extreme care that is required of all PCR-based techniques For a discussion of the stringent laboratory precautions required for accurate diagnosis, *see* **ref. *26*** In diagnostic applications requiring nested PCR, dUTP can be used in both the first and second PCR reactions, but UNG is added to the first reaction only A wide range of different primer sets can be used (*gag, pol, env*) provided that they recognize and bind efficiently to the HIV-1 strains under study
14. An efficient nested PCR using conserved *gag* or *pol* primers, provides reliable detection of HIV-1 from as few as five DBS copies of the HIV-1 provirus/100,000 nucleated cells (***14***, unpublished data) Following amplification and electrophoretic separation on agarose, the PCR product is visualized directly by staining the gel with ethidium bromide This approach is highly sensitive, ensures that the correct size of DNA fragment has been amplified in sufficient amounts, and serves as a simple, low-cost method in regions where budgets and resources are limited In our hands, nested PCR of the *pol* gene region, combined with agarose electrophoretic analysis, has been particularly valuable for confirming the presence of proviral DNA in DBS containing highly divergent strains of HIV-1 Once the presence of HIV-1 has been documented using conserved *pol* primers, a panel of envelope primers can be used to fish out new variants that are not easily recognized by conventional *env* primer sets.
15 Sequencing of DBS specimens provides a rapid screening system to monitor the global spread and emergence of newly identified and previously recognized HIV-1 subtypes (unpublished data, ***17***). The method is particularly well-suited to large-scale epidemiological studies of migratory, isolated, or hard-to-reach populations and should facilitate routine surveillance of breakthrough infections in vaccine field trials

The steps involved in DBS sequencing are common to all applications that employ sequencing techniques to classify, compare, and differentiate between HIV-1 subtypes When performing sequencing applications, a number of choices must be made with respect to the selection of the target sequence, the purification of the PCR and sequencing product, the selection of the sequencing chemistry,

and the fidelity and accuracy of the sequencing enzyme, as well as the overall cost and potential for automation and high volume throughput. No matter what choices are made, the PCR should be fully optimized and both the forward and reverse strands of the DNA should be sequenced, aligned, and edited.

Since our laboratory is interested in large-scale epidemiological studies, we have focused primarily on cycle sequencing using dye-labeled primers *(17)* This sequencing strategy requires the least amount of DNA and the chemistry is robust and versatile, and can be easily applied to the direct population sequencing of a wide range of PCR products. These features of cycle sequencing render the technology suitable for automation and mass screening applications.

16. Although we have successfully sequenced dUTP-containing PCR products, most of our sequence work has been performed on products generated with dTTP Since the potential for carryover contamination is great in the absence of dUTP and UNG, extreme care must be taken to avoid false-positive results and all studies should be stringently controlled to rule out this possibility. In particular, sequencing studies with dTTP should be restricted to regions of the genome that are not amplified for diagnostic purposes, and all new sequences should be extensively compared and screened against current and previous PCR products sequenced in the investigator's laboratory. Some of the most common indicators of laboratory contamination are extensive divergence between linked specimens and identity, or "near-identity" between unlinked specimens or with specimen sequences, molecular clones, and /or PCR products previously studied.

    Reasonable precautions to guard against tube-to-tube carryover of PCR product include performing each step in the amplification and analysis process (sample processing, pre-PCR, first round PCR, nest PCR, and sequencing) in a separate laboratory or containment hood, using designated pipeters and plugged tips, cleaning work areas with bleach after each assay, using disposible gloves that are changed frequently, aliquoting reagents in small volumes suitable for a single PCR assay, and performing first and second round PCR reactions in clean, designated thermocyclers housed in separate rooms

17. In recent studies, we have successfully replaced *Taq* polymerase with Expand™, a new polymerase system that exhibits increased fidelity and enhanced processivity. Using the improved amplification system, outer DBS PCR products of 1 2 kb are now attainable on a regular basis.

18 It is extremely important that PCR reactions be optimized so that only a single, bright band is detectable by agarose electrophoresis. If multiple bands are detected, high-quality sequence date is unlikely, even if the specific band of interest is excised and purified. Although seemingly homogeneous, these bands frequently contain heteroduplexes or artifact PCR products generated by primer oligomerization. If the same primers are used for sequencing, these bands give rise to multiple sets of fragments, rendering the sequencing pattern complex and uninterpretable. Well-designed primers and the use of "hot start" methods that minimize oligomerization and secondary priming are important factors to consider when optimizing PCR reactions. A complete discussion of potential difficulties

is beyond the scope of this chapter, but an excellent overview of problems encountered during automated fluorescent sequencing is presented in **ref. *27***.

19. For successful analysis of PCR products using dye terminator cycle sequencing, it is important to clean up the PCR reaction by removing excess primers and residual nucleotides, even if the same primers are to be used for the sequencing reaction. Although a variety of different methods exist for purifying PCR products, we routinely separate our reactions on 1.5% agarose to determine the size and quality of the product High-quality, sequence-grade agarose is required to avoid carryover of fluorescent contaminants that are often present in lower grades of agarose.
20. Following electrophoresis, the PCR product band is excised on a UV transilluminator and further purified using a QIA Quick Gel Extraction kit according to manufacturer's recommendations. The band should be cut out as quickly as possible under low intensity UV light to avoid damaging the DNA With respect to the QIA columns, some adjustments of the time and speed of centrifugation may be required for optimal purification.
21 After purification, the DNA needs to be accurately quantified A simple method is to reanalyze by agarose electrophoresis using a reference standard to quantify the PCR product The amount of PCR product added to the sequencing reaction is critical. Too little product will lead to weak signal strength, while too much product results in off-scale data and short sequence reads.
22 A wide range of different software programs are available to assist in the analysis and interpretation of the data. Many of these procedures are well described in the ABI user manuals accompanying the software packages ***(27)*** and in the literature relating to phylogenetic analysis ***(28,29)***. In our hands, direct sequencing of the PCR product amplified from DBS has been particularly successful in the pediatric setting and in seroconverters and early-stage asymptomatic patients where the viral repertoire is relatively homogeneous. Using this approach, minor variants are also detected provided that they represent >10% of the total virus populations. However, in patients dually infected with different HIV-1 subtypes, and in patients infected with more than one major variant of the virus, in which one of the variants contains an insertion or deletion, cloning of the PCR product may be required prior to sequencing. We have found that, in the vast majority of HIV-1-infected patients, most *gag*, *pol,* and C2V3 *env* gene regions can be sequenced directly from the PCR product. In contrast, the V1V2 *env* region of HIV-1 frequently contains large insertions, and, as in other sequencing applications, successful analysis of this region requires molecular cloning of the PCR product, followed by sequencing of the individual clones
23. Recent studies in our laboratory, and others, indicate that accurate quantification ***(30)*** and sequencing of HIV-1 RNA from dried whole blood and dried plasma is also feasible and reliable (unpublished results).

5. Future Directions

The widespread utilization of DBS-PCR for large-scale newborn screening and surveillance programs (**Tables 2** and **3**) will depend on increased

Table 2
Advantages and Disadvantages of DBS-Based Genetic Screening[a]

Advantages
Ease and economy of sample collection, storage, and shipment
Noninfectious transport medium
Stability of sample—no need for refrigeration or on-site sample processing
Allows for centralization of testing facilities.
Facilitates systematic, unbiased surveillance.
Widely applicable—facilitates follow-up of migratory and hard-to-reach populations
Disadvantages.
Cannot provide live virus for study in culture.
May be more difficult to obtain long PCR fragments (>1 2 kb)

[a] Table taken from **ref. *19***

Table 3
Current and Potential Applications of DBS to Clinical Diagnosis and Field Surveillance of HIV-1[a]

Perinatal
Early differential diagnosis of infected vs uninfected infants.
Precise determination of perinatal transmission rates in specific populations over time
Assessment of interventive strategies
Genetic characterization of vertically transmitted strains.
Therapeutic
Screening for drug-resistant mutations
Evaluating the risk for mother-to-child transmission of resistant genotypes
International
Determining frequency and distribution of HIV-1 subtypes.
Tracking the spread of HIV-1 subtypes between continents.
Assessing the rate of virus evolution at the individual and population levels.
Initial screening for genetic recombinants

[a] Table taken from **ref. *19***

automation and improved efficiency of sample throughput. It is anticipated that because of the intense effort being put into the Human Genome Project, it may be possible at some point in the near future, to increase DBS-PCR and sequencing throughput by interfacing with robotic workstations. Hopefully, these refinements will lead to increased standardization and reduced costs, making it economically feasible to conduct mass screening and surveillance studies in developing countries where the pandemic is most severe.

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9

PCR for the Detection of Influenza Viruses in Clinical Material

Joanna S. Ellis and David W. G. Brown

1. Introduction

Influenza viruses are segmented, negative-stranded RNA viruses belonging to the family *Orthomyxoviridae*. They are classified into influenza A, B, and C on the basis of different epitopes on the nucleoprotein (NP) and matrix proteins (M). Influenza A viruses are further divided into subtypes H1N1, H2N2 and H3N2, based on differences in the hemagglutinin (HA) and neuraminidase (NA) genes. The ability of influenza viruses to undergo antigenic change in HA and NA enables the virus to evade the immune response of the host and to cause repeated infections and epidemic spread.

1.1. Influenza Infection

Influenza is a major cause of mortality and morbidity throughout the world. In the United Kingdom each winter up to 20,000 deaths are attributable to influenza, in an epidemic year. These occur mostly among the elderly. The clinical picture of influenza infection is very variable, from subclinical infection to severe lower respiratory tract disease. A typical case presents with malaise, fever, myalgia, and sore throat. Often, illness is prolonged for more than 1 wk. After inhalation, virus replicates in the columnar epithelium of the upper and lower respiratory tract. Virus is detectable 24 h before the onset of illness and peak virus titers are reached 24–48 h after the onset of symptoms. Thereafter, virus titers rapidly decline and the disease typically resolves within a week *(1)*.

1.2. Laboratory Diagnosis

Rapid diagnosis of influenza virus infection is important since treatment with amantadine and rimantadine is only effective if started soon after the onset

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of disease. Early diagnosis also aids surveillance of circulating strains and enables the early vaccination or prophylactic treatment of high-risk groups. Laboratory diagnosis of influenza can be made by the detection of virus in respiratory secretions or by serological methods. Serological confirmation of influenza infections are not helpful for management. Influenza viruses are usually detected by isolation of the virus from respiratory specimens in tissue culture cells, generally primary monkey kidney cells or the MDCK cell line. The presence of virus is confirmed by hemadsorption of guinea pig red blood cells or by hemagglutination tests. Although cell culture is sensitive, it requires viable virus and is slow, since it may take 5–10 d for a cytopathic effect to be observed. Faster results can be obtained by centrifugation-enhanced (shell vial) cultures, although these are less sensitive than conventional tube culture *(2)*. More rapid diagnostic tests for influenza viruses based on detection of NP antigen, such as, immunofluorescent (IF) staining and enzyme-linked immunosorbent assays (ELISA) are also widely used *(2–6)*. The disadvantage of the IF test is that it requires good specimen collection and smear preparation to ensure the presence of intact, influenza-infected cells. Where ELISA has been used to detect influenza viruses, reports of the sensitivity and specificity of this technique have differed widely between laboratories *(2–6)*.

1.3. Polymerase Chain Reaction (PCR) for Influenza

The development of PCR *(7)* has provided a highly specific and sensitive method for the detection of viral genomes. PCR assays utilize two oppositely orientated primers flanking a specific DNA region, which by repeated cycles of heat denaturation, annealing, and extension of the primers with *Taq* polymerase allow the amplification of the specific target sequence within the virus genome. Recently, several groups have carried out reverse transcriptase-polymerase chain reaction (RT-PCR) for the detection of influenza virus RNA in clinical material and cell culture fluids. Influenza A HA has been amplified from nasopharyngeal lavages *(8)* and throat swabs *(9)* and influenza BHA from throat swabs *(10)*. Other investigators have amplified influenza A and B M gene regions from nasal washes *(11)* and conserved regions of the nonstructural genes of influenza A, B and C from nasopharyngeal aspirates and swabs *(12)*. In these studies either a nested PCR was used, or a single round of PCR and the specificty of the reaction confirmed by hybridization with product-specific probes. In all cases the primers were specific for the gene targets and no amplification of non-homologous DNA was observed. Zhang and Evans *(13)* used nested PCR assays with primers specific for the M gene and the various human HA and NA genes to type and subtype cultured influenza viruses. In our laboratory, we have used the primers described by Zhang and Evans to detect influenza A M and influenza B HA genes in nasopharyngeal and throat swabs, nasopharyn-

geal aspirates, and throat washes. Nested PCR and detection of amplicons can be performed within 24–36 h. Although PCR is not as rapid as IF or ELISA, which can be performed in a few hours, it is considerably more rapid than cell culture of virus. Furthermore, the sensitivity of PCR has been demonstrated to be comparable to that of isolation of virus by cell culture *(11,12)*.

2. Materials

2.1. Specimen Collection

1. Sterile cotton swabs
2. Sterile polyethylene suction tube with a disposable aspiration trap
3. Viral transport medium: Aseptically add 100 mL 10X Hanks Base Single Strength, 10 mL 20% bovine serum albumin and 10 mL penicillin (10^5 U)/streptomycin (0.1 g) solution to 900 mL sterile distilled H_2O. Aseptically adjust the pH to exactly 6.7 with sterile 4.4% sodium bicarbonate (Life Technologies Ltd, Paisley, Scotland). Dispense 3-mL aliquots into sterile bijoux bottles. Store at 4°C

2.2. Viral RNA Extraction

To ensure against contamination with ribonucleases, gloves should be worn when preparing reagents and during all manipulations in the subsequent method sections. To avoid contamination between specimens, pipet tips with filters should be used, or positive displacement pipets (*see* **Note 1**)

1. Lysis buffer L6: 120 g guanidinium thiocyanate (GuSCN) dissolved in 100 mL 0.1 *M* Tris-HCl, pH 6.4. Add 22 mL 0.2 *M* ethylenediaminetetraacetic acid (EDTA), pH 8.0, and 2.6 g Triton X-100.
2. Wash buffer L2. 120 g GuSCN in 100 mL 0.1 *M* Tris-HCl, pH 6.4. Stir L6 and L2 buffers overnight, at room temperature in the dark. L6 and L2 buffers are stable for 3–4 wk if stored in the dark.
3. Silicon dioxide: place 60 g silicon dioxide in 500 mL deionized water for 24 h at room temperature. Remove 430 mL of supernatant and resuspend the pellet in 500 mL deionized water. Stand at room temperature for 5 h. Remove 440 mL of supernatant and then add 600 µL of concentrated HCl to pH 2.0. Aliquot, autoclave, and store at room temperature in the dark.
4. 70% Ethanol.
5. Acetone.
6. RNase-free H_2O. Prepare by treating deionized water with 0.001% diethylpyrocarbonate overnight, then autoclave.
7. Ribonuclease inhibitor: RNasin (40,000 U/mL). Store at –20°C.

2.3. Reverse Transcription of Viral RNA

All reagents for reverse transcription are stored at –20°C.

1. 10X PCR buffer: 160 m*M* $(NH_4)SO_4$, 670 m*M* Tris-HCl, pH 8.8, 0.1% Tween-20.
2. 50 m*M* $MgCl_2$.

3. 10 m*M* dNTP mix (each dNTP at 10 m*M*).
4. Random hexamer pd(N)$_6$ mix. Sodium salt (2.5 µg)
5. Ribonuclease inhibitor: RNasin (40,000 U/mL)
6. Murine Moloney leukemia virus (Mu-MLV) reverse transcriptase (RTase) (200 U/mL).

2.4. PCR Assay

All reagents for PCR assay are stored at –20°C, unless stated otherwise.

2.4.1. Primary Amplification

1. 10X PCR buffer (as in **Subheading 2.2.**).
2. 50 m*M* $MgCl_2$
3. Outer primers, each at 5 pmol/µL (*see* **Note 2**).
   For influenza A: AMPA 5'CCGTCAGGCCCCCTCAAAGC
   AMPDII 5'GACCAGCACTGGAGCTAGGA
   For influenza B: BHAA 5'GTGACTGGTGTGATACCACT
   BHADII 5'TGTTTTCACCCATATTGGGC
   Primers are complementary to the virus, or Roman numeral II indicates primers complementary to the cDNA
4. *Taq* polymerase (5000 U/mL).
5. Deionized, sterile H_2O Store at room temperature
6. Mineral oil Store at room temperature

2.4.2. Secondary Amplification

1. 10X PCR buffer (as in **Subheading 2.2.**)
2. 50 m*M* $MgCl_2$.
3. dNTP 10 m*M* mix
4. Inner primers, each at 25 pmol/mL
   For influenza A: AMPB 5'CAGAGACTTGAAGATGTCTT
   AMPCII 5'TGCTGGGAGTCAGCAATCTG
   For influenza B. BHAB 5'CATTTTGCAAATCTCAAAGG
   BHACII 5'TGGAGGCAATCTGCTTCACC
5. *Taq* polymerase (5000 U/mL).
6. Deionized, sterile H_2O. Store at room temperature.
7. Mineral oil. Store at room temperature

2.5. Analysis of PCR Products

1. Molecular biology grade agarose.
2. 1X TBE running buffer: Prepare 5X stock by dissolving 54 g Tris base, 27.5 g boric acid in 800 mL deionized H_2O. Add 20 mL 0.5 *M* EDTA pH 8 0 and make up to 1 L Dilute to 1X with deionized H_2O
3. 5X Loading buffer: 25% Ficoll, 1% orange G. Store at 4°C.
4. DNA molecular weight markers. size range 75–1636 bp. Store at 4°C.

5. Ethidium bromide (EtBr, 10 mg/mL) Prepare a working solution of 0 5 μg/mL (*see* **Note 3**)

3. Methods

3.1. Specimen Collection

Influenza viruses are always looked for in samples from the respiratory tract *(14)*. Most commonly nose and throat swab samples are collected, but nasopharyngeal aspirates produce good samples in young children and bronchioalveolar lavage has been described. We have detected influenza RNA by PCR in throat washes, throat swabs, nose swabs, and nasopharyngeal aspirates.

1. Specimens should be collected during the first 3 d of symptoms.
2 Throat swabs and nose swabs should be obtained using cotton swabs by trained personnel. The swabs should be placed in viral transport medium and shipped to the laboratory
3. Nasopharyngeal aspirates should be collected into viral transport medium by trained personnel and transported to the laboratory
4. Influenza viruses are labile and it is necessary to transport specimens to the laboratory rapidly at 4°C and to store samples carefully. Specimens should be stored at –70°C if they are not to be processed rapidly.

3.2. Isolation of Influenza Viral RNA

The method used here is that previously described by Boom et al. *(15)*. A negative control should be included and treated in the same manner as the test samples.

1. Add 50 μL specimen to 900 μL L6 buffer. Vortex for 10 s.
2 Add 40 μL silica (resuspended prior to use by vortexing).
3 Vortex for 10 s, then leave to stand at room temperature for 10 min
4 Vortex for 10 s, then centrifuge for 20 s at 12,000*g*. Discard the supernatant (*see* **Note 4**).
5. Add 1 μL L2 and vortex for 10 s. Centrifuge at 12,000*g* for 20 s and remove the supernatant.
6. Repeat **step 5**.
7. Repeat **step 5** with 70% ethanol, twice.
8. Repeat **step 5** with acetone, once.
9. Dry for 10 min in a heating block at 56°C with the lid open.
10. Add 50 μL RNase-free water. Vortex for 10 s and incubate at 56°C for 10 min to elute the RNA.
11. Vortex to resuspend and centrifuge at 12,000g for 2 min.
12. Transfer 40 μL supernatant to a fresh tube, taking care not to transfer any silica Residual silica may inhibit the subsequent reverse transcription reaction (*see* **Note 5**)

3.3. Reverse Transcription of Viral RNA

The volumes given are for one reaction, multiply these volumes according to the number of samples to be tested

1 Prepare a reaction mix containing 4 µL 10X PCR buffer, 4 µL 50 m*M* $MgCl_2$, 4 µL dNTP, 4 µL H_2O, 0 4 µL random hexamer mix, 0 4 µL RNasin, and 1 µL Mu-MLV RTase
2 Add 22.2 µL RNA and leave to stand at room temperature for 10 min Then incubate at 37°C for 45 min, 100°C for 5 min, and place on ice

3.4. PCR Assay

3.4.1. Primary Amplification

1 Prepare a reaction mix containing: 8 µL 10X PCR buffer, 2 µL 50 m*M* $MgCl_2$, 1 µL each outer primer (AMPA and AMPDII; or BHAA and BHADII), 67 7 µL H_2O and 0.3 µL *Taq* polymerase Overlay with 2 drops of mineral oil
2 Add 20 µL cDNA, below the oil
3 Place in a thermocycler and heat to 95°C for 2 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min

3.4.2. Secondary Amplification

1 Prepare a reaction mix containing 5 µL 10X PCR buffer, 2 5 µL $MgCl_2$, 1 µL dNTP mix, 1 µL each inner primer (AMPB and AMPCII; or BHAB and BHACII), 38 25 µL H_2O, and 0.15 µL *Taq* polymerase Overlay with 2 drops of mineral oil
2 Add 2 µL of products from the primary amplification
3 Place in a thermocycler and heat to 95°C for 2 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min.

3.5. Analysis of PCR Products

1. Prepare a 1% agarose gel by melting 1 g agarose in 100 mL 1X TBE, in a microwave oven Cool to 50°C and then pour the melted agarose into a gel tray. Leave at room temperature for 1 h.
2. Mix 15 µL secondary PCR products from each amplification with 4 µL loading buffer. Mix 1 µL DNA markers with 14 µL H_2O and 4 µL loading buffer.
3 Load each sample into a well in the gel and electrophorese in 1X TBE at 5–20 V/cm until electrophoresis is complete.
4 Stain the gel in EtBr for 20 min (*see* **Note 3**), wash with 1X TBE and view under shortwave UV illumination to observe amplification products. The influenza A M primers amplify a 401-bp fragment and the influenza BHA primers a 767-bp fragment (**Fig. 1**).

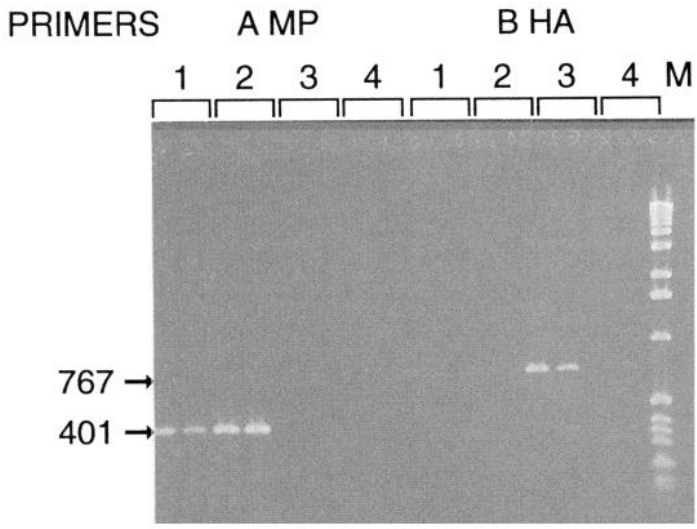

Fig. 1. Agarose gel electrophoresis of influenza PCR products. Specimens 1–3 (1; nasopharyngeal aspirate, 2; throat swab, 3; nasopharyngeal aspirate) and 4 (negative control) were tested in duplicate. Nucleic acid extraction and reverse transcription were performed as described in **Subheadings 3.2.** and **3.3.** Samples were then amplified in nested PCR reactions with primers specific for the matrix protein of influenza A (A M), or the hemagglutinin gene of influenza B (B HA). 15-µL aliquots of the secondary amplification products were analyzed on a 1.2% agarose gel and visualized by staining with ethidium bromide.

4. Notes

1. To avoid contamination between specimens, it is important that separate rooms should be used for reagent preparation, nucleic acid extraction/PCR, and post-PCR procedures. If this is not possible, then separate workstations at suitable sites in the laboratory should be used.
2. Repeated freezing and thawing cycles can result in the deterioration of primers. Commercially available oligonucleotide primers are usually obtained freeze-dried. Primers can be resuspended in sterile deionized H_2O to produce a stock solution and then aliquots of the appropriate working dilutions made and stored at –20°C.
3. Ethidium bromide is a powerful mutagen. A mask and gloves should be worn when handling the powder, and gloves when using the solution.
4. Upon contact with acids, GuSCN can produce a toxic gas (HCN). GuSCN-containing waste can be collected into 10 *M* NaOH, in such an amount that the final concentration does not drop below 0.3 *M*.

5. If it is suspected that some silica has been carried over into the RNA, the sample can be centrifuged again for 2 min before the RNA is transferred to the reverse transcription mix
6. The sensitivity of the PCR was reported to be 3×10^5 viral particles by Zhang and Evans *(13)* and 3.5×10^3 virions in our hands.

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10

Lyssaviruses

Special Emphasis on Rabies Virus

Hervé Bourhy

1. Introduction

1.1. Importance of Laboratory Diagnosis

Lyssavirus infection, better known as rabies, in animals or in humans, is characterized by a clinical picture of viral encephalitis. According to the WHO estimations, it is responsible for more than 50,000 human deaths each year. The diagnosis of lyssavirus infection in animals, which may be responsible for human infections are of utmost importance: if positive, for starting or follow-up of postexposure rabies treatments, or if negative, for the nonprescription or interruption of the treatments. The diagnosis of lyssavirus infection in humans, despite the lack of antivirus treatment after the onset of the disease, eliminates the expense and discomfort of unnecessary investigative tests and inappropriate therapy, and encourages the early initiation of specific epidemiologic control measures such as maintenance of strict isolation precautions and education of employees. This helps to reduce significantly the number of postexposure prophylaxis in hospital personnel. However, the signs and symptoms of the rabies encephalitis may vary greatly according to species and individuals. The clinical differential diagnosis with other viral encephalitis may often be difficult if not impossible. Therefore, laboratory methods are necessary to confirm lyssavirus infection. Nevertheless, some requirements are necessary to ascertain the reliability of the laboratory results: good quality of specimens, good transport conditions to the laboratory, the short delay necessary to obtain the results, and the quick communication of the results.

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1.2. Relationship Between Structure and Diagnosis of Lyssaviruses

The *Lyssavirus* genus is a member of the *Rhabdoviridae* family. It has been divided into 4 serotypes and 6 genotypes **(Table 1)**. The lyssavirus virion has a bullet shape (75 nm wide, 180 nm long) and consists of a helically wound nucleocapsid surrounded by a lipid-containing bilayer envelope of cellular origin. This envelope comprises two viral membrane proteins. the matrix protein (M) on the internal side (its location was shown to be inside the nucleocapsid in the vesicular somatitis virus) and the transmembrane glycoprotein (G) that protrudes to the outside. The nucleocapsid is comprised of the genomic ribonucleic acid (RNA) associated with three proteins: the nucleoprotein (N) closely linked to the RNA, the RNA-dependent RNA polymerase (L), and the phosphoprotein. The genomic RNA (about 12,000 nucleotides) is a linear, nonadenylated, unsegmented negative-stranded RNA *(1)*. During viral replication a large amount of viral nucleocapsids are produced but few of them bud; the rest accumulate in the cytoplasm of the infected cells where they form inclusions Lyssavirus detection in specimens can be performed by demonstrating the presence of infectious particles, the nucleocapsids inclusions, or the viral RNA in the infected tissue. Lyssavirus-specific antibodies do not always appear or may appear only near the end of the clinical phase in human serum or cerebrospinal fluid (CSF). Their detection will not be discussed in this chapter.

1.3. Safety Measures

Level 3 of biological safety is recommended for the handling of all potentially rabies-infected material. All procedures that could generate aerosols, such as, grinding tissues, must be performed in a biosafety hood. As the opening of the skulls of animals is difficult to perform under a safety hood or within an isolator, the technician performing this work must be protected with a face mask, goggles, gloves, and apron. Because the pathogenicity for man of viruses of the genotypes 2, 3, 4, 5, and 6 is not yet well-known, all work with specimens suspected of containing these viruses must be performed within a biosafety hood. Pre-exposure vaccination against rabies is compulsory for all members of the laboratory.

1.4. Specimens for Diagnosis

Specimens collected *intra vitam* may often result in negative results, which do not exclude a diagnosis of rabies. It is therefore recommended to collect as many different specimens as possible and to repeat the tests *(2)*. The following specimens must be collected and shipped to the laboratory under refrigeration: saliva collected by aspiration or by using a cotton swab, CSF, skin biopsies

Table 1
***Rhabdoviridae* Family–*Lyssavirus* Genus**

Genotype	Serotype	Geographic distribution	Animal species
Rabies	1	Worldwide, except Australia, British Islands, Ireland, New Zealand, Japan, Antarticatica, Scandinavia, Hawaii	Humans, wild and domestic carnivores and herbivors, bats
Lagos-bat	2	Nigeria, Central African Republic, South Africa, Zimbabwe, Guinea, Senegal, Ethiopia	Frugivorous bats, cats, dogs
Mokola	3	Nigeria, Central African Republic, Zimbabwe, Cameroon, Ethiopia	Humans, shrews, cats, dogs rodents
Duvenhage	4	South Africa, Zimbabwe	Humans, insectivorous bats
EBL 1	?	European countries	Humans, insectivorous bats *(genus Eptesicus, Pipistrellus)*
EBL 2	?	European countries	Humans, insectivorous bats *(genus Myotis)*

(nape of the neck), and corneal smears performed by touching the cornea with a microscopic glass slide. The slides are marked, air-dried, and individually wrapped in aluminium foil. In most cases, the laboratory diagnosis of rabies is performed on brain specimens. Some parts of the brain contain a greater amount of specific inclusions than others, particularly the Ammon's horn (hippocampus) and the brain stem. A piece of cortex is also collected. In some circumstances, it may be difficult or impossible to open the skull for collection of the brain: epizootiological surveys on a large number of animals; collection of animal specimens in primitive field conditions; or when the patient necropsy is impossible because of religious or customary reasons. Simple techniques have been devised to face these difficulties, based on the collection of a brain cylinder by means of the introduction of a plastic tube inside the skull through the occipital foramen *(3)* or the posterior wall of the eye socket (perforated with a trocar) *(4)*.

1.5. Principle of the Techniques

Two efficient laboratory techniques have been used for a long time in the diagnosis of rabies. The older one is the immunofluorescent technique or fluo-

rescent antibody test (FAT) In the rabies virus-infected cells the specific inclusions are made of nucleocapsids Polyclonal antibodies from laboratory animals (rabbits) immunized with purified nucleocapsids will, after conjugation to fluorescein isothiocyanate, specifically stain these inclusions in a direct immunofluorescence test. Antinucleocapsid murine monoclonal antibodies may also be used in a similar manner, or in an indirect immunofluorescence reaction This technique was first developed by Goldwasser et al. *(5)* more than 35 yr ago and has since been improved *(6,7)*. It is used as the reference technique for postmortem examination. The second technique is virus isolation in cell culture or rabies tissue-culture infection test (RTCIT). It may be useful for confirmation of the results of the antigen detection techniques or for further identification and characterization of the isolate. Neuroblastoma cell lines are particularly susceptible to rabies virus, which produce specific inclusions detectable by FAT in less than 20 h postinoculation *(8,9)*. These two techniques will not be detailed here. This does not mean that they should no longer be used. According to our experience they remain the most useful methods for rabies diagnosis. However, in the last 10 yr some interesting developments in molecular biology were applied to the diagnosis of rabies.

Two immunoassays have been designed for the diagnosis of rabies by detection of the rabies nucleocapsid in brain specimens. In the first one, the RREID (rapid rabies enzyme immunodiagnosis), the microplates used for the detection of rabies antigen are sensitized with antilyssavirus nucleocapsid of genotype 1 IgG, diluted in carbonate buffer *(10)*. Specimens, homogenized in buffer solution or tissue culture medium, are clarified by centrifugation and incubated in microplates. Captured antigens are subsequently identified by adding peroxidase-conjugated antirabies nucleocapsid globulin and the chromogen substrate. Comparison, for a given specimen, of the coloration observed with naked eye, or of the absorbance measured with a photometer, with the positive and negative controls permits conclusions to be made about the presence or absence of rabies antigen *(9,11)*. The minimum amount of nucleocapsid antigen of serotype 1 detectable is 0.8–1.0 ng/mL. The sensitivity of the immunoenzymatic test may also be extended in order to make the diagnosis of genotypes of lyssaviruses other than genotype 1. With antibodies raised against Mokola virus (genotype 3), EBL1 (genotype 5) and the vaccinal strain PV (genotype 1), used for the immunocapture of the antigens (coating of the microplates) and for the antigen detection (conjugated to biotin), it is possible to reach a threshold of detection of 0.1–0.2 ng/mL whatever the lyssavirus concerned. This most recently developed enzyme immunoassay (RREID-lyssa) *(12)* will also be presented.

The detection of the viral nucleic acids extracted from the specimen may be achieved directly by molecular hybridization with nucleic acids complementary to the selected genomic region ***(13)***. However, this method is not sensitive. It should preferably be used after a preliminary amplification step. For this, it is necessary to first perform the reverse transcription of the viral RNA into complementary DNA (cDNA) ***(14)***, then to amplify this cDNA by the polymerase chain reaction (PCR) ***(15)***. The nucleic acids are transferred onto a filter and hybridized using the complementary probe. The revelation system is enzymatic (cold probe). A positive reaction indicates the presence of nucleic acids of viral origin complementary to the sequence used as a probe. The quality of the specimen is of greatest importance when performing the nucleic acids detection techniques. The rapid techniques for collection of specimens are strongly recommended because they prevent contamination by exogenous nucleic acid material.

The amplification of viral cDNA by PCR may also be very useful for the characterization of isolates among the *Lyssavirus* genus in epidemiological studies (in addition to the monoclonal antibody studies) ***(16,17)***. This technique can be easily expanded to allow a precise typing method based on restriction fragment length polymorphism or on nucleotide sequences. Primers allowing the amplification of the nucleoprotein, glycoprotein, and the respective N–M1 and G–L adjacent noncoding sequences have been defined ***(18)***. Considering that the N gene was the most conserved in the lyssaviruses (except some domains of the L protein gene) and that the sequence data concerning this gene were the most exhaustive ***(19)***, it has been chosen for the diagnostic applications of PCR. It should be noted that the technique presented here allows the amplification of a fragment of the N gene on the genomic RNA. This amplification was found to be more sensitive than that of the N mRNA. This may be due to the fact that the encapsidated genomic RNAs are more stable than the nonencapsidated mRNAs. This technique is currently under evaluation and will come into routine use for the *intra vitam* diagnosis after minor improvements have been made.

2. Material

2.1. Enzyme Immunoassays

2.1.1. Buffers and Reagents

1. 0.05 *M* Carbonate buffer, pH 9 6
2. Distilled water
3 Sodium hypochlorite.
4 BSA-Sucrose solution· 0 3% Bovine serum albumin 0 3 and 5% sucrose in 0.05 *M* carbonate buffer, pH 9 6.

5 Washing solution: phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween-20.
6 Immunoglobulins directed against the nucleocapsid of Mokola virus (genotype 3), EBL1 (genotype 5), and PV strain (genotype 1).
7 Immunoglobulins directed against the nucleocapsid of the PV strain (genotype 1), Mokola virus (genotype 3), and EBL1 (genotype 5) conjugated to biotin.
8 Immunoglobulins directed against the nucleocapsid of the PV strain (genotype 1) conjugated to peroxidase
9 Peroxidase conjugated to streptavidin
10 Flat-bottom 96-well microplates
11. Negative control antigen (10% normal mouse brain suspension, clarified by centrifugation at 5000*g* for 30 min).
12. Positive control antigen (10% brain suspension of rabies- [CVS] infected mice, clarified by centrifugation at 5000*g* during 30 min, and inactivated by adding 1/4000 β-propiolactone)
13. Chromogen substrate solution: 0.05 *M* citrate buffer solution, pH 6.0 containing 0.03% hydrogen peroxide.
14 Chromogenic agent. o-phenylenediamine dissolved in chromogen substrate solution (2 mg/mL)
15. Stop solution. 4 *N* sulfuric acid solution

2.1.2. Equipment

1 Automatic or semiautomatic, adjustable or preset pipets or multichannel pipets, able to measure and dispense 50 and 200 µL.
2 Graduated cylinders. 25, 100, 1000 mL.
3 Broek-type glass homogenizers or glass rods adapted to the centrifuge tubes.
4 Biohazardous waste containers.
5. Water bath thermostatically set at 37°C or heating plate
6. Centrifuge.
7 Microplate washer
8. Microplate reader equipped with a 492-nm filter
9. Paper towels or blotting paper.

2.2. Detection of Nucleic Acids After Gene Amplification (PCR)

2.2.1. Reagents

1 TE buffer: 10 m*M* Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0
2. Chloroform.
3 Phenol-hydroxyquinoline, pH 4.0, (12.1)-chloroform (v/v)
4 Absolute ethanol
5. 70% Ethanol
6. 3 *M* Sodium acetate, pH 5.2.
7. Pyrolyzed water
8. 20X SSC buffer: 3 *M* NaCl, 0.3 *M* sodium citrate pH 7.0, SDS 0.1%.

9. Nylon transfer membrane Hybond (Amersham, Buckinghamshire, UK), Saran wrap.
10. Two sets of specific oligonucleotide primers.
    N1 (+) sense: (587) 5'-TTTGAGACAGCCCCTTTTG-3' (605) and
    N2 (–) sense: (1029) 5'-CCCATATAGCATCCTAC-3' (1013),
    N7 (+) sense: (55) 5'-ATGTAACACCTCTACAATG-3' (73) and
    N8 (–) sense: (1585) 5'-AGTTTCTTCAGCCATCTC-3' (1568)
11. Proteinase K and buffer: 0 2 *M* Tris-HCl, pH 7.5, 25 m*M* EDTA, 0.3 *M* NaCl, 2% SDS RNasin 40 U/μL (Promega-Biotec, Madison, WI).
12. Superscript reverse transcriptase (200 U/μL) (Gibco-BRL, Cergy Pontoise, France) and buffer: 250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 50 m*M* dithiothreitol (DTT), 15 m*M* $MgCl_2$, DNA.
13. *Taq* polymerase (Gibco).
14. Solutions (10 m*M*) of nucleotide triphosphates: ATP, GTP, CTP, TTP
15. Washing buffer. 0.1 *M* maleic acid, 0.15 *M* NaCl, pH 7 5, Tween-20 (0.3% w/v).
16. Blocking solution (10X) (Boehringer-Mannheim, Mannheim, Germany) 0.1 *M* maleic acid, 0 15 *M* NaCl pH 7.5, blocking reagent (10% w/v).
17 Detection buffer: 0.1 *M* Tris-HCl, 0.1 *M* NaCl, 50 m*M* $MgCl_2$, pH 9.5, anti-DIG-AP (Boehringer-Mannheim)
18 DIG dUTP 1 m*M* (Boehringer-Mannheim).
19. CSPD (Boehringer-Mannheim).
20 DNA labeling and detection kit, nonradioactive (Boehringer-Mannheim)
21 Total RNA from infected (positive control) and uninfected (negative control) mouse brains

2.2.2. Materials

1. 1.5-mL Polypropylene Eppendorf microtubes and pestles.
2. Variable Gilson pipets with sterile tips.
3 Tips with filter (Dutscher, Brumath, France).
4. Refrigerated microcentrifuge 14,000*g*.
5 UV transilluminator (312 nm).
6. Water bath 37°C.
7 PCR apparatus
8. Multi wells filtration unit (Biodot Apparatus, BioRad).
9. X-ray film (X-OMAT AR Kodak), radiography cassette.

3. Methods

3.1. Enzyme Immunoassays

3.1.1. Sensitization of Microplates for the Detection of Rabies Nucleocapsids

1 Adjust the antinucleocapsid IgG solution to 5 μg/mL 0.05 *M* in carbonate buffer, pH 9.6.
2. Distribute 200 μL IgG solution in each well of the microplate (about 1 0 μg IgG/well).

3. Incubate at 37°C for 3 h, then overnight at 4°C
4. Eliminate and fill the wells with the BSA-sucrose solution and incubate for 30 min at 37°C
5 Wash the wells five times with the PBS-Tween solution
6 Keep the plates at –20°C or store them in vacuum-sealed pouches

3.1.2. Immunocapture

1. RREID and RREID-lyssa are performed on the supernatants of brain suspensions from suspected animals. For each brain specimen to be tested, small pieces from different areas (brain stem, hippocampus, cerebellum, cortex) are collected A 30% suspension is made in the PBS washing solution or in tissue culture medium (if virus isolation in cell cultures is to be performed) This suspension is clarified by centrifugation at 5000*g* for 30 min and the supernatant is harvested
2 Wash the microplate (prepared as in **Subheading 3.1.1**) twice with the washing solution and dry by inverting on blotting paper.
3 Distribute 200 µL of each supernatant (positive and negative control, and specimens to be tested) in the wells of the plate, according to a carefully established distribution plan If reading of the reaction is to be done with an automatic plate-reader, the first well ("blank") should receive 200 µL of washing solution Cover the plate with an adhesive plate sealer.
4. Incubate the microplate at 37°C for 60 min.
5. Remove the plate sealer Aspirate the contents of each well into a flask containing sodium hypochlorite solution, fill each well with washing solution, then aspirate Repeat up to a total of five washings, then dry the plate by inverting it on blotting paper (an automatic plate-washer may be used)

3.1.3 Rapid Rabies Enzymes Immunodiagnosis (RREID)

1 Distribute 200 ml of conjugate solution into each well. Seal with a fresh adhesive plate-sealer and incubate the microplate at 37°C for 60 min.
2 Remove the plate sealer, aspirate the contents of each well, and wash 6 times, as above Dry the plate on a blotting paper, as above.
3. Prepare extemporaneously the chromogenic agent solution, avoiding exposure to direct light. Distribute 200 µL in each well and incubate for 30 min at room temperature (18–25°C), in the dark
4 Stop the reaction by adding to each well 50 µL of stopping solution, with the same distribution sequence as for the previous reagent.

3.1.4. Rapid Rabies Enzymes Immunodiagnosis-Lyssa (RREID-Lyssa)

1 Distribute in each well 200 µL antinucleocapsid globulin conjugated to biotin and incubate at 37°C for 60 min
2 Aspirate the biotin-conjugate and wash 6 times.
3 Distribute in each well 200 µL peroxidase-streptavidin conjugate and incubate at 37°C for 30 min.

4 Aspirate the peroxidase-streptavidin conjugate and wash 6 times
5. Into each well, distribute 200 µL chromogen substrate, freshly prepared, and incubate at room temperature in the dark for 5–10 min.
6. Stop the reaction by adding to each well 50 µL of stop solution

3.1.5. Results

1 Naked eye reading. The positive control well is strongly orange-yellow, the negative control is absolutely colorless. Any specimen giving a more or less intense orange-yellow color is considered as positive. This naked eye reading is often sufficient to make a diagnosis.
2 Photometer reading Carefully wipe the plate bottom Read with a 492-nm filter The positive control must give an optical density (OD) superior to 1.5 U and the negative control an OD inferior to 0.1 U. The cutoff value (COV) is established at· OD of negative control + 0 08 and 0.2 OD U for RREID and RREID-lyssa, respectively Any specimen is considered as positive if its OD is equal or greater than the COV

3.2. Detection of Lyssavirus Nucleic Acids After Gene Amplification (PCR)

3.2.1. Extraction of Total RNA

1 Put 0.5 cm^3 of the specimen (e g , animal brain) or 200 µL biological fluid (saliva or cerebrospinal fluid) into a 1 5-mL plastic tube; add 200 µL proteinase K (200 ng/mL) and 200 µL Proteinase K buffer Incubate for 2 h at 37°C.
2. Mix with an equal volume of phenol-chloroform. Centrifuge at 4000*g* for 10 min.
3. Harvest the upper (aqueous) phase and mix it with an equal volume of chloroform
4. Harvest the upper (aqueous) phase, add 3 *M* sodium acetate (to a final concentration of 0.3 *M*) and mix with 2.5 vol absolute ethanol
5 Keep 30 min at –20°C, centrifuge at 4°C at 14,000*g* for 30 min
6. Drain the tubes and rinse three times with 70% ethanol
7. Drain the tubes and resuspend the pellets in 100 µL of pyrolyzed water

3.2.2. Reverse Transcription

1. Hybridize 1 µL primer A1 (100 ng/µL) with 2 µL of RNA (1 µg) per microtube at 65°C for 3 min and chill on ice.
2. Add 4 µL of a mix containing each nucleotide triphosphate (10 mmol); 0.6 µL RNasin (15 U); 1 µL DTT, 2 µL of Superscript reverse transcriptase buffer, 0.5 µL of Superscript reverse transcriptase (100 U). Incubate at 37°C for 90 min
3. Dilute 10 times in TE buffer, store the tube at –20°C.

3.2.3. Polymerase Chain Reaction

1. Dilute 5 µL of RNA/cDNA hybrid in 50 µL containing 1 µ*M* N7 and N8 primers, 200 µ*M* each nucleotide triphosphate, 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, and 2 U of *Taq* polymerase. Incorporate a negative water control where pyrolyzed water replaces the RNA/cDNA hybrids.

2 Cover with 100 µL mineral oil.
3. Set the PCR apparatus (each primer set has its own adjustments to obtain the optimal working conditions); the following program is given as an example
   a 1 cycle: 94°C, 60 s; 48°C, 90 s; 72°C, 90 s;
   b 30 cycles: 94°C, 30 s, 48°C, 60 s, 72°C, 60 s, and
   c. 1 cycle· 94°C, 30 s; 48°C, 90 s, 72°C, 5 min.

3.2.4. Fixation of DNA onto Filter

1. Place the nylon filter in the multiwell filtration unit and orient it.
2. Dilute 5 µL DNA solution into 95 µL pyrolyzed water, denature 3 min at 95°C, and chill on ice.
3 Quickly distribute the samples in the wells.
4 Let the membrane dry at room temperature.
5 Irradiate the membrane for 3 min on a UV transilluminator, protecting it with Saran wrap

3.2.5. DIG Labeling of Complementary Probe

1 Dilute 2 µL N7–N8 PCR-amplified product in 50 µL containing 1 µ*M* N1 and N2 primers; dATP, dCTP, dGTP (200 µ*M* each), 26 µM dTTP, 13 µ*M* DIG dUTP, 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1 5 m*M* $MgCl_2$, and 2 U of *Taq* polymerase
2. Cover with 100 µL mineral oil.
3 Set the PCR apparatus (same working conditions as in **Subheading 3.2.3.**)
4 Control the positive reaction by gel electrophoresis of the product
5. Store the probe at –20°C

3.2.6. Hybridization with a Nonradioactive Probe

1. Incubate the membrane under agitation for 1 h in 20 mL hybridization buffer at 68°C.
2 Eliminate the hybridization buffer.
3 Incubate the membrane under agitation at 68°C for 16 h in 10 mL of hybridization buffer containing 5 µL digoxigenin-labeled DNA probe denatured for 3 min at 95°C
4. Wash the membrane twice for 5 min at room temperature with 50 mL 2X SSC/SDS 0 1%
5. Wash the membrane twice for 15 min at 68°C with 50 ml 0.1X SSC/SDS 0.1%

3.2.7. Detection by Chemiluminescence

1. Wash the membrane for 1 min at room temperature with 50 mL washing buffer.
2 Incubate the membrane for 30 min at room temperature in 100 mL blocking solution
3 Incubate the membrane for 30 min at room temperature in 20 mL detection buffer containing 76 mU/mL anti-DIG-AP conjugate.

4. Wash twice for 15 min with 100 mL washing buffer.
5 Equilibrate 2 min in 20 mL detection buffer.
6 Incubate the membrane for 5 min in 10 mL detection solution containing Lumigen PPD (R) 0.1 mg/mL.
7. Place the membrane between two transparency sheets for laser printer and press to eliminate the excess solution and air bubbles.
8 Put the membrane and transparency sheets in a hybridization bag and seal it.
9. Incubate for 15 min at 37°C to enhance the luminescent reaction.
10 Expose the membrane to an X-ray film in a radiography cassette at room temperature. Develop the film after 20 min.

3.2.8. Results

Dark spots on the film correspond to positive reactions, while the film remains translucent in front of the noninfected samples. Check that the positive control gives a definite reaction and the negative and pyrolyzed water controls give no reaction. Then register the results for the specimens under test.

4. Notes

4.1. Enzyme Immunoassays

It is important to check that the OD of the controls are correct; otherwise the test is not valid. The RREID-lyssa is far more sensitive than the RREID to the quality of the washings. Respect the number of washings. If the background intensity level of OD in the test is high, then increase the number and the efficiency of the washings.

4.2. Detection of Lyssavirus Nucleic Acids After Gene Amplification

Gloves should be worn whenever samples and reagents are handled. To avoid cross-contamination between specimens, the organization of the laboratory should fulfill the requirements of the PCR protocols.

The RNA, cDNA, and DIG-labeled probe should be stored at –20°C. The protocol given for the synthesis of the labeled probe was not optimized and could sometimes lead to weak amplifications. The N7–N8 primer set are suitable for the diagnosis of all lyssaviruses so far studied *(19,20)*. This confirms the results of Kwok et al. *(21)*, showing in other models that the primers may have few differences with the sequences to be detected, without impairing the PCR results. However, results obtained with some isolates, particularly those not belonging to the genotype 1, may be weak. This problem will almost certainly be resolved by further analysis of the increasing sequence data bank concerning N gene of wild isolates and by finding more conserved sequences

suitable for the design of new primer sets. The direct observation of the amplified product on agarose gel is not specific and sensitive enough. Nonspecific bands are sometimes indistinguishable from specific ones by their molecular weights. Furthermore, some weak reactions may be invisible. The threshold of detection is dependent on the size of the probe. You can lower it by using a larger probe than the one described here. This is particularly important for the *intra vitam* examination.

5. Conclusion

The respective specificity and sensitivity of the FAT, the RREID, and RTCIT have been evaluated during several consecutive years of routine practice in the Rabies Diagnosis Laboratory of the Pasteur Institute, Paris Taking the FAT as the reference technique, these results confirm the rapidity and specificity of RREID and its excellent sensitivity for lyssaviruses of geno-type 1 ***(9,11,22–24)***. Furthermore, RREID may easily be used in connection with the newly proposed techniques for the rapid collection of brain specimens. We recommend the use of the RREID in laboratories that start a rabies diagnosis activity ***(2)***. Furthermore, the development of RREID-lyssa has improved the performances of the immunoenzymatic detection of rabies antigen. The modified enzyme-immunodiagnosis test, RREID-lyssa, is more sensitive than the RREID (0.1–0.2 ng/mL of nucleocapsid whatever the lyssavirus concerned against 0.8–1.0 ng/mL for genotype 1 lyssaviruses and 10–20 ng/mL for other genotypes, respectively) ***(12)***.

The newly developed molecular virology techniques have not yet been applied to the routine laboratory diagnosis of rabies. Their comparative specificity and sensitivity have only been evaluated for a limited number of post-mortem specimens and further studies are necessary ***(15,25)***. About 30 h are necessary to perform rabies diagnosis by PCR. This is longer than the other laboratory techniques, however this technique allows easy and rapid typing of the isolate. After amplification, the nucleoprotein gene may be characterized in a very specific manner by employing a reduced panel of routinely used restriction enzymes. After migration on agarose gel of the fragments resulting from the restriction digest, it is easy to distinguish between the lyssavirus isolates ***(18,26)***. Another very interesting application of this technique concerns the *intra vitam* diagnosis of rabies. The detection of rabies virus in the saliva and in the cerebrospinal fluid of infected humans and animals has been investigated, and the threshold of detection determined in our lab was less than 10 virus particles. This certainly provides an important perspective for an early diagnosis of rabies.

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11

Methods for Detecting Antimeasles, Mumps, and Rubella Virus Antibodies

Ilkka Julkunen, Irja Davidkin, and Christian Oker-Blom

1. Introduction

Measles, mumps, and rubella (MMR) virus infections are common during childhood throughout the world. Measles and mumps viruses belong to the *Paramyxoviridae* family with an RNA genome of negative polarity and a similar overall viral structure at the molecular level *(1,2)*. Rubella virus is a member of the *Togaviridae* family containing a positive-stranded RNA genome encoding both nonstructural and structural viral proteins *(3)*. Schematic representations of the MMR viruses, their genome structures and structural proteins are shown in **Fig. 1**. Measles virus infection is characterized by a generalized exanthema, fever, and occasionally also central nervous system (CNS) symptoms. Measles virus is highly contagious and causes high morbidity. Typically mumps virus causes parotitis, but occasionally complications, such as, meningitis (encephalitis), orchitis, pancreatitis, and some other more rare symptoms, are seen. The symptoms of rubella virus infections are usually very mild with generalized maculopapular rash and low fever. Often the infection goes unrecognized. A special danger associated with rubella virus infections is its ability to cause fetal infection and subsequently severe birth defects known as congenital rubella syndrome.

The application of MMR vaccination campaigns has greatly reduced the incidence of these diseases among vaccinated children as well as in the community as a whole *(4)*. In spite of an efficient MMR vaccine, the need for specific and sensitive diagnostic methods for detecting antibodies against these viruses is, however, utterly important. This is because vaccination coverage may be low, failures may exist, and the vaccine-induced immunity wanes within time *(5)*. Therefore, reliable methods to differentiate measles and rubella

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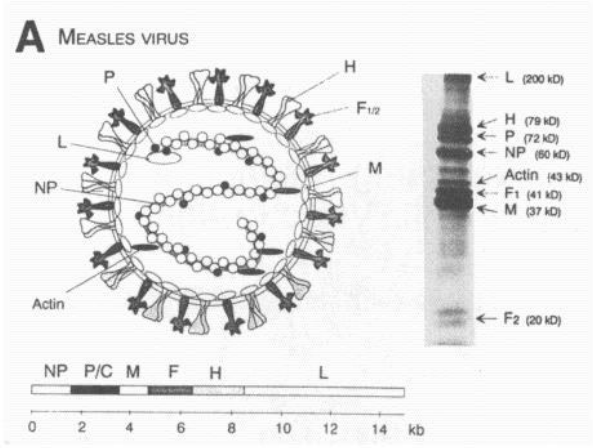
A MEASLES VIRUS
P
H
F1/2
L
M
NP
Actin
L (200 kD)
H (79 kD)
P (72 kD)
NP (60 kD)
Actin (43 kD)
F1 (41 kD)
M (37 kD)
F2 (20 kD)
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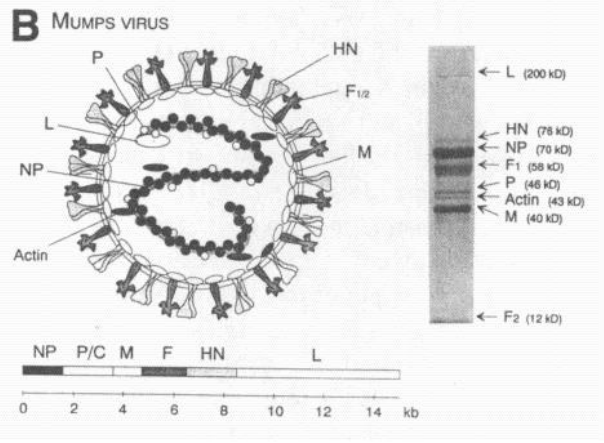
B MUMPS VIRUS
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HN
F1/2
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M
NP
Actin
L (200 kD)
HN (78 kD)
NP (70 kD)
F1 (58 kD)
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M (40 kD)
F2 (12 kD)
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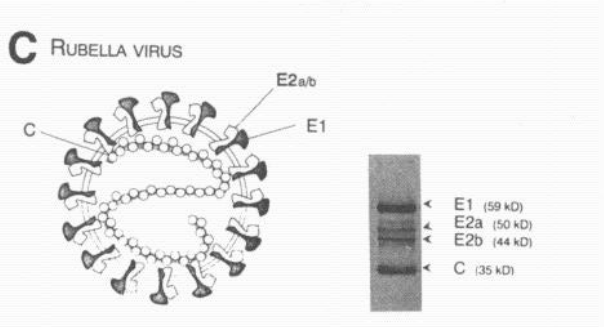
C RUBELLA VIRUS
E2a/b
C
E1
E1 (59 kD)
E2a (50 kD)
E2b (44 kD)
C (35 kD)

Fig. 1.

infections from other exanthemas or to differentiate mumps infection from parotitis and central nervous system infections caused by other infectious agents are needed. In addition, methods to estimate vaccine-induced anti MMR antibody levels have to be sensitive enough to evaluate the potential need for a revaccination. Here, we describe methods for both diagnosing acute viral infections caused by measles, mumps or rubella viruses as well as provide alternatives for estimating long-term immunity often seen years after initial MMR vaccination.

2. Materials

2.1. Equipment

1. Cell culture facilities and incubators.
2. Roux bottles, spin culture flasks, plastic cell culture vials
3. Ultracentrifuge
4. Mini gel electrophoresis and electrotransfer apparatuses (e.g., Hoefer Scientific Instruments, San Francisco, CA or BioRad Laboratories, Hercules, CA)
5. Columns and other chromatography equipment.
6. Multichannel photometer (Titertek, Multiscan, Labsystems, Helsinki, Finland)

2.2. Reagents

1. Host cells for viral cultures (Vero, *Spodoptera frugiperda, Trichoplusia ni,* American Type Culture Collection, Rockville, MD)
2. Vero cell culture media, Minimal Essential Media (MEM) (Gibco/BRL, Life Technologies, Paisley, UK)
3. Insect cell media, TNM-FH (Gibco/BRL).
4. Fetal calf serum, inactivated.
5. Antibiotics for cell culture (penicillin, streptomycin, amphotericin B).
6. Monkey red blood cells
7. Embryonated hen's eggs
8. Flat-bottomed (Polysorb; Nunc, Roskilde, Denmark) and U-bottomed 96-well plates (Nunc).

Fig. 1. *(previous page)* Schematic representation of measles, mumps, and rubella viruses, their genome structure, and SDS-PAGE analysis of viral structural protein. **(A)** Measles virus and autoradiography of metabolically labeled virus-infected Vero cells (kindly provided by Dr R. Vainionpaa). **(B)** Mumps virus and Coomassie blue-stained gel of purified mumps viruses (from **ref. 5**). **(C)** Rubella virus and immunoblot of purified rubella virus stained with rabbit antirubella virus antibodies (Oker-Blom et al , unpublished results) L, viral polymerase; HN, hemagglutinin-neuraminidase; H, hemagglutinin; P, phosphoprotein, polymerase, NP, nucleoprotein; F. fusion protein; F1/2, proteolytically cleaved F protein; M, matrix protein; E1, E2, and C, envelope glycoproteins and nucleocapsid protein of rubella virus, respectively Gene structures as descri-bed (**refs. *1–3***).

9. Phosphate-buffered saline (PBS): 10 m*M* $NaHPO_4$, pH 7 4, 140 m*M* NaCl
10 Tris-buffered saline (TN), 10 m*M* Tris-HCl, pH 7.4, 140 mM NaCl.
11. Sucrose solutions, 30% and 50% (w/w) in TN.
12. Concanavalin A Sepharose 4B gel (Pharmacia, Uppsala, Sweden)
13 Concanavalin A Sepharose binding buffer, 0.5 *M* NaCl, 20 m*M* Tris-HCl, pH 7.4, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$.
14. Phenylmethylsulfonylfluoride (PMSF) (Boehringer Mannheim, Mannheim Germany)
15 Saturated ammonium sulphate in H_2O
16 Gelatin or bovine serum albumin (BSA) (Sigma, St. Louis, MO)
17 Peroxidase- or alkaline phosphatase-conjugated goat or rabbit antihuman IgG and IgM immunoglobulins (Sigma, Bio-Rad, Dako)
18 Enzyme substrate for immunoblotting, 3-amino-9-ethylcarbazole (for peroxidase, Sigma).
19. Enzyme substrate for alkaline phosphatase in enzyme-linked immunosorbent assay, *o*-paranitrophenyl phosphate (Sigma).

3. Methods

3.1. Antimeasles Virus Antibody-Specific Methods

Several diagnostic methods can be applied to determine measles virus-specific antibody responses including neutralization (NT), hemagglutination inhibition (HAI), and complement fixation tests *(6)*, as well as many modifications of ELISA methods. Below we describe antimeasles virus-specific NT and HAI tests, whereas a standard ELISA is described for antimumps virus antibodies. ELISA is well adjustable to antimeasles antibody determinations as long as pure (or crude) viral antigens are available. All these methods can be used for diagnosing acute measles virus infections or measuring vaccine-induced immunity. The complement fixation test is suitable for diagnosing a disease but it is too insensitive for immunity surveys.

3.1.1. Neutralization Test (NT)

1 To produce virus stock infect Vero cells in Roux or other large cell culture vials with a suitable dilution (e.g., 1·100) of reference tissue culture-adapted (e g , Edmonston strain) measles virus. Add 10–20 mL virus stock and after 1–2 h adsorption remove the virus inoculum.
2. Add 40 mL MEM supplemented with 2% fetal or newborn calf serum.
3. Grow the monolayers until a full cytopathic effect (CPE) is seen, usually after 7–10 d.
4 Collect the supernatant and clarify by low-speed centrifugation (20 min, 400*g*) to remove cell debris.
5. Store the viral antigen at –70°C in small aliquots.
6. To determine the virus titer, make serial dilutions of the stock virus and infect confluent monolayers of Vero cells, e.g., in 96-well plates.
7. Add 0.1 mL/well and grow in a humidified chamber at 37°C.

8. Cultures are observed daily for CPE, but usually 10–14 d are needed for full CPE. The log 10 tissue culture infections dose 50 ($TCID_{50}$) titer of the virus stock is determined as the mean of last dilution giving full CPE and the first dilution lacking any CPE.
9. To perform antimeasles NT incubate serial twofold (starting at 1:2 or 1.4) dilutions (0.1 mL) of the tested serum with a dilution corresponding to 100 $TCID_{50}$/ 0.1 mL of stock virus (0.1 mL) at 4°C for 1 h.
10. Add serum-virus mixture to cells and incubate for 10–14 d. The last serum dilution preventing CPE is considered as the neutralizing antibody titer. Include infectivity controls (100, 10, and 1 $TICD_{50}$ in 0.2 mL) and a standard positive serum in each series. A modified shorter incubation assay, e.g , 7-d assay, can also be used, but the virus titer has to be determined accordingly In acute measles virus infections, neutralizing antibody levels may reach several thousands and postvaccination sera may also be relatively high (1:32–1 256)

3.1.2. Hemagglutination Inhibition (HAI) Test

1. For HAI tests the cell culture virus preparation described above may be used, but higher antigen titers can be obtained from virus-infected cells
2 Collect measles virus-infected cells 1–2 d after full CPE, resuspend them in a small vol of PBS, and rapidly freeze the cells on dry ice. Thaw the cells and incubate at 50°C for 30 min to inactivate the virus
3 Remove cell debris by centrifugation for 20 min at 10,000*g*, collect the supernatant and store in small aliquots at –20°C or preferentially at –70°C
4. To titrate the viral antigen make serial twofold dilutions in PBS in U-shaped microtiter plates, resulting in a vol of 0.025 mL well. To each well add 0.025 mL of 0.5% monkey red blood cells in PBS, shake, and incubate for 1 h at 37°C. The highest dilution of antigen giving full hemagglutination is the end-point titer and is considered to contain 1 HA unit.
5. The tested serum specimens should be pretreated with 1.5% (final concentration) monkey red blood cells (RBC) in 1:4 serum dilution in PBS for 60 min (or overnight if convenient) on ice followed by centrifugation for few seconds in an Eppendorf microfuge. Collect the supernatant.
6. To perform HAI test, make serial dilutions of serum specimens in PBS starting at 1·8 dilution (or lower if immune status is analyzed)
7 Add 4 HA units of measles antigen in 0.0.25 mL followed by 0.05 mL of 0 5% monkey RBC.
8. Shake the mixture and incubate at 37°C for 1 h. The titers are determined as the last serum dilution giving full HAI. Controls should include lowest serum dilution without antigen and with control antigen (uninfected cell extract), which should be devoid of HA.

To diagnose an acute measles virus infection a fourfold or higher increase in paired serum specimens (first specimen taken at the onset of the disease and the second specimen 10–14 d later) is considered diagnostic. Both of the

described methods are specific for antimeasles virus antibodies and therefore any detectable antibody level is an indication of immunity (past or vaccine-induced). ELISA methods (see **Subheading 3.2.2.**) with either purified or partially purified measles virus antigens are also sensitive and specific for antimeasles virus antibodies. In ELISA measles virus-specific antibodies can be determined in various immunoglobulin classes (IgG, IgM, and IgA) as well as in immunoglobulin subclasses (IgG1–4).

3.2. Antimumps Virus Antibody-Specific Methods

3.2.1. Mumps Virus Antigen

Mumps virus can be grown either in cell culture (Vero cells) or in embryonated hen's eggs, which give the highest yields.

1. Infect 8-d-old embryonated eggs with a low dilution (1 1000–1:100,000) of mumps stock virus (egg-adapted Enders strain) Allantoic fluid is collected 5 d postinfection and clarified by low-speed centrifugation (Sorwall GSA rotor for 20 min at 5000*g*)
2. Concentrate allantoic fluid 5- to 10-fold by using, e.g , a hollow-fiber liquid concentrator (Amicon).
3. Purify viruses by centrifugation (SW28 rotor for 1.5 h at 120,000*g* at +4°C) between 30% (w/w; 5 mL) and 50% (2 mL) sucrose cushions.
4. Collect the visible virus band light gray band) between the sucrose cushions and dilute 1.3 in TN
5. Further handling of the virus (5a or 5b):
   a. Concentrate virus by pelleting (1 5 h, 120,000*g*) through a 5-mL 30% sucrose cushion. Resuspend the virus pellet for 16 h in a small vol of TN, measure the protein concentration, and store the virus in suitable aliquots at –70°C Analyze a small aliquot (5–10 μg) 10% SDS-PAGE *(7)* and stain with Coomassie blue to visualize the purity of the virus (*see* **Fig. 1B**) This virus preparation is now suitable for ELISA.
   b. For further purification of the virus, take the diluted mumps virus from step 4 (avoid pelleting since it aggregates viruses) and concentrate it on top of a 50% sucrose cushion (in SW28 rotor, for 1 5 h at 120,000*g*). Collect the virus on top of the cushion, dilute 1:4 in TN, apply it (5–10 mL) on top of a 15–30% (20 mL, w/w) sucrose gradient, and centrifuge for a short time (20 min at 25,000 rpm). Virus is visualized as a abroad band in the middle of the gradient Aggregated virus is sedimented on the bottom (or on top of a 2-mL 50% sucrose cushion) and can be recovered by resuspension. Viruses can be concentrated by pelleting and the purity of the preparation is analyzed as in **step 5a**. Other viral components such as viral envelope glycoprotein micelles free of detergents and viral nucleocapsids can be purified according to methods described in detail previously *(8)* and they work well in antimumps virus-specific ELISAs.

3.2.2. Antimumps Antibody ELISA

1 Coat 96-well microtiter plates (Nunc Polysorb) with purified virus or viral antigens at 2 μg/mL (1–5 μg/mL depending on antigen) in PBS for 16 h at 22°C. Prior to dilution and coating it is advisable to sonicate the antigen for 30 s on ice, since mumps viruses have a tendency to form aggregates, which decreases their coating efficiency.
2 Saturate the wells with 0 5% gelatin in PBS for 2 h at room temperature and wash the plates at least twice with PBS + 0.1% Tween-20. The plates can be stored dry at +4°C for several mo (even 1 yr) without any detectable loss of activity in ELISA BSA can also be used for saturation but some individuals may have low antibody levels against BSA
3. Make serial serum dilutions (e g., 1.100–1·10,000) or a suitable single dilution (e.g., 1· 1000) of serum specimens in PBS + 0 2 % gelatin (or BSA) and incubate in viral antigen-coated wells (50 μL/well) for 16 h at +4°C or for 2 h at +22°C
4. After three washings with PBS-Tween-20 (2–5 min each), add alkaline phosphatase (AFOS) conjugated antihuman IgG or IgM antibodies (50 uL/well) and incubate for 2 h at 37°C Most commercial enzyme-conjugates are suitable for routine assays but optimal concentrations have to be determined within the laboratory using a set of positive and negative controls. The mean for negative controls should also be below absorbance values of 0.200–0.300 in the lowest serum dilution (*see* **Fig. 2**).
5 After three washings with PBS-Tween-20, and once with distilled water, the substrate *o*-paranitrophenyl phosphate (2 mg/mL, 100 μL/well) is added
6. After 30-min incubation with the substrate the enzyme reaction is stopped by adding 50 μL of 1 *M* NaOH to each well.
7. The intensity of the color reaction is measured with a multichannel photometer at 405 nm. Alternatively horse radish peroxidase (HRP)-conjugated anti-IgG or anti-IgM, *o*-phenylenediamine substrate (0.4 mg/mL + 0.2 mg/mL H_2O_2 in phosphate-citrate buffer, pH 5.5), and measurement at 492 nm can be used in HRP-based assays.

Antimumps antibody titers can be estimated either by analyzing serial serum dilutions or by using a single dilution system. In all assays it is, however, very important to have adequate negative and positive control sera. **Figure 2** describes the general idea of calculating antibody titers in the serial serum dilution method using antimumps virus-specific ELISA ***(5,8)*** mean absorbance levels and their standard deviations (mean + SD) in different serum dilutions of negative control sera (at least 10–20). The mean + 2 SD units is often chosen as the cutoff value in each serum dilution, but using at least 0.200–0.300 absorbance values as the minimum cutoff level. The absorbance values of positive specimens are plotted and virus-specific antibody titers are estimated as the intersection (cutting point) of the extrapolated dilution curve and the cutoff level (*see* **Fig. 2**). This can be performed manually of by the use of suitable

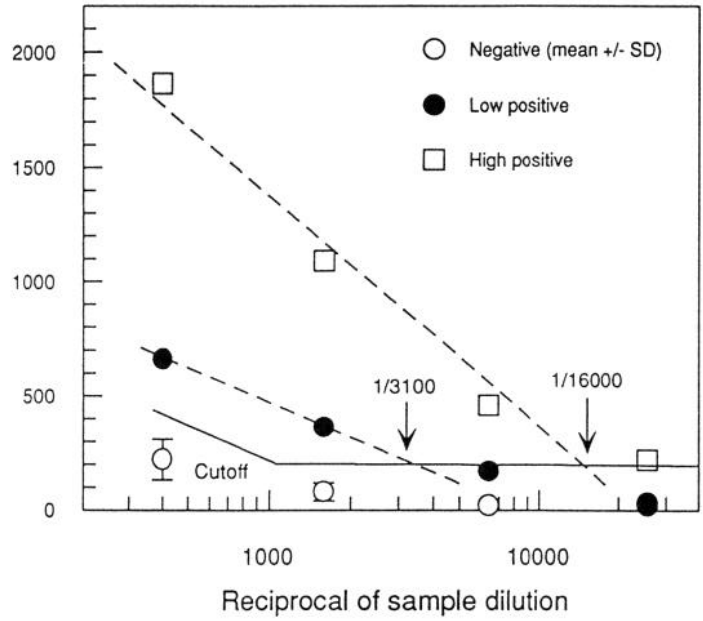

Fig. 2. Interpretative presentation of determination of anti-mumps virus antibody titers by ELISA. Negative control serum specimens (n =1 21) were tested in different serum dilutions in antimumps virus IgG antibody-specific ELISA using purified whole mumps viruses (**Fig. 1B**) as antigen. The arithmetic mean absorbance values ± 1 SD units of negative specimens are shown for each serum dilution. Values below the mean + 2 SD units or at least an absorbance value of 0.200 is considered negative and is shown as a solid line, which represents the cutoffvalue for each serum dilution. The absorbance values of low and high positive postvaccination serum specimens in different dilutions are shown as dots. The point where an extrapolated line drawn between the sample absorbance values and the background (cutoff) line cross (shown as arrows) represents the reciprocal of the antibody titer. In the figure they have been converted to titers of 1:3100 for low positive and 1:16,000 for high positive serum specimens.

computer programs. The internal variation in ELISA is usually low and twofold differences in antibody titers are significant, however, a fourfold increase (or decrease) can be considered diagnostic. The reliability of the assay is also increased, if antibodies against several different viral antigens are measured from the same samples. This eliminates simple dilution errors, since usually diagnostic rises should be seen only to one virus.

In one dilution assay the results are expressed either as absorbance values or as relative units enzyme immunoassay units (EIU). A change in absorbance value of at least 0.200 is often considered significant. In the EIU method strongly positive (P) and negative (N) specimens are included and the absor-

bance values of the samples are converted to units (sample EIU = 100 × [sample absorbance - N absorbance]/[P absorbance – N absorbance]). This calculation gives the value 100 for the positive control (P) and specimens range from 0 to >100. Changes in EIU values of at least 20 can be considered significant.

The standard ELISA described above for antimumps antibodies can basically be applied to any viral antigen, especially for measuring antibodies in the IgG class. IgM antibody determinations can be carried out similarly and can be used to diagnose recent or acute measles, mumps, or rubella infections. Recently, capture IgM *(9)* and antibody avidity *(10)* assays have been described, but these assays are more difficult to standardize and they often require some special reagents, such as, monoclonal antiviral antibodies and recombinant viral antigens. Methods for measuring antibody avidity are based on differential binding strength of antibodies after recent or past infection *(10,11)*. These methods use urea to detach weakly binding antibodies (recent infection) and compare the difference between urea-treated and -untreated serum dilution curves. Low avidity IgG antibody index is indicative of a recent (<6 mo) infection *(10)*.

3.3. Antirubella Virus Antibody-Specific Tests

3.3.1. Production of Rubella Virus

Rubella virus can be grown in B-Vero cells according to procedures that have been described in detail previously *(12)*. However, when grown in cell culture, the rubella virus is relatively unstable, producing poor yields. This problem can partially be overcome by using roller bottles and by harvesting the cells at 8–12-h intervals to avoid virus inactivation. Further, as the genome of rubella virus has been cloned and sequenced it is possible to produce viral antigens for diagnostic purposes by utilizing recombinant DNA technology.

3.3.2. Production of Antigenic Rubella Virus Proteins in Insect Cells

The baculovirus expression vector system offers a good alternative for high-level production of various viral proteins including the glycoproteins of enveloped viruses. We have used this methodology to produce the different structural proteins of rubella virus for both diagnostic and research purposes. The entire 24S cDNA coding for all structural proteins as well as cDNA sequences encoding the individual viral components E1, E2, and C (*see* **Fig. 1C**) were expressed in insect cells using baculovirus vectors *(13–15)*. Briefly, 24S cDNA was digested with suitable restriction enzymes or modified by using the polymerase chain reaction (PCR) and subcloned into baculovirus transfer plasmids under the polyhedron gene promoter. The transfer plasmid constructs were used to cotransfect insect cells with wild type baculovirus DNA to generate the recombinant viruses as previously described *(16)*.

1 For the production of rubella virus structural proteins using the baculovirus system, insect cells *Spodopterafrugiperda* (Sf9 or Sf21) or *Trichoplusia ni* (High Five) monolayer or suspension cell cultures are infected with recombinant baculovirus at multiplicity of infection of 1–10 Spinner flasks or shaker bottles can be used for amplification of the production. Infect the cells (e.g., 500×10^6 cells) with the stock virus (100 mL of undiluted stock virus) for 3–4 h and add fresh culture medium (e.g., 500 mL of TNM-FH + 5% fetal calf serum) to give a final cell density of $1.0–1.5 \times 10^6$ cells/mL. Higher cell density usually reduces the production of recombinant protein unless the conditions are optimized for large scale.
2. Harvest cells at 48–72 h after infection by low-speed centrifugation (400*g*, 5 min). The supernatant, containing the extracellular infectious viruses can be stored at +4°C for further use as stock viruses
3. For purification of the E1 and E2 glycoproteins, wash the cells twice with PBS (e.g., 500 mL) followed by suspension in TN buffer containing 1 m*M* EDTA, 1% Triton X-100, and 0.2 m*M* PMSF. Use 1 mL (or less) of buffer for 2×10^7 infected cells (e g., 25 mL for 500×10^6 cells).
4 Incubate on ice for 30 min, sonicate 3 times (5 s), and clarify the extracts by centrifugation for 10 min at 10,000*g* at 4°C.
5 Collect the supernatant fractions and precipitate with saturated ammonium sulfate at 10% intervals (up to 70%) for 2 h at 4°C. The precipitates at each interval are collected by centrifugation for 20 min at 12,000*g* at 4°C.
6 Dissolve the precipitates in Concanavalin A affinity chromatography binding buffer and dialyze at 4°C for 16 h in a dialysis tube against the same buffer.
7. Pool fractions containing rubella virus E1 and E2 proteins (determined by immunoblotting) and apply to Concanavalin A Sepharose 4B gel (Pharmacia) equilibrated with the binding buffer containing 0.01% Triton X-100
8. Allow E1 and E2 proteins to bind to the resin by recirculation for 12 h at 4°C. Elute glycoproteins using a gradient of methyl-α-D-mannoside (Sigma) with starting buffer containing 10 m*M* EDTA. Detect fractions containing E1 and E2 by SDS-PAGE and immunoblot analysis. Immunoaffinity chromatography can also be used *(17)*, but for those procedures antirubella virus specific monoclonal antibodies are recommended.
9. For production of rubella C protein, the corresponding cDNA is modified by PCR to contain 6 histidine (His) residues at the *N*-terminal portion of the protein The histidine tag allows binding of the protein to metal (e.g., Ni) affinity column.
10. Infect Sf9 cells with recombinant C-His baculovirus. Carry out infection and disruption of cells as described above (**steps 1–4**). Apply clarified cell extracts to immobilized metal affinity chromatography (IMAC).
11. Elute metal chelate-bound C-His with a 10–500 m*M* imidazole gradient, pH 7.4 (**Fig. 3C**). Visualize eluted capsid protein by SDS-PAGE followed by Coomassie blue staining or immunoblotting.
12. In order to monitor the protein contents or to analyze the antibody responses against the recombinant viral proteins, SDS-PAGE and immunoblot analyses can

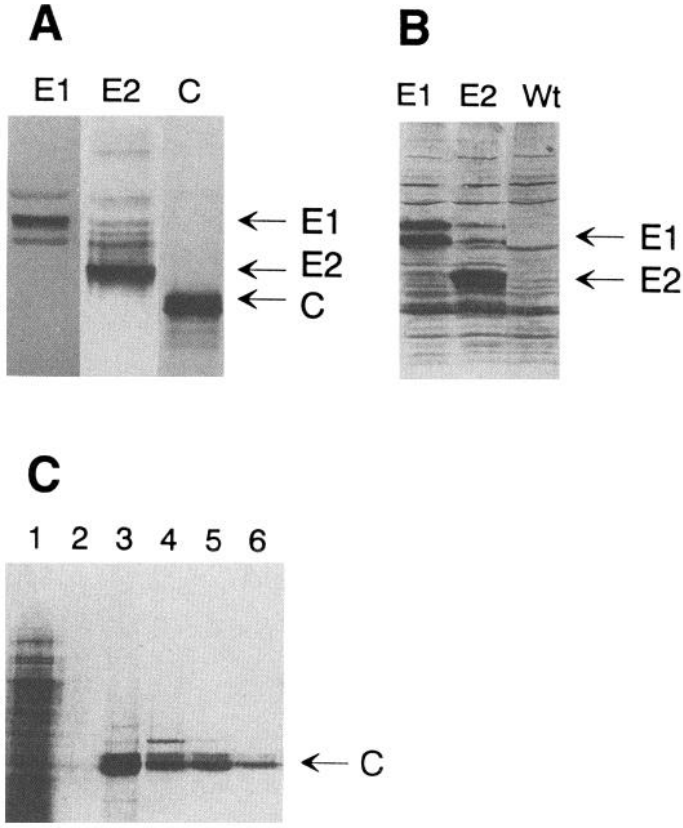

Fig. 3. Synthesis of rubella virus-specific structural proteins by using the baculovirus expression system. *Spodoptera frugiperda* insect cells were infected with recombinant baculoviruses producing rubella virus E1, E2, and C proteins. The E1 and E2 constructs lack the membrane anchor sequences. Cells were collected, soluble cell extracts prepared, and protein samples were separated on SDS-PAGE followed by transfer to Zeta-Probe membrane and immunoblotting with rabbit antirubella virus antibodies **(A)** or human postinfection antiserum **(B)** as described in **Subheading 3.** **(C)** describes the expression of C-His fusion protein in insect cells (lane 1) and immobilized metal affinity chromatography purlfication of the recombinant protein. A sample from the final wash (lane 2) and different fractions of the imidazole-eluted C-His protein (lanes 3–6) are shown. Cell extracts and recombinant viral proteins as indicated in the figure. Coomassie blue-stained gel.

be conducted. For Coomassie blue staining, separate proteins to be analyzed (1–10 μg/lane) by SDS-PAGE ***(7)*** and stain the gel with Coomassie Brilliant Blue (2%) for 30 min followed by destaining in 10% acetic acid in 25% methanol. For immunoblotting ***(18)***, proteins separated by SDS-PAGE (0.1–1 mg/lane)

are electrophoretically transferred (1 h, 200 mA) to nitrocellulose or polyvinylidene difluoride membranes Minigel and minielectrotransfer apparatuses are recommended Wash the membranes with PBS and saturate with 5% nonfat milk in PBS for 30 min at room temperature For primary antibody binding, the membrane sections are incubated with appropriately diluted serum for 1 h at 37°C in PBS/5% nonfat milk. After two 5-min washings a secondary peroxidase-conjugated antihuman (or other species) (Sigma, Bio-Rad) antibodies are added and incubated for 1 h at 37°C After thorough washing the bands are visualized using the ECL chemiluminescence system (Amersham, Buckinghamshire, UK) and autoradiography or by addition of a color substrate such as 3-amino-9-ethylcarbazole *(18)*

13. Purified (or partially purified) recombinant rubella virus proteins can be successfully used in an ELISA assay *(15,17,19)*, using practically the same assay principles described above for the antimumps antibody ELISA (**Subheading 3.2.2.**) In addition, purified or unpurified rubella virus protein containing cell extracts from recombinant baculovirus infected cells can be used in immunoblotting assays (**Fig. 3**)

Other more conventional methods such as hemagglutination inhibition tests *(6)* can also be used for detecting antirubella virus antibodies.

4. Notes

Some of the methods that are applicable for determination of antimeasles, antimumps and antirubella virus antibodies are described above. Practically, the diagnosis of acute infections caused by MMR viruses has to be based on serological assays since these viruses or viral antigens are rarely recovered or detected from infected individuals. Basically, any specific method capable of determining an antibody rise can be used as a diagnostic assay. The sensitivities of different assays do, however, vary tremendously. Neutralization tests, which can be considered as gold standard in immunity determinations, are tedious to carry out and require good standardization. Hemagglutination inhibition tests are easy to carry out and are specific for measles and rubella virus antibody determinations, while antibodies to mumps virus give rise to crossreactivity with other paramyxovirus infections *(20,21)*.

During the past 20 yr, ELISA methods using either crude or purified viral antigens and more recently, also certain recombinant viral proteins have become available for diagnostic purposes. ELISAs (or radioimmunoassays) have certain advantages over the other methods. They are very easy to perform and standardize, they show relatively low variability making interassay variation low, antibodies of different immunoglobulin classes or subclasses or avidity differences can be determined, and large serum materials can be analyzed

with reasonable costs. There are, however, a number of drawbacks; sometimes, nonspecific binding of immunoglobulins is seen resulting in false-positive results. The specificity of a positive result can be confirmed by another assay, e.g., immunoblotting assays have become the confirmatory tests in anti-HIV antibody determinations. In addition, problems with nonspecific IgM antibody binding can be circumvented with capture IgM assays, which have been developed for antimeasles and antirubella virus antibody determinations *(9,19)*. It is also noteworthy that antibodies measurable by ELISA represent a pool of antibodies against all possible epitopes of viral antigens and are by no means indicative of vaccine-induced immunity. Antibody responses against viral envelope glycoproteins (**Fig. 1**) correlate to immunity and therefore ELISAs based on purified viral glycoproteins could be a better way to measure the immune status of an individual. However, a good correlation with neutralizing and overall ELISA antibody levels has been observed *(21–23)* suggesting that ELISA methods are also applicable for large scale immunity surveys *(5)*.

The entire genomes of MMR viruses have been cloned and sequenced. Thus if cloning the expression and purification of recombinant proteins is successful, large-scale production can be used . This has the advantage of providing standardized material for large-scale ELISA applications. This is an alternative welcome for producing rubella and measles virus antigens, since they are relatively tedious and expensive to grow in cell culture. All the rubella virus structural proteins have been produced in recombinant systems (*see* **Subheading 3**), whereas in the case of measles only hemagglutinin, neuraminidase, and nucleoprotein expression has been described *(9,24)*. Mumps virus grows very well in eggs and therefore an urgent need for recombinant protein production is not evident. Recombinant materials are also free of infectious MMR viruses, which is an important aspect of laboratory safety. Sometimes, it may be difficult to produce and purify a recombinant viral protein. The baculovirus expression system and fusion gene constructs like the one described for rubella C-His (**Fig. 3**) may go some way to resolve these problems. However, antibody responses are usually directed against all viral structural proteins and therefore a single recombinant protein may not produce an assay of the required sensitivity and specificity. It is presumable that a mixture containing several viral proteins may be required.

In the present review we have limited the description of anti-MMR antibody specific methods to the most applicable ones for each virus. The literature is, however, full of descriptions of diagnostic methods for detecting MMR virus infections and to further their understanding we recommend that the reader becomes acquainted with these.

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12

Papillomaviruses

Sandra H. Kirk and David T. Y. Liu

1. Introduction

1.1. Papillomaviruses

Both epidemiological and experimental evidence suggests sexual activity contributes to the etiology of cervical cancer. Searches for a responsible agent have pointed to an association with human papillomavirus (HPV). These viruses are nonenveloped, approx 55 nm in diameter, contain a double-stranded DNA genome of approx 8 kb, and are grouped with the *Papovaviridae*. To date 70 types are known, the subtypes with the early (E) genes E6 and E7 having the capacity for involvement with cellular transformation *(1)*.

HPVs are epitheliotropic, with both cutaneous and mucosal cells being permissive to infection, although the tendency is toward selectivity. There is considerable molecular heterogeneity of HPV isolates in infection between populations, within populations and in the same individual. The mode of viral entry into epithelial cells is not known, but abrasion has been discounted as a necessary event *(2)*. *In situ* hybridization and polymerase chain reaction (PCR) analysis have shown that anogenital dysplastic lesions are usually associated with HPV 16 and 18. Amplification of the genome only occurs in the stratum spinosum epidermidis and stratum granulosum epidermidis. E gene expression is found throughout the epithelium, whereas late (L) gene expression (for productive viral infection) is confined to terminally differentiated keratinocytes, resulting in koilocytosis, nuclear enlargement, and dyskeratosis. Viral DNA can persist in cells that do not support infection, either as an extrachromosomal element or as an integral of the host genome. Such persistence may also be associated with transformation into high-grade cervical intraepithelial neoplasia (CIN) or carcinoma. Furthermore, infection within columnar epithelium may represent this variant of infection.

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1.2. Papillomavirus and Clinical Disease

Between 3 and 30% of the population are carriers of HPV, with prevalence more likely after the age of 35 *(3)*. Progression from HPV infection through CIN to carcinoma of the cervix has been demonstrated, and is reflected in clinical findings of latent, subclinical, and clinical lesions.

In the anogenital area there is a close association between HPV subtype and the severity and type of lesion observed *(2)*. HPV 6 and 11 are associated with condylomata acuminata and CIN I, and 16 and 18 with high-grade CIN and carcinoma *(4)*. These clinical observations have led to the classification of HPV into low- (e.g., types 6 and 11), intermediate- (e.g., 31 and 33), and high-risk (e.g., 16 and 18) types. Although a large number of HPV infections regress naturally *(5)*, infection with types 16 and 18 substantially increases the likelihood of development of CIN II or CIN III *(6)*; evidence also suggests that HPV 18 is more likely to be associated with glandular epithelial cells. The presence of HPV increases the risk of development and progression of CIN by up to 16-fold, and encourages earlier onset of clinical lesions by up to 10 yr *(5)*. Detection of the viral genome by Southern blotting and the more sensitive PCR technique has thus proven useful over recent years in studies of the involvement of the various viral subtypes in cervical cancer and other lesions *(7–9)*. The latter methodology has been shown to be more sensitive and rapid than the former, and may easily be developed for use in screening programs. The work described below forms part of a project carried out in our laboratory to identify the presence of HPV subtypes in samples from a human semen donor bank (Kirk, S. H. and Liu, D. T. Y., in preparation). This is of significant clinical importance because of the use of the material for direct introduction into the cervix in the treatment of infertility.

1.3. PCR Amplification of Viral DNA

Major problems in identification of HPV in clinical samples are the large number of subtypes and the high degree of conservation between genomes in terms of gene organization and sequence. For accurate analysis and to overcome potential problems of nonsubtype-specific amplification, careful choice of PCR primers is necessary. The approach described here was determined by Tham et al. *(9)* and involves the use of subtype-specific pairs of primers derived from known genome sequences designed to produce amplification products of different discrete lengths from individual viral subtypes. Other workers have designed consensus primers to amplify material from any HPV present *(7,10,11)*. This latter approach is coupled with sequencing of or specific oligonucleotide hybridization with the amplified product to determine subtype. This is particularly useful in studies where

the precise subtype associated with a clinical condition are uncertain or where more than one viral subtype may be involved, or for identification of novel genotypes.

In this chapter we describe the PCR method we have used to identify HPV 11, 16, and 18 in human semen, as a model for donor bank screening. Numerous methods for DNA extraction from clinical samples are available involving cell lysis, protein removal, and DNA recovery by precipitation *(12)*, but the use of semen has some particular problems. The highly crosslinked outer surface of sperm is a requirement for their protection, but creates problems in cell lysis for DNA extraction. We have overcome this by modifying existing extraction protocols to include incubation with high concentrations of dithiothreitol (DTT), and have modified the salt concentrations of buffers to allow for the ionic composition of semen *(13)*. Additional phenol extraction steps are also employed to remove excess proteinaceous material. The amplification protocols used are standard, but with 40 cycles to ensure detection of very low level viral DNA. Our studies have indicated that this high cycle number under the conditions described below allows efficient detection of less than 0.1 pg viral DNA against a background of 0.5–1 μg human DNA without nonspecific amplification.

The primers used (**Table 1** *[9]*) are from the open reading frame of the E6 regions of the HPV subtypes. The E6 and E7 genes of oncogenic genital HPV types, such as 16 and 18 encode proteins involved in transformation to the cancerous state, although they encode other functions in other subtypes, e.g., the nononcogenic HPV 11. Detection and typing of these genes is therefore of importance in determining oncogenic potential of infecting particles.

2. Materials

All reagents used should be of molecular biology grade, and used solely for this work.

2.1. Isolation of DNA from Human Semen

2.1.1. Phenol/Chloroform/Isoamyl Alcohol/8-Hydroxyquinoline (50:50:1:0.1, v/v/v/w)

1. Phenol
2. Chloroform.
3. Isoamyl alcohol
4. 8-Hydroxyquinoline
5. 0.5 *M* Tris-HCl, pH 8.0
6. 0.1 *M* Tris-HCl, pH 8.0.
7. Chloroform/isoamyl alcohol, 24:1 (v/v).

Table 1
Primers Used for Amplification of HPV 11, 16, and 18 DNA

HPV subtype	Primer name	Sequence[a]	Length of amplification product
HPV 11	HPV 11p (forward primer)	GCCTCCACGTCTGCAACATC	424 bp
	HPV 11q (reverse primer)	CTTCCATGCATGTTGTCCAG	
HPV 16	HPV 16r (forward primer)	GCGACGTGAGGTATATGACT	334 bp
	HPV 16s (reverse primer)	GGTTTCTCTACGTGTTCTTG	
HPV 18	HPV 18g (forward primer)	TACCGCATGCTGCATGCCAT	154 bp
	HPV 18c (reverse primer)	GGTTTCTGGCACCGCAGGCA	

[a]Sequences are written in the 5'-3' direction.

2.1.2. DNA Extraction Method 1

1. 2 *M* DTT in distilled water. Store in aliquots at –20°C
2. Suspension buffer 0.3 *M* sodium acetate, 20 m*M* Tris-acetate, pH 7.5, 1 m*M* ethylenediaminetetraacetic acid (EDTA) Make up as required.
3. 10% (w/v) Sodium dodecyl sulfate (SDS).
4. Phenol/chloroform/isoamyl alcohol/8-hydroxyquinoline (50 50.1 0.1, v/v/v/w), prepared as described in **Subheading 3.1.1.**, and stored at 4°C in a light-proof bottle. This is useable until the 8-hydroxyquinoline indicator changes to a yellow color indicating oxidation of the phenol
5. Chloroform/isoamyl alcohol (24 1, v/v), stored at 4°C.
6. Absolute ethanol.
7 70% (v/v) Ethanol.
8 TE: 10 m*M* Tris-HCl, pH 7 6, 1 m*M* EDTA.
9 15-mL Polypropylene centrifuge tubes.

2.1.3. DNA Extraction Method 2

1. Nucleon™ I DNA extraction kit (Scotlab, Strathclyde, UK) This kit is stable for at least 2 mo at room temperature
2. 5 *M* DTT, stored at –20°C in aliquots
3. Buffer 1 800 m*M* Tris-HCl, pH 8 0, 120 m*M* EDTA, 180 m*M* NaCl, 2% (w/v) SDS Store at room temperature.
4. Chloroform (stored at –20°C).
5. Absolute ethanol.

6 70% (v/v) Ethanol.
7. TE (*see* **Subheading 2.1.2.**).
8. Phenol/chloroform/isoamyl alcohol/8-hydroxyquinoline (50:50:1:0.1, v/v/v/w).
9. Chloroform/isoamyl alcohol (24:1, v/v).
10. 1.5-mL Eppendorf tubes

2.2. PCR Amplification

2.2.1. Cloned HPV Sequences

1. Plasmids (pBR322) containing HPV 11, 16, and 18 sequences were obtained from Dr. E -M. de Villiers, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany.
2 Restriction endonucleases and associated buffers.
3. 1 5-mL Eppendorf tubes.
4. Low melting point agarose, e.g , from Sigma, Poole, Dorset, UK.

2.2.2. PCR Reagents

1. Primers: Supplied in solution directly from an ABI DNA synthesizer and stored at –20°C until purified The primers used are described in detail in ref *(3)*, and are shown in **Table 1**
2. 3 *M* Sodium acetate.
3. Absolute ethanol.
4. 80% (v/v) Ethanol.
5 Sterile deionized water
6 *Taq* DNA polymerase, Life Technologies Ltd, Paisley, UK (5 U/µL)
7. dNTP mix. Individual 100 m*M* dATP, dTTP, dCTP, and dGTP solutions (Pharmacia Ltd , St. Albans, UK) diluted in sterile deionized water to give a mixture containing 2 m*M* each deoxynucleotide. Stored in aliquots at –20°C.
8 10 m*M* $MgCl_2$
9. 10X PCR buffer: 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 1 mg/mL gelatin. This must be heated to allow dissolution of the gelatin, and is stored in aliquots at –20°C.
10. Mineral oil.
11 Diethyl ether
12. Nitrogen gas.
13. 0 5-mL Thin-walled Eppendorf tubes.
14 250 µg/mL λ DNA (Pharmacia), stored at –20°C.
15. 1 µg/µL 100-bp DNA ladder (Pharmacia), stored at –20°C.
16. Filtered Gibson pipet tips (e.g., Aerosol Resistant Tips from Life Technologies).

3. Methods

3.1. DNA Extraction from Human Semen

Two methods have been used in our laboratory. The first to be described in **Subheading 3.1.2.** is a modification of a standard method used for DNA

extraction from whole blood, and the second (**Subheading 3.1.3.**) a modification of a commercially available kit marketed for DNA extraction from whole blood. Modification to each method was necessary as sperm have an outer coating highly cross-linked by disulphide bonds which is refractory to many standard treatments used for cell lysis. The incubation steps with high concentrations of DTT are used to overcome this (*see* **Note 1**).

3.1.1. Preparation of Phenol/Chloroform/Isoamyl Alcohol Solution

Both DNA extraction methods involve the use of this solvent system. Phenol and chloroform are toxic. You should wear gloves and safety glasses at all times when handling this mixture and the raw chemicals.

1. To 100 g phenol and 0.1 g 8-hydroxyquinoline in a bottle, add 100 mL 0.5 *M* Tris-HCl, pH 8.0. Shake until all the phenol has dissolved.
2. Allow the phases to separate, then aspirate and discard the top aqueous layer.
3. Add 100 mL 0.1 *M* Tris-HCl, pH 8.0, shake well, and allow to settle.
4. Aspirate top layer and check its pH.
5. If the pH is less than 7.6 repeat **steps 3** and **4** until the pH reaches or exceeds this.
6. To the phenol (lower) phase add an equal volume of chloroform/isoamyl alcohol (24:1, v/v).
7. Shake well and store at 4°C in a light-proof bottle. It is the lower phase of this mix that is used.

3.1.2. DNA Extraction Method 1

1. To a 15-mL polypropylene tube add 0.5 mL semen and 0.5 mL 2 *M* DTT. Mix thoroughly and incubate at room temperature for 5 min.
2. Add 1 mL suspension buffer and 100 µL 10% SDS and mix thoroughly to lyse cells.
3. Extract the lysate by mixing gently but thoroughly (i.e., by hand, not using a vortex mixer) with an equal volume (2.1 mL) phenol/chloroform/isoamyl alcohol/8-hydroxyquinoline (50.50:1:0.1, v/v/v/w) for at least 5 min. This lengthy extraction time is needed for efficient protein removal.
4. Centrifuge at 2000*g* in a bench centrifuge for 5 min to separate phases.
5. Remove the upper aqueous phase to a fresh tube using a wide-bore pipet tip, or a standard tip with the last 2 mm or so removed. Avoid the milky interface between the two phases, as this contains a lot of protein.
6. Repeat **steps 3–5**.
7. Extract the aqueous phase with 2 mL chloroform/isoamyl alcohol (24:1, v/v) to remove any residual phenol and centrifuge as above.
8. Precipitate the DNA from the aqueous (upper) layer by adding 2 vol (4.2 mL) absolute ethanol. The DNA should appear as fine white strands on mixing the tube contents gently.
9. Recover the DNA by centrifugation as above, and rinse gently in 2 mL 70% ethanol. Recentrifuge (*see* **Note 3**).

10. Remove all the supernatant and dissolve the DNA in 100 µL TE by rotary mixing overnight at 4°C (*see* **Note 4**) It is important to remove all the alcohol or the DNA will not dissolve efficiently in the TE. To ensure removal of the alcohol, centrifuge the tube again for a further 2 s after removal of the bulk of the supernatant and remove residue with a fine pipet tip Leaving the tube open in the fume hood for 5 min will allow evaporation of the alcohol, although it is important to be aware of any potential sources of contamination while the samples are left open.
11 Determine the concentration of DNA in the sample by measuring absorbance at 260 nm (absorbance of 1 corresponds to a DNA concentration of 50 µg/mL) Check the quality of the preparation by comparing absorbances at 260 and 280 nm, and running a 0.2-µg sample on a 0.3% agarose gel alongside 0 2-µg of λ DNA An absorbance ratio (A_{260}:A_{280}) greater than 1.5 is satisfactory. Less than this indicates significant contamination with protein, and the samples should be subjected to further phenol/chloroform/isoamyl alcohol extraction for its removal.
12 Store samples at –20°C

3.1.3. DNA Extraction Method 2

This is based on the Nucleon™ I DNA extraction kit (Scotlab), but with modified early incubations for reasons described in the Introduction, and with additional phenol/chloroform/isoamyl alcohol extractions because of the large amount of protein and other contaminants in human semen.

1. Add 140 µL semen and 35 µl 5*M* DTT to an Eppendorf tube on ice, mix, and incubate for 5 min at 4°C.
2. Add an equal volume of Buffer 1 (used in place of kit Reagents A and B) and vortex mix.
3. Add 100 µL 5 *M* sodium perchlorate and incubate in a shaking water bath for 20 min at 37°C
4. Transfer to a 65°C shaking water bath and incubate for a further 20 min
5. Add 580 µL cold chloroform and shake at room temperature for 20 min.
6. Transfer in 0 5-mL aliquots into a Nucleon™ tube (Eppendorf tube with an insert midway down that restricts flow of sample through the tube, and provides a region where the silica used below forms a layer between aqueous and organic phases), centrifuging for a few seconds after each addition to ensure that the sample flows through the insert to the bottom of the tube Centrifuge at low speed (1300*g*) in a bench microfuge for 1 min.
7. Add 45 µL Nucleon™ silica suspension (*see* **Note 2**) and centrifuge at 1300*g* for 4 min.
8. Without disturbing the silica interface, pour or pipet off the aqueous layer into a fresh Eppendorf tube.
9. Extract the lysate by mixing gently but thoroughly (i.e., by hand, not using a vortex mixer) with an equal volume phenol/chloroform/isoamyl alcohol/ 8-hydroxyquinoline (50:50.1.0.1, v/v/v/w) for at least 5 min.

10 Centrifuge at high speed in a microfuge for 5 min.
11. Remove the upper aqueous phase to a fresh tube using a wide-bore pipet tip, or a standard tip with the last 2 mm or so removed, avoiding the milky interface.
12. Repeat **steps 9–11**.
13 Extract the aqueous phase with 440 µL chloroform/isoamyl alcohol (24:1, v/v) and centrifuge as above
14. Add 880 µL absolute ethanol, and invert to precipitate DNA Centrifuge at 4000*g* (high speed) for 5 min and discard supernatant
15. Rinse pellet with 1 mL 70% ethanol, and recentrifuge as above.
16. Discard supernatant, resuspend DNA in 50–100 µL TE and determine concentration and quality as described in **Subheading 3.1.2.**

3.2. PCR Amplification (see Note 5)

3.2.1. Preparation of Standard HPV-DNA

HPV-DNA inserts are excised from plasmids for use in PCRs as positive controls.

1. Digest approx 0.3 µg of each plasmid with an appropriate restriction enzyme for 1 h at 37°C to release the HPV sequence
2 Separate inserts from vector sequences by electrophoresis on a 0 6% low melting point agarose gel alongside 0.25 µg λ DNA digested with *Hin*dIII (*see* **Note 6**).
3 Determine approximate amounts of HPV-DNA in gel bands by comparison of band intensity to that of λ standards The 23.1-kb λ band contains 120 ng DNA, the 9.4-kb band, 50 ng, and the 6 6-kb band, 35 ng If available, use of a gel scanner will provide a semiquantitative estimate.
4. Using a scalpel, excise the HPV-DNA band in the smallest amount of gel possible, and transfer to a preweighed Eppendorf tube Determine the weight of the gel slice, and assuming that 1 g of gel corresponds to a volume of 1 mL, determine the concentration of DNA in the slice in µg/µL. Water can be added to the gel slice to give a stock of 1 µg/mL viral DNA
5 Store gel slice at –20°C, thaw, and melt by incubation at 65°C when required. Mix thoroughly before removing aliquots to ensure complete dissolving of agarose.

3.2.2. PCR Primer Purification (see Note 7)

Primers are purified by precipitation and washing.

1. Place 200 µL primer in a sterile Eppendorf tube on ice. Store the remaining primer at –20°C in 200-µL aliquots for future use.
2 Add 20 µL 3 *M* sodium acetate and mix.
3. Add 2 vol (440 µL) absolute ethanol and mix by inversion Place in a –70°C freezer for at least 1 h.
4. Centrifuge at 4000*g*, 10 min, 4°C Decant supernatant carefully, avoiding disturbing any pellet (*see* **Note 8**)
5. Rinse pellet by adding 0 5 mL 80% ethanol and centrifuge as above for 5 min.

6. Decant supernatant and allow pellet to air dry, taking precautions to avoid contaminants entering tube
7. Resuspend primer in 100 µL sterile deionized water.
8. Determine concentration of a 1 in 400 dilution of small aliquot of primer by A_{260} assuming that an absorbance of 1 corresponds to a primer concentration of 20 µg/mL. To determine the molar concentration of each primer, assume that an individual nucleotide residue has a molecular weight of 330
9. Dilute purified primer to give a 5 µ*M* working stock. This is stored at –20°C in aliquots.

3.2.3. PCR Amplification Procedure

The aim of the procedure described is to detect trace amounts of viral genome against a human genetic background. Thus a high number of amplification cycles are used. For each amplification series a negative and a positive control are run alongside any samples. These consist of the PCR mixes without any added template and with 10 ng purified HPV-DNA, respectively. It is essential to include these controls to ensure that false-positive or false-negative results are not obtained as a result of contamination of reagents or of failure of amplification (*see* **Note 9**).

1 The reagents (**Subheading 2.2.2.**) are added to thin-walled 0 5-mL microtubes on ice in the order given (*see* **Note 10**), using fresh filtered Gilson pipet tips for each addition:

10X PCR buffer	5 µL
Water	to give total volume of 50 µL
10 m*M* $MgCl_2$	7.5 µL
dNTP mix	15 µL
Primer 1	15 µL
Primer 2	15 µl
DNA template	0.5–1 µg semen extract/10 ng HPV-DNA

2. Samples are mixed, centrifuged for 2 s, overlaid with 25 µL mineral oil, and placed in a Perkin-Elmer thermocycler.
3 The thermocycler is programmed at 94°C for 7 min to fully denature the template.
4. While samples are still hot, add 1.25 U *Taq* DNA polymerase and begin PCR cycling as follows.

94°C	1 min	(denaturation of template)
65°C	3 min	(annealing primers and template)
72°C	3 min	(primer extension)

5. Program machine to repeat cycle 40 times, and then to maintain samples at 4°C (*see* **Note 11**).
6. Remove oil from samples: Add two drops of diethylether to lessen viscosity, and pipet off as much of the organic (upper) layer as possible. Add an additional two drops and repeat.

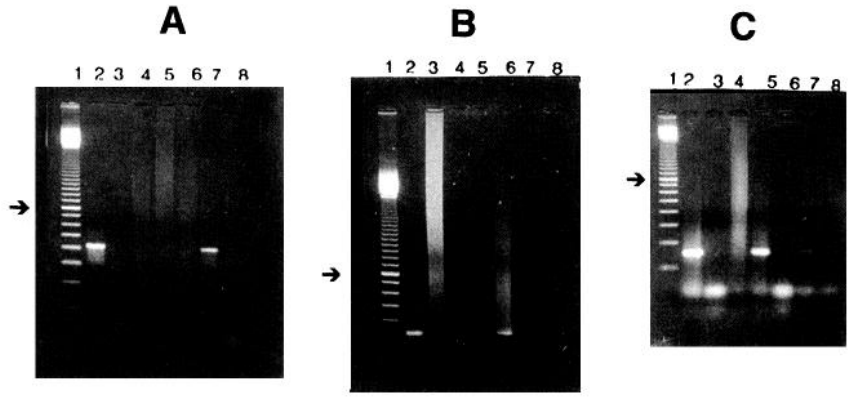

Fig. 1. PCR amplification products using HPV subtype-specific primers. PCR amplification of human semen DNA extracts was carried out as described in **Subheading 3.2.3.**: **(A)** using HPV 11 primers; **(B)** HPV 16 primers; **(C)** HPV 18 primers. In each case: lane 1, 100-bp DNA ladder (arrow shows 800-bp band); 2, positive control; 3, negative control; 4–8, semen DNA extracts.

7. Evaporate remaining ether by gently blowing nitrogen gas over the surface of the sample (*see* **Note 12**).
8. Run 15-µL aliquots of each sample on a 1.5% agarose gel alongside 1 µg 100-bp DNA ladder. Typical results are shown in **Fig 1**.

4. Notes

4.1. Preparation of DNA

1. DNA extraction procedures are based on the principle of cell lysis, protein removal and DNA precipitation, and many methods have been published and kits marketed. In this chapter we used semen as a starting material, and found that modifications of existing methods are necessary to obtain good quality DNA. Initial extracts using published procedures were found to have low A_{260}:A_{280} ratios (values of 1.0–1.1), and low yields. The resilience provided by the outer coating of sperm is likely to have been responsible for this, so modifications of standard and kit methods have been devised as described in **Subheadings 3.1.2.** and **3.1.3.** Extraction method 1 gives a better DNA yield (500 µg/mL semen compared to 150 µg/mL), although method 2 gives better quality preparations in terms of A_{260}:A_{280} ratio (typically 1.3–1.5 compared to 1.2). Using both methods we have shown that extraneously added HPV-DNA can be efficiently recovered from semen samples and detected by PCR amplification as described, and samples containing endogenous HPV were identified using both extraction methods. The requirement for repeated phenol/chloroform extractions with both methods is unfortunate in that it increases the handling time, and probably reduces yield.

Without this, however, very impure preparations result The purity achieved is still not optimal, and it may be possible to improve the procedures by using an ammonium acetate incubation step following sample resuspension in TE (**steps 10** and **16** for methods 1 and 2, respectively). This involves adding 0.5 vol 7 5 *M* ammonium acetate to precipitate any protein and centrifuging the mixture. DNA is precipitated from the supernatant by addition of 3 vol absolute ethanol, and rinsed and resuspended as described in **Subheading 3.1.2**

2 When using the modified Nucleon™ method (**Subheading 3.1.3.**) it is important to shake the silica suspension well before use because of settling during storage

3 In the methods described, we have used centrifugation as a means of recovering genomic DNA precipitates. An alternative for the dextrous is to wind the DNA strands onto a glass rod, and blot dry on filter paper

4. TE is used for resuspension of DNA, as EDTA chelates the magnesium ions required for nuclease activity, and thus the buffer confers protection against these. The use of buffer at pH 8.0 protects against deamidation of the DNA, which would interfere with PCR efficiency and fidelity

4.2. PCR Amplification

5 PCR amplification of DNA samples is a relatively simple technique, but requires care so that contamination is avoided. This is particularly true where clinical samples are being used, and the results have implications, as in this case, for the use of particular donor samples. It is good practice in any DNA diagnostic laboratory to have a defined "one-way system" for the processing and testing of materials to prevent contamination problems This involves physical separation of areas for reception and storage of unprocessed samples, storage of DNA extracts, preparation and storage of PCR reagents, setting up PCR mixes, storing completed reaction mixes, and so on.

6. When preparing the λ/*Hin*d III marker DNA it should be heated to 65°C for 5 min and quenched on ice directly prior to use. This melts any association between the 23.1- and 4.4-kb fragments that occurs on storage owing to these fragments containing the 12 nucleotide overhangs forming the bacteriophage *cos* site

7. On obtaining stocks of primers, it is recommended that they be divided for storage, as repeated freezing and thawing can cause damage resulting in markedly reduced amplification yields with time.

8. As the quantity of primer DNA is very small, you may not be able to see the pellet produced on ethanol precipitation during purification, thus retain the supernatant as a precaution in case the pellet has dislodged.

9. The inclusion of positive and negative controls in each PCR batch is essential, and running occasional samples spiked with added HPV is useful The latter controls show that the DNA extracts themselves do not contain material that interferes with the PCR. Using the methods described this has not been a problem in our hands.

10. It is important to add the reagents to the microtubes in the order shown This prevents the sample DNA being subjected to high ionic strength solutions which

may cause damage prior to amplification. Also, addition of the sample DNA to the tubes last (before enzyme) reduces risks of cross-contamination between tubes.

11 It may be possible to reduce the overall time for PCR (approx 5.5 h, allowing for time taken to ramp between cycle temperatures) by reducing the annealing and extension times of the cycle to 1 min each. This should be ample for the short lengths of DNA to be amplified, and result in the process taking only approx 3 h

12 A quicker alternative to removing mineral oil with ether is to pipet samples through the oil and dispense them as discrete spots onto parafilm. The parafilm absorbs any oil passing over with the sample Add loading buffer to the sample spots, mix by pipeting up and down, and load onto gel

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13

Diagnosis of Parvovirus B19-DNA by Dot-Blot Hybridization

Karen E. Hicks, Stuart Beard, Bernard J. Cohen, and Jonathan P. Clewley

1. Introduction

Parvovirus B19 was discovered at the Virus Reference Laboratory in 1975 by Cossart and colleagues *(1)*. First found in healthy blood donors, parvovirus B19 infection usually manifests as erythema infectiosum (fifth disease) in children. In adults the infection can cause acute arthritis. The virus replicates in erythroid progenitors, and patients with an underlying hemolytic anemia can experience a transient aplastic crisis. Anaemia may occur in immunocompromised patients due to persistent B19 infection and this can be treated with immunoglobulin. Infection during pregnancy can result in fetal anemia, hydrops fetalis, and intrauterine death.

In immunocompetent individuals viremia is rapidly cleared following the production of anti-B19 antibodies and diagnosis is most often made by specific IgM serology. However, in some cases it is important to be able to detect the virus. For instance, in immunocompromised patients anti-B19 antibody levels may be too low to permit a diagnosis. In susceptible high-risk individuals, such as patients with hematological disorders and pregnant women, it may be important to be able to detect B19 infection before antibody appears.

As B19 virus is not readily grown in cell culture it must be detected by other means. B19 antigen can be detected by counterimmune electrophoresis, radioimmunoassay, immune electron microscopy, and Western blot, but these methods may lack sensitivity. B19-DNA can be detected by dot-blot hybridization and this method gives a level of sensitivity appropriate for diagnosis *(2)*. B19-DNA can be amplified by the polymerase chain reaction (PCR) but this

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technique may be too sensitive for routine diagnostic use in acute resolving cases, as it can remain positive beyond the point of clinical significance. PCR can be useful when IgM serology is doubtful and when blood products have to be screened for the presence of B19. PCR is also useful for monitoring patients after immunoglobulin treatment so that in the case of a relapse, virus can be detected before symptoms return. PCR is an expensive procedure and is relatively complex technically, especially if nested primers are used. With the dot-blot assay large numbers of samples can be screened simultaneously and the method is quick, simple, and specific.

Hybridization assays require a specific probe. If this is prepared from cloned B19-DNA there is a possibility of cross-hybridization between residual bacterial plasmid and/or host chromosomal DNA and bacterial DNA in a contaminated sample (vector homology), even if the viral insert is gel purified *(3,4)*. This problem may not be entirely solved by using a PCR product as the probe if plasmid DNA is present in the template because even very small amounts of labeled plasmid DNA can produce a false-positive reaction if there is homologous DNA in the sample. The probe used in the dot-blot hybridization assay described in **Subheading 2.1.** is therefore produced by PCR using viral DNA as the template. This DNA is purified from B19-positive serum, which can be identified by screening blood donors (as described in **Subheading 3.3.**).

The primers used produce an amplicon of 4.5 kb, representing about 80% of the B19 genome. This PCR product is purified using silica and labeled with digoxigenin by random priming. Several PCRs can be carried out at the same time and a stock of DNA ready for labeling prepared, standardized, and stored. One PCR produces enough DNA for several probes and each probe can be used several times. Only a small amount (15 ng) of viral DNA template is used in each PCR. Moreover, if viral DNA supplies are low, a nested PCR could be used with the initial PCR product as template to increase the number of probes produced from one viral DNA preparation.

As an alternative the amplicon can be labeled directly by incorporating the digoxigenin labeled nucleotide in the PCR reaction. However, the PCR is less efficient with digoxigenin-labeled dUTP than with unlabeled TTP, and less amplified DNA is produced per PCR. For routine work, in which the need for a new probe is known in advance and consistency is important, it is simpler to label an aliquot of DNA from a standardized stock of purified PCR product by random priming than to carry out a direct labeling PCR from viral DNA. The development of this protocol is discussed elsewhere *(5)*.

The samples to be tested are applied to a nylon membrane. Serum samples can be used directly but tissue samples must first be processed to extract the DNA. Cloned B19-DNA is applied to the membrane as a positive control dilution series. The membrane is hybridized with the labeled probe. Bound probe

is detected using an alkaline phosphatase-conjugated antidigoxigenin antibody and a chemiluminescent reaction. Chemiluminescence is preferable to a color reaction because some samples (e.g., tissue extracts and hemolyzed blood) discolor the membrane, interfering with the interpretation of results.

The method can be adapted to produce a quantitative assay by analyzing the exposed X-ray film by modified scanning densitometry. The details of this will depend on the particular scanning equipment and analysis programs available. We will describe our approach.

A shortened protocol for screening blood donors for B19-DNA is also described, using a color reaction for digoxigenin detection. This is relatively insensitive, but as it is used for identifying positive sera from which to purify B19 antigens and DNA, only donations containing high titers of B19 are of interest. This method was applied to screening blood donors at the North London Blood Transfusion Centre from January to April 1993 inclusive, in an epidemic year for parvovirus B19. During this time 18,800 donations were screened by dot-blot hybridization, and 3 were positive. All reactions were confirmed by immune electron microscopy, which showed the presence of parvovirus particles.

2. Materials

The following materials are used in several of the methods described:

1. TE: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* ethylenediaminetetraacetic acid (EDTA)
2. Sterile distilled water (sdH_2O).
3. 1% Agarose gel
4. TBE running buffer: 87 m*M* Tris-HCl, 87 m*M* boric acid, 2 m*M* disodium-EDTA, pH 8.5.
5. DNA molecular weight markers 1 kb ladder, Gibco-BRL-Life Technologies, (Paisley, UK)

2.1. Probe Preparation

2.1.1. Viral DNA Purification

1. 15, 20, 25, and 30% (w/v) Sucrose made up in 20 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 150 m*M* NaCl
2. Ultracentrifuge, swing-out rotor and tubes, e.g., Beckman SW50.1 (Beckman, Fullerton, CA).
3 5% Sodium dodecyl sulfate (SDS)
4. 0.5 mg/mL Proteinase K, made up in sdH_2O, stored at –20°C.
5. Redistilled phenol:chloroform, 1:1 (v:v).
6. Chloroform.
7 3 *M* Sodium acetate, pH 4 5.
8 Absolute ethanol and 80% ethanol, stored at –20°C.

2.1.2. PCR

1. 10X PCR buffer: 100 m*M* Tris-HCl (pH 8.8), 500 m*M* KCl, 15 m*M* $MgCl_2$, 1% Triton X-100.
2. 1 m*M* dNTPs a mix containing 1 m*M* each of dATP, dCTP, dGTP, and dTTP
3. Primers: forward, 5'CCC GCC TTA TGC AAA TGG GCA G3' (residues 217–238 of the B19-Au DNA sequence; ***[6]***); reverse; 5'TTG TGT TAG GCT GTC TTA TAG G3' (residues 4893–4872). Diluted in sdH_2O to 5 pmol/μL
4. 1% Gelatin.
5. 5 U/μL *Taq* polymerase.
6. Paraffin oil.
7. Thermocycler
8. Geneclean kit (Bio 101, La Jolla, CA) containing: 6 *M* sodium iodide, silica suspension, "NEW"wash concentrate (a concentrated solution of NaCl, Tris and EDTA)

   Store **items 1–5** at –20°C

2.1.3. Probe Labeling and Purification

1. Digoxigenin-DNA-labeling kit (Boehringer Mannheim, Lewes, UK), includes:
   a. 50-μL Labeled control DNA: linearized pBR328 DNA labeled with digoxigenin, containing 1 μg template DNA and approx 260 ng synthesized labeled DNA.
   b. DNA dilution buffer. 1 mL herring sperm DNA (50 mg/mL), in 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0
   c. 10X Hexanucleotide reaction mixture.
   d. 10X dNTP labeling mix containing 1 m*M* each of dATP, dCTP, and dGTP, 0.65 m*M* dTTP, 0 35 m*M* digoxigenin-dUTP, pH 7.5.
   e. Klenow enzyme, labeling grade, 2 U/μL All these components should be stored at –20°C
2. Digoxigenin high prime kit (Boehringer Mannheim). The kit consists of a single tube containing a 5X reaction mix of random primer mixture, 1 U/μL Klenow enzyme, 1 m*M* dATP, 1 m*M* dCTP, 1 m*M* dGTP, 0 65 m*M* dTTP, 0 35 m*M* digoxigenin-11-dUTP and 5X stabilized reaction buffer in 50% glycerol (v/v). Store at –20°C.
3. PCR digoxigenin-labeling kit (Boehringer Mannheim) containing:
   a. 10X PCR digoxigenin mix. dATP, dCTP, dGTP (2 m*M* each); 1.3 m*M* dTTP, 0.7 m*M* digoxigenin-11-dUTP, alkaline labile, pH 7.0
   b. 10X PCR buffer. 100 m*M* Tris-HCl, 500 m*M* KCl, pH 8.3
   c. 25 m*M* $MgCl_2$
   d. 5 U/μL *Taq* DNA polymerase
   e. Control template: 20 pg/μL plasmid DNA in TE, pH 8 0
   f. Control PCR primer mix 50 pmol each of primers 1 and 2.

   Store at –20°C
4. Oven, 120°C or 80°C.

5. Nick columns (Sephadex G-50, Pharmacia, Uppsala, Sweden).
6. Nylon membrane, positively charged (Boehringer Mannheim) (*see* **Note 1**).

2.2. Dot-Blot Preparation, Hybridization, and Detection

1. Hand homogenizer (e.g., Dounce or Griffith grinder) for homogenization of tissue samples
2. 2X SSC (20X SSC stock solution. 3.0*M* NaCl, 0.3 *M* trisodium citrate, pH 7 0).
3. Denaturing solution. 100 µL 2 *M* NaCl, 1 *M* NaOH.
4. 96-Well microtiter plates
5. Manifold apparatus such as Minifold (Schleicher and Schuell, Keene, NH), (*see* **Note 2**).
6. Vacuum source.
7. Nylon membrane, positively charged (Boehringer Mannheim) (*see* **Note 1**).
8. Blotting paper (Schleicher and Schuell or Whatman 3MM paper)
9. Oven, 120°C or 80°C
10. Hybridization solution: Easy Hyb (Boehringer Mannheim) (*see* **Note 3**).
11. Hybridization oven (e g , Appligene, Illkirch, France, minihybridization oven, Techne hybridizer, Techne, Cambridge, UK HB-1) (optional).
12. Posthybridization wash solutions: 2X SSC, 0.1% SDS; and 0.1X SSC, 0.1% SDS.
13. Digoxigenin nucleic acid detection kit (Boehringer Mannheim), includes
    a. Antidigoxigenin-AP conjugate· polyclonal sheep antidigoxigenin Fab-fragments, conjugated to alkaline phosphatase, 750 U/mL.
    b. NBT· Nitroblue tetrazolium salt, 75 mg/mL in 70% dimethylformamide (v/v)
    c. X-phosphate 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, 50 mg/mL in DMF
    d. Blocking reagent

    All these components should be stored at –20°C except the antibody conjugate which, once opened, should be stored at 4°C. The blocking reagent can also be stored at 4°C or room temperature
14. Solutions for digoxigenin detection:

    Buffer 1· 100 m*M* maleic acid, 150 m*M* NaCl, pH 7.5.

    Buffer 2: 1% blocking reagent (**step 13d**) in buffer 1 (heat to dissolve), make up fresh or store at –20°C.

    Buffer 3: 100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, 50 m*M* $MgCl_2$

    Washing buffer: buffer 1 + 0.3% Tween-20.
15. Chemiluminescent substrate such as CSPD (Boehringer Mannheim), stock solution 10 mg/mL, store at 4°C
16. X-ray film (e.g., Amersham hyperfilm-MP, Amersham, Little Chalfont, UK).
17. Developing and fixing solutions (e.g., Kodak LX-24 & FX40 Kodak) and dark room facilities.
18. Stripping solution: 0.2 *M* NaOH, 0.1% SDS.
19. 100% ACS grade *N*,*N*-dimethylformamide (DMF)

3. Methods

3.1. Probe Preparation

3.1.1. Viral DNA Purification

The starting point is B19-positive serum. The virus is first isolated by ultracentrifugation through a sucrose gradient, then the DNA is purified *(7)*.

1. Set up a sucrose gradient as illustrated in **Fig. 1**
2. Ultracentrifuge in a swing-out rotor at 200,000*g* (40,000 rpm with a Beckman SW50.1 rotor in an L8-80 centrifuge) for 4 h.
3. Discard the supernatant and resuspend the pellet in 100 µL TE at 4°C overnight, then disperse pelleted virus by sonication in a bath sonicator
4. Add 10 µL 5% SDS and 10 µL 0.5 mg/mL proteinase K, and incubate at 50°C for 30 min
5. Add 120 µL phenol:chloroform, vortex, and microfuge for 1 min Remove the top aqueous phase to a clean tube Repeat
6. Repeat **step 5** with 120 µL chloroform
7. Precipitate the DNA by the addition of 12 µL 3 *M* sodium acetate and 300 µL absolute ethanol Leave on ice for 10–20 min
8. Microfuge for 15 min Remove the supernatant, wash the pellet with 1 mL 80% ethanol, and spin for a further 5 min
9. Remove all the ethanol and allow the pellet to dry, either on the bench or in a vacuum desiccator
10. Resuspend the pellet in 20–30 µL TE.
11. Estimate the yield by measuring the absorbance at 260 nm of a 1 in 100 dilution (1 absorbance unit is equivalent to a concentration of 50 µg/mL) and analyze 1 µL on a 1% agarose gel with molecular weight markers (*see* **Note 4**) A band of about 5 5 kb should be seen.

3.1.2. Polymerase Chain Reaction

A PCR is set up with the purified viral DNA as template. A hot start method is used to prevent mispriming, i.e., the *Taq* polymerase is added after the reaction mixture has reached the denaturation temperature.

1. Mix the following components in a 0.5-mL microfuge tube:

Components	µL
10X PCR buffer	4 0
Forward primer	5.0
Reverse primer	5.0
1 m*M* dNTPs	12 5
1% Gelatin	0 5
15 ng Template DNA (**Subheading 3.1.1., step 11**)	x
sdH_2O	y
	40.0

Overlay with a drop of paraffin oil

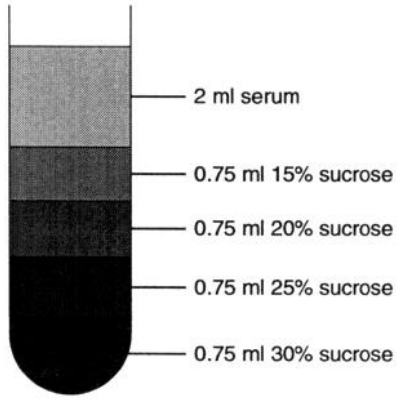

Fig. 1. 2mL B-19 positive serum is layered onto a 15–30% (w/v) sucrose gradient.

2. In a separate tube mix:

Component	µL
sdH_2O	8.5
10X buffer	1.0
Taq polymerase	0.5
	10.0

3. Program the thermocycler as follows:

Increment	94°C for 2 min
10 cycles of Denaturation at	95°C for 10 s
10 cycles of Annealing at	58°C for 30 s
10 cycles of Elongation at	72°C for 3 min
20 cycles of Denaturation at	95°C for 10 s
20 cycles of Annealing at	58°C for 30 s
20 cycles of Elongation at	72°C for 3 min plus 30 s increment per cycle

4. Place the tube from **step 1** in the thermocycler and start. When the temperature reaches 94°C pipet the mixture from **step 2** into this tube, below the paraffin oil.
5. When the cycling is complete, analyse 5 µL on a 1% agarose gel, with molecular weight markers, to ensure amplification has occurred. A band of 4.5 kb should be seen.

3.1.2.1. Purification of PCR Products

PCR products can be purified using silica with a Geneclean kit or equivalent system (*see* **Note 5**). Volumes given are for one 50-µL PCR product. If several

PCRs are carried out simultaneously up to 300 µL can be purified in one 1.5-mL microfuge tube and volumes should be scaled up accordingly (except for NEW wash).

1 Add 150 µL 6 *M* sodium iodide to the DNA solution in a 1 5-mL microfuge tube
2 Add 10 µL of silica suspension, vortex to mix thoroughly, and incubate at room temperature for 5 min, mixing occasionally.
3. Pellet the silica in a microfuge for 10 s and remove the supernatant
4 To wash the pellet add 200–700 µL cold NEW wash Vortex to mix, then pellet the silica in a microfuge for 10 s Remove the wash solution and repeat this step twice more. (To prepare the NEW wash solution add the contents of the NEW concentrate to 140 mL distilled water and 155 mL 100% ethanol and mix thoroughly Store the prepared solution at –20°C)
5. Elute the DNA from the silica with 10 µL TE. Pipet to mix, incubate at 56°C for 2–3 min then microfuge for 30 s.
6 Collect the supernatant, repeat the elution procedure, and pool the eluates Before pipeting from this solution microfuge briefly to pellet any residual silica (*see* **Note 5**)
7 Estimate the DNA concentration in the pooled eluate by measuring the absorbance at 260 nm of a 1 in 100 dilution Absorbance at 280 nm can also be read to check that it is DNA giving the absorbance reading, the reading at 280 nm should be approximately half that at 260 nm

3.1.3. Probe Labeling and Purification

The DNA can be labeled either by traditional random prime labeling, High Prime labeling, or by direct incorporation of digoxigenin-dUTP into the PCR.

3 1 3.1. Random Prime Labeling

Using the digoxigenin DNA labeling kit (*see* **Note 6**).

1 Make the volume of 1 µg of the purified PCR product to 15 µL with sdH_2O Denature by heating to 95°C for 10 min, then quench on ice
2 Add 2 µL of hexanucleotide mixture, 2 µL dNTP mixture and 1 µL of Klenow enzyme, mix well
3. Incubate at 37°C for 20 h.
4 Stop the reaction with 2 µL 0.2 m*M* EDTA, pH 8.0

3 1.3 2 High Prime Labeling

The digoxigenin High Prime kit contains all the components of the standard random prime labeling reaction in a single tube 5X reaction mix (*see* **Note 6**).

1. Make the volume of 1 µg of the purified PCR product to 16 µL with sdH_2O, denature by heating to 95°C for 10 min, then chill rapidly on ice.
2 Add 4 mL of the digoxigenin High Prime mix and incubate the reaction at 37°C for 20 h
3. Stop the reaction with 2 µL 0 2 *M* EDTA, pH 8 0

3.1.3.3. Direct Incorporation of Digoxigenin into PCR

Use a PCR digoxigenin-labeling kit. The first time this procedure is carried out it is advisable to also set up a control reaction with the control DNA and primers provided in the kit.

1 Mix the following components in a 0.5-mL microfuge tube:

Components	µL
10X PCR buffer	4.0
25 m*M* $MgCl_2$	3 0
Forward primer (**Subheading 2.1.2.**)	5.0
Reverse primer (**Subheading 2.1.2.**)	5.0
PCR digoxigenin mix	5.0
15 ng Template DNA (**Subheading 3.1.1.**)	x
sdH_2O	y
	40 0

Overlay with a drop of paraffin oil.

2. As in **Subheading 3.1.2., steps 2–4.**
3. Analyze 10 µL on an agarose gel. The molecular weight of the labeled DNA is increased compared to that of the unlabeled fragment and a clear band may not be seen.

3.1.3.4. Removal of Unincorporated Nucleotides

Unincorporated nucleotides can be removed from probes by gel filtration using Nick columns. This may not be necessary for probes made by PCR incorporation (*see* **Note 7**).

1. Remove the top cap and pour off the excess liquid.
2. Rinse once with TE
3. Remove the bottom cap.
4. Support the column over a waste container and equilibrate the gel with approx 3 mL TE.
5. Allow the TE to completely enter the gel bed.
6. Add the sample to the column
7 Add 400 µL TE and let it enter the gel bed
8. Place a 1.5-mL microfuge tube under the column for collection.
9. Elute the sample by adding a further 400 µL TE.

3.1.3.5. Test Strip

To show that the labeling was successful, and to estimate the amount of labeled DNA, dilutions of the probe DNA are spotted onto a nylon membrane alongside control labeled DNA provided in the DNA labeling and detection kit. Visualization in this case is by color reaction. If the probe was not purified

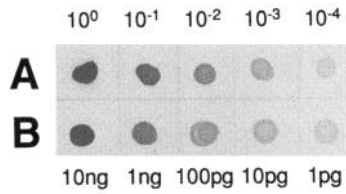

Fig. 2. Serial dilutions of **(A)** random prime digoxigenin-labeled B19-DNA probe and **(B)** control labeled DNA spotted onto a nylon membrane and visualized by a color reaction.

as above make the volume up to 400 µL with TE so that the volumes used are comparable (*see* **Note 8**).

1. Make 10-fold serial dilutions of the labeled control DNA in DNA dilution buffer, 1 in 10 to 1 in 100,000. Spot 5 µL of each dilution onto a strip of nylon filter.
2. Make 10-fold serial dilutions of the labeled probe in DNA dilution buffer, 1 in 10 to 1 in 10,000 and spot 4 µL of neat probe and of each dilution alongside the control DNA spots, as in **Fig. 2**.
3. Allow the spots to dry, then bake the membrane at 120°C for 20 min or 80°C for 1 h to bind the DNA (*see* **Note 9**).
4. Visualize the digoxigenin by a color reaction as described in **Subheading 3.2.4.**
5. After a few min the more concentrated spots will appear as a purple color and within 30 min all 5 controls will be visible. The number of spots visible for the probe shows the relative efficiency of probe labeling compared with the control. If 1 µg template DNA is labeled for 20 h by random prime labeling the amount of labeled probe DNA produced is approximately equal to the amount contained in the control DNA, i.e., 260 ng (*see* **Note 8**) (**Fig. 2**).

3.2. Dot-Blot Preparation, Hybridization, and Detection

3.2.1. Extraction of DNA from Tissue Samples

1. Produce a 10% (v/v) homogenate from a small piece of tissue in 2–3 mL TE (depending on the volume of tissue available) using a hand homogenizer (Griffith grinder).
2. Extract the DNA from 300 µL homogenate, using the Geneclean kit, with 900 µL sodium iodide and 20 µL silica, as described in **Subheading 3.1.2.1., steps 3–7**, eluting twice with 20 µL TE.

3.2.2. Application of Specimens to Nylon Membrane

1. To each well of a microtiter plate add 90 µL 2X SSC and 100 µL denaturing solution.
2. Add sample or control (10 µL serum, control DNA or tissue extract) to each well and allow 15 min to denature. For positive controls make 10-fold serial dilutions of cloned B19 from 1 ng/µL to 10 fg/µL. Normal human serum can be used as a negative control.

3. Cut the membrane and blotting paper to fit the manifold (*see* **Note 2**) and mark the membrane to identify orientation. Immerse the membrane and blotting paper in 2X SSC.
4. Place the blotting paper over the lower half of the manifold, then lay the membrane on top ensuring there are no air bubbles trapped Apply the lid to the manifold and secure using diagonally opposite clips.
5. Transfer the contents of the wells of the microtiter plate to the corresponding wells of the manifold. Apply a gentle vacuum for a few min, until all the sample has passed through into the vacuum chamber of the apparatus.
6 Remove the membrane, rinse briefly in 2X SSC, and bake at 120°C for 20 min or 80°C for 1 h (*see* **Note 9**). If the membrane is not to be used immediately it can be stored dry at 4°C

3.2.3. Hybridization and Washes

Hybridization temperature is usually 68°C in standard hybridization solution or 42°C in 50% formamide. Easy Hyb solution allows the hybridization to be carried out at 42°C without the use of formamide (*see* **Note 3**). Hybridization can be carried out in a hybridization oven, if available, or in a sealed plastic bag in a shaking water bath. Two membranes can be hybridized, back to back, in the same bag.

1 Incubate the membrane in 20 mL Easy Hyb solution at 42°C for a minimum of 1 h.
2 Denature the probe (400 µL eluate from Nick column or PCR product from direct incorporation) by boiling for 10 min, quench on ice, then add to 10 mL Easy Hyb solution.
3 Incubate the membrane in this solution at 42°C overnight (*see* **Note 10**)
4. Recover the probe after hybridization and store at –20°C Before reuse denature by heating to 68°C, for 10 min (*see* **Note 11**).
5. Wash the membrane twice for 5 min in 2X SSC, 0 1% SDS at room temperature, then twice for 15 min in 0 1X SSC, 0.1% SDS at 68°C

3.2.4. Digoxigenin Detection

The membrane is incubated with the antidigoxigenin conjugate. Bound antibody is detected with either a color or a chemiluminescent reaction.

The following steps are carried out at room temperature.

1 Wash the membrane briefly in washing buffer.
2 Incubate for 30 min in 20 mL buffer 2 (blocking reagent)
3 Dilute the antidigoxigenin-AP conjugate to 75 mU/mL, 4 µL in 20 µL buffer 2, and incubate the membrane in this solution for 30 min
4. Wash in washing buffer three times for 15 min.
5 Rinse briefly in buffer 3.

3 2.4.1. Color Detection

This is used for developing test strips (**Subheading 3.1.3.5.**) and for the rapid screening method (**Subheading 3.3.**).

1. Make up 10 mL color solution as follows: 10 mL buffer 3, 45 µL NBT, 35 µL X-phosphate Seal the membrane into a hybridization bag with this solution and place in the dark until the spots are visible. Do not shake while the color is developing. Strong signals will be visible as a purple color within 5–10 min. Weaker signals appear by 30 min.
2. Stop the reaction by washing the membrane in distilled water, allow to dry, and seal in a plastic bag for permanent storage

3.2.4 2 Chemiluminescent Detection

This method is used for routine dot blots.

1. Dilute the CSPD stock solution 1 100, 200 µL in 20 mL buffer 3, and incubate the membrane in this solution for 5 min. The solution can be saved, stored at 4°C in the dark, and reused three times
2. Allow excess liquid to drain from the membrane, seal the damp membrane in a plastic bag and incubate at 37°C for 15 min.
3. Expose the membrane to X-ray film for between 15 s and 1 h (*see* **Notes 11** and **12**)
4. To develop, immerse the film in developer for 1–3 min, rinse in water, then immerse in fixing solution and allow the film to clear Rinse with water and allow to dry.

Figure 3 shows an example of a typical diagnostic dot-blot. With a probe made by random prime labeling 1 µg DNA for 20 h, 1 pg B19-DNA can be detected in this assay (*see* **Note 13**).

It is possible to follow chemiluminescent detection with the color reaction by washing off the CSPD substrate with buffer 3 for 5 min, then adding the color solution as normal.

3.2.5. Stripping Membranes for Reprobing

Nylon membranes can be stripped and reprobed. Nitrocellulose membranes are dissolved by the solutions needed to remove color and cannot be used with chemiluminescence. If a membrane is to be stripped and reprobed it must not be allowed to dry out after color or chemiluminescent detection.

3.2.5.1. Stripping Chemiluminescence-Detected Membranes

1. Rinse the membrane in H_2O.
2. Incubate twice for 10 min in 0.2 *M* NaOH, 0 1% SDS at 37°C
3. Rinse thoroughly in 2X SSC
4. Proceed with prehybridization and hybridization as normal or store wet in 2X SSC in a sealed bag

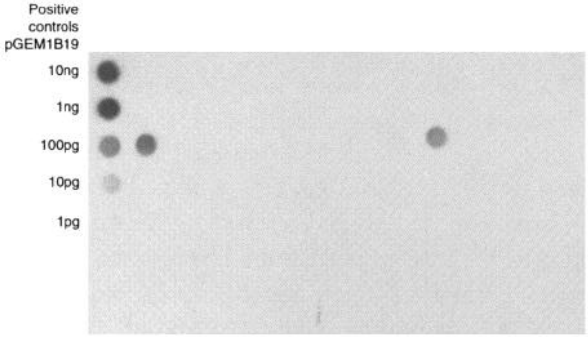

Fig. 3. Autoradiograph of a routine B19 dot-blot hybridization assay.

3.2.5.2. Stripping Color-Detected Membranes

1. Using a water bath in a fume hood, heat a large glass beaker of dimethylformamide to 50–60°C.
   Caution: Dimethylformamide is volatile and flammable, the flash point is 67°C.
2. Incubate the membrane in the heated DMF until the color has been removed. Changing the solution frequently will increase the speed of decolorization.
3. Rinse the membrane thoroughly in H_2O.
4. Incubate twice for 20 min in 0.2 *M* NaOH, 0.1% SDS at 37°C.
5. Rinse thoroughly in 2X SSC.
6. Proceed with prehybridization and hybridization as normal or store in 2X SSC in a sealed bag.

3.3. Rapid Method for Screening Blood Donors

In this method incubation times are shortened wherever possible to create a protocol suitable for processing large numbers of samples in one day. This is possible here because a strong positive is required. We describe the samples being blotted onto the membrane prior to denaturation so that the membranes can be prepared at the blood bank; however, if this is not the case, the samples can be applied as usual.

1. Blot 10 µL serum samples directly onto a nylon membrane.
2. Denature by floating the membrane on denaturing solution for 15 min.
3. Rinse in 2X SSC for 5 min, then bake at 120°C for 15 min.
4. Prehybridize in Easy Hyb for 3 min.
5. Hybridize for 2 h.
6. Wash the membrane twice for 5 min in 2X SSC, 0.1% SDS at room temperature, then twice for 15 min in 0.1X SSC, 0.1% SDS at 68°C.
7. Detect digoxigenin using the color reaction as described in **Section 3.2.4**. Incubations can be shortened as follows: 30 to 20 min and 15 to 10 min. Strong positives will appear within 5–10 min of adding the color solution.

3.4. Quantification by Scanning Densitometry

Positive signals may be quantified by scanning the X-ray film or colored membrane using a dedicated imaging densitometer, such as, those available from Molecular Dynamics or Bio Rad (Hercules, CA). Alternatively, a relatively inexpensive grayscale scanner may be connected to a PC and used with appropriate software packages to give a satisfactory means of quantifying and electronically recording the results as described by Shea *(8)*. For hardware, we use a ScanMaker IIG flatbed gray scanner (Microtek, Redondo Beach, CA) connected to a Macintosh computer (Apple Computer, Cupertino, CA); for software, OmniScan (Caere Corporation), Scan Analysis (Biosoft, Cambridge, UK or Ferguson, MO), and Excel (Microsoft, Caere, Los Gatos, CA). This scanning hardware setup can also be used for pulse-field gel electrophoresis (PFGE) and restriction fragment length polymorphism (RFLP) type gels with the Taxolab software from L'Institut Pasteur (Bureau des Relations Industrielles, Institut Pasteur, Paris, France, e-mail inquiries to pgrimont@pasteur.fr). The public domain program NIH Image (available via anonymous FTP at many Mac software sites) will prove to be a useful asset for image manipulation.

1. Orientate the film or filter in the top right-hand corner of scanner
2. Start Image Asst GS Microtek (see OmniScan manual).
3. Select Scan Image from the File menu.
4. Deselect all Smart Controls Check that the Image Type is Gray Scale—this can be changed in the Settings dialog box
5. Preview scan
6. Optional select Adjust Brightness and Contrast
7. Scan.
8. Save—select only no compression in TIFF options.
9. Quit Image Asst GS Microtek.
10. See the Scan Analysis manual It may be necessary to increase the amount of memory available to Scan Analysis to be able to open some TIFF images (Use Get Info from the File menu in the Finder and increase the Preferred Size to, for example, 2400k)
11. Open the TIFF image in Scan Analysis and process according to the manual Import the results into a spreadsheet (e.g., Excel) if desired

4. Notes

1. Nylon and nitrocellulose membranes should be handled with blunt-ended forceps and gloved hands and handling should be kept to a minimum. Nitrocellulose membranes can be used with color detection but not with chemiluminescence (due to a drastic reduction in sensitivity)
2. If a vacuum manifold is not available for the dot-blot, samples can be pippeted directly onto the membrane. In this case 5-µL samples are applied prior to dena-

turation, allowed to dry, then the membrane floated, sample-side up, on denaturation solution for 15 min and rinsed in 2X SSC.

3. Many hybridization solutions contain carrier DNA (e.g., salmon sperm DNA) and Denhardt's solution (BSA, PVP and Ficoll); however, we have found these to be unnecessary Standard hybridization solution contains 5X SSC, 1% blocking reagent (Boehringer Mannheim or nonfat powdered milk, e.g., Marvel or Carnation), 0.1% *N*-lauroylsarcosine sodium salt, 0 02% SDS In this solution probes may be re-denatured by boiling for 10 min
4. Yields of B19-DNA from positive sera may vary greatly We have obtained 2–8 μg B19-DNA per mL of serum.
5. This method of DNA purification is limited by the DNA binding capacity of the silica; the manufacturers recommend 5 μL silica for up to 5 μg DNA, then a further 1 μL per 0.5 μg. Also, the presence of silica in a DNA solution can interfere with optical density readings, hence it is important to microfuge the DNA solution briefly before use to pellet any residual silica. You may prefer to substitute this with your favorite DNA purification method.
6. The amount of labeled DNA synthesized depends on the amount and purity of the template DNA and the length of incubation The incubation time can be varied from 1 to 20 h 1 μg of DNA labeled for 20 h was chosen as a convenient standard for reproducible results and maximum incorporation of label
7. The manufacturers state that the removal of unincorporated nucleotides is not necessary when labeling with the High Prime kit However, we found that purification by gel filtration did make a noticeable improvement in the background. Purification of directly labeled PCR products does not appear be necessary
8. The labeled control DNA is in a volume of 50 μL 5 μL of a 1 in 10 dilution is spotted onto the membrane. If the probe is in a volume of 400 μL but contains the same amount of DNA as the 50 μL control DNA, 4 μL of neat probe will contain the equivalent amount of DNA to the 5 μL diluted control
9. The membrane is baked to fix the DNA to the membrane. This can also be done by UV-crosslinking
10. Hybridization is complete after approx 5 h, but it is more convenient to hybridize overnight (approx 18 h).
11. Probes can be reused 10 times or more by gradually increasing the length of exposure to X-ray film With a new probe an initial exposure of 15 s will show up strong positives and a further exposure of about 5 min will reveal all 5 controls As the probe is reused these times will need to be increased until a probe used about 10 times will require an initial exposure of 10–15 min and perhaps a further exposure of 1 h. Digoxigenin-labeled probes are stable at –20°C for at least 1 yr without loss of activity.
12. For the briefest exposure to X-ray film, the chemiluminescent reaction should be at steady state. This is reached in 15 min at 37°C or 7–8 h at room temperature Further exposures can be obtained from the blot for up to 2 d after the addition of the chemiluminescent substrate

13 1 pg B19-DNA is equivalent to about 10^5 B19 genomes The titer of B19 virus in clinical specimens is frequently as high as 10^9–10^{11} genomes/mL *(9)*

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14

Polioviruses

Concurrent Serotyping and Intratypic Differentiation of Polioviruses

David J. Wood

1. Introduction

Human enteroviruses include polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses designated enteroviruses 68–71. They are classified as one genus of the *Picornaviridae*; rhinoviruses are a separate genus of the same virus family. All picornaviruses are small, icosahedral viruses 20-30 nm in diameter. Virons lack a lipid membrane and encase a single strand of positive-sense RNA about 7.5 kb long. Enteroviruses multiply in the alimentary tract but can spread to other organs and cause a variety of diseases, which depending on the individual enterovirus type, include poliomyelitis, aseptic meningitis, rashes, respiratory illness, eye disease, and cardiac disease. Rhinoviruses multiply in the respiratory tract and are one cause of the common cold.

Isolation of virus in cell culture remains the gold standard for diagnosis of enterovirus and rhinovirus infection. Molecular biological approaches are used to characterize isolates made in cell culture and to provide alternative diagnostic methods for direct detection of viruses in clinical specimens. This chapter will describe characterization of poliovirus isolates using a poliovirus-selective transgenic mouse cell line (L20B) and neutralizing monoclonal antibodies.

Polioviruses exist in three distinct serotypes designated type 1, type 2, and type 3, and each serotype can cause the disease poliomyelitis. In developed countries poliomyelitis has been controlled by vaccination, usually but not exclusively with the live attenuated vaccines developed by Sabin. The World Health organization has declared its intention of eliminating the disease from the world by the year 2000. As countries approach and achieve eradication there

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is a need to characterize poliovirus isolates as wild-type or vaccine-derived. The method described below is a simple variation of standard poliovirus diagnostic procedures that achieves concurrent serotyping and intratypic differentiation of isolates (*see* **Note 1**).

Polioviruses and other human enteroviruses grow in a variety of human or primate cell lines. Poliovirus cytopathic effect (cpe) is indistinguishable from cpe caused by nonpolio-enterovirus, and serotyping with neutralizing antisera is required to identify a poliovirus isolate. Where poliovirus and nonpolio-enteroviruses are present in the same isolate, resolution of poliovirus from the mixture can be difficult with conventional cell lines Isolation of the gene for the human cellular receptor for polioviruses *(1,2)* led to the development of transgenic mouse cell lines (L20B *[1]*, Lα *[2]*) that are susceptible to infection with polioviruses but very few other human enteric viruses *(3,4)*. Isolates that show enterovirus cpe in conventional cells can be serotyped in L20B or Lα cells, and polioviruses will be selectively identified even in the presence of other enteroviruses.

The major procedures involved in this protocol are neutralization of isolates with polyclonal and monoclonal antibody mixtures followed by inoculation of L20B cells. After incubation cells are fixed, stained, and scored for presence or absence of virus neutralization.

2. Materials

2.1. Viruses and Assay Medium

The cpe produced by polioviruses in conventional cell lines such as Hep 2, RD, and primary monkey kidney is indistinguishable from cpe produced by other enteroviruses. This consists of rounding up and detachment of cells from the culture substrate (**Fig. 1**). Cultures (or the culture fluids harvested from cultures) showing enterovirus cpe can be stably stored at –20°C (or lower) prior to concurrent serotyping/intratypic differentiation.

Assay medium is used for dilution of viruses, polyclonal, and monoclonal antibodies, and for resuspension of L20B cells. The following composition should be freshly prepared on the day of assay

1 87 5 mL Eagle's Minimum Essential Medium (Eagle's Salt Base)
2. 10 mL Fetal calf serum.
3 1 mL Penicillin/streptomycin solution
4. 5 mL Sodium bicarbonate solution (7.5%)

dMEM and sodium bicarbonate stock solutions should be stored at +2 to +8°C. Fetal calf serum and penicillin/streptomycin solutions should be stored at –20°C.

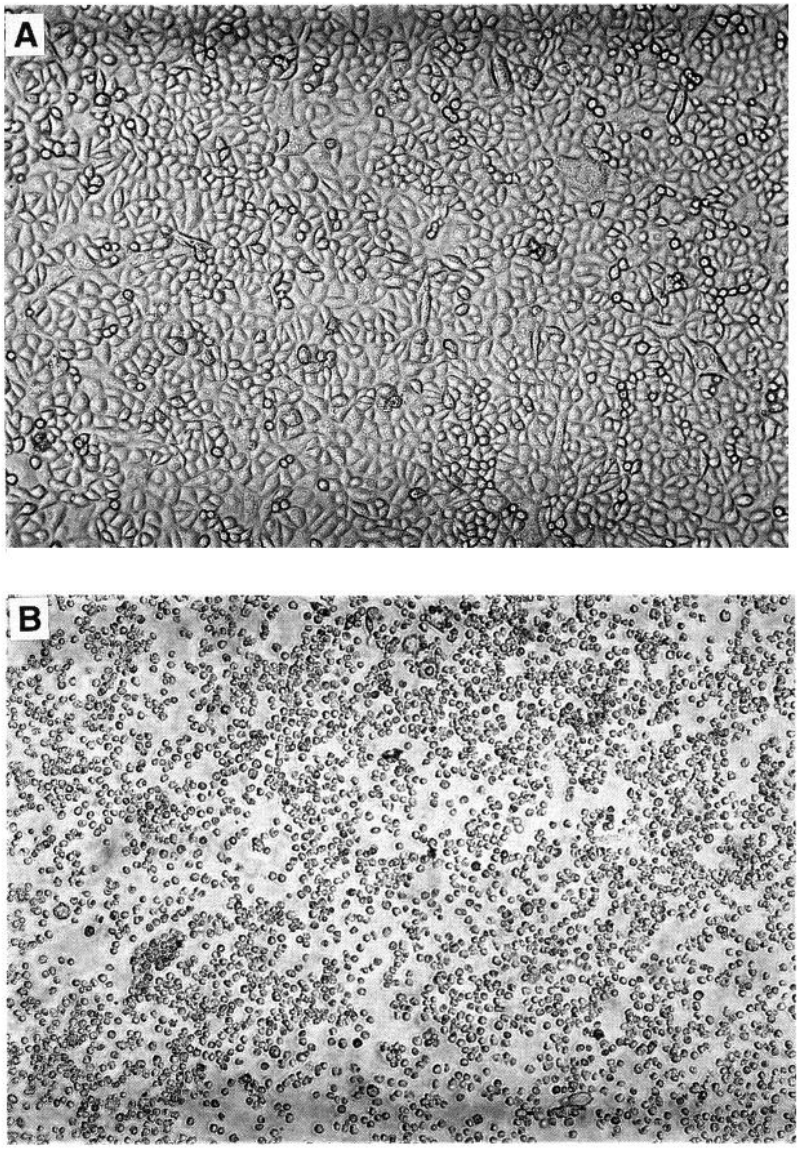

Fig. 1. Poliovirus cpe in L20B cells. **(A)** Control uninoculated cells **(B)** cells infected with poliovirus type 1 (Sabin) showing typical rounding of cells (×125).

2.2. Polyclonal and Monoclonal Antibodies

High-titer monospecific polyclonal antisera to poliovirus types 1, 2, and 3 are required (*see* **Note 2**). As recent vaccinees may excrete more than one poliovirus type it is necessary to use combined antiserum pools to identify isolates.

Four pools of poliovirus antisera (diluted in assay medium according to the suppliers' instructions) are freshly prepared as follows:

1. Antisera type 1 + antisera type 2
2. Antisera type 2 + antisera type 3.
3. Antisera type 1 + antisera type 3.
4. Antisera type 1 + antisera type 2 + antisera type 3.

All vials must be clearly labeled with the antisera contents and date made.

A trivalent mixture (i.e., antisera to poliovirus types 1, 2, and 3) of high-titer monoclonal antibodies that neutralizes only the Sabin strains of poliovirus is also freshly prepared according to instructions from the suppliers (*see* **Note 3**).

2.3. L20B Cells

Stock cultures of L20B cells should be maintained in a virus-free laboratory (*see* **Note 4**). The growth medium is described above (**Subheading 2.1.**). For concurrent serotyping/intratypic differentiation the cells should be suspended in assay medium in a microtiter plate at a concentration found by prior experiment to produce a confluent monolayer within 3 d. Single-cell suspensions of L20B cells should be used as soon as possible, but can be stored at +2 to +8°C for up to 3 h.

2.4. Other Reagents and Equipment

Flat-bottomed microtiter plates of tissue culture grade are required (*see* **Note 5**). Pressure sensitive film is used to seal plates for the neutralization step and after addition of cells.

Naphthalene black is used to stain plates at the end of the incubation period. This is made as follows

1. 1 g Naphthalene black.
2. 13.6 g Sodium acetate.
3. 60 mL Acetic acid.

Make up to 1 L in glass-distilled water. This reagent is stable when stored at room temperature. Other equipment includes tubes and 1-mL pipets for making virus dilutions and plugged 50-µL droppers for quick dispensing of the virus dilutions and polyclonal and monoclonal antibody mixtures.

3. Methods

3.1. Dilution of Viruses

Four arbitrarily selected dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-6}) have been found in practice to yield results with most cell culture-derived poliovirus isolates. The

use of four dilutions enables detection of virus mixtures in which one component is present in considerable excess over the other. Make the dilutions as follows.

1. Dispense 9.9 mL assay medium into a tube labeled 10^{-2} and 4 5 mL assay medium into 4 further tubes, labelled 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}, respectively
2. With a plugged pipet add 0.1 mL of the virus isolate into the 10^{-2} tube. Discard the pipet
3. Using a fresh pipet aspirate the contents of the 10^{-2} tube several times to mix, and transfer 0.5 mL to the 10^{-3} tube Discard the pipet.
4. Repeat step 3 with the 10^{-3}, 10^{-4}, and 10^{-5} tubes. Aspirate to mix the contents of the 10^{-6} tube
5. Always include three control viruses, one per poliovirus type, in each assay

3.2 Antibody Neutralization

1. Unwrap the number of plates required for the test. One isolate is tested per plate. Label all plates with a code, and date and record separately the isolate number corresponding to the code
2. Virus dilutions and antiserum pools should always be added to the same positions in the microtiter plate (**Table 1**). It is advisable to mark the grid pattern onto each plate
3. Starting with the 10^{-6} dilution, add 0.05 mL to 4 wells/row of the appropriate 6 rows of the microplate plate. Using the same plugged pipet or dropper add, in turn, the 10^{-4}, 10^{-3}, and 10^{-2} dilutions to the appropriate rows of the microtiter plate The 10^{-5} dilution made in **Subheading 3.1.** is *not* used.
4. Working across the microtiter plate from left to right add 0.05 mL of the antiserum mixtures described in **Subheading 2.2.** to all the wells of a row, as follows (and *see* **Table 1**).

Rows 1 and 7	Polyclonal antipoliovirus types 1 + 2
Rows 2 and 8	Polyclonal antipoliovirus types 2 + 3
Rows 3 and 9	Polyclonal antipoliovirus types 1 + 3
Rows 4 and 10	Polyclonal antipoliovirus types 1 + 2 + 3
Rows 5 and 11	Assay medium only
Rows 6 and 12	Monoclonal antipoliovirus (Sabin) types 1 + 2 + 3

5. Seal the plate with pressure-sensitive film and incubate at +35°C (±2°C) for 3 h (±15 min) to neutralize the poliovirus

3.3. Culture in L20B Cells

1. During the neutralization step, prepare a suspension of L20B cells from a healthy monolayer, as described in **Subheading 2.3.**
2. At the end of the neutralization step, remove the pressure-sensitive film, being careful not to cause splashes, and add 0.1 mL of cell suspension to each well of the plate. Reseal with fresh pressure-sensitive film.

Table 1
Layout of Microtiter Plate for Concurrent Serotyping and Intratypic Differentiation

	Microtiter Plate							
Antiserum[a]	H	G	F	E	D	C	B	A
po 1 + 2	1							
po 2 + 3	2							
po 1 + 3	3		10^{-6}			10^{-4}		
po 1 + 2+ 3	4		virus			virus		
assay medium	5		dilution			dilution		
mo 1 + 2 + 3	6							
po 1 + 2	7							
po 2 + 3	8		10^{-3}			10^{-2}		
po 1 + 3	9		virus			virus		
po 1 + 2+ 3	10		dilution			dilution		
assay medium	11							
mo 1 + 2 + 3	12							

[a]po, polyclonal antipoliovirus antisera, mo, monoclonal antipoliovirus (Sabin) reagents

3 Incubate the plates at +35°C (±2°C) for 4–5 d, then fix and stain in naphthalene black as described below (*see* **Note 6**).
4 Remove pressure-sensitive film and discard culture medium from plates into a suitable disinfectant (e.g., 2% chloros). Fix cell sheets by immersion in formaldehyde for 30 min (*see* **Note 7**) Decant fixative from plate
5. Stain plate by immersion in naphthalene black for 30 min (± 5 min) (*see* **Note 7**) Decant naphthalene black from all wells and wash under running water to rinse free stain from the plate Dry plates on blotting paper
6 Fixed and stained plates are stable at room temperature and therefore results can be recorded when convenient. Over 4–5 d poliovirus growth will destroy the entire cell sheet of a microtiter plate well. Where virus has been neutralized a confluent monolayer of cells will have formed.
7 Score each well as virus-positive (+) (no cells left) or virus-negative (–) (confluent monolayer) Record details in a laboratory notebook that also records the complete specimen number of each sample

3.4. Interpretation of Results

1. The overall assay is valid if the control viruses have grown and given the expected serotyping results and if there are confluent cell monolayers in wells where the control viruses are neutralized

Table 2
Examples of Neutralization Patterns

Sample	Antiserum[a]	Virus positive (+) or negative (–) at virus dilution 10^{-6}	10^{-4}	10^{-3}	10^{-2}	Conclusion
A	po 1 + 2	– – – –	– – – –	– – + –	+ + + +	Poliovirus type 2,
	po 2 + 3	– – – –	– – – –	– + – –	+ + + +	Sabin-like Neutral-
	po 1 + 3	+ + + +	+ + + +	+ + + +	+ + + +	izing capacity of
	po 1 + 2 + 3	– – – –	– – – –	– + + –	+ + + +	antisera exceeded
	assay medium	+ + + +	+ + + +	+ + + +	+ + + +	at 10^{-3} and 10^{-2}
	mo 1 + 2 + 3	– – – –	– – – –	– + + –	+ + + +	virus dilutions
B	po 1 + 2	– – – –	– – – –	+ + – +	+ + + +	Poliovirus type 1
	po 2 + 3	+ + + +	+ + + +	+ + + +	+ + + +	Intratypic character-
	po 1 + 3	– – – –	– – – –	+ – – –	+ + + +	ization inconclusive
	po 1 + 2 + 3	– – – –	– – – –	– – + –	+ + + +	Neutralizing capacity
	assay medium	+ + + +	+ + + +	+ + + +	+ + + +	of antisera exceeded
	mo 1 + 2 + 3	+ + + +	+ + + +	+ + + +	+ + + +	at 10^{-3} and 10^{-2} virus dilutions
C	po 1 + 2	– – – –	– – – –	– – – –	– – – –	Poliovirus types 1
	po 2 + 3	+ + + +	+ + + +	+ + + +	+ + + +	and 2 Type 1 pre-
	po 1 + 3	– – – –	+ + + +	+ + + +	+ + + +	sent at higher titer
	po 1 + 2 + 3	– – – –	– – – –	– – – –	– – – –	Both serotypes
	assay medium	+ + + +	+ + + +	+ + + +	+ + + +	Sabin-like
	mo 1 + 2 + 3	– – – –	– – – –	– – – –	– – – –	

[a]po, polyclonal antipoliovirus antisera, mo, monoclonal antipoliovirus (Sabin) reagents

2. When poliovirus is present in an isolate, virus growth should occur at one or more of the dilutions tested (*see* **Note 8**) Select the highest virus dilution at which all four wells that received isolate plus assay medium only (rows 5 and 11, **Table 1**) are scored as virus-positive. At that dilution, neutralization patterns with the polyclonal antiserum mixtures will identify the poliovirus serotype(s) present in the sample (*see* examples in **Table 2**).
3. At the same dilution identified in **step 2** above, the neutralization pattern with the monoclonal antibody mixture will provide intratypic characterization Examples are given in **Table 2**. Neutralization in all four wells shows that the serotype is Sabin-like. Virus growth in all four wells suggests either that the serotype is wild-type or that it is a Sabin strain with changes in the antigenic epitope recognized by the monoclonal antibody (*see* **Note 9**). Further studies with different methods are required to characterize such isolates.
4 Results at dilutions other than that identified in step 2 may reveal the presence of a second (or third) serotype present as a minor component (Table 2). Results with the Sabin-specific monoclonal antibodies may also show the vaccine-like character of such minor components.

5 If the sample has a high titer of poliovirus the neutralizing capacity of the antisera may be exceeded at some virus dilutions (**Table 2**)

4. Notes

1 A number of alternative methods are available for intratypic differentiation of polioviruses These can be based on antigenic properties of the virus (e.g., ELISA using cross-absorbed polyclonal sera) or on nucleic acid methods (e.g , probe hybridization, restriction fragment length polymorphism, or PCR). A WHO Collaborative Study recently compared these methods *(5)*.

2 Polyclonal antisera to poliovirus types 1, 2 and 3 can be obtained commercially Diluted bivalent antiserum pools (i.e , pools 1, 2, and 3 in **Subheading 2.2.**) should be checked for lack of neutralization of the absent poliovirus type. For example, a poliovirus type 3 reference virus should be titrated in the presence and absence of antiserum pool 1 (antipoliovirus types 1 and 2). The difference in titer in the presence and absence of antiserum should be no more than 1 0 $\log_{10}$ tissue culture infectious doses $TCID_{50}$ for sera with no cross-reactivity.

3. Sabin-specific neutralizing monoclonal antibodies are available on application to the author.

4. L20B cells are available on application to the author. A master cell bank and a working cell bank should be established on receipt These cell banks should be stored in gaseous or liquid nitrogen. Careful records should be maintained and routine subculturing of stock cultures limited to 15 passages This minimizes the risk of *Mycoplasma* contamination and also the theoretical risk of loss of poliovirus sensitivity from overgrowth of poliovirus receptor-expressing cells by cells not expressing the poliovirus receptor.

5 The use of microtiter plates for typing of poliovirus isolates requires careful attention to technique to prevent cross-contamination between wells

6. Alternatively, each well of the plate can be read microscopically. Poliovirus gives a typical cpe in L20B cells (**Fig. 1**) and wells showing such cpe should be scored positive

7 During immersion fixation and immersion staining it is important to ensure that all wells are filled with fixative or stain. This will not occur if there are air bubbles in any of the wells

8. L20B cells are highly selective for polioviruses but some other viruses, e.g., reoviruses, will grow in this line *(3)* However, polioviruses grow much quicker and are the only viruses likely to cause complete cpe in the 4–5 d assay period. If no growth occurs in L20B cells the most likely explanation is that the isolate is not a poliovirus. However, as the sample is diluted 10^{-2} or more, it is advisable to check that the sample does not contain a very low amount of poliovirus. This is done by inoculation of undiluted sample into L20B cells If no growth occurs after 4–5 d, the sample does not contain a poliovirus.

9. Antigenic variants are known to occur during multiplication of the Sabin vaccine strains in vaccinees *(6)*. Some of the variants can have changes in the antigenic sites recognized by the monoclonal antibodies. However, studies at the National

Institute for Biological Standards and Control (Potters Borough, Herts, UK) show that 80–90% of poliovirus isolates can be satisfactorily characterized with the available reagents (unpublished data).

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15

Poxviruses

Hermann Meyer, Susan L. Ropp, and Joseph J. Esposito

1. Introduction

Poxviruses are large, double-stranded DNA viruses that replicate in the cytoplasm of infected cells. The family *Poxviridae is* divided into two subfamilies, the *Entomopoxvirinae* of insects and the *Chordopoxvirinae* of vertebrates; the latter consists of eight genera and several unclassified viruses. The genus *Orthopoxvirus* comprises morphologically and antigenically closely related viruses, including the now eradicated variola (smallpox) virus and several pathogens of veterinary and zoonotic importance.

According to the current taxonomy *(1)*, orthopoxviruses (OPVs) are allocated into 11 species (**Table 1**). DNA maps and various other molecular biologic features have been determined to varying extents for 10 of these; the 11th, Uasin Gishu African horsepox virus, was last studied in the early 1970s *(2)*. Identification and differentiation of the 10 currently available species have been achieved by various methods, including restriction endonuclease cleavage site mapping (*3,4*) which has provided definitive information for virus classification in support of immunologic and biologic criteria. Recently, polymerase chain reaction (PCR) methods have been applied to rapidly identify and subtype available OPVs (*5–7*).

In this chapter, three methods are described for the preparation of OPV-DNA (**Subheadings 3.1., 3.2., and 3.3.**) and three methods are described for PCR identification of OPV (**Subheadings 3.4., 3.5., and 3.6.**). DNA prepared according to the first method (**Subheading 3.1.**) is more suited for restriction enzyme cleavage analysis as depicted in **Fig. 1**, which shows that variations in patterns are useful for OPV identification. Isolates or strains of a given species can often be differentiated by observing relative minor changes in the electropherograms. DNA prepared by all three methods is suitable for PCR analysis.

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Table 1
Old World Eurasian-African and New World North American Orthopoxviruses

Species	Host Range	Occurrence
Old world		
Variola	Human	Eradicated
Vaccinia	Buffalopox in milking buffalo, dairy cattle, and humans. Rabbitpox in Utrecht & Rockefeller Institute laboratory rabbits	Worldwide use as smallpox vaccine and laboratory virus Buffalopox in India, Egypt Indonesia, Pakistan
Monkeypox	Humans, various monkeys, and arboreal squirrels *Funisciurus anerythrus and Heliosciurus rufobrachium*	African rainforest
Camelpox	Camel	Africa, Asia
Cowpox	Bovine, human, rodent, feline, various zoo animals	Europe, Asia
Ectromelia	Mouse	Worldwide in laboratory mice
Gerbilpox	*Tatera kempii*	Africa (Dahomey)
Uasin Gishu	Horses	Africa (Kenya)
New world		
Raccoonpox	*Procyon lotor*	Maryland, USA
Volepox	*Peromyscus, Microtus*	California, USA
Skunkpox	*Mephitis mephitis*	Washington, USA

Particularly, DNA can be quickly prepared from infected chick embryo chorioallantoic membranes, various clinical samples (e.g., crusted scab, dried vesicle fluid of lesions) by **Subheading 3.2.**, or very rapidly from infected cell cultures by **Subheading 3.3.**

The three PCR protocols presented here should enable relatively simple and accurate detection and differentiation of OPVs from sometimes devastating infections judging from a limited number of analyses with field samples to date. The ATI-PCR assay *(5)* described in **Subheading 3.4.** was accomplished by the selection of two oligonucleotides that correspond to sequences within the gene that encodes the acidophilic, or A-type, inclusion protein (ATI) of cowpox virus. The primer pair described is based on sequences flanking a region that exhibits various deletions in a comparison of corresponding sequences

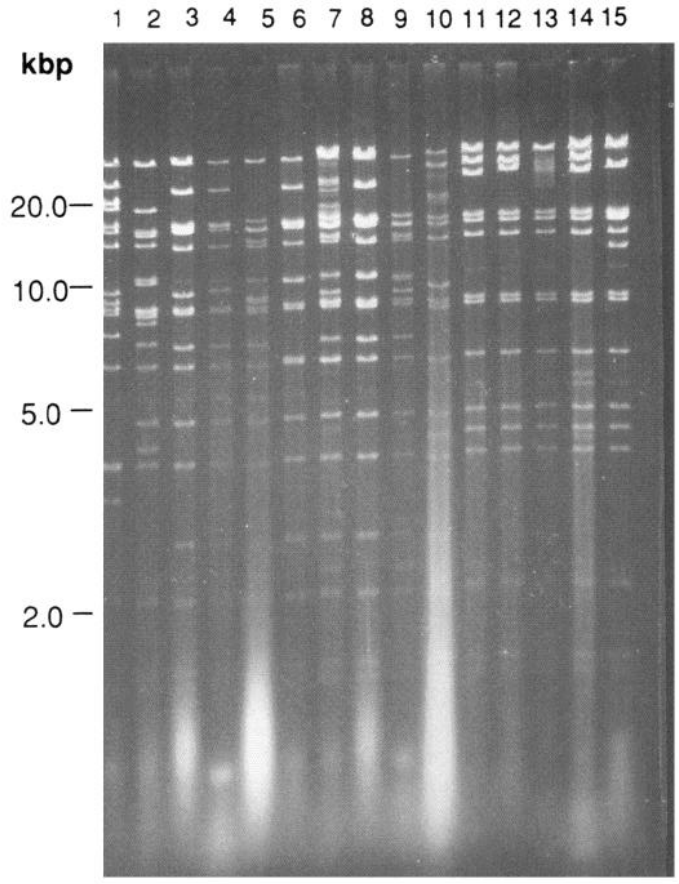

Fig. 1. Electropherogram of orthopoxviruses genome DNAs digested with *Hin*dIII. (1) cowpox virus Brighton (CPX-BRT), (2) camelpox virus Somalia (CML-SOM78), (3) variola virus Harvey (VAR-HAR44), (4) variola virus Somalia (VAR-SOM77), (5) variola virus Sierra Leone (VAR-SLN68), (6) variola virus Bangladesh (VAR-BSH75), (7) variola virus Butler (VAR-BUT52), (8) variola virus Congo (VAR-CNG70), (9) variola virus Brazil Garcia (VAR-GAR66), (10) vaccinia virus Wyeth high egg passage (VAC-CV178), (11) vaccinia virus Venezuela red pock (VAC-VCX), (12) vaccinia virus Venezuela smallpox vaccine (VAC-VNV), (13) vaccinia virus Temple of Heaven (VACVTH), (14) vaccinia virus Wyeth Dryvax New York Board of Health (VAC-NYBH), (15) vaccinia virus Lister (VAC-LIS).

determined for vaccinia, mousepox, monkeypox, and camelpox viruses. Thus, depending on which of the five species is being examined, the ATI-PCR method provides a DNA fragment of distinct size. The protocol described in **Subheading 3.4.** combined with BglII digest electrophoresis of the resultant

Table 2
DNA Fragment Sizes[a] After 56 Orthopoxvirus DNAs Were Separately Amplified by PCR Using Primer Pair ATI-low-1 and ATI-up-1 and After the PCR Products Were Cleaved with *Bgl*II

Species	Size of PCR product	Size of *Bgl*II fragments from product	Samples tested
Cowpox	1672	522, 466, 293, 165, 154, 72	22
Vaccinia	1596	470, 444, 291, 165, 154, 72	7
Ectromelia	1219	519, 345, 145, 93, 70, 47	6
Camelpox	881	399, 165, 154, 91, 72	20
Monkeypox	1500	475, 470, 180, 165, 154, 64	1

[a]Sizes of fragments based on sequencing agreed well with sizes determined by electrophoresis in gels *(5)*

fragment has enabled correct species assignment of 56 different isolates already known to belong to the five species (**Table 2**), and new data indicate that smallpox variola virus can also be distinguished by ATI-PCR.

The hemagglutinin gene PCR assay (HA-PCR) described in **Subheading 3.5.** targets the open reading frame coding for the hemagglutinin (HA) glycoprotein *(6)*, the major component of the HA antigen, an infected cell membrane complex that distinguishes OPVs from all other poxvirus genera. A consensus sequence primer pair was devised to amplify an HA-DNA fragment from genome DNA of known New World (i.e., North American) OPVs: raccoon, skunk, and volepox viruses. A second pair of consensus primers was devised to amplify an HA-DNA fragment from the genome of Old World (i.e., Eurasian-African) OPVs: variola, vaccinia, cowpox, monkeypox, camelpox, ectromelia mousepox, and gerbilpox virus (Note: no virus or DNA has been available for Uasin Gishu virus, thus it remains unstudied). Using HA-PCR, RsaI digest electrophoresis of the HA-PCR product from the North American OPVs enhances resolution of fragment differences. TaqI digestion enables identification and differentiation of the seven Eurasian-African OPVs; to date, 79 isolates have been verified (**Table 3**). Interestingly, use of primers with no mismatches has further enabled precise distinction of individual virus DNAs; we were able to amplify and discern individual virus DNAs in deliberately cross-contaminated samples *(6)*.

Table 3
DNA Fragment Sizes[a] After 83 Orthopoxvirus DNAs Were Separately Amplified by PCR Using Primer Pair NACP1+2 or EACP1+2 and After the PCR Products Were Cleaved with *TaqI* or *RsaI*

Species	Size PCR fragment using Primer		Size digest fragment	Samples tested
	NACP1+2	EACP1+2		
New World			*RsaI*	
Raccoonpox	652	None	194, 192, 153, 113	2
Volepox	580	None	467, 113	1
Skunkpox	658	None	264, 202, 113, 71, 8	1
Old World			*TaqI*	
Variola	None	942	536, 406	33
Vaccinia	None	948	451, 295, 105, 97	5
Camelpox	None	960	474, 331, 90, 75	4
Monkeypox	None	942	451, 220, 105, 91, 75	24
Ectromelia	None	846	343, 220, 111, 97, 75	3
Cowpox	None	942	303, 289, 115, 96, 91	9
Gerbilpox	None	960	342, 331, 97, 80, 75, 35	1

[a]Sizes of the fragments determined by sequencing agreed with sizes determined by fragment electrophoresis *(6)*

The third PCR assay described in **Subheading 3.6.**, also targets the HA gene; however, in this assay a small region within the HA open reading frame is amplified, and the fragment size designates the species. For this method, a set of primers and modified PCR conditions had to be devised from a comparison of multiple aligned HA base sequences for 50 different OPV isolates and by various titrations to establish suitable PCR reagent concentrations. **Figure 2** illustrates the primer annealing locations within the HA sequences and the expected size fragment to distinguish species.

2. Materials

2.1. Preparation of Poxvirus DNA: Extraction of Cytoplasmic OPV-DNA

1. TSE buffer: 10 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 5 m*M* disodium ethylenediaminetetraacetic acid (Na_2EDTA).
2. TKE buffer: 10 m*M* Tris-HCl, pH 8.0, 10 m*M* KCl, 5 m*M* Na_2EDTA
3. 10% Triton X-100. Filter through 0.45-μm nitrocellulose membrane.
4. 14.3 *M* 2-mercaptoethanol.
5. TE buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* Na_2EDTA.
6 54% (w/w) Sucrose.

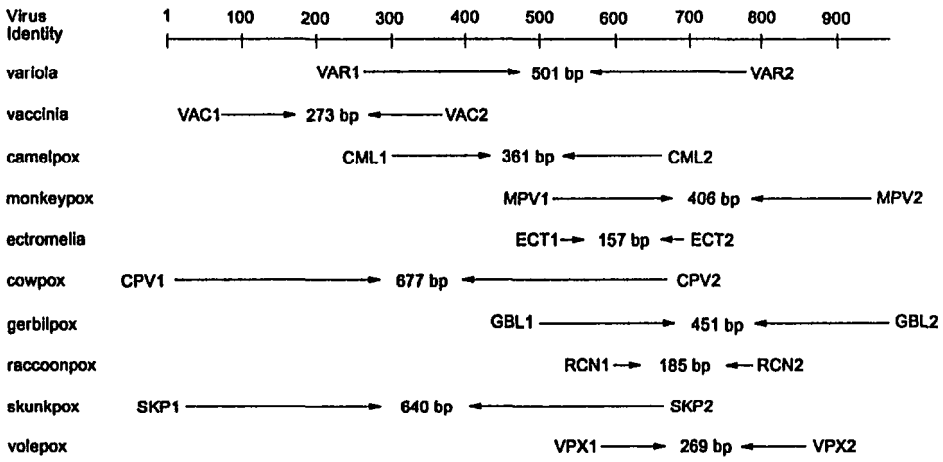

Fig. 2. Annealing location and base pair (bp) size of amplification product expected by using primers variola (VAR1&2), vaccinia (VAC1&2), camelpox (CML1&2), monkeypox (MPV1&2), ectromelia (ECT1&2), cowpox (CPV1&2), gerbilpox (GBL1&2), raccoonpox (RCN1&2), skunkpox (SKP1&2), and volepox (VPX1&2)

7 10 mg/mL Proteinase K solution (store in aliquots at –20°C)
8 10% Sodium dodecyl sulfate (SDS, genetic-technology-grade).
9 5 *M* NaCl.
10. 25.24:1 Phenol:chloroform:isoamyl alcohol mixture (genetic-technology-grade phenol is washed thrice with TE buffer and then combined with chloroform and isoamyl alcohol).
11. 24:1 Chloroform: isoamyl alcohol mixture
12. BPB dye. 0 25% bromphenol blue in 40% (w/v) sucrose.
13. SeaKem GTG agarose (FMC BioProducts, Rockland, ME).
14 TAE buffer: 40 m*M* Tris-HCl, 5 m*M* sodium acetate, 1 m*M* Na_2 EDTA, pH 8.0, prepared from 50X concentrate (prior to use, for 1X buffer, add ethidium bromide solution (3 mg/mL) to 0.5 μg/mL, wear gloves)
15. 1-kb Ladder DNA marker (Gibco-BRL, Gaithersburg, MD).
16. Restriction endonuclease *Hin*dIII (store at –20°C) and appropriate 10X reaction buffer (Boehringer Mannheim, Germany).
17. 301-nm Light source to visualize and photograph DNA in gels.

2.2. Preparation of Poxvirus DNA: Preparation of DNA from Clinical Samples

1 Microfuge tube pestle (Kontes, Inc , Vineland, NJ).
2. Lysis solution: 50 m*M* Tris-HCl, pH 8.0, 100 m*M* Na_2EDTA, 100 m*M* NaCl, 1% SDS.
3. 20 mg/mL Proteinase K (stored in aliquots at –20°C)

4. 25.24:1 Phenol:chloroform.isoamyl alcohol mixture (genetic-technology-grade phenol is washed thrice with TE buffer and then combined with chloroform and isoamyl alcohol).
5. 24:1 Chloroform:isoamyl alcohol mixture.
6. 100% Ethanol.
7 70% Ethanol.

2.3. Preparation of Poxvirus DNA: Lysis of OPV-Infected Cells for Use in PCR

Lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* Na_2EDTA, 0.5% Tween-20; prior to use add proteinase K to give a final concentration of 50 µg/mL.

2.4. Detection and Differentiation of OPV by Using PCR: ATI-PCR Assay

1. DNA prepared as described above.
2 Primers ATI-up-1: 5'-AATACAAGGAGGATCT-3' and ATI-low-1. 5'-CTTAAC-TTTTTCTTTCTC-3'
3. GeneAmp PCR Reagent Kit (Perkin-Elmer, Norwalk, CT)
4. Mineral oil (if needed for thermocycler; Perkin-Elmer Model 9600 requires no oil).
5. NuSieve GTG and SeaKem GTG agarose (FMC BioProducts, Rockland, MD).
6 *Bgl*II (store at –20°C) and appropriate 10X reaction buffer (Boehringer Mannheim).

2.5. Detection and Differentiation of OPV by Using PCR: Hemagglutinin-PCR Assay (HA-PCR)

1. DNA prepared as described above.
2. Eurasian-African OPV-HA consensus primers EACP1: 5'-ATGACACGATT-GCCAATAC-3'; EACP2· 5'-CTAGACTTTGTTTTCTG-3'.
3. North American OPV-HA consensus primers NACP1: 5'-ACGATGTCGT-ATACTTTGAT-3', NACP2: 5'-GAAACAACTCCAAATATCTC-3'.
3. GeneAmp PCR Reagent Kit (Perkin-Elmer, Norwalk).
4. Mineral oil.
5. NuSieve GTG and SeaKem GTG agarose (FMC BioProducts).
6. RsaI and TaqI (store at –20°C; Boehringer Mannheim).

2.6. Detection and Differentiation of OPV by Using PCR: Species-Specific PCR

1. DNA prepared as described above.
2 25 m*M* $MgCl_2$.
3. GeneAmp PCR Reagent Kit (Perkin-Elmer).
4. Mineral oil.
5 NuSieve GTG and SeaKem GTG agarose (FMC BioProducts).
6. Variola-specific primers VAR1: 5'TAAATCATTGACTGCTAA-3'; VAR2· 5'GTA-GATGGTTCATTATCATTGTG-3'.

7 Vaccinia-specific primers VAC1 5'-ATGCAACTCTATCATGTAA-3', VAC2 5'-CATAATCTACTTTATCAGTG-3'
8. Camelpox-specific primers CML1: 5'GCCGGTACTTATGTATGTGT-3', CML2: 5'GATCTTCTTCTTTATCAGTG-3'.
9 Monkeypox-specific primers MPV1: 5'CTGATAATGTAGAAGAC-3'; MPV2: 5'-TTGTATTTACGTGGGTG3'.
10. Ectromelia-specific primers ECT1· 5'CATACAGTCACAGACACTGTTG-3'; ECT2. 5'GATGCTTTCTACAGTTGTTGGTA-3'.
11 Cowpox-specific primers CPV1. 5'ATGACACGATTGCCAATACTTC-3'; CPV2· 5'CTTACTGTAGTGTATGAGACAGC-3'
12 Gerbilpox-specific primers GBL1: 5'CGTCGGTATTCGAAATCGCGAA-3'; GBL2· 5'GTTTTGTATTTACGTGAACGG-3'
13. Raccoonpox-specific primers RCN1: 5'GATGATACGCAATATAATGT-3'; RCN2. 5'TCTACCGTTGTTGGTATCGAG-3'.
14 Skunkpox-specific primer pair SKP1: 5'AGTTCTGCTAATATCGCTAG-3'; SKP2· 5'AGTGGTTGTGGGAGCAGTGG-3'
15 Volepox-specific primers VPX1: 5'CCATCACCAGAAGTAGTTGCAG-3'; VPX2: 5'ATATGTGCTCCATATGAACT-3'

3. Methods

3.1. Preparation of Poxvirus DNA: Extraction of Cytoplasmic OPV-DNA

1. Infect ten 150-cm^2 tissue culture monolayers (monkey kidney or other suitable cell line) at 95% confluence with a virus multiplicity of 0.1–0.01 PFU/cell; incubate at 37°C until severe cytopathic effect is evident (usually 3 d)
2. After chilling cultures at 4°C for 2–16 h, scrape infected cells from the tissue culture flask.
3 Transfer cell suspension to conical 50-mL centrifuge tubes, collect cells by centrifugation for 10 min at 500*g* at 23°C.
4 Suspend cells in 100 mL TSE buffer and pellet as in **step 3**
5. Suspend the pellet in 18 mL TKE buffer at 4°C for 10 min, every 5 min gently vortex mixture for 15 s.
6. Add 2 mL 10% Triton X-100, mix gently, add 50 µL 2-mercaptoethanol, then mix gently and chill on ice.
7 Vortex chilled mixture slowly after 5 min and again at 10 min; observe cells microscopically for cytoplasmic lysis
8. Remove nuclei by centrifugation for 10 min at 1500*g* at 4°C.
9. Transfer the supernatant fluid (cytoplasmic extract) to a fresh polycarbonate tube; concentrate viral cores by centrifugation for 30 min at 20,000*g* and 4°C
10. Aspirate the supernatant fluid and suspend the pelleted viral cores in 0.8 mL cold TE buffer; use a syringe with a 20-gauge cannula attached.
11. Add in order by gentle mixing. 1.4 mL 54% sucrose, 15 µL 2-mercaptoethanol, and then 50 µL proteinase K solution. Incubate on ice for 15 min.

12. Place tubes at 37°C, add 250 µL 10% SDS, mix gently, and incubate at 37°C overnight or at 50°C for 2–4 h
13 Add 0.4 mL 5*M* NaCl; mix gently.
14 Extract DNA from the digested lysate thrice with an equal vol of phenol.chloroform·isoamyl alcohol mixture; use gentle manual shaking for several minutes or use motor-driven rocker to avoid shearing the DNA Separate the extraction phases at 8000*g* for 1 min, remove phenol (bottom) phase using a Pasteur pipet.
15. Aspirate final phenol phase completely, gently extract the aqueous phase twice using an equal vol of chloroform:isoamyl alcohol mixture
16. Transfer aqueous phase to a small, wetted dialysis tubing; dialyze TE buffer overnight with at least two buffer changes. Transfer dialyzed DNA solution to a microfuge tube; add a few drops of chloroform to preserve DNA solution at 4°C.
17 Estimate the DNA concentration by optical density at 260 nm (1 OD_{260} ~ 45 µg DNA). DNA prepared by this method is suitable for restriction enzyme analysis or PCR assay.
18. Prepare a restriction digest reaction using approximately 2 µg DNA in a total volume of 20 µL (*Hin*dIII digest patterns are often used for comparison with digest pattern in **refs. *3*** and ***4***). Digest for 3 h to overnight at 37°C; add 2 µL of BPB dye solution prior to separating DNA fragments in a 0.6% agarose gel submerged run in TAE buffer at 0.7 V/cm/20 h. Visualize gel stained with ethidium bromide; photograph.

3.2. Preparation of Poxvirus DNA: Preparation of DNA from Clinical Samples

1 Suspend a small aliquot of crusted scabs in 90 µL lysis solution, add 10 µL proteinase K, and digest for 10 min at 37°C
2. Disrupt the scab with a microfuge tube pestle.
3. Add another 350 µL lysis solution and 50 mL proteinase K, mix gently, and then incubate for 2 h at 37°C.
4. Extract the lysed suspension twice with an equal volume of phenol:chloroform:isoamyl alcohol mixture Separate the extraction phases at 8000*g* for 1 min
5. Aspirate the phenol (bottom) phase completely; extract the aqueous phase twice with an equal vol of chloroform:isoamyl alcohol mixture.
6. Transfer aqueous phase to a new tube; add 2 vol cold absolute ethanol.
7. Place tube at –70°C for about 30 min to precipitate DNA.
8. Collect the DNA precipitate by centrifugation at 15,000*g* for 5 min at 4°C.
9. Aspirate the supernatant fluid completely; wash the pellet with 70% ethanol by centrifugation as in **step 4**.
10 Air dry DNA for a few minutes at room temperature; dissolve the DNA pellet in 10 µL H_2O

3.3. Preparation of Poxvirus DNA: Lysis of OPV-Infected Cells for Use in PCR

1. Infect a 25-cm^2 tissue culture monolayer (monkey kidney or other suitable cell line) at 95% confluence with a virus multiplicity of 0.1–0.01 PFU/cell and incubate at 37°C until severe cytopathic effect is evident (~3 d).
2. Harvest infected cells from the surface by scraping into the medium, mix 100 µL with an equal vol of lysis buffer.
3. Incubate at 56°C for 1 h.
4. Inactivate the proteinase K in the lysate by incubating at 100°C for 15–20 min; chill on ice.
5. Use 1–2 µL of digest for each PCR assay.

3.4. Detection and Differentiation of OPV by Using PCR: ATI-PCR Assay

1. Prepare template DNA from virions, chemical samples, or infected cells as outlined above (**Subheadings 3.1., 3.2.,** and **3.3.**).
2. In a 0.5-mL microcentrifuge tube, mix into a total reaction volume of 100 µL: 2 µL of each dNTP, 10 µL of 10X PCR buffer, 60 ng each of ATI-up-1 and ATI-low-1, and appropriate vol H_2O; then add 50 ng template DNA, and 2.5 U *Taq* polymerase.
3. Centrifuge at 1000*g* for 15 s, and overlay with 50 µL mineral oil if necessary.
4. Place sample in an automatic thermocycler. Cycle as follows:
   a. 6 min at 94°C (once),
   b. 1 min at 94°C; 1 min at 45°C; 2.5 min at 72°C (repeat 24–29 times);
   c. 10 min at 72°C (once), and
   d. Store at 4°C until analysis.
5. Mix 10 µL reaction mixture with 1 µL BPB dye solution and load onto 1% SeaKem-GTG submerged agarose gel in TAE buffer. Load a parallel track with DNA-marker ladder. Separate the reaction products at 100 V until BPB dye has migrated about 5 cm.
6. Visualize DNA and photograph.
7. To enhance the resolution of fragment-size differences, mix 10 µL of the PCR products with 10 U *Bgl*II restriction endonuclease, add 2 µL 10x restriction enzyme buffer and water to give a total vol of 20 µL. Incubate for 2 h at 37°C.
8. Perform electrophoresis in a 3% NuSieve GTG agarose—1% SeaKem GTG submerged agarose gel. Visualize and photograph as above (*see* **Note 1**).

3.5. Detection and Differentiation of OPV by Using PCR: Hemagglutinin-PCR Assay (HA-PCR)

1. Prepare template DNA as outlined above.
2. Set up the reaction mixture as in **Subheading 3.4., step 2**; use either 60 ng of each primer NACP1 plus NACP2 or EACP1 plus EACP2, respectively (add mineral oil if necessary).

3. Place sample in an automatic thermocycler. Cycle as follows:
   a. 6 min at 94°C (once);
   b. 1 min at 94°C; 2 min at 55°C; 3 min at 72°C (repeat 24 times);
   c. 10 min at 72°C (once); and
   d. Store at 4°C until analysis.
4. Mix 15 µL of reaction mixture with 1.5 µL BPB dye solution, then load onto submerged gel (3% NuSieve GTG agarose—1% SeaKem GTG agarose). Load a parallel lane with DNA-ladder size marker. Separate by electrophoresis in TAE buffer at 100 V until BPB dye has migrated about 5 cm.
5. Visualize DNA and photograph.
6. To enhance the resolution of fragment-size differences, add 5 U *Rsa*I (for North American OPVs) and *Taq*I (for Eurasian-African OPVs) to 30 µL of the resulting PCR mixture; incubate 2 h at 37°C for *Rsa*I or at 65°C for *Taq*I. Perform electrophoresis in 3% NuSieve GTG agarose—1% SeaKem GTG agarose submerged gel (include DNA size marker); visualize and photograph as above (*see* **Note 1**)

3.6. Detection and Differentiation of OPV by Using PCR: Species-Specific PCR

1. Into 10 separate microcentrifuge tubes add 10 µL each of 10X reaction buffer and appropriate amounts of dNTPs, $MgCl_2$, and the respective OPV specific primer pair as follows:

Primer pair	m*M* dNTP	m*M* $MgCl_2$	ng each primer
MPV1 + 2	800	2 5	500
RCN1 + 2	800	2.5	500
VPX1 + 2	800	2 5	500
ECT1 + 2	800	1.5	500
VAR1 + 2	25	1.5	500
SKP1 + 2	25	1.5	100
GBL1 + 2	8	1.5	500
CPV1 + 2	8	1.5	250
CML1 + 2	8	1.5	25
VAC1 + 2	8	1.5	25

2. Add H_2O, 50 ng template DNA, and 2.5 U *Taq* polymerase to a total volume of 100 µL.
3. Place sample in automatic thermocycler (e.g , Perkin-Elmer, Model 9600). Cycle as follows:

a. 6 min at 94°C (once),
b. 1 min at 94°C; 2 min at 55°C, 3 min at 72°C (repeat 24 times),
c. 10 min at 72°C (once),
d 4°C until analysis.

4 Mix 15 µL of the reaction products with 1.5 µL BPB dye solution; load onto 3% NuSieve GTG agarose—1% SeaKem GTG agarose submerged gel In one lane load 1-kb DNA ladder as size marker. Perform electrophoresis (100 V) in TAE buffer.
5 Visualize and photograph (*see* **Note 2**).

4. Notes

1. In applying the present protocols, it is suggested that the PCR be done first using consensus primers, i e , the ATI- or HA-primer pair (**Subheading 3.4.** or **3.5.**). This procedure allows a differentiation of species based on restriction fragment length polymorphism (RFLP) of the resulting PCR products. The paper by Ropp et al. ***(6)*** also describes an alternative method of amplifying the HA open reading frame of different OPVs with a set of primer pairs of more exact sequence specificity than the consensus primers described here. The RFLP obtained after using the set of primers with no mismatches were comparable to those with the consensus primers, although more reactions are needed, greater specificity is gained.
2 We currently suggest that the results obtained with the third method (**Subheading 3.6.**) should be interpreted with caution because it has not been evaluated as extensively as the HA- or ATI-PCR methods; such work is in progress The specificity of the primers relies on the precise use of the PCR conditions described It is also suggested that serologic and biologic tests such as electron microscopy, virus growth morphology on chorioallantoic membranes of 12 d-old hens eggs, and antigenic analysis ***(8,9)***, in addition to direct sequencing of the products from the three PCR methods here should always be considered for reference identification and verification of new orthopoxvirus isolates. The primers we developed appeared to work well with a variety of purified virus DNA preparations and they have been successfully used to examine a limited number of clinical samples (**Tables 2** and **3**). However, further validation with various specimens is needed to gain confidence in the procedures and provide further information on the extent of efficacy and accuracy of the assays described in this chapter

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16

Respiratory Syncytial Virus and Other Pediatric Respiratory Virus Infections

G. L. Toms

1. Introduction

1.1. Respiratory Virus Infection of Infants

Infants and young children undergoing their primary infection with common human respiratory viruses are at risk of serious, even life-threatening, lower respiratory tract infection. A multiplicity of viruses infect the human respiratory tract but a relatively small number are responsible for the majority of significant illness. Of these the most commonly diagnosed in the pediatric population is respiratory syncytial virus (RSV), which infects essentially all children in their first or second year of life, bringing approx 1% into the hospital with bronchiolitis or pneumonia *(1)*. Children with underlying cardiac or pulmonary disease, or born prematurely are particularly at risk. The virus also causes problems in the immunosuppressed and mortality rates are alarmingly high for RSV pneumonia after bone marrow transplantation *(2)*. The availability of therapy with ribavirin *(3)* or high titer anti-RSV γ-globulin *(4)*, which may be of benefit in these at risk groups, places a premium on rapid and accurate, but cost effective, diagnosis.

Significant lower respiratory tract disease may also result from infection with the parainfluenza viruses, the influenza viruses, and adenoviruses. Measles virus, currently relatively rare in countries operating a successful vaccination regime, may also be regarded as an important respiratory pathogen. Influenza C virus, the coronaviruses, the rhinoviruses, the reoviruses, and the enteroviruses, also commonly demonstrable in respiratory secretions, are generally considered less significant respiratory pathogens, mainly restricted to

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the upper respiratory tract. Nonetheless all may be associated, at least occasionally, with serious lower respiratory tract disease.

1.2. Virus Diagnosis

There are a number of routes to the diagnosis of a respiratory virus infection, not all of which are suitable for routine pediatric use. Serological diagnosis has proved unreliable in infants, the younger of whom still possess transplacentally acquired maternal antibodies that can interfere with or mask serological responses *(1)*. The detection of viral nucleic acid in nasopharyngeal secretions by polymerase chain reaction (PCR) offers great sensitivity, but the precautions required to prevent false-positive results, and the greater expense and time taken to achieve a diagnosis make it currently unattractive for routine use *(5)*.

Demonstration of infectious virus by inoculation of secretions into cell cultures of high virus susceptibility, although necessarily laborious and slow, was for a long time the gold standard against which other techniques were measured. Although this approach can be speeded up by the immunofluorescence staining of early cultures with virus-specific monoclonal antibodies (MAbs), culture can never match the speed of direct antigen detection in specimens using either immunofluorescence or enzyme immunoassay. Although in most studies virus culture shows marginally greater sensitivity than the rapid techniques, occasionally antigen detection may succeed where virus culture fails. Whether antigen detection methods alone, without the backup of virus culture, are adequate to provide a reliable diagnostic service remains a controversial issue.

Enzyme-linked immunosorbent assay (ELISA) techniques are amenable to automation and attractive to laboratories with a large throughput. Although ELISA techniques are often relatively slow and of poor sensitivity, some rapid and sensitive commercial kits are now available that should be considered where the cost can be justified *(6)*.

Immunofluorescence staining of virus antigens in exfoliated cells collected from nasopharyngeal secretions is the most widely used technique for the demonstration of respiratory viruses in pediatric populations. Where suitable antibodies, evaluated directly on clinical material, are used, sensitivity and specificity are comparable with any other technique, with the possible exception of PCR *(5,7,8)*. In addition, the direct observation of the specimen provides useful visual feedback on specimen quality allowing negative results to be reported with greater confidence Using direct immunofluorescence with conjugated MAbs, results can be available within an hour with only minor sacrifice of sensitivity. Furthermore, suitable antibodies are available for all of the major and many of the minor viral respiratory pathogens (**Table 1**) which can thus be tested for on a single multiwell slide offering great flexibility at reasonable cost. It is this technique, therefore, that will be detailed here.

Table 1
Commercially Available Monoclonal Antibodies to Respiratory Viruses[a]

Virus	Biosoft	Dako	Novo
Respiratory syncytial virus[b]	I, D	D	I, D
Influenza A virus	I, D	D	I, D
Influenza B virus	I, D	D	I, D
Influenza C virus			
Adenovirus group	I, D	D	I, D
Parainfluenza viruses[c]	I, D	D	
Coronavirus group	I, D		
Rhinovirus group[d]			
Enterovirus group		I	I, D
Reovirus group			
Measles virus	I, D		

[a]I, unconjugated antibodies for use in indirect immunofluorescence, D, antibodies conjugated to fluorescein for direct immunofluorescence, Biosoft, Biosoft/Argene antibodies, Parc Technoloqique (Varines, France) Dako, Dako Ltd , Glostrup, Denmark, Novo, Novocastra Laboratories, Newcastle upon Tyne, UK.

[b]Two antigenically distinct subgroups of RSV isolates, A and B can be differentiated with monoclonal antibodies. The clinical relevance of sub-grouping has yet to be established but a subgroup A-specific MAb is available from Biosoft

[c]MAbs to parainfluenza virus types 1, 2, and 3 are generally available but not to type 4

[d]A group-specific rhinovirus antigen has been described *(9)* but no monoclonal antibodies to this antigen are currently available.

1.3. Immunofluorescence Diagnosis of Respiratory Virus Infections in Infants and Children

Diagnosis is made by demonstrating the presence of virus-infected respiratory epithelial cells desquamated into the nasopharyngeal mucus of the infected child. Infected cells are sedimented from samples of mucus, fixed onto a glass slide, and stained either with virus specific antibodies conjugated to fluorescein (the direct technique) or unconjugated antibodies subsequently labeled with a fluorescein-conjugated secondary anti-immunoglobulin (the indirect technique). Fluorescing infected cells are then visualized under a UV microscope.

Immunofluorescence staining is also valuable in identifying viruses isolated in cell culture in tubes or shell vials, and may be carried out 1–2 d postinoculation, which is often some time before cytopathic effect is evident *(10)*.

The detailed descriptions of these techniques below are essentially those of Gardner and McQuillin *(11)*, with minor modifications to take account of more recent, commercially available reagents.

2. Materials

1 Sterile plastic mucus extractor (Henley's Medical Supplies, London, UK)
2. Sterile polythene feeding tube, size 8 (Sherwood Medical, Crawley, UK)
3. Vacuum pump. Hospital wards commonly have a suitable vacuum supply. The Laerdal Suction Unit (Laerdal Medical, Orpington, Kent, UK) is a convenient, portable, battery-operated pump with a maximum negative pressure of 600 mmHg (12psi)
4 Virus transport medium consisting of Hank's balanced salt solution (Oxoid/Unipath, Basingstoke, UK) containing 0 2% bovine albumin (Sigma Chemical, Poole, Dorset, UK), 100 IU/mL of penicillin and 100 μg/mL streptomycin (GibcoBRL, Paisley, Scotland), adjusted to pH 7.2 with 4.4% $NaHCO_3$
5 Sterile phosphate-buffered saline (Dulbecco 'A') (Oxoid/Unipath Ltd , Basingstoke, UK).
6. Teflon-coated multiwell glass slides with the required number of wells for cell preparations (Hendley Essex Ltd. Loughton, Essex, UK).
7 Moist box. Slides may be stained on two parallel swab sticks 2 5 cm apart in a plastic Petri dish containing a wet pledget of cotton wool or moistened sponge rubber to provide a humid atmosphere
8. Evan's blue counter stain: 0 5% in PBS (Sigma Chemical), diluted 1/1000 in PBS for use.
9. Fluorescein isothiocyanate (FITC)-conjugated antimurine IgG (Dako, Glostrup, Denmark)
10. FITC-conjugated antirabbit IgG (Dako, Glostrup, Denmark)
11 Antiviral antibodies Indirect immunofllorescence with carefully prepared polyclonal antisera, appropriately absorbed to remove nonspecific staining and extensively evaluated on positive and negative clinical material gives optimal sensitivity and specificity of viral antigen detection Such high quality reagents, however, are not generally available, and where they are interpretation of fluorescence staining requires an experienced eye. For the preparation of such antisera the reader is referred to Gardner and McQuillin *(11)*. MAbs, carefully selected for sensitivity and specificity on clinical material, are commercially available (**Table 1**) and offer an acceptable alternative in many situations (*see* **Note 1**) Direct immunofluorescence with fluorescein-labeled MAbs provides a simpler, more rapid, and easily interpretable test, but MAbs rarely offer the sensitivity achievable with polyclonal antisera. The lack of sensitivity may be improved by using a pool of MAbs to different virus epitopes, preferably on different proteins, which may both increase the number of cells stained and improve the level and pattern of staining within individual cells.
12. Fluorescence microscope. The fluorescence microscope must be equipped with a lamp and an excitation and barrier filter system capable of illuminating the speci-

men with light at close to 490 nm, the peak of absorption by fluorescein, and of transmitting only apple green fluorescent light of wavelengths around 517 nm to the eyepiece (*see* **Note 2**). The most suitable modern fluorescence microscopes are equipped for incidence light fluorescence with high numerical aperture low-power and high-power (×50 or ×63) oil immersion objectives (*see* **Note 3**).

3. Methods

3.1. Collection of Nasopharyageal Secretions

Secretions collected from the nasopharynx of children with respiratory infections are the material of choice for the identification of virus as they contain a larger number of infected cells than nose and throat swabs and are more easily available than secretions from the lower respiratory tract. The latter, however, are preferable if available. Here, the simple aspiration of nasopharyogeal secretions is described although some laboratories instill a small volume of buffered saline into the nose prior to aspiration *(12)*. Cough and nasal swabs are collected simultaneously as suction for nasopharyngeal secretions is not always productive.

Nasopharyngeal secretions, cough, and nasal swabs will generally be collected by ward staff who may require some training to produce material optimal for virus diagnosis (*see* **Note 4**).

1. Swab one of the child's nostrils and break the swab into 4 mL of transport medium held on melting ice
2. Swab the back of the throat until the patient gags and coughs onto the swab Break this swab into the same bottle of transport medium which is held in melting ice
3. Attach a sterile polythene feeding tube to the inlet of a sterile plastic mucus extractor. Attach the mucus extractor to a portable vacuum pump which has a maximum suction pressure of –26psi (239 kPa)
4. Switch on the pump and insert the feeding tube into the child's nasopharynx via each nostril in turn. Some children may show signs of distress, particularly if the nose is dry and secretions sparse and viscous. Secretions collect in the mucus extractor, although if few are present in the child's nose, they may lodge in the feeding tube and will require washing through with a little sterile PBS
5. Place the mucus extractor and the cough and nasal swabs in a vacuum flask on melting ice and transport to the laboratory with minimum delay.

3.2. Preparation of Infected Cell Suspensions (see Note 5)

3.2.1. Swabs and Secretions

1 Repeatedly pipet the medium over the swabs to remove adherent secretions. Remove the fluid containing the secretions to a centrifuge tube and centrifuge at 380*g* for 10 min at 4°C to pellet the cells. Remove the supernatant, which may be used for virus isolation.

2 Resuspend the cells in 3 mL of PBS by gentle pipeting and recentrifuge as above to obtain a cell pellet.

3 Centrifuge the mucus extractor containing nasopharyngeal secretions at 380*g* for 10 min at 4°C to collect all the secretion at the base of the vessel A small aliquot may be removed for virus isolation Add 2–3 mL of PBS to the remainder and disperse the mucus by gentle pipeting with a wide-bore pipet. Add further aliquots of PBS to about 10 mL, pipeting after each aliquot until the majority of the mucus is broken up Transfer the suspension, leaving any remaining mucus lumps, to a centrifuge tube and pellet the cells at 380*g* for 10 min at room temperature
4 Resuspend cell pellets (derived from swabs or from nasopharyngeal secretions) in a minimum volume of PBS to obtain a free running solution. The volume of PBS added will depend on the amount of mucus left on the cells The aim is to produce the most concentrated cell suspension achievable while diluting any remaining mucus beyond the point where it renders the suspension sticky. Mucus stains nonspecifically and also interferes with access of the staining reagents to the cells

*3.2.2. Infected Cell Cultures (see **Note 5**)*

1 Remove and discard medium from infected cultures of adherent cells.
2. Wash the cells gently with PBS and discard (*see* **Note 6**) the washings. Add a volume of PBS approximately equivalent to the original volume of the culture and scrape the cells into this using a Pasteur pipet for tubes or a plastic cell scraper for larger vessels. Pellet the cells by centrifugation at 380*g* for 5 min at room temperature
3 Discard the supernatant and resuspend the cells in PBS at approx 10^6 cells/mL

3.3. Preparation of Fixed Cells on Glass Slides (see Note 5)

1. Rinse Teflon-coated glass multiwell slides in absolute alcohol and wipe dry with a lint-free cloth. Engrave identification details on the free end and wipe again with a watermoistened cloth.
2. Place sufficient cell suspension on each well to cover the glass surface (15–35 µL depending on the size of the well). Larger wells, allowing more cells to be examined, will give an increase in sensitivity.
3 Allow to air dry (a hairdryer on a cold setting or a fan may be used to speed drying).
4 Immerse slides in cold acetone (4°C) for 10 min (*see* **Note 7**)
5 Dry at room temperature and stain immediately or store at or below –40°C indefinitely. (Samples stored next to the door can be repeatedly freeze/thawed on opening and closing)

3.4. Indirect Staining of Mixed Cell Preparations for Immunofluorescence

1 Place freshly prepared fixed cell preparations or those removed from –40°C storage in a moist box and allow to equilibrate to room temperature
2 Carefully spread one drop (10–15 µL) of relevant primary antibody or control reagent (*see* **Note 8**), diluted to the recommended working dilution in PBS, over each cell preparation (*see* **Note 9**). A bacteriological loop may be used to ensure that the whole well is covered.

3. Incubate for 30 min at 37°C in a moist box.
4 Gently rinse antibody from each slide with PBS applied via a Pasteur pipet
5. Immerse the slide in PBS in a Coplin jar or staining trough and soak for 10 min (*see* **Note 10**).
6 Discard and replenish the PBS and soak for a further 10 min Repeat, for three 10-min soaks total
7. Drain the slides and air dry.
8. Staining of the immunoglobulin-coated cells with the appropriate FITC-conjugated antiglobulin is carried out immediately as described below **(Subheading 3.5.)** for direct staining of cells.

3.5. Direct Staining of Fixed Cells for Immunofluorescence

This procedure may be applied to fixed ceils already reacted with unconjugated antibodies by the indirect technique described in **Subheading 3.4.** using the appropriate species-specific FITC-conjugated anti-γ-globulin. Alternatively it may be used with FITC-conjugated antiviral monoclonal or polyclonal antibodies for single-step visualization of virus-infected cells. Suitable control reagents must be included in each test to ensure the specificity of the fluorescent antibody staining observed (*see* **Note 11**).

1. Dilute the relevant FITC-conjugated antibody to the recommended working dilution in Evan's blue counterstain (*see* **Note 9**) The diluted conjugate may be kept at 4°C but only so long as it remains sterile. Long-term storage of diluted reagent is not recommended
2. Carefully spread one drop of FITC-conjugated antibody over each cell preparation.
3 Incubate for 30 min (*see* **Note 12**) at 37°C in a moist box
4 Rinse as in **steps 4–7** of **Subheading 3.4.**
5 Immerse slides in distilled water and soak for 1–2 min to remove PBS, which will crystallize if allowed to dry on the slide
6 Allow to dry in air and either immediately examine microscopically immediately or store at 4°C in the dark in a closed container for examination the next day. Quality of staining deteriorates on storing of unmounted preparations, particularly if immersion oil has been applied Mounting in Fluokeep (Biosoft; TCS, Buckingham, UK) under a coverslip not only increases the intensity of fluorescence but protects the cells from the deleterious effects of immersion oil

4. Notes

1. A vast range of MAbs to viral antigens are available commercially and through other channels, but very few of these are likely to be suitable for diagnostic purposes. Some currently available MAbs or MAb pools designed for antigen detection in clinical material are listed in **Table 1**. Also noteworthy are the polyvalent pools of antibodies to RSV, the influenzaviruses, the parainfluenza viruses, and adenoviruses produced by some manufacturers. These allow one-test screening

for significant respiratory virus pathogens, reducing the costly screening of negative samples against multiple type specific reagents.

2. For more information prior to purchasing consult E O. Caul *(13)*
3. Not all objectives are suitable for fluorescence microscopy owing to autofluorescence of some components or excessive internal light scattering. Manufacturers should be able to give advice on those most suitable.
4. Adequate specimens must contain a reasonable number of respiratory epithelial cells derived from the nasopharyngeal epithelium Squamous epithelial cells, derived from the anterior air passages, are not generally infected
5. It is advisable to carry out the handling of material likely to generate airborne pathogens in a class 2 containment cabinet.
6. Cell cultures exhibiting extensive cytopathic effect may detach on washing In this case, the cells should be scraped and pipeted directly into the culture medium. Cells are pelleted at 380*g* for 5 min at room temperature, resuspended to original volume in PBS to wash, and recentrifuged to obtained a washed cell pellet. A similar procedure is adopted for virus-infected cultures of nonadherent cells
7. Acetone should not be stored in conventional refrigerators as it poses an explosion hazard A Coplin jar of acetone in an ice bath is convenient for fixing a small number of slides. For cell culture preparations, which are free from mucus, fixation may be reduced to 5 min.
8. Specificity of reagents cannot be taken for granted, particularly when clinical material is being investigated and adequate controls must be included to reveal non-specific reactions. The following should be included for the indirect test·
   a. A cell preparation similar to the test specimen but known to be negative for the virus in question, stained in parallel with the test specimen Once sure of all the reagents and methods and familiar with the patterns of staining they produce, experienced workers may choose to omit this control.
   b. A duplicate preparation of the test specimen stained with a negative antibody as similar as possible to the virus-specific antibody in use, followed by the FITC-conjugated antiglobulin. Where a polyclonal antiserum from an animal is employed, the ideal control would be the preimmune serum from the same animal. This is rarely available for commercial products and here a serum from an animal immunized by a similar protocol with an antigen likely to be absent from the specimen (e g., another virus) must suffice.Where MAbs are employed a MAb of the same immunoglobulin class but specific for an irrelevant antigen known to be absent from the specimen should be used
   c. A duplicate preparation of the test specimen stained with PBS, instead of the virus specific antibody, followed by the FITC-conjugated antiglobulin.
9. Although the maker's instructions or advice from other laboratories may be taken as a guide, optimal working dilutions vary widely from laboratory to laboratory *(14)*. It is always advisable to titrate new antibody preparations in your laboratory. Normally, testing preparations fivefold either side of the recommended dilution on known positive control material will suffice.

10. For cell culture preparations, in which nonspecific staining is less troublesome, 5-min soaks will suffice.
11. In the direct test the controls should include:
    a. A cell preparation similar to the test specimen but known to be negative for the virus in question and stained in parallel with the test specimen.
    b. A duplicate preparation of the test specimen stained with a negative control conjugated antibody as similar as possible to the virus-specific conjugated antibody to be employed. Where a polyclonal antibody from an animal has been purified and conjugated, the most suitable control would be the preimmune serum from the same animal similarly purified and conjugated. Where such is not available, a similar conjugated antiserum from the same species but specific for an antigen known to be absent from the test specimen will suffice. Where conjugated MAbs are employed, a conjugated MAb of the same immunoglobulin class but specific for an antigen known to be absent from the test specimen should be used.
    The specificity of fluorescent staining can be confirmed in a subsequent blocking test. Here, preparations of the test specimen are preincubated with either unconjugated virus-specific antibody or unconjugated negative-control antibody. On subsequent staining with conjugated antibody, specific fluorescent staining will be blocked by the latter but not the former
12. In the direct immunofluorescence test this incubation period may be reduced to 15 min for many conjugated MAbs in line with manufacturer's instructions.

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Detection and Molecular Epidemiology of Rotavirus by RNA Gel Electrophoresis

Lennart Svensson

1. Introduction

Rotavirus has been recognized as the major etiological agent of acute gastroenteritis in infants and young children. The rotaviruses contain a genome of 11 segments of double-stranded RNA that can be separated into distinct bands by electrophoresis. The migration pattern of the 11 genome segments following electrophoresis of the viral RNA is called the RNA electropherotype. Electrophoretic separation of the segmented genome has gained popularity as a method not only for detection of rotavirus but also for molecular epidemiological studies *(1,2)*. Most molecular epidemiological studies have analyzed rotaviruses by their electropherotype, since this marker is both characteristic and constant for a given virus strain, i.e., rotaviruses from different animal species including human exhibit distinct electropherotypes. RNA gel electrophoresis is not only the most feasible way to detect and distinguish between different serogroups (A–G) of rotaviruses *(3)*, but the technique can also be used to:

1. Characterize virus strains in large outbreaks;
2. Trace nosocomial outbreaks;
3. Determine how many virus strains circulate in a family, hospital, city, or country; and
4. Determine if specific virus strains are associated with specific disease.

The accuracy and validity of RNA gel electrophoresis is highly dependent on the resolution achieved for the distinction of individual RNA segments in a gel system. For higher sensitivity and best segment resolution, agarose gels

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and ethidium bromide have been replaced by polyacrylamide gels and silver staining. This chapter describes protocols for separation and characterization of rotavirus RNA by polyacrylamide gel electrophoresis and silver staining.

1.1. Preparation of Samples for Viral RNA Gel Electrophoresis

Viral RNA can be extracted from infected cells or from 10% fecal suspensions in phosphate-buffered saline (PBS). Methods for purification of viral RNA have included either partial purification of virus particles from stool samples followed by purification of the RNA from these purified particles or direct phenol extraction of viral RNA from cell or stool material followed by RNA precipitation with ethanol. RNA has also been isolated from purified virus by:

1. Deproteinization with proteinase K;
2. Deproteinization with phenol,
3. Deproteinization with both proteinase K and phenol; or
4. Simple lysis of the purified particles by boiling in a buffer containing sodium dodecyl sulfate (SDS) and a reducing agent *(1)*.

1.2. Polyacrylamide Gel Electrophoresis (PAGE)

Viral RNA may be analyzed by agarose gel electrophoresis but is most commonly analyzed by electrophoresis in 7.5% polyacrylamide gels and a Tris-glycine buffer system described by Laemmli *(4)*. In contrast to the original method for separation of proteins by PAGE *(4)*, SDS does not need to be included in gel preparation for RNA separation *(5)*.

1.3. Silver Staining

Nucleic acids separated by PAGE can be detected by ethidium bromide, silver staining, or radio labeling of RNA segments at their 3' end with [γ^{32}P] and T4 RNA ligase. Since radiolabeling procedures are rarely used and ethidium bromide is toxic and less sensitive, silver staining has become the method of choice. Silver staining was introduced more than a decade ago as a sensitive procedure to detect small amounts of proteins in polyacrylamide gels. Since then silver staining has been used to detect nucleic acids in PAGE gels. Silver staining follows one of two methods. One uses diamine or ammoniacal silver solutions for gel impregnation and dilute acid solutions of formaldehyde for image development. The other method impregnates with silver nitrate in a weakly acidic milieu and uses formaldehyde to reduce silver under alkaline conditions. Acidic methods are more sensitive and work better with thin gels, while diamine alkaline methods are better suited for thicker gels. Silver stained thin PAGE gels make it possible to detect nucleic acids at the picogram level. For further reading on silver staining (*see* **ref. *6***).

Table 1
Bio-Rad Mini-Protean II Electrophoresis Cell

	Separation gel 5 cm (7.5%)	Spacer gel (4 5%)
40% Acrylamide	1 86 mL	0.43 mL
2% Bis	1 mL	0.23 mL
8X LA	1 25 mL	—
8X LB	—	0 48 mL
dH_2O	5 70 mL	2.62 mL
10% AMPS	60 µL	30 µL
TEMED	10 µL	6 µL

2. Materials

2.1 RNA Extraction

1. Solution A. 10 m*M* Tris-HCl, 10 m*M* NaCl, 10 m*M* ethylenediaminetetraacetic acid (EDTA), (Boehringer Mannheim, Bromma, Sweden, cat. no 808 261), 0 5% SDS, pH 8.0
2. Proteinase K (Sigma, St. Louis, MO, P-4914) diluted in solution A to 1 mg/mL Aliquot and store at –20°C
3. Phenol solution. Melt phenol (JT Baker Inc., Phillipsburg, NJ) at 68°C and extract once with water and several times with an equal volume of 1.0 *M* Tris-HCl, pH 8 0 followed by 0 1 *M* Tris-HCl, pH 8.0 The phenol solution can be stored at 4°C for up to 1 mo.
4. RNA sample buffer (4X)· 50 m*M* Tris-HCl, 5 m*M* EDTA, 5% SDS, 5% 2-mercaptoethanol, 40% glycerol, 1 mg/mL phenol red, pH 7 6
5 1,1,2-Trichloro-1,2,2-tri-fluoroethane (Sigma, cat. no T 5271) Commercial names are Genetron and Freon
6 Chloroform-isoamyl alcohol (24:1).

2.2. PAGE Gel Preparation (Table 1)

1. 40% Acrylamide gel solution (Bio-Rad, Hercules, CA, cat no 161 0140), 2% Bis (*N,N'*-methylene-bis-acrylamide) solution (Bio-Rad cat 161-161-0200). Acrylamide and Bis are toxic and should be handled and disposed with care. 10% Ammonium persulfate (AMPS, Bio-Rad cat no 161-0700), in deionized water (dH_2O), N,N,N,N'-tetra-methylethylene diamine (TEMED) (Bio-Rad cat. no. 161-0800).
2 Separation buffer (8X LA). 3 *M* Tris-HCl, pH 8.8
3. Spacer buffer (8X LB): 1 *M* Tris-HCl, pH 6.8.
4. Running buffer (10X LC)· 0.25 *M* Tris, 1 92 *M* Glycine
5. Butanol (Sigma, cat. no. BT-105).

6 PAGE gel equipment such as Sturdier SE 400 (Hoefer, San Francisco, CA) for 11–14-cm gels. For shorter gels Mini-Protean II electrophoresis system (Bio-Rad cat no 165-2940) is suitable

2.3. Silver Staining

1 95% Ethanol
2 NaOH.
3 Silver nitrate ($AgNO_3$, Merck, Darmstadt, Germany, 1 01512): Store silver nitrate shielded from light. $AgNO_3$ is toxic and should be handled and disposed with care.
4 37% Formaldehyde (formalin, HCHO) solution (Sigma, cat no. F 1635)
5 Sodium thiosulfate, $Na_2S_2O_3$ (Sigma, cat no S1648)
6 7 5% Acetic acid in dH_2O

2.4. Fixation Solution

For **Subheading 3.3.1.**: 35% ethanol/10% acetic acid
For **Subheading 3.3.2.**: 7.5% acetic acid.

2.5. Washing Solution

For **Subheading 3.3.1.**: 10% ethanol/10% acetic acid
For **Subheading 3.3.2.**: dH_2O

2.6. Silver Nitrate Solution

For **Subheading 3.3.1.**: 0.35 g $AgNO_3$ in 200 mL dH_2O
For **Subheading 3.3.2.**: 0.15 g $AgNO_3$, 0.056% HCHO in 100 mL dH_2O

2.7. Developing Solution

For **Subheading 3.3.1.**: 7.5 g NaOH, 2 mL HCHO in 250 mL dH_2O.
For **Subheading 3.3.2.**: 3 g Na_2CO_3, 0.056% HCHO, 40 μg $Na_2S_2O_3$.

2.8. Stop Solution

For **Subheading 3.3.1.**: 5% acetic acid in dH_2O.
For **Subheading 3.3.2.**: 7.5% acetic acid in dH_2O

3. Methods

3.1. Extraction of Viral RNA

1. Mix 500 μL of a 10% stool suspension or infected cell cultures together with an equal vol of 1,1,2-trichloro-1,2,2-trifluorethane
2. Collect supernatant after centrifugation for 3 min at 12,000*g* and mix with 10 μL of proteinase K (1 mg/mL) and 100 μL of solution A
3. Incubate at 56°C for 15–20 min.

4. Add 500 µL phenol solution. Shake vigorously until solution turns white
5. Centrifuge at 12,000*g* for 2–3 min. If supernatant is still opalescent repeat **step 4** (*see* **Note 1**).
6. Collect supernatant and add 250 µL phenol solution and 250 µL chloroform-isoamyl alcohol. Shake vigorously Centrifuge at 12,000*g* for 2–3 min (*see* **Note 2**)
7. Collect supernatant and mix with sample buffer (1 vol sample buffer/3 vol sample). If supernatant not is clear, repeat chloroform-isoamyl alcohol extraction once more before mixing with sample buffer (*see* **Note 3**)

3.2. PAGE Gel Setup

1 Assemble gel equipment according to the manufacturer's instructions. Prepare a 7.5% polyacrylamide gel with a 4.5% spacer gel. Use 0 75-mm (or 1-mm) spacers (*see* **Note 4**).
2 Start by mixing the separation gel (in order from top to bottom) and pour into the gel cast Add to about 2 5 cm from the top. Overlay the gel surface with butanol and allow to polymerize for about 1 h at room temperature. When the gel has polymerized, pour off (not in the sink) the butanol and wash the gel surface three times with tap water.
3. Prepare and load the spacer gel (2.5 cm) and insert the Teflon comb (0.75-mm, 10-well) immediately. Allow the spacer gel to polymerize for 1 h.
4. Assemble the electrophoresis equipment and make sure there is no leaking. Load the prepared samples into the wells (10–25 µL/well). Prepare the running buffer. 1 vol (10X LC) and 9 vol dH_2O (350 mL will be enough). Start the run with 100 V for 15 min and increase to 160 V for another 70 min. Disassemble and fix the gel (*see* **Notes 4** and **5**)

3.3. Silver Staining

Two protocols are given for silver staining. A critical step with both protocols is the use of high purity chemicals with solutions prepared in dH_2O.

3.3.1. Protocol (Modified from **ref. 5**)

1. Fix gel in 200 mL fixation solution for 45 min by agitation. The fix solution can be stored at room temperature
2. Replace with 200 mL washing solution for 45 min
3. Drain and replace with freshly made silver nitrate solution and agitate for 30 min
4. Drain and rinse gel in 100 mL dH_2O for 10–15 s
5. Drain rapidly and replace with 250 mL developing solution Add about 50 mL of the 250 mL, and agitate for 30 s When the solution turns dark brown, drain rapidly and add the remaining 200 mL of developer. When bands have turned dark brown or black, drain off solution and add 50–100 mL dH_2O for 10 s , drain again, and add stop solution.

3.3.2. Protocol (Modified from ref. 6)

1 Fix gel (0 75-mm thickness) for 15 min in fixation solution
2. Wash gel three times for 2 min in dH_2O.
3. Drain and add 100 mL of freshly made silver nitrate solution for 20 min.
4. Drain and rinse gel in dH_2O for 15 s.
5 Rapidly drain and develop 2–10 min in 100 mL developing solution (*see* **Notes 6** and **7**)
6 When bands are distinct, drain and replace with stop solution (*see* **Note 8**)

4. Notes

1. An important technical note is that failure to deproteinize RNA prior to analysis may result in the retardation of some of the genome segments
2 If the RNA concentration is low (no RNA bands can be identified after silver staining) then it should be concentrated by salt-ethanol precipitation. This can be achieved by adding 1/10 vol of 3*M* NaAc + 3 vol of ethanol (95%) to the extracted RNA mix and incubating for 1 h at –70°C or on dry ice The RNA is then pelleted by centrifugation at 12,000*g* for 15 min at 4°C Decant the supernatant carefully and dissolve the pellet in water or 1X sample buffer
3 The extracted RNA can be stored at –20°C either in sample buffer or as supernatant
4. Use a safety cabinet and gloves during PAGE preparation, as polyacrylamide is highly toxic
5. Do not lift the 0 75-mm gel by hand as it will crack Carefully remove one of the glass plates and mark a corner, then turn the gel upside down and let the gel float off by itself into a tray of fixative
6. Sodium thiosulfate effectively reduces nonspecific background staining Decreasing sodium carbonate concentration causes higher background staining and poor image contrast, probably by decreasing the overall rate of silver reduction
7 Formaldehyde concentrations between 0 03 and 0 1% (by volume) provide an optimal stain Lower formaldehyde concentrations have the general effect of reducing sensitivity while higher concentrations increase sensitivity but also background staining.
8 Stop solutions can cause image fading when using acetic acid concentrations higher than 7.5%–10% The stop reactions are also best controlled by using cold (4°C) acetic acid

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Detection of PCR-Amplified Sandfly Fever Sicilian Virus RNA by Colorimetric Enzymatic Immunoassay

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1. Introduction

Sandfly fever Sicilian (SFS) virus (genus *Phlebovirus,* family *Bunyaviridae*) is a member of the sandfly fever virus serogroup, which also includes sandfly fever Naples and Toscana viruses. These viruses are transmitted by *Phlebotomus* sandflies in the Mediterranean and the Middle Eastern regions *(1,2)* and are associated with large epidemics with significant morbidity and mortality *(3)*. The prevalence of sandfly fever (SF) disease appeared to decline during the 1950s and 60s following insecticidal campaigns, but recent seroepidemiological data indicate that the disease still occurs in endemic areas *(4–9)*.

Like other viruses in the family *Bunyaviridae*, SFS has a three-segmented, single-stranded RNA genome. The large (L) segment encodes the viral polymerase in the virus complementary-sense RNA; the medium (M) segment encodes the two envelope glycoproteins, G1 and G2, in a continuous open reading frame in the cRNA; and the S segment encodes the nucleocapsid protein in the cRNA and a nonstructural protein (NSs) in the virus sense RNA.

Diagnosis of sandfly fever and virus identification are performed routinely by serological techniques, e.g., indirect fluorescent antibody, hemagglutination inhibition, complement fixation, plaque-reduction neutralization (PRN), and enzyme-linked immunosorbent assays (ELISA). Of these techniques, the PRN is the most specific in distinguishing viral serotypes, but even this technique is not entirely satisfactory given the number and diversity of the phleboviruses *(10)*.

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The polymerase chain reaction (PCR) technique has been used successfully to detect bunyaviruses *(11)* as well as other viruses *(12,13)*, bacteria *(14,15)*, and parasites *(16)*. The most commonly used method for detecting PCR products depends on molecular size analysis of PCR products by agarose gel electrophoresis and ethidium bromide staining. Although this is the method of choice in research laboratories, it is not ideal for use in routine diagnosis because it lacks the ability to authenticate the amplified sequence. Therefore, specific and sensitive detection methods are needed. Restriction fragment length polymorphism (RFLP), sequence analysis, or membrane-based probe hybridization are more specific, but they are technically demanding. With careful selection of target sequence, primers, and probes, solution-based probe hybridization methods should provide the high levels of specificity required to authenticate PCR products. Several probe-based hybridization methods using isotopic and nonisotopic labeling and detection of nucleic acids have been described. Recently, several nonisotopic, colorimetric or chemiluminescent enzymatic assays using solution hybridization in a microtiter plate format have been reported. Generally, these assays are referred to as PCR-enzyme-linked immunosorbent assay (PCR-ELISA) or PCR-enzyme immunoassay (PCR-EIA).

Here, we describe a PCR-ELISA that combines the high sensitivity of RT-PCR amplification and the specificity of probe hybridization to detect SFS viruses. The assay uses commercially available reagents and material for labeling and detecting amplified nucleic acids. In this assay, a hapten (digoxigenin) is incorporated into the PCR products during amplification by partially substi-tuting dTTP with digoxigenin-11-dUTP. The digoxigenin-labeled amplicons are hybridized in solution to a biotinylated probe, the hybrid is immobilized onto a streptavidin-coated microtiter plate, and the PCR product is detected with enzyme-linked antidigoxigenin antibody and a color substrate. A schematic of the assay is shown in **Fig. 1**.

Because the assay uses colorimetric enzymatic microtiter plate format, it is compatible with existing ELISA technology and can be automated, thus allowing multiple sample processing. The assay described herein was tested with four SFS virus strains (Sabin, OSS-114, R-18, and R- 1286) and five other phleboviruses (Naples, Punta Toro, Corfu, Toscana, and Chagres). The utility of the assay was evaluated with simulated serum samples and experimentally infected sandflies. The detection limit of the assay (2.5 PFU) was 10-fold higher than the detection limit of ethidium bromide-stained agarose gels.

2. Materials

2.1. Equipment and Consumables

1. Refrigerated microcentrifuge.
2. Thermal cycler, e.g., GeneAmp PCR System 9600 or PTC-100 (available from Perkin Elmer [Foster City, CA] or MJ Research [Watertown, MA], respectively).

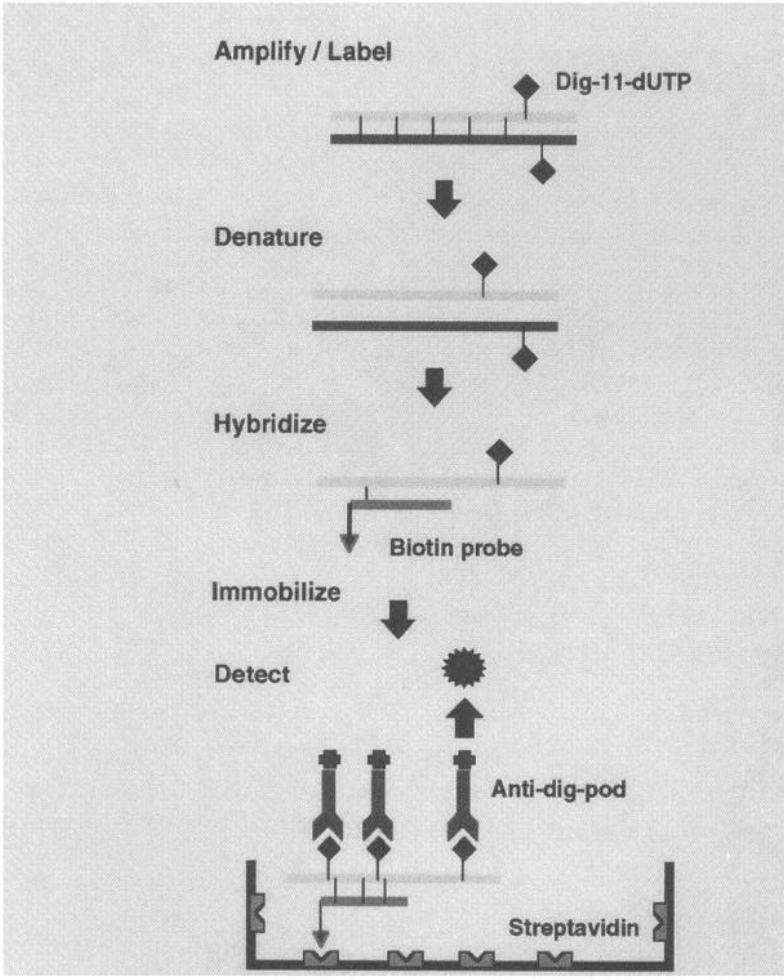

Fig. 1. Diagram of PCR-ELISA. DIG-labeled PCR products are denatured, hybridized to a biotin-labeled probe, captured onto streptavidin-coated microwell, and detected with anti-DIG-pod antibody and a chromogenic substrate.

3. ELISA reader, e.g., MR5000 (Dynatech, Chantilly, VA) or equivalent.
4. Microplate washer (recommended for large-scale assays).
5. Heat blocks (55 and 95°C).
6. Water bath (42°C).
7. Vortex mixer.
8. Pipetman (P2, P10, P20, P100, P200, P1000) or equivalent.
9. Multichannel pipetor (25–200 µL).
10. Sterile aerosol barrier pipet tips (for RT-PCR), and general purpose pipet tips.

11 Sterile 1.5-mL microfuge tubes, and thin-walled 200-µL MicroAmp PCR tubes and caps (Perkin Elmer) or other appropriate PCR tubes.
12 Reagent reservoirs.
13 Microtiter plates
14. Sterile gloves

2.2. Virus Source

1 The phleboviruses tested with the present protocol included SFS Sabin and SFN Sabin (American Type Culture Collection [ATCC], Rockville, MD), SFS R-18, SFS OSS-114, Punta Toro MSP-3, Corfu Pa Ar-81, and Toscana ISS PH1 3 (Yale Arbovirus Research Unit [YARU], New Haven, CT); and Chagres 204102 and SFS 1286 (USAMRIID, Ft Detrick, MD)
2 The viruses were propagated at 37°C for 5–7 d in Vero E-6 cells maintained in a medium that contained Eagle's minimal essential medium (EMEM), 5% fetal bovine serum, 1% penicillin-streptomycin, 1% tricin, and 0 2% amphotericin B
3 Viral titers were determined by plaque assay.

2.3. RNA Extraction

1 Trizol (Life Technologies, Gaithersburg, MD)
2 Water-saturated chloroform.
3 Isopropanol
4 Absolute ethanol and 75% ethanol
5 RNase-free water or diethylpyrocarbonate (DEPC)-treated water (*see* **ref. *17***).
6 Glycogen (1 µg/µL) (optional)

2.4. Reverse Transcription

1 Superscript II RNase H-reverse transcriptase (200 U/µL) (Life Technologies)
2. Random hexamers (300 ng/µL) (Life Technologies).
3 Human placental ribonuclease inhibitor (10,000 U/µL) (Life Technologies)
4. 5X RT buffer· 250 m*M* Tris-HCl, pH 8 3, 375 m*M* KCl, 15 m*M* $MgCl_2$ (Life Technologies)
5 Mixed dNTP stock 10 m*M* each dATP, dGTP, dCTP, and dTTP (Life Technologies)
6. 100 m*M* Dithiothreitol (DTT) (Gibco BRL).

2.5. Oligonucleotide Primers and Probes

1. The selection of primers and probes is facilitated by a primer design software program, e g , Oligo (National Biosciences, Plymouth, MN).
2. In the protocol described here, we used the primers SFSS1 (321/5'-GAGGGCAGA-CCAGGCAGGAA-340/3') and SFSS2 (575/5'-GGAAAACATGAACAAGA-AAA-556/3') to amplify a target fragment of 236 bp encoding a portion of the nucleocapsid (N) protein gene of SFS virus.
3 As a capture probe, we used the oligonucleotide SFSP1 (385/5'-GCAGGGA-GCACAATGGACCGCA-3'/406), synthesized with a biotin molecule at

the 5'- end using ABI 394 oligonucleotide synthesizer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA). Unincorporated biotin molecules are removed from the probe by sodium acetate/ethanol precipitation *(17)*

The nucleotide position numbers indicated in the primers and probe correspond to the published sequence of SFS-RNA (GenBank, accession# J04418).

2.6. PCR Amplification

1. Ampli*Taq* DNA polymerase (5 U/μL) (Perkin-Elmer).
2. 10X PCR buffer: 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 15 m*M* $MgCl_2$, 0.01% gelatin (Perkin-Elmer).
3. PCR DIG-dNTP labeling mix. 2 m*M* dATP, dCTP, dGTP each, 1.9 m*M* dTTP, 0.1 m*M* digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN)
4. PCR master mix: 1 μL of each primer (100 pmol), 5 μL 10X PCR buffer, 5 μL DIG-dNTP labeling mix, 0.5 μL Ampli*Taq* DNA polymerase and 35.5 μL autoclaved dH_2O per reaction

2.7. ELISA Detection

1. Anti-DIG-horseradish-peroxidase (POD) conjugate (1 mU/μL) (Boehringer Mannheim).
2. Streptavidin-coated microtiter plate modules (Boehringer Mannheim).
3. Alkaline denaturation solution (Boehringer Mannheim).
4. Hybridization solution (Boehringer Mannheim).
5. Washing tablets (Boehringer Mannheim).
6. 2,2'-Azino-di[3-ethyl-benzthiazoline sulfonate(6)] (ABTS) color substrate tablets. final concentration 1 mg/mL ABTS in substrate buffer (Boehringer Mannheim)

3. Methods

3.1. RNA Extraction

Extract RNA by using a guanidine isothiocyanate-based procedure *(17,18)* or other appropriate procedures, following the necessary precautions to maintain the integrity and purity of RNA. We found that extraction with Trizol reagent (Life Technologies), a mixture of guanidine isothiocyanate and phenol, gave consistent results with virus-infected cell culture, clinical or arthropod specimens. We routinely extract RNA from 100 μL-aliquots of virus-infected cell suspensions or culture supernatants containing approx 10^5–10^6 PFU/mL. The following procedure is based on the manufacturer's instructions with minor modifications:

1. In a microfuge tube, mix by vortexing 100 μL of virus-infected cell culture suspension or culture supernatant containing virions with 1 mL of Trizol reagent. Incubate at room temperature for 5 min.

2. Add 200 µL water-saturated chloroform. Mix by hand or by vortexing and incubate at room temperature for 2 min.
3. Centrifuge at 12,000*g* for 15 min at 4°C.
4. Carefully remove the upper (aqueous) phase to a fresh rmicrofuge tube avoiding the interface. For samples that contain high levels, mix the aqueous phase with 1 mL Trizol and repeat **steps 2** and **3** RNA recovery can be improved by back extraction of the organic phase with 250 µL RNase-free water
5. Add equal vol (about 500 µL) isopropanol and mix gently by inverting the tube several times Incubate at room temperature for 10 min, then centrifuge at 20,000*g* for 10 min at 4°C. To enhance RNA recovery in small samples or samples containing low virus concentration add 5–10 µL (5–10 µg) of glycogen.
6 Carefully remove and discard the supernatant without disturbing the often invisible pellet.
7. Add 1 mL of ice-cold 75% ethanol and mix by gentle vortexing. Centrifuge at 7500*g* for 5 min at 4°C. Carefully remove and discard the supernatant without disturbing the pellet
8 Allow the pellet to air dry completely at room temperature.
9 Dissolve RNA in 10 µL RNase-free (or DEPC-treated) dH_2O by heating at 55°C for 10 min, briefly spin in a microcentrifuge, place on ice, and proceed immediately with reverse transcription. Alternatively, RNA samples can be stored as dry pellets at –70°C (for several months) until needed.

3.2. Reverse Transcription

1. Mix 10 µL RNA solution with 1 µL (300 ng) random hexamers. Heat at 95°C for 3 min, chill on ice, spin briefly in a microfuge, and return to ice.
2 Add 1 µL human placental ribonuclease inhibitor, 4 µL 5X RT buffer, 1 µL dNTP mix, 2 µL DTT, and 1 µL Superscript II RNase H-reverse transcriptase
3. Incubate at 42°C for 60 min, heat at 95°C for 3 min, chill on ice, spin briefly in a microcentrifuge, and return to ice Proceed with PCR amplification. Alternatively, the cDNA can be stored at –20°C until needed

3.3. PCR Amplification

1. PCR reactions are carried out in 50-µL volumes in appropriate PCR tubes (*see* **Subheading 2.1.**).
2. Mix 2 µL of the RT reaction containing first-stranded cDNA with 48 µL freshly prepared PCR master mix (*see* **Subheading 2.6.**).
3 Perform 35 cycles, each consisting of 94°C for 15 s, 50°C for 15 s, and 72°C for 30 s with a final extension step at 72°C for 7 min

3.4. ELISA Detection of PCR Products

1. In wells of a microtiter plate, mix 10 µL of the digoxigenin-labeled PCR products with 20 µL of alkaline denaturing solution and incubate at room temperature for 10 mm

2. Add 200 µL of the biotinylated oligonucleotide probe (50–125 ng/mL in hybridization solution). Mix several times by gently pipeting up and down without creating bubbles.
3. Transfer the mixture to wells of streptavidin-coated microtiter plate strips Incubate at 37°C for 1–3 h.
4. Remove unbound molecules by washing the strips three times with washing buffer (*see* **Subheading 2.7.**).
5. To each well, add 200 µL of 1:100 dilution of antidigoxigenin-POD conjugate (diluted in conjugate dilution buffer). Incubate at 37°C for 30 min
6. Wash the strips three times with 1X washing buffer and three times with dH_2O
7. To each well, add 200 µL of color substrate. Incubate at 37°C in the dark for 15–60 min
8. Measure the optical density with an ELISA reader at dual wave length 410/490 nm at 15, 30, and 60 min.
9. Determine the cutoff values from the mean of three no-template negative controls plus three times the standard deviation.

4. Notes

1 The general method described here was tested with SFS viruses, but it is applicable to other infectious disease agents. Variations of this method have been used to detect *Salmonella* sp. ***(19)***, *Mycoplasma pneumoniae* ***(20)***, HIV ***(12)***, and other pathogens. The most important requirement is the selection of a target sequence in which highly conserved (crossreactive) PCR primers and a highly specific hybridization probe sequence can be obtained. The crossreactive primers should be selected to amplify target sequences of 150 to 1000 bp in a family of closely-related sequences The specific (homologous) hybridization probes should be selected to identify individual sequences
2. To ensure high specificity, we selected the PCR primers from a highly conserved region of the SFS virus genome after aligning all available sequences of SFS and other related phleboviruses. The probe was selected from a highly homologous SFS sequence. In the probe sequence, only one nucleotide mismatch existed with one of the four SFS virus strains. whereas four to five mismatches existed with other phleboviruses. When the primers were tested with RNAs extracted from four different SFS strains (Sabin, OSS114, R-18, and R-1286) and five other phleboviruses (SFN, Punta Toro, Corfu, Toscana, and Chagres viruses), and the PCR products were analyzed by ethidium-bromide stained agarose gels, a single band of the predicted size of 236 bp was detected only in SFS virus strains (**Fig. 2**)
3. The assay involves sample preparation. nucleic acid amplification, and detection of the amplified products The sample preparation step is extremely important, particularly when RNA is the target. Guidelines and protocols for handling and isolating nucleic acids from a variety of samples are described elsewhere ***(17)***. and a variety of nucleic acid extraction reagents are available from commercial sources (Qiagen, Promega, Life Technoligies, Boehringer Mannheim, and others). The protocols used herein for RT-PCR, and nucleic acid labeling and detec-

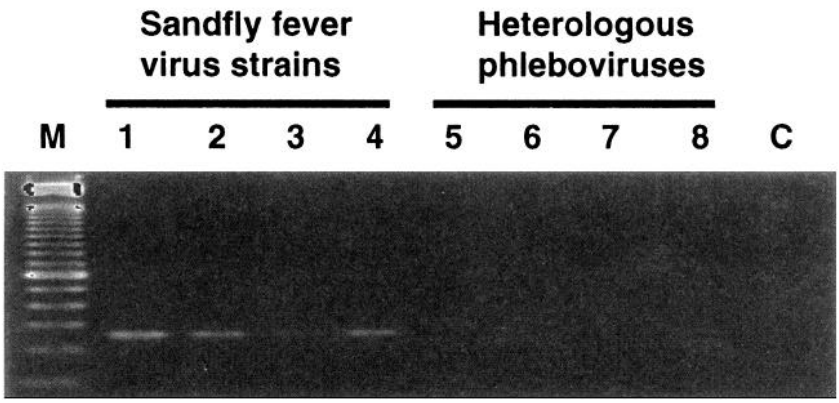

Fig. 2. The specificity of the PCR primers SFSS1 and SFSS2 were tested by using RNA extracted from four different SFS virus strains: Sabin (1), OSS114 (2), R-18 (3), and R-1286 (4), and four other phleboviruses: SFN (5), Punta Toro (6), Corfu (7), and Toscana (8). A single band of the expected size of 236 bp was detected only in SFS virus. M is 100 bp DNA ladder; C is a no-template negative control.

tion were adapted from the manufacturers' recommendations (Life Technologies, Perkin-Elmer, and Boehringer Mannheim) for the SFS virus. Optimization of the RT-PCR, labeling, and detection conditions are necessary in other laboratories using different target sequences, primers, probes, RT and *Taq* polymerase enzymes, buffers, or thermal cyclers. For example, the manufacturer recommends up to 50 ng/mL of a 20-mer oligonucleotide probe but we found that 125 ng/mL was optimal in our system.

4. The nucleic acid labeling and ELISA detection reagents and material are readily available as kits from Boehringer Mannheim (PCR ELISA DIG labeling kit, cat. no. 1636 120; PCR ELISA DIG detection kit, catalog# 1636 111). Some reagents can be prepared in the laboratory (PCR DIG labeling mix, streptavidin-coated microtiter plates, conjugate dilution buffer, and substrate buffer). PCR DIG labeling mix can be prepared as described in **Subheading 2.6.** In this labeling mix, the concentration of DIG-11-dUTP relative to dTTP is 5%. Increasing the DIG-11-dUTP concentration up to 30% may improve the assay sensitivity. Higher concentrations may reduce the PCR yield and increase nonspecific signals.
5. Streptavidin-coated microtiter plates are available from the manufacturer (Boehringer Mannheim) in high- or low-binding capacities. In the assay described herein we used the low-capacity modules. Streptavidin-coated microtiter plates can be prepared in the laboratory as follows: coat microtest III flexible microtiter plates (Becton Dickinson, Franklin Lakes, NJ) with 10–100 µg/mL streptavidin diluted in phosphate-buffered saline (PBS), pH 7.4, overnight at 4°C. Wash the

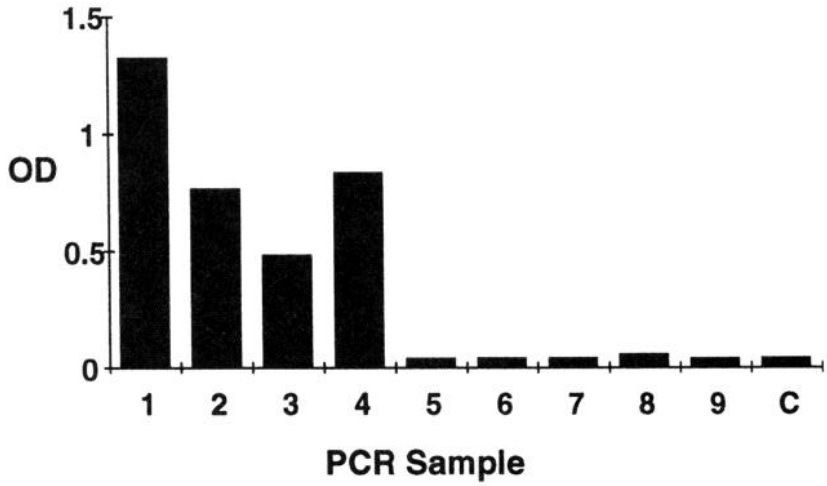

Fig. 3. DIG-labeled PCR products shown in **Fig. 2** were analyzed by PCR-ELISA using SFS-specific probe. Only SFS viruses (1, 2, 3, 4) developed colorimetric reactions with OD values (410/490 nm) higher than the cut-off value (C). Each OD value represents the mean of three replicates. The cutoff OD value was determined from the mean of three negative control replicates plus two standard deviations.

plates three times with washing buffer (100 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 0.05% Tween-20) and block with a blocking buffer (wash buffer containing 2% bovine serum albumin, 1% nonfat dry milk, and 100 µg/mL sonicated calf thymus DNA) for 1 h at room temperature. After blocking, wash the plates three times with wash buffer and store at 4°C until needed. We have used plates that were stored at 4°C for up to 3 mo without significant loss in sensitivity.

6. The buffer (100 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 0.05% Tween-20) can be used as a substitute for manufacturer's conjugate dilution and substrate buffers.
7. All stock solutions of ELISA detection should be stored at 4°C. Avoid repeated freezing thawing of the anti-DIG-POD conjugate. Once reconstituted, the conjugate solution should be divided into aliquots and stored at –20°C, and when thawed, it should be stored at 4°C until used.
8. When the ELISA was used to analyze the PCR products shown in **Fig. 2**, all four SFS strains developed colorimetric reactions whose values were significantly higher than the background, whereas the values obtained with non-SFS phleboviruses were similar to or near that of the threshold (**Fig. 3**). The detection limit of the assay was evaluated with triplicate 10-fold serial dilutions of virus-infected cell culture. The results (**Fig. 4**) show that with ethidium bromide-stained agarose gel, the detection limit was about 25 PFU, whereas the detection limit of ELISA was 2.5 PFU.

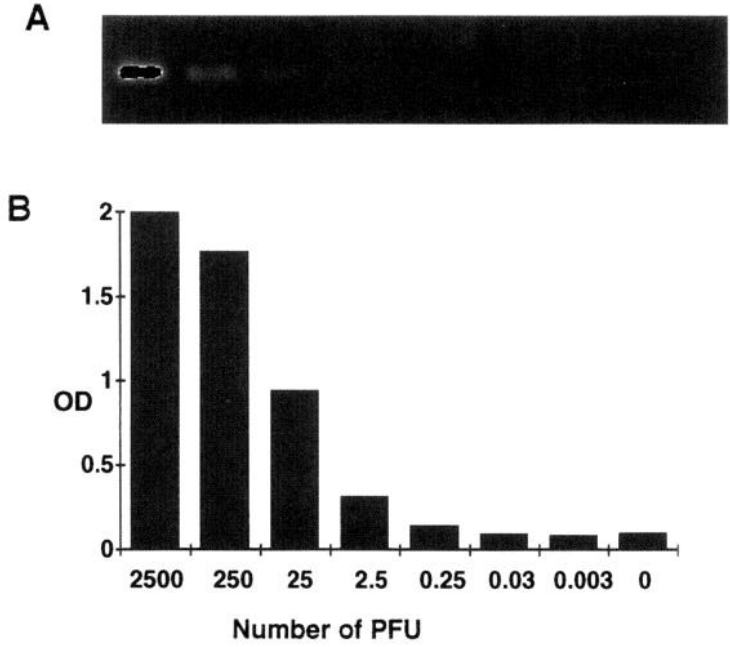

Fig. 4. The detection limit of PCR-ELISA was compared with that of agarose gel using 10-fold serial dilutions of RNA extracted from predetermined SFS virus titer. The detection limit of ethidium bromide-stained agarose gell was approx 25 PFU, whereas the detection limit of PCR-ELISA was approx 2.5 PFU. Each OD value (410/490 nm) represents the mean of three replicates. The cutoff OD value was determined from the mean of three negative control replicates plus two standard deviations.

9. The smallest quantity of PCR product that could be detected by ELISA was determined by using serial twofold dilutions of 1 µL (approx 10 ng) of DIGlabeled SFS PCR products. The results (**Fig. 5**) show that the colorimetric EIA detected 150 pg, whereas the ethidium bromide-stained gels detected about 1500 pg.
10. In conclusion, probe-based ELISA assays are useful for the specific detection of SFS virus from cultures as well as clinical samples. These assays are easy to perform, more specific, and are at least 10-fold more sensitive than agarose gel electrophoresis. Because the assays use the microtiter plate format. they are also suitable for automation. allowing processing of large numbers of samples.

Acknowledgments

David Swenson contributed to the development of the assay; Joe Magniafico and Peter Summers provided virus stocks; Art Anderson, David McClain, and

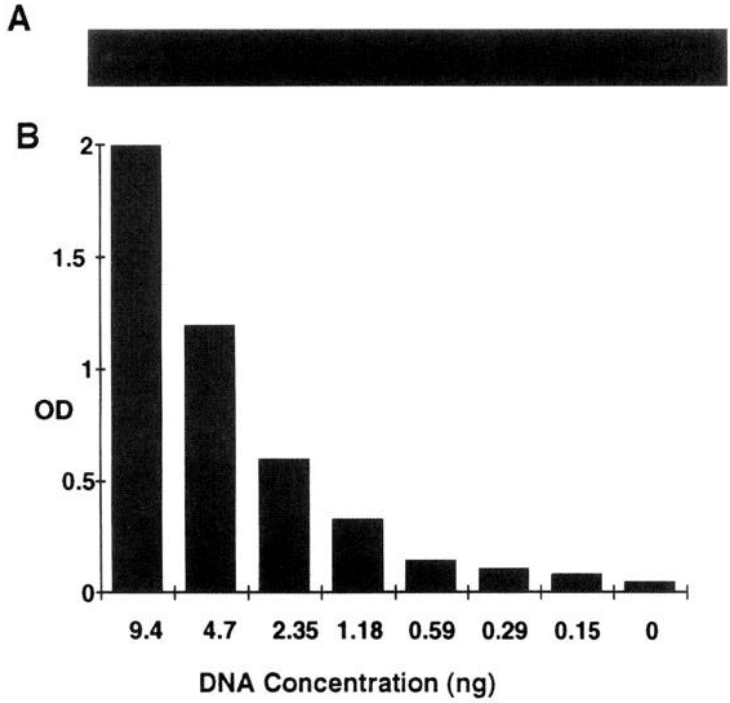

Fig. 5. The amount of amplified DNA detectable by agarose gel and PCR-ELISA were compared by using twofold serial dilutions of 1 μL (~9.4 ng) dIG-labeled SFS PCR product. PCR-ELISA detected approx 150 pg of PCR-amplified DNA, whereas ethidium bromide-stained agarose gel detected approx 1200 pg. Each OD value (410/490 nm) represents the mean of three replicates. The cut-off OD value was determined from the mean of three negative control replicates plus two standard deviations.

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19

Time-Resolved Fluorescence

Pekka Halonen and Timo Lövgren

1. Introduction

The principle of time-resolved fluorometry and its application to solid-phase immunoassays (TR-FIAs) was invented in the 1980s in Turku, Finland at the Wallac Biochemical Laboratory and University of Turku *(1,2)*. One of the early diagnostic applications was the detection of viral antigens with immunoreagents prepared from either polyclonal or monoclonal antibodies *(3–7)*. Viral antigen detection using time-resolved fluorescence combined with one-step immunoassay has resulted in the highest sensitivities obtained with any solid-phase immunoassays. In the test, Eu-labeled monoclonal antibody reacts quickly and efficiently in liquid-phase with the viral antigen of the test specimen and the formed antigen-antibody complex is adsorbed to another monoclonal antibody attached to solid-phase (**Fig. 1**). The bound Eu-chelate is then measured with a single-photon-counting fluorometer designed to measure only the specific lanthanide fluorescence with a long decay time. This is achieved when the background fluorescence reaches an insignificant level. The specificity of fluorescence is further increased by the large difference between the excitation and emission wavelengths of lanthanide compounds (Stoke's shift).

Another emerging application of time-resolved fluorescence to diagnostic virology is in the liquid-phase hybridization for the detection of PCR amplicons *(8,9)*. The test format that has been used most recently is similar to the one-step monoclonal TR-FIA *(10)*. In the test, Eu-labeled oligonucleotide probe is hybridized quickly in liquid-phase with the specific sequences in single-stranded PCR amplicons, and this hybrid is bound in the second step to the biotinylated probe on the solid phase. After washing off the nonbound detector

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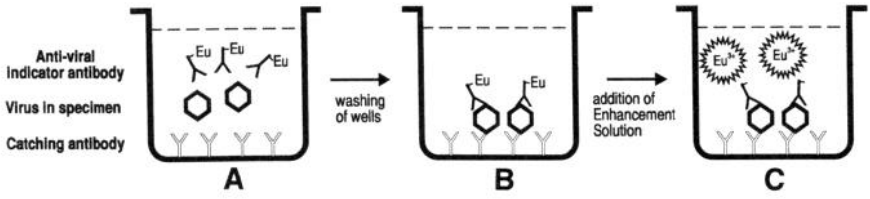

Fig. 1. Principle of the monoclonal one-step time-resolved fluoroimmumoassay for the detection of viral antigen. **(A)** Addition of reagents and incubation; **(B)** removal of reagent excess; **(C)** measurement with time-resolved flourometry.

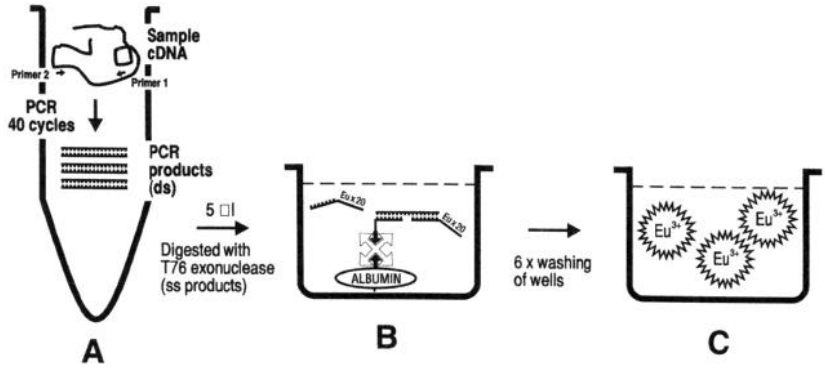

Fig. 2. Principle of liquid-phase by time-resolved fluorometry in detection of PCR products. Eu-labeled probe (Eux20); biotinylated probe (↙); streptavidin (▒); biotinylated albumin (ALBUMIN). **(A)** PRC; **(B)** liquid-phase hybridization and collection of hybrids on solid phase; **(C)** development of flourescence by enhancement solution.

probe the Eu-chelate is measured by time-resolved fluorometry (**Fig. 2**). Compared with the detection of amplicons with gel electrophoresis the advantage of liquid phase hybridization is higher specificity; adaptation of the technology to micro-titration strips also allows easy handling and high throughput in standard diagnostic laboratories. A review on the use of lanthanide chelate labels in a number of alternative nucleic acid hybridization assays has been published recently ***(11)***.

The details of Eu-chelate labeling of monoclonal antibodies and DNA oligonucleotide probes and of test protocols of one-step monoclonal TR-FIA and liquid-phase hybridization assays are reported in this chapter.

2. Materials

2.1. Reagents

2.1.1. Reagents for TR-FIA

1. Polystyrene microtitration strips, 12-well format, high quality (Maxisorp strips, Nunc, Roskilde, Denmark).
2. Washing solution (Wallac Oy, Turku, Finland) 5 m*M* Tris-HCl, pH 7.75, 0.15 *M* NaCl, 0.05% Tween-20, 0.05% NaN_3.
3. Coating solution for monoclonal antibody: 0.1 *M* $NaHCO_3$, pH 9.6
4. Postcoating solution: 50 m*M* Tris-HCl, pH 7.75, 0.15 *M* NaCl, 0.05% NaN_3, containing 0.1% gelatin and 20 µ*M* *N*'-diethylene triaminepentaacetic acid
5. Assay buffer: 50 m*M* Tris-HCl, pH 7.75, 0.15 *M* NaCl, 0.05% NaN_3, 0.5% gelatin, 0.01% Tween-40, and 20 µ*M* N'-diethylene triaminepentaacetic acid (DELFIA® assay buffer, Wallac Oy).
6. Specimen diluent: 20% inactivated fetal calf serum, 2% Tween-20 in phosphate-buffered saline, pH 7.2
7. DELFIA enhancement solution (Wallac Oy)
8. Eu-labeling kit: Europium chelate of N'-(*p*-isothiocyanatobenzyl)diethylenetriamine-N_1,N_2,N_3,N_3-tetraacetic acid (Wallac Oy)
9. Biotinylation reagent: Biotin-amidocaproate *N*-hydroxysuccinimide ester (Sigma, Deisenhofen, Germany), 10 mM in dry *N,N*-dimethylformamide
10. Chromatography buffer 50 m*M* Tris-HCl, pH 7.8, 0.9% NaCl, 0.05% NaN_3

2.1.2. Reagents for Liquid-Phase Hybridization

1. Polystyrene microtitration strips, 12-well format, high quality (Maxisorp strips, Nunc)
2. Coating solution for biotinylated bovine serum albumin (BSA): 50 m*M* K_2HPO_4, 0.05% NaN_3, 0.9% NaCl.
3. DELFIA assay buffer, *see* **Subheading 2.1.1., item 5**
4. 2X Hybridization solution: DELFIA assay buffer with an additional 2.92 g NaCl per 50-mL bottle.
5. DELFIA wash solution, *see* **Subheading 2.1.1., item 2.**
6. 1 *M* Na_2CO_3.
7. 10 m*M* Tris-HCl, pH 7.5, 50 µ*M* ethylenediaminetetraacetic acid (EDTA).
8. Biotinylated BSA. Dissolve 10 mg BSA in 50 m*M* Na_2CO_3. Add a 50-fold molar excess of biotin-amidocaproate *N*-hydroxysuccinimide ester in 200 µL dry *N,N*-dimethylformamide. Incubate at room temperature (RT) for 3 h. Purify the biotinylated BSA twice on PD-10 columns (Pharmacia Biotechnology, Uppsala, Sweden) using 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, containing 0.05% NaN_3 and 0.9% NaCl as the eluent.
9. Streptavidin (Boehringer Mannheim GmbH, Mannheim, Germany)
10. Reagents for probe biotinylation: Biotin-amidocaproate *N*-hydroxysuccinimide ester, Act-biotin (Sigma), or other equivalent amino-reactive biotin derivative

11 Reagents for Eu labeling of oligonucleotide probes: Amino-modified phosphoramidite, 5'-O-dimethoxytrityl-*N*4-(trifluoroacetylamidohexyl)-deoxycytidine-3'-O-(beta-cyanoethyl-diisopropylamino)-phosphoramidite (modC) and europium chelate of 4-(2-(4-isothiocyanatophenyl)ethyl)-2,6-bis(*N,N*-bis(carboxymethyl) aminoethyl)pyridine (Wallac Oy)
12 DELFIA enhancement solution (Wallac Oy)

2.2. Instrumentation

1 DELFIA plate dispense (Wallac Oy).
2. DELFIA platewash (Wallac Oy)
3. DELFIA plateshake (Wallac Oy)
4. DELFIA research fluorometer (Wallac Oy)
5 Oligonucleotide synthesizer, with option to use additional phosphoramidites

3. Methods

3.1. One-Step Monoclonal Time-Resolved Fluoroimmunoassay for Viral Antigen Detection

3 1.1. Europium Labeling of Monoclonal Antibodies

1 Reconstitute the labeling reagent (Eu-labeling kit, Wallac Oy) with 500 µL distilled water The Eu concentration of the solution is 3 mM.
2. A 1:25 molar IgG/Eu ratio is used in labeling. As 1 mg IgG corresponds to 6 25 nmol, 26 µL labeling reagent solution is used for 0.5 mg IgG (total volume 0.5 mL)
3 Adjust pH to 8 6 with 1·10 (v/v) vol of 0.1 *M* borate buffer, pH 8 6 (*see* **Note 2**), and incubate the labeling reaction overnight at RT.
4. Purify the labeled IgG by gel filtration on a PD-10 column (Pharmacia). If a more purified label is needed, gels with a better separation ability, e.g , Superdex 200 (Pharmacia), should be used.
5. Equilibrate the PD-10 column with 25 mL of chromatography buffer. After loading the sample, collect 0 5-mL fractions into 3-mL tubes by adding further chromatography buffer onto the column Collect 10 tubes
6. To detect the IgG fractions, prepare a 1:100 dilution from each fraction to the enhancement solution Transfer 200 µL in triplicate to empty microtitration wells and measure the fluorescence with the DELFIA research fluorometer Pool the fractions containing IgG

3.1.2. Characterization of the Labeled Antibody

1 Measure the Eu concentration in the pool by making a 1:10,000 dilution from pool and a 1:100 dilution from 100 n*M* Eu standard (std) to the enhancement solution. Measure fluorescence as above and calculate the Eu concentration (C_{Eu}; in µ*M*) by using the equation:

$$C_{Eu} = F\,(\text{sample})/F\,(\text{std}) \times \text{dilution}$$

where F = fluorescence signal.
If the signal of the pool exceeds 2×10^6 cps, make further dilutions

2. Calculate the IgG concentration (C_{IgG}) from absorbance at 280 nm after subtracting the absorbance of the formed thiourea bond using the equation.

$$C_{IgG}\ (\text{g/L}) = \frac{Abs - 0.008 \times C_{Eu}}{1\ 34}$$

where Abs = Absorbance at 280 nm

$$C_{IgG}\ (\text{mol/L}) = \frac{C_{IgG}}{\text{MW}_{\text{IgG}}}$$

where C_{IgG} = IgG concentration (g/L)
MW_{IgG} = molecular weight of IgG (160,000 g/mol).

3. Calculate the yield of Eu bound to the IgG

$$\text{yield (Eu/IgG)} = \frac{C_{Eu}}{C_{IgG}}$$

4. Calculate the recovery of labeled antibody.

$$\text{Recovery} = \frac{C_{IgG}\ \text{recovered} \times \text{pool volume}}{C_{IgG}\ \text{labeled}} \times 100$$

5. Add 1 mg/mL of purified BSA, filter through a 0.22-μm filter (e.g., Millipore Millex), and store at +4°C (*see* **Note 3**).

3.1.3. One-Step Monoclonal Time-Resolved Fluoroimmunoassay

3.1.3.1. Coating of Microtitration Wells

1 Add 200 μl purified monoclonal antibody in optimal dilution (e.g., 0.5 μg/well) in carbonate buffer, pH 9.6, to the wells in single 12-well strips. The antibody is adsorbed to the wells in sealed strips overnight at ambient temperature in a moist chamber.
2 Wash wells three times with the platewash using the wash solution.
3. Postcoat the wells by adding 250 μL postcoating solution as described above, again with overnight incubation at ambient temperature
4. Wash wells twice as described above and store the sealed strips in sealed plastic bags with moist paper for several months at 4°C.

3.1.3.2. One-Step Immunoassay

1. Add the specimens (e.g., mucus aspirate diluted originally 1:5 in the specimen diluent, sonicated for 2 min and diluted further 1:2 with the assay buffer; the final dilution of the specimen is 1:10) in 100-μL volumes, followed by 100 μL of appropriately diluted (with the assay buffer) Eu-detector antibody to duplicate wells coated and postcoated as described above (*see* **Note 4**). Include three wells of positive controls and five wells of diluent controls.

2 Shake the strips for 2 min on a plateshaker and incubate for 1 h at 37°C.
3 Wash the strips carefully six times (*see* **Note 5**), and add 200 µL of enhancement solution per well with the plate dispenser (*see* **Note 6**).
4 Shake the plates on the plateshaker for 25 min at ambient temperature.
5. Place in the fluorometer for counting.
6. The fluorometer is programmed to calculate the mean and coefficient of variation of 12 reagent blanks and to take the mean and coefficient of variation of the duplicates of each specimen minus the reagent blanks. The printed data can be further analyzed by computing the mean and standard deviation of all the initially negative specimens run in the same test and using this mean + 3 standard deviations as the cutoff value for the positive specimens.

3.2. Detection of Viral-Specific PCR Amplicons by Liquid-Phase Hybridization Using Time-Resolved Fluorescence

3.2.1. Europium Labeling of Oligonucleotide Probes (see **Notes 7–9**)

1. Dissolve the modC in dry acetonitrile to a final concentration of 0.1 *M*. Attach the modC solution to the synthesizer at the appropriate site Upon completed synthesis, the deprotection and removal of the oligonucleotide from the support takes place in concentrated ammonia overnight at 55°C according to standard procedures (instruction manual of the synthesizer)
2 Purify the oligonucleotide containing the tail of modCs on the 5' end by urea polyacrylamide gel electrophoresis (PAGE), and subsequently elute the product according to standard procedures ***(12)***. Change the buffer of the eluted oligonucleotide to H_2O by using NAP-5/NAP-10 columns. The amount of pure oligonucleotide is determined by absorbance measurement at 260 nm, 1 OD_{260} = 33 µg/mL
3. Dry 1.5–10 nmol of the oligonucleotide in H_2O in a Speed-Vac centrifuge (Savant Instruments, Inc , New York). Redissolve the oligonucleotide in 50 µL H_2O. Adjust the pH to 9 5 by adding 2.5 µL 1 *M* Na_2CO_3.
4 Dissolve about 0.5–4 µmol Eu-chelate in 100 µL H_2O. Quantitate by making serial dilutions (1.100, 1.10, etc.) into 1 mL enhancement solution, and measure the fluorescence in a time-resolved fluorometer against a 1 n*M* Eu-standard. The exact concentration of Eu-chelate [Eu] is calculated using the formula.

$$[\text{Eu}]\ (\text{n}M) = \frac{y \cdot z}{x}$$

where y = fluorescence in cps of Eu-chelate-containing sample
x = fluorescence in cps of the 1 n*M* Eu-standard
z = dilution factor.

Add to the oligonucleotide solution a molar excess of the Eu-chelate based on the following formula:

$$\text{Eu-chelate excess (nmol)} = A \times B \times 10$$

where A = nmol oligonucleotide
B = number of modC per oligonucleotide

5. Incubate the reaction mixture overnight at 4°C, and purify the Eu-labeled oligonucleotide by gel filtration. When less than 200 nmol of Eu-chelate is used in the labeling reaction, the labeled oligonucleotide can be rapidly purified over a NAP-5 or NAP-10 column. When larger amounts of oligonucleotide are labeled, a bigger column is required for the purification (e.g , 1 × 50 cm, Sephadex G-50 column). Elution is carried out in 10 m*M* Tris-HCl, pH 7.5, containing 50 μ*M* EDTA. It is recommended that absorbance be recorded at 260 nm and the Eu profile determined by diluting 1-μL aliquots of each fraction into 1 mL enhancement solution and measuring Eu content in a time-resolved fluorometer. Identify the first peak based on the UV and Eu profile and pool the fractions containing the Eu-labeled oligonucleotide.
   The final product is analyzed spectrophotometrically to determine the amount of oligonucleotide recovered The Eu content of the pooled fraction is measured and the Eu/oligonucleotide ratio is obtained Calculate the total amount of Eu-chelate (in nmol) :n the pooled fractions by the following formula·

$$Eu_{TOT}\ (nmol) = [Eu] \times V \times 10^{-3}$$

   where V = the volume of the pooled fractions in mL
   [Eu] is in n*M*

6 Record the absorbance at 260 nm using the elution buffer as reference The Eu-chelate absorbs at 260 nm; thus, absorbance measured at 260 nm for the oligonucleotide cannot be used for the estimation of the amount of oligonucleotide recovered. The oligonucleotide concentration is given by:

$$C = \left[A_{260} - \left\{ \frac{[Eu]}{50} \times 0\ 83 \right\} \right] \times 33$$

   C = concentration of oligonucleotide in μg/mL
   [Eu] is in μM.
   The amount of oligonucleotide (nmol) is calculated according to the following formula:

$$\text{Oligonucleotide}_{\ TOT}\ (nmol) = \frac{C \times V \times 1000}{MW}$$

   where C = concentration of oligonucleotide in μg/mL
   V = volume of the pooled fractions in mL
   MW = molecular weight of the oligonucleotide (length × 330 g/mol)

   The Eu:oligonucleotide ratio is obtained from the formula

$$\text{Eu.oligonucleotide} = \frac{Eu_{TOT}\ (nmol)}{\text{Oligonucleotide}_{\ TOT}\ (nmol)}$$

7. To obtain a usable concentration of the probe, dilute part of the Eu-labeled oligonucleotide in the elution buffer to a concentration of 1 μg/mL and store at 4°C.

The solution is stable for 6 mo The stock solution is stored at –20°C and is stable for at least 1 yr

3.2.2. Preparation of Biotinylated Oligonucleotide Probes (see **Note 10**)

1 Dry 5 nmol oligonucleotide in H_2O containing a single modC at the 3' end in a Speed-Vac centrifuge Dissolve the oligonucleotide in 50 µL H_2O and add 2 5 µL 1 *M* Na_2CO_3. Then add a 50-fold molar excess of act-biotin (0.11 mg) dissolved in dry *N,N*-dimethylformamide. Incubate at RT for 3 h.
2. Purify the biotinylated oligonucleotide by gel filtration on NAP-5 and NAP-10 columns using 10 m*M* Tris-HCl, pH 7 5, containing 50 µ*M* EDTA as eluent Measure the recovery of the oligonucleotide spectrophotometrically at 260 nm, using the elution buffer as the reference (1 OD_{260} = 33 µg/mL) Dilute part of the biotinylated oligonucleotide to a concentration of 1 µg/mL in 10 m*M* Tris-HCl, pH 7 5, containing 50 µM EDTA. The diluted probe solution is stable for 6 mo when stored at 4°C The stock solution is stable for 1 yr when stored at –20°C.

3 2.3. Liquid-Phase Hybridization

1. For the coating of polystyrene strips, add 400 ng of biotinylated bovine serum albumin in 200 µL of coating solution (pH 9 0) to wells; seal with tape and incubate overnight at ambient temperature in a moist chamber
2 After three washes with wash solution, saturate the wells with 1600 ng of streptavidin in 300 µL assay buffer and incubate the sealed strips for 5 h at ambient temperature
3 Wash again three times as above and add 200 µL biotinylated probe appropriately diluted in assay buffer (*see* **Note 11**) to wells, incubate for 1 h at ambient temperature
4 Wash the wells six times and store the sealed strips in plastic bags with moist paper for several weeks at 4°C.
5. Add single-stranded PCR amplicons (boiled and cooled, or digested with exonuclease; *see* **Note 9**) diluted to 100 µL in 2X hybridization solution (all in ice water bath) and 100 µL Eu-labeled probe appropriately diluted (*see* **Note 11**) in 2X hybridization solution to duplicates of the biotinylated probe-coated wells Include positive and diluent controls in each test
6. Incubate the strips 1 h at ambient temperature, wash six times, and add 200 µL of enhancement solution with the plate dispenser to each well; incubate the strips again at ambient temperature for 25 min on the plate shaker
7. Place in the fluorometer for counting. The cutoff value for positive specimens is 10 times the background value obtained with diluent controls.

4. Notes

4.1. Time-Resolved Fluoroimmunoassay

1. One-step all-monoclonal TR-FIAs are probably the most sensitive assays for the detection of viral antigens, but they require exceptionally high working stan-

dards. Handling of Eu-chelates must be done with similar care as working with radioactive or infectious material. Lanthanides have no known health hazards but contamination of the laboratory and particularly the fluorometer increases the background of all tests to such an extent that TR-FIAs cannot be used in the laboratory before it is cleaned again.

2. The isothiocyanate group of the labeling reagent reacts with the free amino groups on either the protein or the buffer. It is therefore important that the protein to be labeled is in a buffer that does not contain any primary or secondary amino groups. For example, Tris buffer contains primary and HEPES buffer contains secondary amino groups Azide should be avoided as well
3. The stability of Eu-labeled antibody is good and a 100X concentrate can be stored at 4°C. The undiluted label may be stored frozen to avoid microbial contamination.
4. Optimization of coating concentrations with monoclonal catching antibody and label concentrations require box titrations using increasing concentrations of the coating antibody (usually 0.25, 0.5, and 1.0 µg/well) against various concentrations of the Eu-labeled detector antibody (usually 6 25, 12.5, 25, and 50 ng/100 µL) with dilutions of a control antigen
5. Washing steps are critical and only reliable washers should be used The importance of washings is demonstrated by the fact that about 1 million cps are added to the well in the Eu-labeled detector antibody; in the negative specimens without specific binding of the label, the activity should be washed off to a level of a few hundred cps values.
6. The high standard and minimum variation between duplicates requires careful daily and weekly maintenance of all instruments and particularly automatic pipetors and washers must be maintained according to manufacturer's instructions

4.2. Liquid-Phase Hybridization

7. The same high working standards and maintenance of instruments apply to liquid-phase hybridization using time-resolved fluorescence as to TR-FIAs.
8. The primers and probes must be carefully selected for liquid-phase hybridization and these selection criteria may not be the same as for spot hybridization. The size of amplicons should be less than 200 bp and the optimal size may be between 100 and 150 bp. The probes should not interlap with the primers or with each other. Their optimal length may be between 18 and 25 bases. A loop formation of single-stranded amplicons and probes may cause problems in liquid-phase hybridization because the conditions selected for optimal hybridization also favor loop formation. These selection criteria are not unique for time-resolved fluorescence but apply to any liquid-phase hybridizations.
9. Separation of the double-stranded amplicons to single strands can be done by boiling for 10 min and cooling in ice water, or the strand competing with the probes can be digested with T7 gene 6 exonuclease, which requires that the other strand is protected from digestion by phosphorothioate analogs incorporated in the primer ***(10)***.

10. The biotinylation reaction should be at least 90–95% efficient. The biotinylated probe may be checked by HPLC for the presence of biotin *(13)* Unreacted biotin must be carefully removed by gel filtration. If unreacted biotin is present, it will drastically lower the signal level achieved in the hybridization test, since the free biotin will effectively bind to the streptavidin on the solid support.
11. Usable concentrations of biotinylated and Eu-labeled oligonucleotide probes are 2–20 ng/mL. The biotin binding capacity of the solid-phase support limits the amount of biotinylated oligonucleotide that can be used in the hybridization reaction Unspecific binding of Eu-labeled oligonucleotide to the solid support can often be decreased by lowering the concentration of labeled probe.

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20

Enzyme-Linked Immunosorbent Assays

David M. Kemeny

1. Introduction

Sensitive immunoassays were developed in the 1950s and 1960s using radioactive isotopes. The application of enzymes as labels *(1,2)* in the late 1960s increased the potential sensitivity and safety of immunoassays. The widespread popularity of enzyme-linked immunosorbent assays (ELISAs) owes much to the use of microtiter plates *(3)*. It was probably the adoption of microtiter plates that provided ELISA with its greatest advantage—convenience. However, the convenience of plastic microtiter plates carries with it a limitation, the ease with which reagents bind to plastic and the way in which they perform once bound. In this chapter, I will review ELISA procedures and discuss their advantages and disadvantages.

1.1. Types of Assay

There are many different types of ELISAs. All involve different combinations of antigen and antibody. They can be broadly grouped into assays for antibody or antigen and into competitive or noncompetitive assays. The choice depends on the nature of the sample, availability of reagents, and the precision and sensitivity required. Noncompetitive assays in which all the constituents, other than the sample, are in excess will normally be more accurate than their competitive counterparts. However, competitive assays make it possible to obtain an estimate of the amount of a particular antibody or antigen where it is not possible to obtain suitable reagents for noncompetitive assays. They are less precise than noncompetitive assays as more than one component is present at a limiting concentration, but can be used to give an indication of the bioreactivity of the antibody or antigen and can be used to obtain measurements of relative antibody affinity *(4,5)*.

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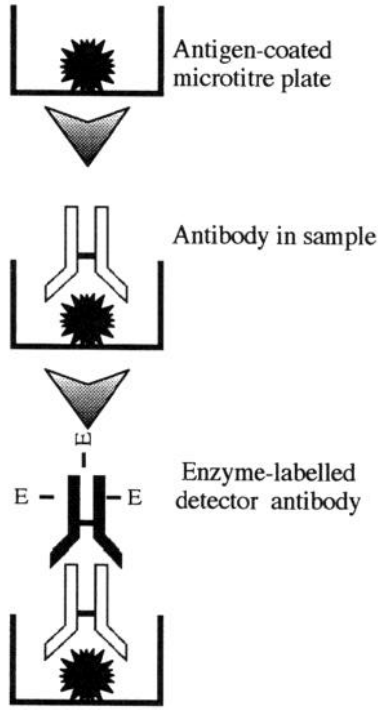

Fig. 1. The antigen-coated plate ELISA. Antigen-coated microtiterplates are incubated with sample containing antibody that binds to the antigen on the plate and is subsequently defected by addition of an enzyme-labeled antibody specific for the bound antibody. The amount of test antibody is proportional to the amount of enzyme bound.

1.2. Assays for Virus-Specific Antibody

The simplest procedure is the antigen-coated plate ELISA for antibody. Here the microtiter plate is coated with antigen. Sample, containing the test antibody, is added. Any test antibody present binds to the antigen on the plate. Bound test antibody is subsequently defected by addition of an enzyme-labeled antitest-antibody (**Fig. 1**). The presence of bound enzyme is determined by addition of a chromogenic substrate that generates color or luminescence. The enzyme-labeled antibody is referred to as the detector antibody, which may be labeled directly or may be detected following addition of a second, enzyme-labeled, antiglobulin antibody. We have successfully used the same antimouse antibody to detect monoclonal antibodies specific for human IgG1–4 antibodies. Alternatively, the detector antibody may be biotinylated and subsequently detected with avidin-labeled enzyme. In these assays all of the reagents except the test antibody should be present in excess.

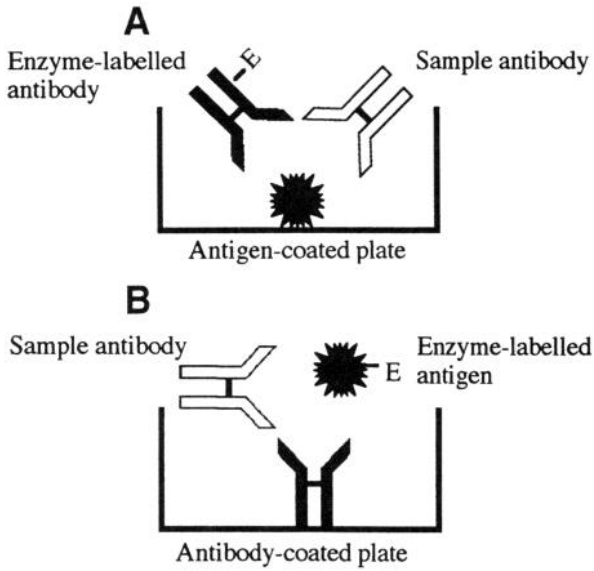

Fig. 2. The competitive ELISA for detection of antibody. In this assay competition is determined between (**A**) the sample antibody and a fixed amount of plate-bound antigen and enzyme-labeled antibody, or (**B**) a fixed amount of plate-bound antibody and enzyme-labeled antigen. The amount of test antibody is inversely proportional to the amount of enzyme bound.

Antibody can be measured by competition with a fixed amount of labeled antibody for solid-phase antigen or in competition with solid phase antibody for a fixed amount of labeled antigen (**Fig. 2**). Here the amount of fixed and labeled reagents must be limited so that small differences in the amount of sample antibody can be seen.

There are times when it is necessary to separate one class of antibody from another before detection of specific antibody activity. IgM antibodies are diagnostic of recent infection with a number of pathogens such as German measles (rubella). However, if the patient has previously been exposed to rubella, there may be many times more IgG than IgM antirubella antibodies. As IgG antibodies persist for years they are not indicative of recent infection with rubella and so are of little diagnostic value. The problem is compounded by the fact that the IgG antibodies present will usually be of much higher affinity and so may disproportionately compete with IgM antibodies for binding to the rubella antigen. In IgM class-capture assays, the microtiter plate is coated with polyclonal or monoclonal antibody specific for IgM. This antiIgM-coated plate is used to capture IgM in the sample and the antirubella antibody activity of the bound IgM is subsequently determined by addition of labeled antigen (**Fig. 3**). It is important

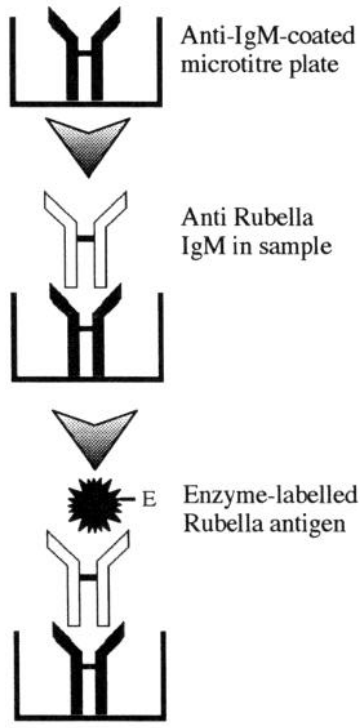

Fig. 3. The class-capture ELISA. Anti-IgM-coated plates are used to bind IgM in the sample. The antibody activity of the captured IgM is then determined using enzyme-labeled antigen. The amount of IgM-antibody is proportional to the amount of enzyme bound.

that most, or at least a substantial proportion, of the IgM in the sample is captured and suitable capture antibodies must be carefully selected *(6)*.

1.3. Assays to Detect Viral Antigen

Sometimes referred to as the two-site or sandwich assay, microtiter plates are coated with capture antibody (so called because it captures the antigen). The sample containing antigen is added and any antigen present binds to the antibody on the plate. Finally the bound antigen is detected by addition of an enzyme-labeled antibody specific for the bound antigen (**Fig. 4**). The detector antibody may be labeled directly or may be detected following addition of an unlabeled antibody specific for the bound antibody followed by a second enzyme-labeled antiglobulin antibody. Alternatively the detector antibody may be biotinylated and subsequently detected with avidin labeled enzyme. The coating antibody and the detector antibody may be the same or

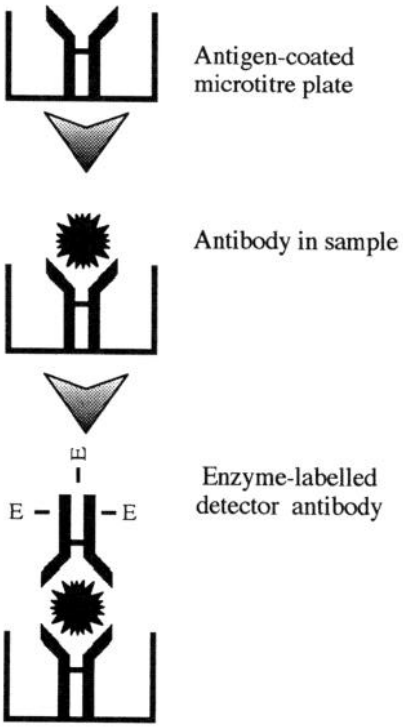

Fig. 4. The two-site or sandwich ELISA. Antibody-coated microtiter plates are incubated with sample containing antigen that binds to the antibody on the plate and is subsequently detected by addition of an enzyme-labeled antibody specific for the bound antigen. The amount of test antigen is proportional to the amount of enzyme bound.

different and they may also be polyclonal or monoclonal, although in the latter ease it is important that they do not compete with each other for binding to antigen. This is usually accomplished by using two monoclonal antibodies that recognize different epitopes although there are examples of monoclonal antibodies that can be used for both capture and detection in the same assay *(7)*.

Antigen can be measured in a competitive ELISA in two ways. In the first, sample antigen competes with a fixed amount of labeled antigen for binding to solid-phase antibody; in the second, sample antigen and solid-phase antigen compete for binding to a fixed amount of labeled antibody (**Fig. 5**). The drawback to the antigen-coated plate method is that a precise, limiting quantity of antigen must be bound to the microtiterplate. Competitive assays work best when competition is carried out in solution where the association and dissociation of antibody and antigen approximates to the law of mass action.

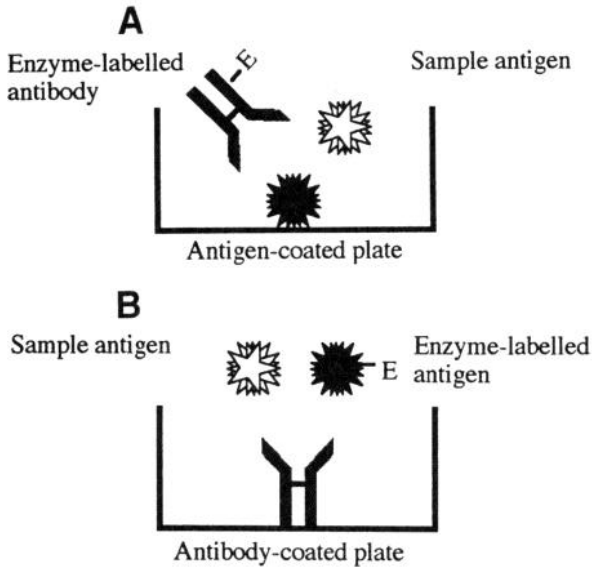

Fig. 5. The competitive ELISA for detection of antigen. In this assay the competition is determined between (**A**) the sample antigen and a fixed amount of plate-bound antigen and enzyme-labeled antibody, or (**B**) a fixed amount of plate-bound antibody and enzyme-labeled antigen. The amount of test antigen is inversely proportional to the amount of enzyme bound.

1.4. Assay Development

The reliability, sensitivity, and specificity of ELISAs is dictated by the quality of the reagents used. Where suitable reagents are not available effort may be focused on cloning or isolating antigen or on the generation of suitable antibodies. Ideally reagents will be available commercially but where they are not it is often possible to obtain them from other investigators. There is little point in expending a great deal of effort and resource when a quick Medline search would have identified a source of reagent. Sample volume is usually 50 μL, for economy; this also gives the best surface area to volume ratio. Temperature is typically ambient and incubation times usually 1–2 h. We generally use the same type of plastic plate, which has a high capacity for antigen, for all our assays.

Coating of the plastic plate with antigen or antibody is of critical importance since failure at this stage renders all other steps useless. Coating can be evaluated in two ways. To test whether a mouse monoclonal antibody has bound to the plate, enzyme-labeled antimouse antibody can be used; to test whether the bound antibody is functional, the appropriate labeled antigen can be added. The optimum coating buffer will differ for different antigens, depending on

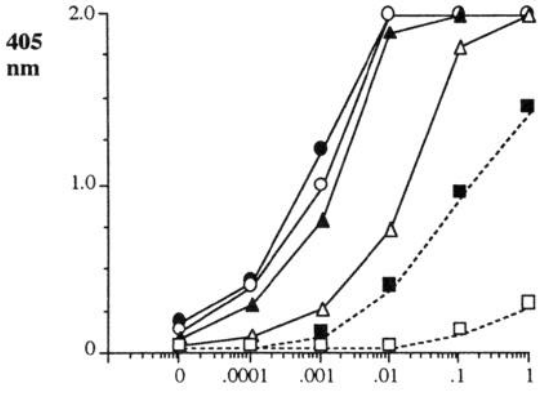

Fig. 6. The optimal concentration of coating antigen is determined by coating the microtiter plate with increasing concentrations of antigen, here bee venom. The optimal concentration is 3 μg/mL as a small increase or decrease has little effect on the amount of signal generated. Antigen coating concentrations: (—●—) 10 μg/mL; (—○—) 3 μg/mL; (—▲—) 1 μg/mL; (—△—) 0.3 μg/mL; (---■---), 0.1 μg/mL; (---□---), 0 μg/mL.

their isoelectric point. The concentration of antibody or antigen used to coat the plate is important. Too much and the loss of bound protein will cause a prozone effect ***(8)***, too little and the sensitivity and robustness of the assay will be adversely affected. Wherever possible it is best to use a concentration where a small increase or decrease has little effect (**Fig. 6**).

By far the easiest method of coating the microtiter plate is by passive adsorption to the surface of the plastic. However, proteins may be damaged by binding to the plate ***(9)*** and may be specifically orientated, for example, through the binding of hydrophobic regions to the plastic, so that some antigenic determinants fail to be expressed. Antigen can be desorbed or leach off the plastic ***(10–12)***. Such difficulties may be overcome by polymerization of the coating material with crosslinking agents such as glutaraldehyde or carbodiimide ***(13–16)***, or with specific antibody, which also has advantages in terms of reproducibility, sensitivity, and specificity ***(10,17,18)*** (**Fig. 7**). Most antibodies will retain their ability to bind antigen when coated to plastic, although there is little doubt that a substantial amount of antibody activity is lost. Alternatively, antibodies can be biotinylated and immobilized with avidin-coated plates. Plates can be coated with antibodies to haptens such as tri-nitrophenyl phosphate (TNP) ***(19)***, which can then be used to bind hapten-labeled antigens. To reduce nonspecific binding of the sample to the plate some authors have added a blocking step but we

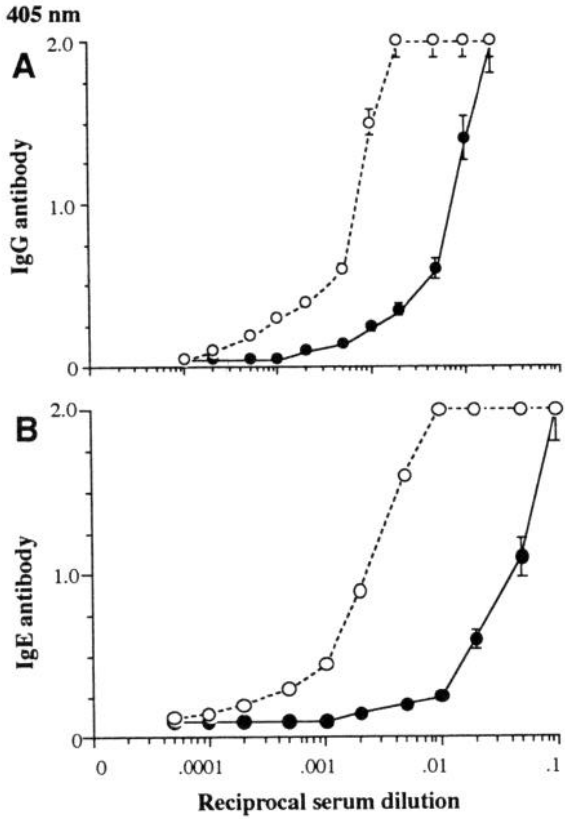

Fig. 7. Comparison of IgE (**A**) and IgG (**B**) antibody binding to microtiter plates coated directly (○) and indirectly (●) with antigen (bee venom phospholipase A_2). The amount of antibody in the reference serum bound by plates coated with antigen indirectly is much greater than with plates coated with antigen directly.

and others have observed that blocking can either increase or decrease background activity ***(20,21)***. Part of the problem may be that proteins do not evenly coat the plastic surface but exist as small islands ***(22)*** and additional protein simply binds to these foci.

Just as there is a limited range of protein concentrations that binds effectively to plastic, there is a limit to the amount of sample that can be measured. In practice this is rather similar between assays and typically covers a concentration range of 1–1000 ng/mL. Very low concentrations require special modifications and higher concentrations need dilution. In practice it is rarely possible to assay serum samples at a dilution of less than 1/50 because the high protein content of serum often interferes with the assay. The composition of

the sample buffer too is important. The addition of 1% animal serum and 0.1% Tween-20 helps to reduce nonantibody-antigen interactions. To ensure low assay backgrounds it is a good idea to use serum from the same species as the enzyme-labeled antibody. To make it easier to check which wells have been filled, I find it particularly helpful to add colored dyes to the assay diluent *(23,24)*.

In immunoassay, a "detector" is any molecule that has specificity for the sample and that is used to detect it. It can be labeled directly with enzyme or indirectly. Methods for preparing enzyme conjugates have been extensively reviewed elsewhere *(25–28)*. In noncompetitive assays the detector antibody or antigen should be present in excess. Where more than one detector is used, each should be optimized in turn (**Fig. 8**). The low nonspecific binding of enzyme-labeled reagents to plastic means that they can be used at a relatively high concentration in ELISA. The diluent should normally be the same as for the sample. The detector can be labeled directly or indirectly using an enzyme-labeled anti-detector antibody *(29,30)* or other ligands such as biotin/avidin *(31)* or FITC/anti-FITC *(32)*. Removal of the Fc portion of the antibody yields conjugates with increased tissue penetration (for histology) and lower background binding *(7,33)* and greater sensitivity when amplified substrate systems are used (**Fig. 9**). The rate of color development with alkaline phosphatase can be increased using an amplified substrate system *(7,34–36)*.

1.5. Quantitation

The ways in which we express the results of ELISA procedures should be fairly straightforward. During optimization experiments and methodological development it is acceptable to express the data as units of optical density (OD) (color). But for most ELISAs there is not a simple numerical relationship between signal (color) and analyte concentration, and there can be variation in enzyme activity from day to day. To take account of this, the amount of color generated must be calibrated against a standard—a sample whose content is known, or which has been assigned arbitrary units. Once plotted, the data forms a reference or standard curve. The reference material can be a pool of positive sera, cell culture supernatant, or a recombinant protein. It is usually stored frozen in aliquots to reduce damage resulting from freezing and thawing.

Many ELISA plate-readers come with suitable software for plotting curves and calculating results. Where such facilities are not available the results can be plotted by hand on a semilogarithmic paper as most reference curves cover a relatively wide (2–3-$\log_{10}$) concentration range. The data are plotted as OD against the reciprocal of the reference material (**Fig 10**) such that a 1/10 dilution is plotted at 0.1, 1/20 at 0.05, 1/50 at 0.02, etc. Results are then read from the reference curve. They will then need to be multiplied by the appropriate dilution factor. The lower limit of the assay must be defined. In our laboratory

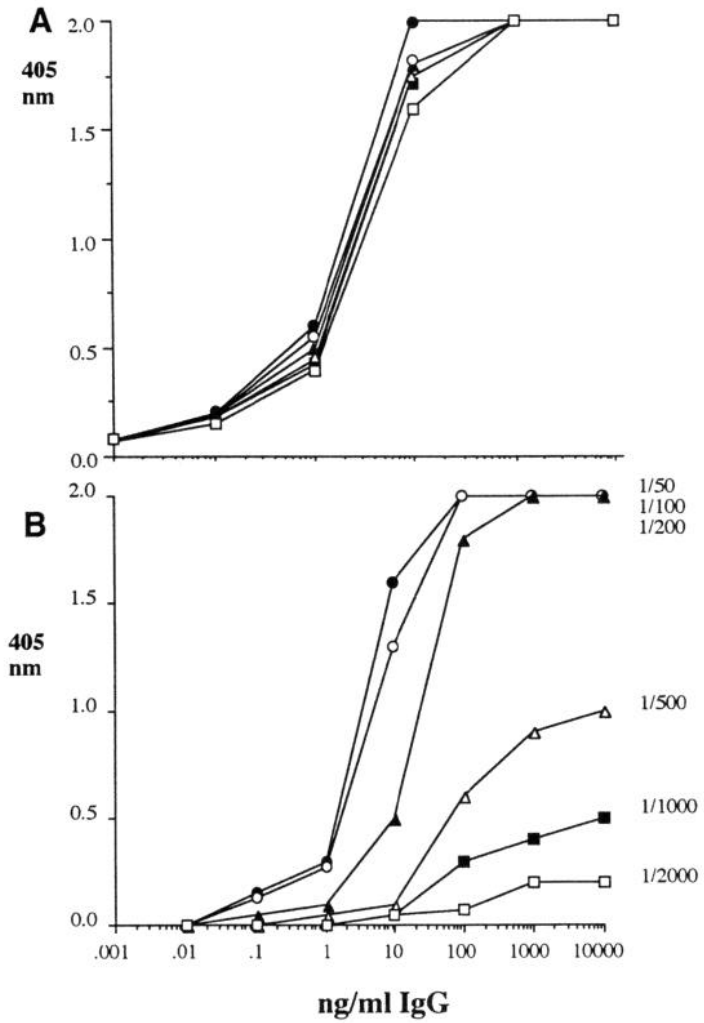

Fig. 8. Optimization of (**A**) detector antibody (monoclonal anti-IgG) and (**B**) enzyme-labeled antimouse antibody. Different concentrations of antihuman IgG-monoclonal antibody are used in the assay. All give similar results and 1/1000 is chosen. Once this has been optimized, the second enzyme-labeled antibody that detects the bound monoclonal antibody optimized in (A) is added at different concentrations. Here there is a much bigger effect of concentration and 1/100 is optimal. ●, 1/100; ○, 1/200; ▲, 1/500; △, 1/1000; ■, 1/2000; □, 1/5000.

we use a lower limit of 1.5 times the background value. If the background is high then the working range of the assay will be inadequate and the assay will clearly require further development in order to reduce this.

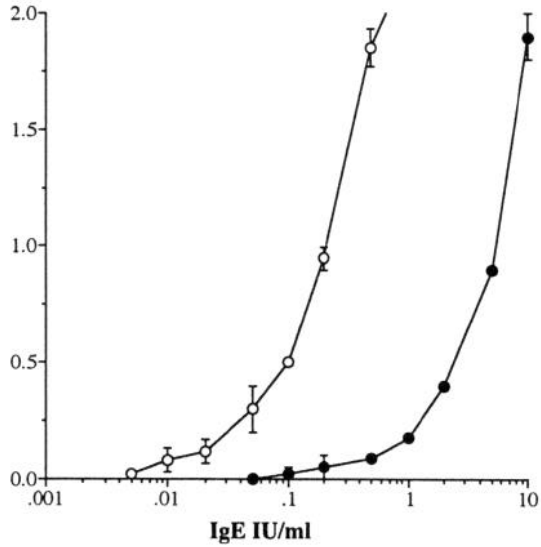

Fig. 9. Detection of human IgE by a two-site ELISA using whole anti-IgE-AP (●) (405 nm) and Fab' antiIgE-AP (○) (492 nm) conjugates. The color was generated using PNP or the amplified substrate *(7,34–36)*.

Another way in which assay results can be expressed is as a titer. This is the lowest dilution of the sample that can be detected at a predetermined cutoff. Originally used to score red cell agglutination assays, titers can provide a considerable amount of information about the sample. Unlike using a reference curve, where values will be recorded over a range of antibody/antigen ratios, the end-point titer will have a constant antibody/antigen ratio. End points that are too close to the background are unreliable because very small differences in concentration give equally small changes in color. An end point 0.2 OD units above background (wells with diluent alone) is usually adequate.

In order to ensure that comparable results are obtained in different assays it is necessary to run quality controls. These are simply the same samples that are run in each assay. They should be run at a minimum of three dilutions to cover the upper, middle, and bottom parts of the standard curve (**Fig. 11**). By running quality controls in each essay it is possible to determine the coefficient of variation (CV). This is a measure of the variation between assays and the formula for this is:

$$CV = (\text{Standard deviation}/\text{Mean}) \times 100$$

Intra (within) -assay and inter (between) -assay variation should be monitored. At least four or five assays need to be run before the interassay CV can be properly determined. If the results obtained with these standards fall outside acceptable limits then the assay will need to be repeated. Acceptable limits will

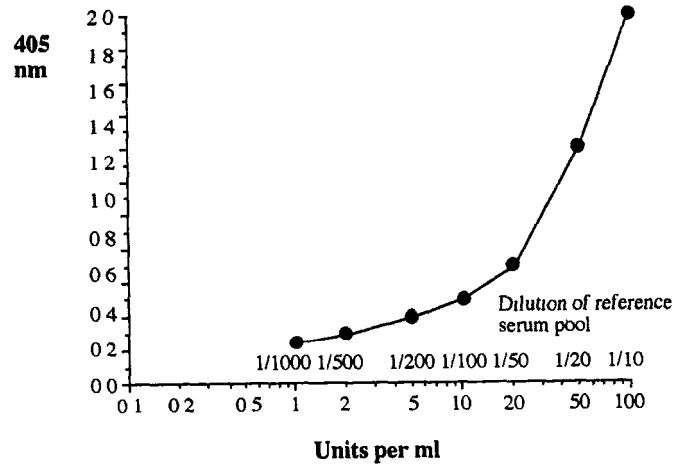

Fig. 10 Calibration of the reference curve. For convenience the results are plotted on semilog arithmic graph paper. Assuming the reference has an arbitrary value of 1000 U/mL, a value of 1 corresponds to a 1/1000 dilution and a value of 20 to a 1/50 dilution. The results can be calculated by comparing the amount of color in the sample wells against the curve and reading the units on the horizontal axis.

differ from assay to assay but are typically within 10%. With new batches of reagent one sometimes sees a change in the quality control.

2. Materials

1. Coating buffer. 0.1 *M* carbonate/bicarbonate, pH 9 6. Take 4 24 g Na_2CO_3 and 5 04 g $NaHCO_3$ and make up to 1 L with distilled H_2O and check pH. Store at 4°C for no more than a few weeks.
2. 0 2 *M* citrate/phosphate buffer, pH 5.0: Make up stock solutions of 21.01 g citric acid ($C_6H_8O_7$ H_2O, 0.1 *M*) and 28 4 g of Na_2HPO_4 (0.2 *M*) each in 1 L of H_2O Add 48.5 mL of 0.1 *M* citric acid to 51.5 mL of 0.2 *M* Na_2HPO_4, producing a pH of approx 5 0. Check and adjust as necessary.
3. 0.05 *M* Diethanolamine buffer, pH 9 8: Dissolve 101 mg of $MgCl_2$ in 800 mL distilled water. When dissolved, add 97 mL diethanolarnine, mix thoroughly, and adjust the pH to 9 8 with concentrated HCl Make up to 1 L with H_2O, add 200 mg of NaN_3, and store in the dark at 4°C.
4. 0.05 *M* Phosphate-buffered saline (PBS), pH 7.4: Dissolve 16.7 g Na_2HPO_4, 5.7 g NaH_2PO_4, 85 g NaCl, and 100 mg of NaN_3 in distilled H_2O, make up to 10 L with H2O, and check pH. Store at room temperature
5. 0.5 *M* Tris-HCl, pH 8.0: Dissolve the Tris in 800 mL and adjust the pH to 8.0 using 1 *M* HCl. Make up to 1 L and store at 4°C.
6. 0.1 *M* Tris-HCl, pH 7 4: Dissolve the Tris in 800 mL and adjust the pH to 7 4 using 1 *M* HCl. Make up to 1 L and store at 4°C.

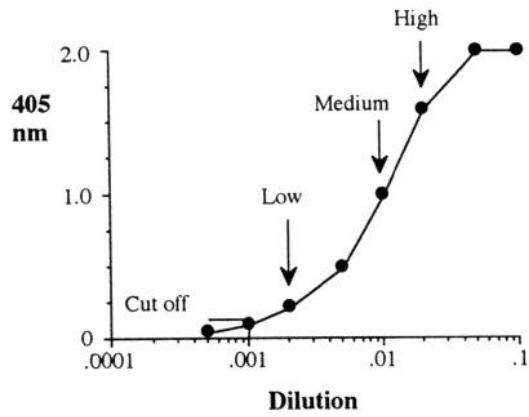

Fig. 11. Quality controls are independent samples that correspond to different parts of the standard curve that are run in each assay and make it possible to assess the reliability of the assay.

7. Amplified ELISA substrate buffer: Prepare 50 m*M* diethanolamine buffer at pH 9.5. Add 1 m*M* $MgCl_2$ 4% v/v ethanol, and 0.1% NaN_3 and check pH.
8. Amplified ELISA amplifier buffer: Prepare 20 m*M* sodium phosphate, pH 7.2. Add 1 m*M* p-iodonitro tetrazolium violet (INT) violet and 0.1% NaN_3 and check pH.
9. Assay diluent: PBS, or 0.1 *M* Tris-HCl, pH 7.4, 1% animal serum, 0. 1% Tween-20. To 98.5 mL of PBS or Tris HCl add 1 mL animal serum, 0.5 mL Tween-20 and 0.5 mL of the red and blue solutions in a 100-mL bottle (*see below*). The particular animal serum used will depend on the reagents and antibodies used in the assay. Horse serum or rabbit serum are commonly used but crossreactivity between serum and reagent antibodies may make it necessary to use another serum such as goat. Phenol red (40 mg/100 mL) and Amido black (60 mg/100 mL) made up in H_2O can be used as colored additives. Add 0.5 mL of each to 100 mL of the buffer being used. Store for no more than 2–3 d. At neutral pH it should be a green color.
10. Washing buffer: Add 5 mL of Tween-20 to 10 L PBS or Tris-HCl, pH 7.4.
11. OPD: Dissolve 0.4 mg *o*-phenylenediamine (OPD) hydrochloride in 10 mL of citratephosphate and adjust the pH to 5.0 with 0.2*M* Na_2HPO_4 or citric acid as necessary. The substrate should be mace up no earlier then 10 min before use and hydrogen peroxide (4 µL of 3% H_2O_2) added just prior to use. It is well worth optimizing the substrate conditions for each batch of reagents. Incubation time with the substrate is about 15 min after which the reaction is stopped by the addition of 50 µL of 2 *M* H_2SO_4. Absorbance must be recorded (at 492 nm) as soon as possible as the substrate product is unstable.
12. TMB: Dissolve 5 mg of 3,3',5,5'-tetramethyl benzidine (TMB) in 2.5 mL of absolute ethanol (warming to 40°C may be necessary). Prepare 0.2 *M* acetate buffer, pH 3.3, and add 5 mL to 92.5 mL of water containing 100 mg of nitroferricyanide. Mix

the two solutions just prior to use Substrate kits for peroxidase using such substrates as TMB are available commercially (Pierce, Wirral, Cheshire, UK) and work well.

13. *p*-Nitrophenyl phosphate (PNP): Add 1 PNP tables to 5 mL of diethanolamine buffer and allow them to dissolve, mixing for 10 min The substrate is stable at 4°C for at least 1 h Substrate incubation time is up to 2 h at 37°C or 24 h at 4°C. The reaction is stopped using 50 µL of 3 *M* NaOH The colored substrate product is very stable at 4°C and plates can be stored for several days in the dark Absorbance should be read at 405 nm.
14. Amplified substrate for alkaline phosphatase. The substrate α-nicotinamide adenine dinucleotide phosphate (NADP) is purified with a suitable ion exchange resin such as DEAE or QAE Sephadex (Pharmacia, Milton Keynes, Bedfordshire, UK) to remove contaminating α-nicotinamide adenine dinucleotide (NAD) and made up as a 100 µ*M* solution in amplified ELISA substrate diluent. Alcohol dehydrogenase (70 mg) and 1% bovine serum albumin (BSA) or similar protein stabilizer is dissolved in 7 mL amplifier diluent and dialyzed extensively against amplifier diluent at 4°C 10 mg diaphorase (NADH dye oxidoreductase EC 1 6.4.3) in 5 mL 50 m*M* Tris-HCl, pH 8.0, and add 1% BSA or similar protein stabilizer. This is then dialyzed against amplifier diluent. The two amplifier enzymes are diluted 10-fold and mixed together immediately prior to use. The optimum ratio can be determined by mixing different ratios of the enzymes from 10:1 to 1:10, although 1:1 is generally satisfactory

3. Methods

3.1. Antigen-Coated Plate Antibody Assay

This assay is used to measure antibodies in animals that we immunize and in immune humans ***(10,18,37,38)*** It can readily be adapted to measure human antibodies of different classes and subclasses. As long as the antigen binds well to the plate this is a simple and reliable procedure.

1 Coat the plate with antigen at 100 µL/well (e.g , bee venom phospholipase A_2) overnight at 4°C or for 1 h at 37°C at a concentration of between 10 and 3µg/mL in bicarbonate coating buffer. Alternatively plates can be coated with antiphospholipase A_2 at 1 µg/mL overnight in bicarbonate coating buffer, washed as in **steps 2, 4**, and **6**, and 10 ng/mL phospholipase A_2 in assay diluent added next day and incubated for 1 h and the assay continued as in **steps 2–10**.
2. Wash three times with 300 µL of wash buffer with 30 s to 1 min between each wash.
3. Add dilutions of reference sample pool (e.g , 1/50–1/50,000), quality controls (e g., 1/100,1/1000,1/10,000), and sample at 1/500 in assay diluent at 100 µL/well Any samples outside the range of the standard curve are repeated at up to 1/50 dilution or as low as necessary. Incubate for 2 h at room temperature.
4. Wash three times with 300 µL of wash buffer with 30 s to 1 min between each wash.
5. Add 100 µL/well alkaline phosphatase-labeled rabbit antirat IgG at 1/300 in assay diluent and incubate for 1 h at 4°C.

6. Wash three times with 300 µL of wash buffer with 30 s to 1 min between each wash.
7. Make up PNP in diethanolamine buffer as described in **Subheading 2.1.3.** Add 100 µL/well and incubate at 37°C for 1–2 h.
8. Stop enzyme reaction by adding 50 µL 3 *M* NaOH/well for PNP or 50 µL 2*M* H_2SO_4/well for OPD or TMB.
9. Measure absorbance at 405 nm for PNP, 492 nm for OPD, and 450 nm for TMB.
10. Read results from the reference curve described above.

3.2. Two-Site ELISA for the Detection of IgE

This is probably the most reliable ELISA format *(7,39)*. Interassay CVs can be as low as 5%. Using the Fab' detection antibody it is one of the most sensitive assays ever described.

1. Coat the plate with 100 µL/well monoclonal anti-IgE at 3 µg/mL overnight at 4°C or for 1 h at 37°C in bicarbonate coating buffer.
2. Wash three times with 300 µL of wash buffer with 30 s to 1 min between each wash.
3. Add 100 µL/well IgE standards, quality controls, and 100 µL/well patient sera at 1/10 and 1/100 in assay diluent. Any samples outside the range of the standard curve are reassayed using dilutions as necessary. Incubate for 3 h or overnight.
4. Wash three times with 300 µL of wash buffer with 30 s to 1 min between each wash.
5. Add 100 µL/well AP or horseradish peroxidase (HRP)-anti-IgE or Fab' anti-human IgE at 1/300 or 1/3000 in assay diluent respectively and incubate for 3 h or overnight.
6. Wash three times with 300 µL of wash buffer with 30 s to 1 min between each wash
7 For alkaline phosphatase make up substrate PNP in diethanolamine buffer For peroxidase make up substrate OPD in citrate phosphate buffer or TMB in acetate buffer Add 100 µL/well and incubate at 37°C for 1–2 h for PNP or 15 min for OPD and TMB.
8. Stop enzyme reaction by adding 50 µL 3*M* NaOH/well for PNP or 50 µL 2 *M* H SO_4/well for OPD or TMB.
9. Measure absorbance at 405 nm for PNP, 492 nm for OPD, or 450 nm for TMB.
10. Read results from the standard curve and express as U/mL (1 U of IgE is approx 2.4 ng).

3.2.1. For Ultra-Sensitive Assay

Follow **steps 1–6** in **Subheading 3.2.** Then proceed as follows:

1. All buffers must be phosphate free.
2. Add 100 µL of NADP to the wells and incubate for 10 min (this can be increased or decreased depending on the sensitivity required).
3. Mix together equal volumes of the amplifier enzymes as detailed above and add 200 µL
4. Monitor the color produced and read the results at 492 nm kinetically or after stopping with 50 µL of 0.5*M* H_2SO_4.

5 Read results from the standard curve and express as U/mL (1 U of IgE is approx 2.4 ng).

3.3. Two-Site ELISA for the Detection of Human IFN-γ

These assays are becoming particularly prominent with the cloning of many new cytokines. It is possible to purchase pairs of antibodies that have been optimized. It is worth calibrating standards using those obtained from other kits so that comparisons are easier to make.

1. Coat the plate with 100 µL/well anti-IFN-γ monoclonal antibody at 1 µg/mL overnight at 4°C or for 1 h at 37°C in coating buffer.
2 Wash three times with 300 µL of wash buffer with 30 s to 1 min between each wash
3 Add 100 µL/well IFN-γ standards, quality controls, and samples in assay diluent. Any samples outside the range of the standard curve are reassayed at an appropriate dilution. Incubate for 1 h at room temperature.
4 Wash three times with 300 µL of wash buffer with 30 s to 1 min between each wash
5 Add 100 µL/well biotin-labeled anti-IFN-γ at 1 µg/mL in assay diluent for 1 h at room temperature
6. Wash three times with 300 µL of wash buffer with 30 s to 1 min between each wash
7 Add 100 µL/well streptavidin-labeled alkaline phosphatase at 1/500 in assay diluent for 1 h at room temperature
8 Make up substrate PNP in diethanolamine buffer, pH 9.8, as described in **Subheading 2.13.** Add 100 µL to each well and incubate at 37°C for 1–2 h
9 Stop enzyme reaction by adding 50 µL 3 *M* NaOH/well
10. Measure absorbance at 405 nm.
11 Record the results as U, ng, µg, or mg/mL from the standard curve

4. Notes

1. Method 1. Antigen-coated plate antibody assay Regularly retitrate the coating antigen (*see* **Subheadings 1.2.** and **3.1.** and **Fig. 1**)
2 Method 2· Two-site ELISA for the detection of IgE This is one of the most robust ELISAs with an interassay CV of approx 5% (*see* **Subheadings 1.2.** and **3.2.** and **Fig. 4**)
3 Method 3: Two-site ELISA for the detection of human IFN-γ. It is worth calibrating your standard and assay against a commercial kit to ensure you are in the right range (*see* **Subheading 1.2.** and **3.3.** and **Fig. 4**)

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21

Dynamic Analysis of Viral Populations by Direct DNA Sequencing and Solid-Phase Technology

Joakim Lundeberg, Jacob Odeberg, and Mathias Uhlén

1. Introduction

The improvements in DNA-sequencing technology in recent years has opened new possibilities, from large scale genome projects to routine diagnostic applications. Among the most important developments towards automated systems has been the introduction of the polymerase chain reaction (PCR) and the replacement of isotopic labels by fluorescent dyes and on-line monitoring of the DNA sequence. The use of PCR for amplification of genetic material allows for direct DNA sequencing, which avoids both time-consuming cloning steps as well as control sequencing necessary to exclude "mutations" introduced by the *Taq* DNA polymerase *(1,2)*. Thus the DNA sequence of a sample can be determined rapidly and will represent the sequence of the sample prior to amplification, as the *Taq* polymerase errors will not significantly contribute to the resulting signal. Different methods can be employed to obtain labeled dideoxy DNA fragments according to the Sanger methodology *(3)*, such as, using fluorescent-labeled primers, dideoxy chain terminators, or fluorescent-labeled dATPs. These possibilities enables many different flexible non-radioactive sequencing alternatives. In principle all commercial instruments are based on the excitation of sequencing bands by a laser beam which then can be detected in the gel during electrophoresis. An important ability with the commercially available instruments is that these enable reliable quantitation of polymorphic and heterozygous positions using different software tools *(4,5)*.

The introduction of solid-phase technology (i.e., magnetic beads) in DNA sequencing has further improved robustness by the increased reproducibility with accompanying higher yields. Automation has also been facilitated since reaction buffers and additional reagents can be rapidly changed without cen-

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trifugation or precipitation steps. The manual protocols for magnetic separation of DNA described below have been used as the basis for developing semiautomated DNA-sequencing systems. The template preparation and the sequencing reactions have been implemented on a Beckman Biomek 1000 workstation (Beckman, Fullerton, CA, *6*) and on an ABI Catalyst workstation (Applied Biosystems, Foster City, CA; *7*).

Solid-phase DNA sequencing, outlined in **Fig. 1**, employs superparamagnetic beads coated with streptavidin that are used to immobilize and purify biotinylated PCR products *(6,8)*. This robust approach for DNA sequencing avoids many of the common problems in the preparation of single-stranded sequencing templates. The principle is based on the use of the biotin and streptavidin interaction, which is extremely strong ($K_d = 10^{-15} M$) and temperature stable (up to 80°C), and is tolerant to alkali treatment (0.1 *M* NaOH). The most preferred method of introducing a biotin label into a double-stranded DNA fragment is to have one of the PCR primers biotinylated. Biotinylation of primers has been significantly simplified by the introduction of biotin phosphoramidites that enables a direct coupling onto the 5'-end during primer synthesis. Immobilization of the biotinylated PCR products onto the streptavidin-coated magnetic beads is achieved by a short incubation (a few minutes). The immobilized DNA is then denatured into two separate strands by the addition of 0.1 *M* NaOH. This results in the elution of the nonbiotinylated strand into the supernatant, while the biotinylated strand remains immobilized to the bead surface. The single-strand template in the supernatant can be recovered by use of a magnet and subsequent removal of the eluate into a separate tube for neutralization. The advantage of the method is that all reaction components are removed, including the complementary strand, enabling optimal sequencing conditions with no reannealing problems. In most of the clinical applications, the T7 DNA polymerase (Sequenase) is the enzyme of choice because of a high process-ivity, which results in more uniform bands compared to the other polymerases. However, the newly introduced enzymes Thermosequenase and *Taq* FS seem to have similarly even peak heights. Peak uniformity is important for accurate base-calling, especially for the detection of heterozygosity and polymorphism *(2,4,9–12)*.

The versatility of solid-phase direct sequencing for analysis of virus populations has been documented for both HIV-1 *(4,10,11)* and hepatitis C *(2,13,14)*. A high degree of sequence heterogeneity is frequently found in samples containing these viruses owing to the error-prone nature of the RNA-directed RNA polymerase of the virus. It is therefore more accurate to describe a virus isolate as a population of closely related viral variants, in which each genome can be unique. Thus, to study an infected patient, a population-based approach, such as direct sequencing, which actually simplifies the handling of a sample

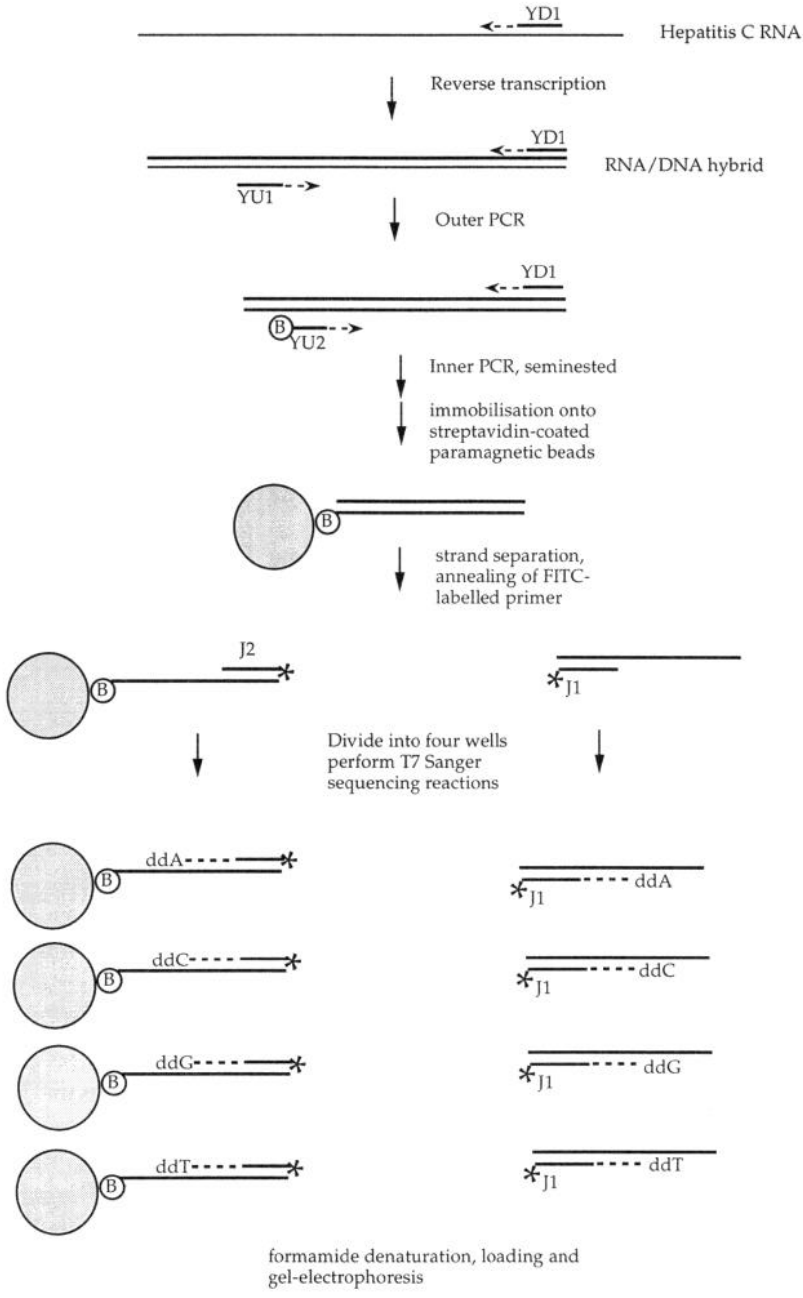

Fig. 1. The solid-phase sequencing concept for analysis of hepatitis C virus.

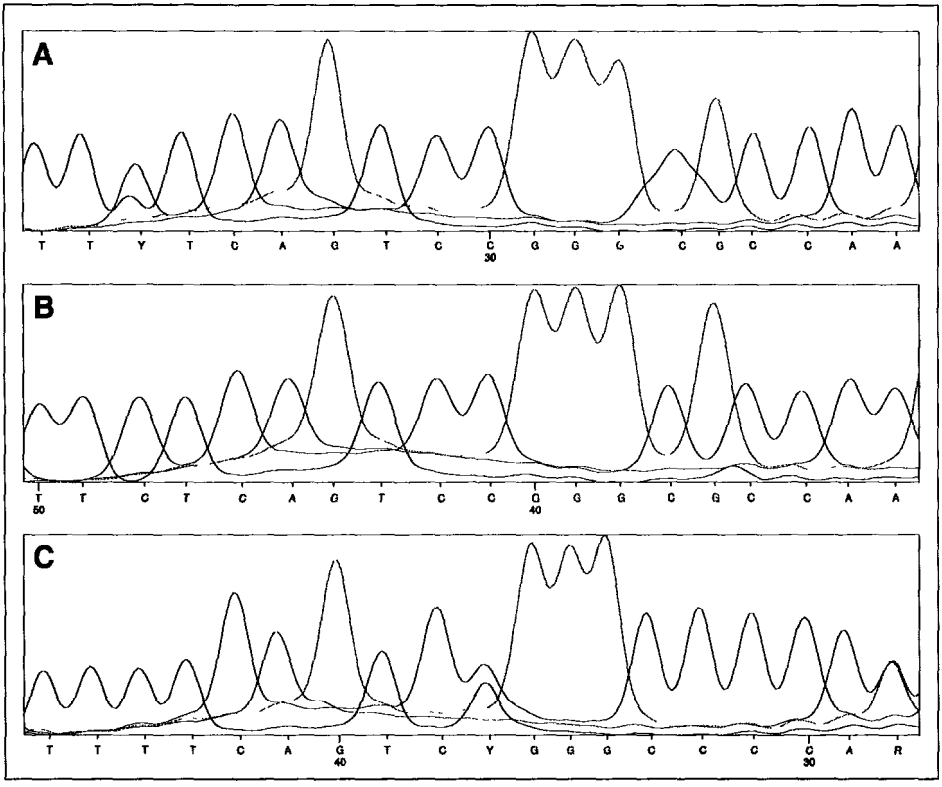

Fig. 2. An example of raw data for part of the HVR-1 from one patient at three different time points during interferon-α-therapy.

as compared to the frequently used method of sequencing a number of individual reverse transcriptase PCR clones is required. Below is the detailed procedure to analyze the hypervariable region 1 (HVR-1) of hepatitis C virus (HCV), which can easily be adapted to other viral targets. HCV has a single positive-strand RNA genome of 9400 nt, which includes a 27-amino acid long HVR-1 region encoding a putative envelope protein with low functional and structural constraints. The region shows a high degree of variation and heterogeneity and appears to contain major neutralizing epitopes. A representative analysis of this region is shown in **Fig. 2**, and presents the dynamic changes of the HVR-1 region in a patient prior to **(A)** and after cessation of 15 mo IFN-α treatment **(B)**, and after 6 mo follow-up **(C)**. The usefulness of the technique as a tool for analysis of heterogenous virus populations is clearly shown by the possibility

to study gradual nucleotide shifts in the major sequence over time in relation to humoral response and antiviral therapy.

2. Materials

1. Denaturation solution: 4 *M* guanidium thiocyanate, 25 m*M* sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 *M* mercaptoethanol.
2. *E. coli* tRNA (Boehringer Mannheim, Mannheim, Germany).
3. RNase inhibitor (Boehringer Mannheim).
4. MMLV reverse transcriptase (Gibco-BRL, Life Technologies, Paisley, UK).
5. 10X PCR Buffer: 100 m*M* Tris-HCl, pH 8.3 (20°C), 20 m*M* $MgCl_2$, 500 m*M* KCl, 1% Tween-20.
6. Thermocycler
7. Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT).
8. Nucleotide solution (2 m*M* of each dNTP).
9. PCR primer solution containing two primers, each having a concentration of 2.5 μ*M*. Outer primer set: YU1 (1350-1369) 5'-CT(G/A) CTC CGG ATC CCA CAA GC-3'; YD1 (1614-1633) 5'-TCA TTG CAG TTC AGG GCC GT-3', Inner primer set: YU2 (1426-1445) 5'-biotin-(T/G)(C/T)T CCA TGG TGG GGA ACT GG and YD1 Annealing sites according to sequence for hcv-1 (Genbank accession number M62321)
10. Dynabeads M-280 Streptavidin (10 mg/mL) (Dynal AS, Oslo, Norway).
11. Neodymium-iron-boron magnet (MPC Dynal AS)
12. Washing/binding solution: 10 m*M* Tris-HCl, pH 7.5 (20°C), 1 m*M* ethylenediaminetetraacetic acid (EDTA), 2 *M* NaCl supplemented with Tween-20 (0.1% final conc), and β-mercaptoethanol (1 m*M*, final conc).
13. 1X TE buffer: 10 m*M* Tris-HCl, pH 7.5 (20°C), 1 m*M* EDTA.
14. 0.10*M* NaOH. Use a 1.000 ± 0.005 *M* volumetric solution of NaOH for accurate dilution. Aliquot and store at –20°C.
15. 0.333 M HCl. Use a 1.000 ± 0.005 *M* volumetric solution of HCl for accurate dilution. Aliquot and store at –20°C.
16. A 6% polyacrylamide sequencing gel and electrophoresis unit or alternatively automated sequencers such as the Pharmacia ALF (Uppsala, Sweden) or ABI Automated 373/377 Sequencers (Foster City, CA).
17. Sequencing primers specific for the HVR-1 region. J1 (1436–1454) 5'-FITC-GGG GAA CTG GGC IAA GGT C-3'and J2 (1604–1624) 5'-FITC-TTC AGI GCI GTI CI(A/G)TTG ATG-3'. FITC denotes fluorescein and I denotes inosine.
18. Annealing buffer for single-labeled sequencing primer: 280 m*M* Tris-HCl, pH 7.5 (20°C), 100 m*M* $MgCl_2$.
19. Extension buffer: 300 m*M* citric acid, pH 7.0 (20°C), 318 m*M* dithiothreitol (DTT), 40 m*M* $MnCl_2$.
20 T7 DNA polymerase with enzyme dilution buffer (Pharmacia, Uppsala, Sweden)
21 Four nucleotide mixes each containing 40 m*M* Tris-HCL, pH 7.5 (20°C), 50 m*M* NaCl, 1.0 μ*M* of each dNTP and 5 0 μ*M* of one specific ddNTP

22 Stop solution: Shake 100 mL formamide with 5g Amberlite MB-1 resin and 300 mg dextran blue for 30 min. Filter through 0.45 μm pore-size filter.

3. Methods

3.1. Preparation of Hepatitis C Virus RNA

Blood-sera were collected from anti-HCV-positive patients, and serum samples were stored at –20°C. RNA extraction from serum was performed as described elsewhere, using a modified acid guanidium thiocyanate-phenol-chloroform method *(15,16)*.

1 Mix 50 μL serum with 500 μL denaturation solution and 10 μg *E coli*-derived tRNA.
2 Add 100 μL 2 *M* sodium acetate, pH 4 0, and 600 μL phenol-chloroform (5.1) saturated with TE buffer (pH 8.0)
3 Vortex and centrifuge at 12,000*g* for 20 min at 4°C
4 Precipitate the aqueous phase (containing the RNA) with isopropanol for 1 h at –20°C
5. Centrifuge at 12,000*g* for 20 min at 4°C.
6 Repeat the precipitation **steps 4** and **5**.
7. Wash the pellet with 75% ethanol.
8. Dissolve the RNA pellet in 22 μL ice-cold diethylpyrocarbonate-treated H_2O containing RNase inhibitor.
9. Freeze the extracted RNA immediately and keep at –70°C until use

3.2. Reverse Transcription and PCR

Reverse transcription and amplification of a target gene can be performed using designed primer. However, obviously, the choice of primers will influence the conditions used in the RT-PCR. For analysis of HVR-1 in hepatitis C, reverse transcription and the outer PCR were performed in a single tube with 50 pmol of each primer, YU1 and YD1 (**Fig. 1**). The outer PCR product was diluted 1000-fold. Inner PCR was carried out using 5 pmol of each primer (YU2 and YD1).

1 Prepare an RT-outer-PCR master mix in a microcentrifuge tube: 10 μL 10X PCR buffer; 10 μL (50 pmol) primers, 10 μL dNTP solution; 5 U of MMLV, 1 U Ampli*Taq* and sterile water to 100 μL. Aliquot into PCR tubes.
2 Add template RNA
3 Cover with light mineral oil, if necessary. RT-outer PCR program: 40°C, 30 min, followed by 35 cycles comprising of 95°C 1 min, 45°C 2 min, 72°C 3.0 min.
4 Prepare an inner PCR master mix in a microcentrifuge tube: 10 μL 10X PCR buffer; 10 μL (5 pmol) primers; 10 μL dNTP solution; 1 U Ampli*Taq*, and sterile water to 100 μL Aliquot into PCR tubes.
5. Add template (outer PCR product).
6 Cover with light mineral oil, if necessary. Inner PCR cycle: 95°C, 30 s; 58°C, 30 s; 72°C, 45 s for 30 cycles. A final extension step at 72°C for 10 min is strongly suggested

7. Analyze the PCR product on an agarose gel. Amplification of HVR-1 results in a 208-bp fragment.

3.3. Preparation of the Solid Support

The streptavidin-coated paramagnetic beads need to be washed prior to immobilization of PCR products. A neodymium-iron-boron permanent magnet was used to sediment the beads.

1 Resuspend the beads by pipeting. Use 20–30 µL (200–300 µg) of resuspended beads per PCR template; pipet the suspended beads into a clean 1.5-mL microcentrifuge tube. The beads may be washed in bulk for the total number of PCR templates which need to be purified.
2. Place the tube in the magnetic holder and allow for beads to adhere to the magnet at the side of the wall Remove the supernatant using a pipet (do not remove the tube from magnetic holder).
3. Add an equal vol of washing/binding solution and gently pipet to suspend
4. Repeat, using the magnetic holder, allow the beads to adhere to the side of the tube, and remove the supernatant.
5. Resuspend the beads in washing/binding solution using twice the original vol. The bead concentration is now 5 µg/µL.

3.4. Immobilization of the PCR Product and Strand Separation

The biotinylated PCR products are directly immobilized to prepared streptavidin-coated paramagnetic beads. Denaturation of the immobilized double-stranded DNA on the beads, followed by elution of the nonbiotinylated strand, yields two single-stranded DNA templates. One is immobilized on the beads and the other is in the supernatant.

1. Take the inner PCR amplification reaction to a fresh 1.5-mL microcentrifuge tube and add 40–60 µL of the pre-washed Dynabeads.
2. Incubate at room temperature for 15 min. Mix during the immobilization reaction once or twice by gentle pipeting or tapping.
3. Collect the beads, by moving the vials to the magnetic holder, and remove the supernatant with a pipet.
4. Wash the beads once with 50 µL washing/binding solution.
5. Wash once with 50 µL 1X TE buffer. Remove the 1X TE buffer carefully, avoiding droplets on the walls and the bottom of the tube.
6. Resuspend the beads in exactly 10 µL 0.10 *M* NaOH.
7 Incubate at room temperature for 5 min.
8. Collect the beads (now with only single-stranded DNA attached) by placing the tube in the magnetic holder and transfer the 10 µL of NaOH supernatant (containing the non-biotinylated strand) to a clean tube. Neutralize the NaOH supernatant with 3 µL 0.333 *M* HCl and mix immediately.

9 Wash the beads once with 50 µL 0 1 *M* NaOH, once with 50 µL washing/binding solution, and once with 50 µL 1X TE buffer. Remove the 1X TE carefully, without leaving any droplets.
10. Resuspend the beads in 16 mL of sterile water (or the appropriate buffer for the sequencing protocol to follow:

3.5. Solid-Phase DNA Sequencing

The sequencing protocol described below is suitable both for manual and automated sequencing using the automated laser fluorescent sequencing apparatus (ALF) (Pharmacia Biotechnology) system. In principle the sequencing primers to be used can be either custom-designed as for HVC (complementary to a sequence inside the target DNA being amplified) or a universal primer complementary to a sequence introduced by a "handle" sequence in one of the PCR primers or one of the primers used in the PCR. The Sanger sequencing reactions can also be carried out using an automated Biomek-1000 robotic work station (Beckman Instruments, Fullerton, CA), following the protocol described by Hultman and co-workers *(6)*. An example of the results of sequencing the HVR-1 of HCV using 5'-end fluorescent-labeled sequencing primer is presented in **Fig. 2**. Processed sequences from the ALF sequencing apparatus are manually edited at the polymorphic sites caused by the heterogeneous virus populations, and can be subjectively evaluated to determine the major consensus sequence and the minor variations at polymorphic sites as earlier described by Leitner and coworkers *(4)*.

1. Add beads with immobilized single-stranded DNA or eluted single-stranded DNA to a fresh tube. Adjust the volume (with sterile water) to 16 µL
2. Add 2 µL (1 pmol) labeled primer; sequencing primers for the HVR-1 region of hepatitis C J1 (immobilized strand) and J2 (eluted strand)
3. Add 2 µL of annealing buffer and mix gently with a pipet. Incubate at 65°C for 10 min. Mix gently and leave to cool at room temperature for at least 10 min, mixing two or three times during cooling.
4. Add 1 µL of extension buffer and mix gently
5. Dilute the T7 DNA polymerase to 1.5 U/µL using cold dilution buffer, 2 µL of this diluted stock solution will be required for each template. Keep the tube with diluted stock solution (1.5U/µL) on ice.
6. Label four new tubes "A", "C", "G," and "T." Dispense 2.5 µL of the corresponding dNTP/ddNTP sequencing mixes into the tubes.
7. Warm the dispensed nucleotide sequencing mixes at 37°C for at least 1 min
8 Add 2 µL of the T7 polymerase diluted stock solution (from **step 5**) to the template mixture (from **step 4**) and mix gently Immediately add 4.5 µL of this mixture to each of the preincubated nucleotide sequencing mixes.
9. Incubate at 37°C for 5 min
10. Add 5 µL of stop solution to each reaction and mix gently

11. Incubate at 95°C for 5 min and then put the tubes on ice
12. Load the samples onto 6% polyacrylamide gel (ReadyMix Gel, Pharmacia Biotechnology) sequencing gel.

4. Notes

1 The biotinylated oligonucleotide needs to be purified from unbound biotin, preferably by reverse-phase FPLC or HPLC, since free biotin will occupy binding sites on the beads and reduce the binding capacity of biotinylated PCR products. Other alternatives to increase binding capacity include: addition of more beads, higher immobilization temperature (up to 50°C), exchange of 2 *M* NaCl to 6 *M* LiCl in the washing/binding buffer

2 The introduction of α-thio nucleotides *(17)* or/and 7-deaza-dGTP (c7dGTP) in the sequencing reactions may resolve band compressions.

3 The sequencing reactions may be stored at –20°C if not loaded immediately Prior to loading, heat the samples to 95°C for 2 min and place on ice.

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22

Robotic Analyzers

Dale R. Pfost and Richard G. Keightley

1. Introduction

In general, three assay types are commonly used in diagnostic virology. Since these are described in other chapters it suffices here just to reiterate the methodology names:

1. Agglutination (complement-fixation tests);
2. Immunological (enzyme-linked immunosorbent assay [ELISA]); and
3. Molecular biological (polymerase chain reaction [PCR] and DNA probes)

Although most virology laboratories undoubtedly will be performing these assays manually, an increasing number are moving to automation. Indeed, since the late 1980s, there has been a number of papers published that describe automated procedures *(1–3)*. This chapter discusses the advantages of automation and describes how this can be achieved using robotic sample-processing systems.

2. The Need for Automation

2.1. Use of 96-Well Plates—Manual Processing

The 96-well plate format has been adopted for a wide variety of diagnostic assays, including those in virology. Although this is a convenient format in which to work manually, the often complicated sample/reagent layout (*see* **Fig. 1**) and the intensity of pipeting steps can lead to a variety of problems caused by normal human error and operator fatigue or boredom. Possible results are summarized below:

1. Misplacement of samples and/or reagents in the test plate;
2 Incorrect liquid volumes pipeted or dispensed; and
3 Accuracy and/or reproducibility errors in pipeting.

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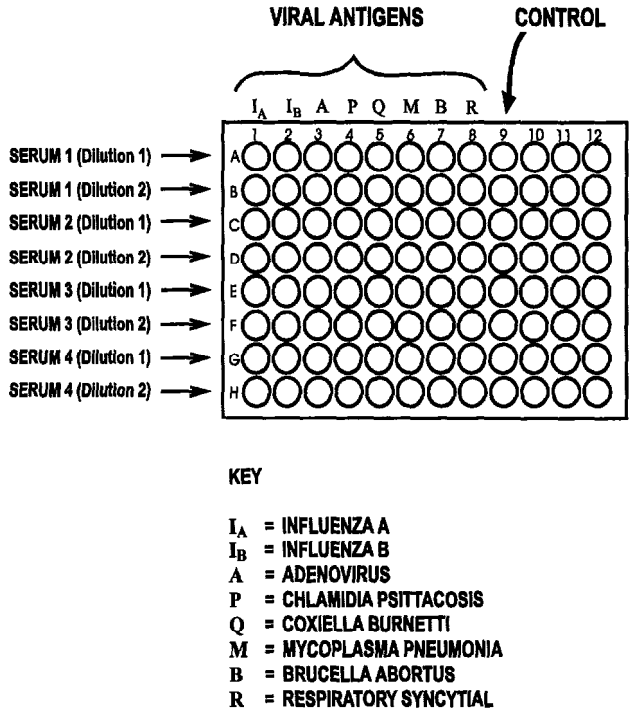

Fig 1 Example of a plate layout for a complement-fixation test

The consequences of such errors could be serious; the emergence of a false-positive or false-negative diagnostic result could have a dire consequence for the patient. Although manual laboratory procedures can be tightened to help reduce errors, the elimination of human error is impossible. It is primarily for these reasons that the virologist will want to consider automating diagnostic assays.

2.2. Improved Performance With Automation

The small size and regular geometry of the 96-well plate makes it a good candidate for automated processing. Robotic automation of ELISA and DNA-based diagnostics has been reviewed frequently in the literature *(4,5)*, and improvements in results from elimination of manual errors have been reported *(6)*. In addition, particularly for DNA-based methods involving PCR, the use of a robotic processing system helps to avoid sample contamination. Whereas ELISA usually requires the use of flat-bottom well plates for photometric measurements, agglutination and DNA-based methods use U-bottom or V-bottom plates. The ELISA methods also have a need for plate- (or rather well-) washing; results have to be read photometrically on a plate-reader.

Although a robotic analysis system should be capable of handling all types of plates (and a variety of other labware too), some systems have optionally integrated plate washing and reading. For ELISA, this is a definite advantage since it provides the possibility of longer periods of unattended operation, and thereby greater automation. However, it should be realized that if the test throughput is particularly high, it may be impossible to handle the whole workload on a single robotic workstation. Therefore, it may be necessary to use an auxiliary robotic arm to transfer plates, sample tubes, etc., to/from labware "hotels" or to use more than one sample-processing station. The latter option is particularly appropriate for ELISA methods in which it may be best to perform the individual stages of the assay on separate, dedicated stations. Although in this case it is necessary to transfer plates between processing stations manually (required for incubation steps anyway), very high levels of throughput (depending on the number of processors) can be achieved as a result.

2.3. Sample and Operator Safety

Performing assays manually can present a threat to sample integrity (as mentioned above) and to the safety of the virologist if samples contain pathogens. In both cases this can be reduced significantly by employing automation. In addition, a suitably sized robotic processor can be situated in a containment cabinet. In this case it is beneficial to use a robotic system that provides a maximum of automation capability (or "walk-away" time) so that the operator's interaction with the processing system is reduced to a minimum. For example, Gulakowski et al. *(7)* used a robotic sample processor to handle all pipeting, dilution, and dispensing in their HIV drug-screening assays, including the transfer of cells and virus solutions. They found that such automated systems are "ideal for work with HIV-infected cells to minimize direct technician involvement, to decrease the possibility of human error and, more importantly, to decrease possible exposure of staff to the lethal pathogen."

Another important consideration is disinfection of the robotic system in order to avoid subsequent sample cross-contamination and danger to the operator. Sample processors can usually be decontaminated *in situ* by wiping the instrument down carefully with a suitable sterilizing solution. The operator should consult the operating manual or the manufacturer for advice on the best material to use. In extreme cases it may be possible to use formaldehyde, but the manufacturer should be consulted before this hazardous chemical is used.

2.4. Time Saving

In addition to the advantages outlined above, automation also relieves the virologist from the tedium of repetitive liquid handling. Therefore, precious time is saved that can be used for more productive or interesting work. This

improves productivity in the virology laboratory. The amount of time saved depends on the complexity of the diagnostic method, the number of tests being performed, and the level of automation used. If the time saving is costed (based on the cost of the operator's time), it is possible to calculate a "payback time" for the robotic analyzer. This is the length of time (since installation) required to make sufficient savings to pay for the cost of the robotic system. The payback time is often about 3 yr for robotic analyzers, but this depends on the usage of the system. Clearly, from the payback point of view, once the robotic system has been installed it should be used to its full capabilities.

3. Types of Robotic Systems

3.1. Robotic System Architecture

In considering the various means of assay automation available, it is useful to review the elements of system architecture. Besides the obvious assay specific figures-of-merit or performance specifications such as through put and levels of cross contamination, the architecture of a given system tends to cast the role of the device in the environment of the laboratory not only at the time of purchase but also as time changes the tasks needed. The subsystems and subissues related to system architecture are listed in **Table 1**.

Perhaps the most important of these are those listed under system philosophies. Specifically, the degree that a system is needed to be flexible and reconfigurable is frequently at odds with the need for the system to be dedicated and streamlined for constant high levels of throughput. Ideally, one would have both. Historically, suppliers of laboratory apparatus have striven to achieve a balance.

The two extremes can be pictured as, first, a class of robotic devices known as anthropomorphic, and second, dedicated but rather more inflexible systems. Anthropomorphic robotic systems are of a similar design, but on a smaller scale, as those robots used for the assembly of automobiles and other industrial applications.

Several such anthropomorphic systems exist for laboratory automation—some with cylindrical coordinates and others with arm, elbow, and wrist layouts. They are very flexible, able to perform a large and varied range of tasks, such as handling powders and centrifugation, but able to be reconfigured (in many cases with considerable programming and customized hardware) to perform completely different tasks. By virtue of their means of accessing space (multijointed arms), the volume taken up by these systems can be very large—mitigating against their use on normal-sized lab benches or in normal-sized containment cabinets. Some laboratories construct specialized rooms for such systems, a luxury many cannot afford. Secondly, one sees many systems

Table 1
Robotic System Architecture

User interface
User control and programming
Report creation
Archiving/databasing
Internal calculations, closed loop interactive processing and sample tracking
Heuristic algorithm
Monitoring kinetics
Bar-code reading
End effectors
Processing
Analytical
Input-output flow/tasking
Discrete vs flow-through (bulk) and effectors
Pipeting—single/multiple; tip changing/fixed, splaying/fixed; level sensing, ganged or individually addressable; septa/open
Motion Control
Coordinate systems: anthropomorphic vs Cartesian
Internal feedback/closed loop vs open loop position
External sample/item related closed loop (tactility)
Sensors / imagers
Safety
Motion related
Sharps
Containment (aerosols, etc.)
Waste systems
System philosophies
Integrated vs modular and dedicated vs flexible
Programming intensive vs turn-key oriented

intended for use in the clinical diagnostic setting, where efficiencies are achieved by specialized design elements, consumables, and specific limited ranges of motion and fluidics paths. These systems tend to be upgradable only through software and new reagent cassettes but not reconfigurable to perform fundamentally new assays.

Another important parameter is the degree of integration of an assay achieved by a given system—the number of steps in the total protocol from sample preparation to report generation that are really automated by the sys-

tem. The extremes are, on the one hand, a totally manual assay or perhaps a set of single-task processors/analyzers linked by human intervention, and on the other, a system capable of the full integration of all aspects of the procedure. The concern with fully integrated systems is that owing to conflicting process times they need to be designed in order not to be held up or slowed down if, for example, an incubation time is complete, and the sample is ready for optical reading, in the middle of a long liquid-handling process being performed on another set of samples. Which activity takes priority? What will be the impact on the subsequent assays? This is a well-known problem in robotics and the traditional multi-tasking automation arena.

As more capability is added to a single stage of a unit this can actually decrease throughput The partitioning of tasks in an integrated system should be reviewed carefully to avoid this form of throughput constipation. Assay design can lead to the favorable situation of incubation times equaling liquid-handling and reading times in which case a whole series or batches of samples can proceed in synchronized march step through the system, unimpeded by "gridlock "

There are some systems that are both flexible and integrated. This is best illustrated in **Fig. 2** in which the axes are "degree of flexibility" and "degree of integration"—the upper right quadrant being desirable for users who wish the speed and performance of tailored systems and who do not wish their systems to be obsolete from the inevitable technological breakthroughs and emerging virology assay designs. Examples of required upgradability include well-established ELISA assays being converted to fluorescence immunoassay (FIA) or even DNA-probe-based assays. Some of the systems are capable of keeping pace with technological change, remaining streamlined, and maintaining high throughput. A typical example of such a system is shown in **Fig. 3**. If the problems highlighted above can be avoided, by such architecture partitioning as keeping the optical reader as a separate device or station, then these flexible and integrated devices probably point the way for the future in many laboratory environments.

3.2. Optional Capability

Table 1 covers many subjects that are more tangible or concrete than system philosophies; one in particular needs, to be reviewed in depth, namely, end effectors. This subject covers many of the optional capabilities available on laboratory systems. The name "end effectors" is adopted from the parlance of industrial robotics, but applies equally well to laboratory automation. Perhaps the most important area is in the liquid-handling end effectors. Again, two major classes can exist on a continuum, with tube-and-valve bulk dispensers (or washers) at one extreme and discrete pipeting systems with tip-changing capabilities at the other. Space does not

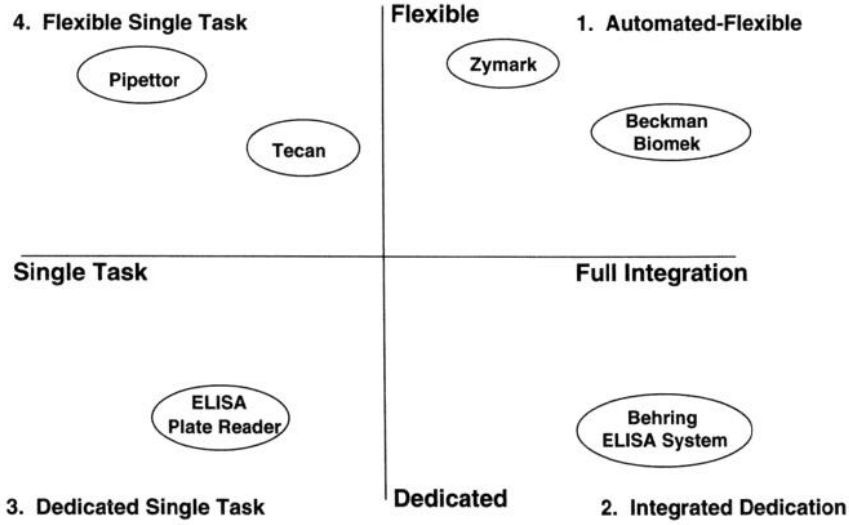

Fig. 2. Integration/flexibility space map—the upper right quadrant is desirable for users who wish the speed and performance of tailored systems yet wish to modify protocols easily.

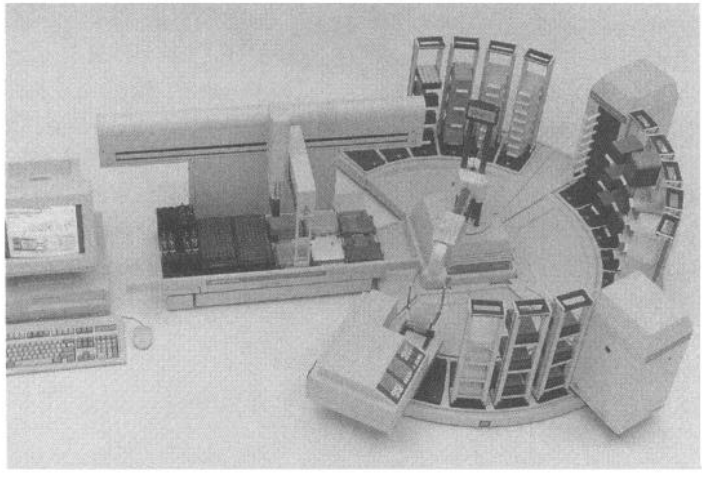

Fig. 3. Beckman Biomek 2000 Robotic Workstation with automatic labware transfer system.

permit a comparative analysis but it has been our experience that tip changing is critical in virology assays, especially with the advent of PCR and the resulting need for minimal cross-contamination. Such issues as accu-

racy and precision, aerosol control, liquid level sensing, and bar-code sample tracking are all important.

Other capabilities not covered in the preceeding paragraph include user interface, data management, incubation and analytical capabilities. It is our experience that a good way of evaluating a particular product is to assess the amount of time it takes from the moment of opening the crate to obtaining useful results. The user interface can render some systems as only programmable by individuals familiar with formal programming languages. Assays, no matter how stable or well established, inevitably require some form of optimization from lab to lab, and thus the system's user-friendliness and flexible programming are important. Systems adopting a graphical user interface that provides a bird's-eye view of the labware can assist in a smooth introduction to the laboratory. If analytical capabilities are incorporated directly into the architecture of a sample processor then there is the added benefit of having little to go wrong in transposing sample results or samples being mislabeled. The direct link possible in such systems also provides better information records appropriate for good laboratory practices (GLP) and ISO 90001 and 9002. Labware management, tracking, and disposal are obviously additional important system considerations.

We have conducted some informal experiments with laboratory technicians in timed pipeting tasks. The subjects were asked in one trial to pick from six sample wells designated on paper by letter and number (row and column). In another trial, a similar test was conducted with the addition of a time constraint. The rate of incorrectly picked wells ranged from about 1.5 to 3.5%. During another series of experiments the simple act of pipeting was found to render actual liquid delivery precision well outside of specifications of the pipetor manufacturer. Investigations found many hard-to-avoid operator errors contributed to the pipeting problems. All of these are improved upon dramatically with most of the automation systems available.

4. Automating the Assay

4.1. Method Planning

The approach to performing a diagnostic assay on an automated system is different from that used for a manual test procedure. This is because the robotic system is an electromechanical device controlled by a computer and every movement (or operation) must be programmed. Although good commercial robotic analyzers do not require an operator to have knowledge of computer programming as such, the nature of any automated system requires the entry of control parameters before operation can proceed. This necessitates careful planning to ensure efficient use of space and time and to avoid errors. For this purpose it is very

useful to have a worksheet on which the stages of the assay can be written with the pipeting (or other) requirements, recorded. An example of such a worksheet is shown in **Fig. 4**. Then the individual liquid handling steps of the assay can be translated into program functions, in the system control software. Each function can represent a stage of the assay, and recording details of the functions as programming proceeds keeps track of progress, helps to avoid mistakes, and provides a permanent record of the program structure for future reference (*see* **Fig. 5**).

4.2. Programming

Earlier robotic analysers relied on DOS-based software which, although quite adequate for controlling the system, had a number of limitations, for example, interfacing with standard data-processing software. Today, most robotic systems use control software operating in Windows™, which provides smooth and convenient links to other software for data transfer, etc. An example of a program screen for such Windows-based software is shown in **Fig. 6**. Another major advantage afforded by Windows is operation of the software using a mouse This provides easy and rapid selection and change of parameters and program screens.

Under certain circumstances it is useful if the software has a program-looping capability so that particular operations can be repeated, e.g., addition of a reagent at timed intervals. Although such functions can be achieved without looping, this capability provides easier and quicker programming. In addition, where pipeting can be linked to result measurement, the two (or more) operations can be linked to create integral decision making, leading to a higher level of automation and greater time saving for the operator.

4.3. Method Testing

In order to ensure that the program performs the assay correctly it is necessary to test it. This is very important since, on the computer screen, the programmed method may seem correct but some parameters inevitably will need fine-tuning in order to produce correct operation of the robotic system and to optimize pipeting accuracy and precision. This testing procedure saves time in the long run and, possibly more importantly, valuable samples and reagents. The test is best performed by running a dummy assay on the system with water (or dye-colored solutions) being substituted for the samples and reagents. If serious mistakes in the program have been made the results will be strikingly obvious! However, more usually, it is a case of fine-tuning the pipetting to achieve the best possible assay results. Any necessary improvements will require editing of the program. In order to locate the exact points in the program where editing is required, it is very useful to have produced a log of the analyser's operation with program steps and timing indicated. Usually, this can be achieved by

BECKMAN

BIOMEK™ 1000
ASSAY PROGRAMMING WORKSHEET

We provide this worksheet to help you organize important data needed in designing your assay for the Biomek™ 1000. Use the Table below to determine the steps needed to complete the assay, the fluids to be used, and the volumes for each. Then fill out your tool and labware requirements. By completing the worksheet, you'll have a convenient outline to refer to while programming Biomek to perform your assay.

Assay Step	Reagent	No. Pipettings x Volume	Volume of Reagent/Step	Total Volume
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

SELECTING TOOLS

Fill in the blanks or place a check by the appropriate entries to determine which tools are needed. Biomek can hold up to four tools at any given time.

Range of volume to be pipetted: _____

Single-tip transfers?
☐ P20 (2-20 μL)
☐ P100 (10-100 μL)
☐ P200/P200L (20-200 μL)
☐ P1000 (100-1000 μL)

Multi-tip (8 tips) transfers?
☐ MP20 (2-20 μL per tip)
☐ MP200 (20-200 μL per tip)

Pipette tips needed?
☐ P250
☐ P1000

Bulk-dispense transfers?
☐ Single channel
☐ Multi channel (8 channels)

Measurement functions?
☐ OD tool

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Fig. 4. Example of an assay programming worksheet.

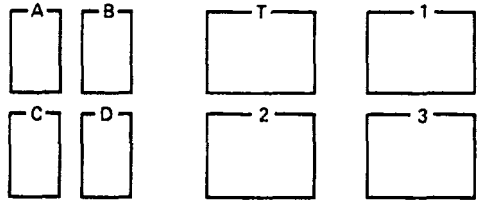
simply selecting a log option in the software. In a real automated assay this log of operations is also useful confirmation of correct operation and in some laboratories e.g., where GLP is in force, it may be mandatory.

BECKMAN

BIOMEK™ **1000** SUBROUTINE WORKSHEET

SUBROUTINE NAME _____ PURPOSE _____

CONFIGURATION CHANGE



	Function	Description
1.		
2.		
3.		
4		
5.		
6		
7.		
8.		
9		
10.		

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Fig. 5. Example of an assay programming record sheet.

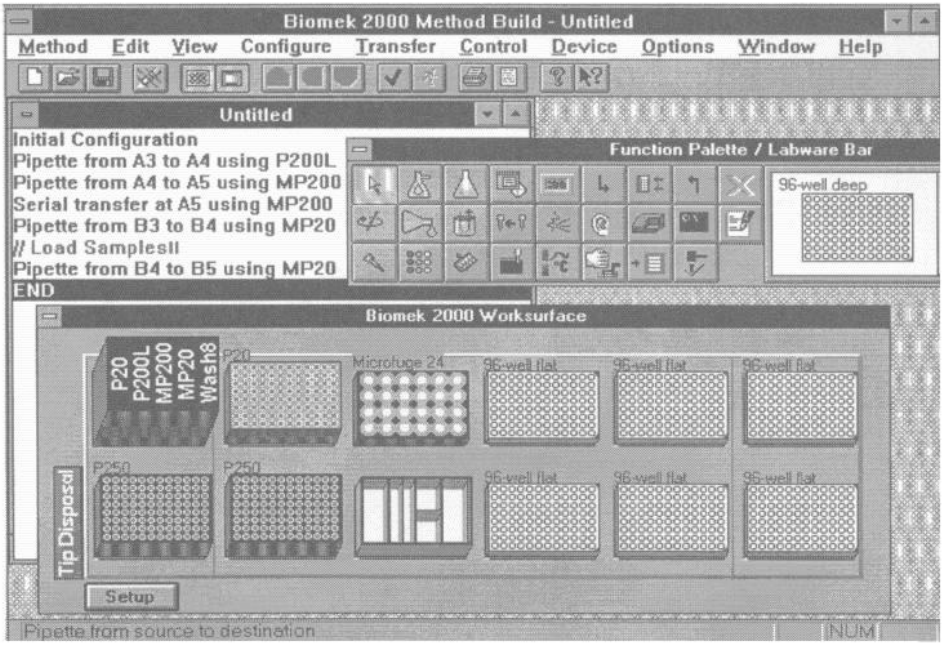

Fig. 6. Example of a programming screen for Windows-based software

5. Typical Results

Robotic analyzers have been used very successfully to automate a variety of diagnostic virology assays. Results for the different assay types and comparison with manually performed methods are discussed below.

5.1. Agglutination Methods

Since complement-fixation tests (CFT) use an agglutination method the usual outcome of a positive test is the appearance of a protein pellet in the bottom of the microtiter well. Although individual instruments are available to read CFT plates automatically, in most cases the plates are read by eye and only qualitative results can be obtained. Therefore, automation is limited to sample processing in the plate but, considering the often complex layout of samples and reagents required for CFTs, automated processing is very desirable since it saves operator time and improves the reliability of the results *(1)*.

5.2. Immunoassay Methods

An ELISA provides a good test of the effectiveness of an automated analyzer because it comprises several distinct steps, for example:

1. Sample pipeting into wells.
2. Dilutions (often serial).
3. Multiple reagent additions
4. Two or more plate washes.
5. Optical plate reading.

Thus, there is plenty of scope for errors that will accumulate as each stage is performed. The complexity of the method means that manual processing will be particularly prone to mistakes and inaccuracy. Improvement in methodology performance using robotic systems is well documented and comparisons of results for manual and automated operation can be very revealing *(8)*.

5.3 Molecular Biological Methods

The advent of PCR and other DNA-based analysis techniques has meant that these genetic methodologies are now being applied to the identification of various viral infections in public health laboratories. For example, automated procedures for HIV and human papilloma virus (HPV) detection using PCR have been reported *(3,6,9)*. Holodniy et al. *(9)* found that both total sample processing time and operator time were reduced by one-third using a robotic system, and no contamination of the PCR reactions was detected. They commented that "when assaying a large number of samples on a routine basis, the savings of time and labour become significant." They also found that the accuracy of results was improved by using automation and that the robotic system's versatility allowed them to automate a variety of different tasks Finally, they concluded that "this type of system should make gene amplification more technically and economi-cally acceptable in the clinical laboratory."

Cuzick et al. *(6)* used a robotic system to automate detection of human papillomavirus DNA using PCR procedures. They found that the results obtained with automation compared well to those obtained manually. Although the speed of pipeting was no faster than that performed manually, they stated that "the robot has the important advantage of eliminating operator error, which can readily occur in any highly repetitive procedure." They also found that the robotic system was suitable for routine clinical screening, requiring minimal labor and technical expertise.

Thus, there appears to be plenty of evidence for the successful application of robotic analyzers to diagnostic viral assays. In an environment of increasing sample throughput and clinical cost-containment, a robotic analyzer represents a realistic alternative, especially where a cost payback can be achieved in a reasonable timescale. As more viral diagnosis is performed as plate-based assays requiring accurate liquid handling, avoidance of contamination, and significant time investment, robotic analyzers will have an increasingly impor-

tant role to play in enabling the virology laboratory to cope with diagnostic workload and demands for greater efficiency and improved reliability.

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23

The Detection of Enteroviruses in Water and Associated Materials Using the Polymerase Chain Reaction

Peter Wyn-Jones and Jane Sellwood

1. Introduction

The environment furnishes a variety of surroundings that may favor the survival of microorganisms. The aquatic environment (which includes marine and fresh water, raw and treated sewage, sludge, and sediments) often provides conditions in which pathogenic viruses released from the body may retain infectivity and cause disease on entry into a new host. Enteric viruses shed from the gut in feces will be transported into the sewerage system and may end up in water used for drinking water abstraction or recreation *(1)*. Enteroviruses, which include vaccine-derived poliovirus, can readily be isolated using cell culture and have therefore been shown to be abundant in raw sewage. Detection of any of the other enteric virus groups is less practical as techniques are more difficult or not available. Even the procedure for enteroviruses, used in many studies of the environment, is laborious, expensive, and slow to produce results. Although not a cause of gastroenteritis, enteroviruses may cause disease including paralysis, meningitis, myocarditis, and cardiomyopathy, and less severe infections, such as, colds and fever, mainly in young children. They are therefore a public health concern, and in order to make proper evaluation of their significance, in water techniques are required that are rapid and reliable and can accommodate large numbers of samples in one test batch. It should then be possible to process the number of samples required to make proper risk assessments *(2,3)*. Further, detection of enteroviruses will demonstrate the presence of sewage and therefore by implication the potential presence of other enteric viruses, such as small, round-structured viruses (Norwalk-like viruses), which do cause gastroenteritis. The European Union (EU) recognizes the detection

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of enteroviruses in bathing waters as a parameter in its Bathing Waters Directive *(4)* and though this currently requires enterovirus detection by infectivity assay, the use of a rapid test to screen out negative samples prior to analysis for infectious virus by cell culture makes the testing process more efficient in time and cost.

The polymerase chain reaction (PCR *[5]*) provides a sensitive, specific, and rapid approach for the detection of viruses in environmental samples. PCR is widely used in identification of viruses in clinical specimens and the transfer of the technique to environmental materials is in principle straightforward. The difference between the two types of samples is the possible presence of substances in environmental materials that inhibit either or both of the enzymes involved in the reaction. These inhibitors are usually soil-derived phenolic or carboxylic acids or fatty acid esters present in humus, or metallic ions that chelate prosthetic groups on the enzymes.

Because of the low numbers of virus particles likely to be present in environmental samples it is essential to test the whole of the sample, not just a subsample, in order to maintain statistical confidence. This requires that the whole concentrate is used in one PCR reaction, and we have developed a concentration technique which permits the RNA from a 10-L water sample to be analyzed in a single PCR sample. It can, however, be of importance to compare the amount of infectious enterovirus in a water sample with the detection of virus by PCR and therefore a method for cell culture isolation has been included in this chapter. In this case a double-size sample may be taken, concentrated to twice the usual volume and the concentrate divided in two equal portions, one being used for cell culture enumeration and the other for PCR analysis. The whole procedure comprises the following stages:

1 Virus concentration. The sample (from 1 to 10 L) is concentrated by adsorption onto microporous filters and the virus eluted with a smaller volume of high protein solution. This protein eluate is flocculated by acidification and the flocculus, with virus adsorbed, is deposited by centrifugation. The flocculus is resuspended in (usually) 10 mL phosphate buffer *(6)*.
2. RNA extraction: Virus in the concentrate is adsorbed to silica and the RNA released by lysis of the capsid with guanidine isothiocyanate by a modification of the method of Boom et al. *(7)*
3 Reverse transcription (RT) and PCR· After washing, the RNA is reverse transcribed and the cDNA is amplified by the PCR. Products can be visualized by agarose gel electrophoresis. A second round of PCR is done using the internal primer P2 and one (P1) of the original primers (seminested PCR) This specifically amplifies the fragment flanked by the primers P1 and P2 and therefore provides confirmation of the identity of the product of the first round and increases the signal-to-noise ratio
4. Southern blotting and hybridization: The PCR product from the first round will contain a sequence complementary to the internal primer P2 Addition of

digoxigenin (DIG)-labeled P2 to this product following its electrophoretic separation and transfer to a membrane will permit the confirmation by size and specificity of the product. Since DIG detection is very sensitive this also gives an increase in signal strength, and first round PCR bands that may be invisible on a gel can be visualized. This may be used as an alternative to blotting and hybridization; it is useful where alternative facilities for second round are not available

5 Cell culture: To assess the amount of infectious virus present the concentrate is mixed with a BGM cell suspension (**Subheading 2.6.**), nutrient medium, and agar containing neutral red stain. Virus infection causes cell death and the formation of plaques after 2–3 d incubation A direct count of the number of virus infectious units present in the concentrate, and hence the original sample, is obtained All 10 mL of the concentrate should be tested.

1.1. Controls

In the detection and assay of viruses from environmental samples it is necessary to include standard positive controls in the cell culture concentration and assay stages, and in the RNA concentration and PCR stages both positive and negative controls are required. It is recommended that the controls comprise the following.

1.1.1. Virus Concentration

Regular use of this method demands that some confirmation of efficiency of virus recovery be made. This can be done by filtering a sample containing a known amount of cell culture-propagated enterovirus and enumerating as for a normal sample. Although the recovery efficiency of adsorption/elution varies between laboratories there should be much less variation between samples in the same laboratory. The control sample should contain a level of virus similar to that anticipated in the environmental samples to be tested, rather than the high titers it is possible to obtain by growing these agents in culture; for recreational waters this should be up to 500 pfu in 10 L.

1.1.2. Cell Culture Assay

Each plaque assay should include a positive control sample containing 1 mL of virus of known count. Ideally this should about 30 pfu/mL.

1.1.3. RNA Extraction and PCR

Cell culture-grown virus samples should be taken through the extraction procedure at the same time as the test materials, and negative samples (uninfected cell culture harvest) also included. Two negative samples should be processed, one at the beginning and one at the end of the test sample series; this will help to determine the source of carryover in the event of false-positive results occurring. A positive sample should also be processed, at the end of the

test sample series. To check the source of any failure in the extraction or PCR, it is useful to include additional positive and negative controls by processing cell culture-grown material through the RT and PCR only. This may be obtained by boiling the cell culture material at 95°C for 5 min to release the RNA, which may then be reverse transcribed and amplified as for test samples.

2. Materials

2.1. General Equipment

1. Centrifuge with 100–400 mL capacity, capable of spinning at 7000*g*
2. 10 L or 20 L stainless steel pressure vessel (Sartorius, Epsom, UK).
3. Sterile pipets. 10 mL, 1 mL
4. Cell counting chamber
5. Appropriate discard facilities.
6. Water bath, steamer or microwave oven, CO_2 incubator.

2.2. Virus Concentration

1. Filters and filter holders: Cellulose nitrate disk membranes, 142 mm or 293 mm diameter having mean pore diameters of 0.45 μm, 1 2 μm, and 5 μm (Sartorius), or fiberglass filter tubes, 8 μm, 25 μm mean pore diameter (Balston/Whatman, Maidstone, UK).
2. 3% Beef extract (w/v) in deionized water.
3. 0.1% Skimmed milk (w/v) in deionized water.
4. One of these solutions is used to elute virus from the microporous filters Though either may be used if only cell culture analysis is to be done, if PCR analysis is be carried out then skimmed milk must be used as eluant since beef extract is inhibitory to the PCR Both eluants must be adjusted to pH 9 5 with 1 *N* NaOH after autoclaving. 0.15 *M* Disodium hydrogen phosphate buffer (Na_2HPO_4) pH 7.0
5. 5 *M* or 1 *M* Hydrochloric acid (HCl)
6. 1 *M* Sodium hydroxide (NaOH)
7. 1 *M* $AlCl_3 \cdot 6H_2O$

2.3. RNA Extraction

All solutions are prepared in deionized, double-distilled water. Molecular biology reagents (enzymes, RNasin, dNTPs, primers) are prepared in molecular biology grade water, which is as above, but treated to destroy any RNase by adding diethyl pyrocarbonate to a concentration of 0.1% (v/v), stirring for 30 min, and autoclaving at 10 psi for 10 min. All glassware is baked at 180°C for a minimum of 5 h.

1. Size-fractionated silica is prepared as follows:
   a. Resuspend 30 g silica (Sigma, Poole, UK) in 250 mL water in a 250-mL measuring cylinder. Cover the top and allow to settle for 24 h at room temperature.
   b. Aspirate off the top 215 mL using a pipet attached to a low-vacuum line and discard

c. Make up to 250 mL with fresh water and resuspend the silica by vigorous shaking. Allow to settle for 5 h.
d. Aspirate off the top 220 mL and discard Add concentrated HCl (approx 300 µL) until the pH is 2.0.
e. Dispense aliquots of 2–4 mL into glass bottles Seal and autoclave at 121°C for 20 min. This may be stored at room temperature in the dark for up to 6 mo.

2. Lysis buffer. 24 g guanidine isothiocyanate (GITC) is dissolved in 20 mL 0 1 *M* Tris-HCl, pH 6.4 containing 4.4 mL 0.2 *M* ethylenedianetetra-acetic acid (EDTA) pH 8 0 and 0 52 g Triton X-100 (*see* **Note 17**)
3. Wash buffer: 48 g GITC is dissolved in 40 mL 0.1 *M* Tris-HCl, pH 6 4. Both solutions are sufficient for about 24 extractions and are stable a maximum of 3 wk in the dark at room temperature.
4. HCl (2 *M*), ethanol, acetone and other general molecular biology reagents.

2.4. Reverse Transcription and PCR

2.4.1. Primers

Several sets of primers for the amplification of enterovirus sequences have been published. In order to act as "catch-all" reagents they are derived from the 5' non-coding region of the enterovirus genome that contains quite long sequences well-conserved among the majority of serotypes. One significant exception is echovirus 22, which is not conserved We use the primers of Tougianidou and Botzenhart ***(8)***. Primers P1 and P3 are opposite sense and flank a region of 396 bp, corresponding to nucleotide positions 160–180 and 558–533, respectively **(Fig. 1)**. Primer P2 is antisense and corresponds to nucleotide positions 473–452. When used in second round PCR in conjunction with P1 a 310-bp fragment is produced.

Primer P3 is used in the reverse transcription and P1 and P3 in the first round PCR. In the seminested PCR primers P1 and P2 are used. All primers are used at concentrations of 20 pmol per reaction.

2.4.2. Reverse Transcription Mix

	µL	
RNasin	1.0	(40 U)
RNA solution	45.0	
10X PCR buffer*	6.0	
$MgCl_2$	3 6	(1.5 m*M* final)
dNTPs	6.0	(125m*M* final each)
Primer P3	0.5	(20 pmol)
MMLV reverse transcriptase	0.6	(8 U)
Total volume	62.7 µL	

*as supplied with *Taq* enzyme.

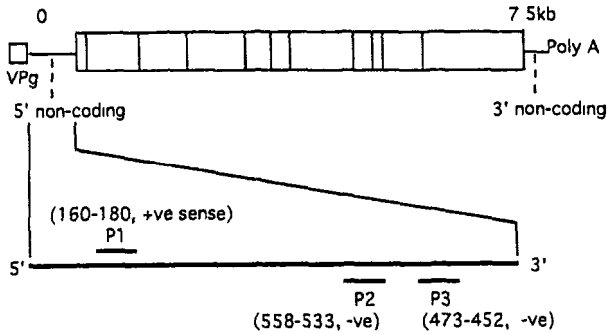

Fig. 1. Poliovirus genome organization to show primer locations

2.4.3. First Round PCR Mix

	µL	
RNA/cDNA hybrid solution	63	
10X PCR buffer*	4 0	
$MgCl_2$	2.4	(1.5 m*M* final)
dNTPs	6 0	(125 µ*M* final each)
Primer P1	0.5	(20 pmol)
Primer P3	0.5	(20 pmol)
Taq polymerase	0.5	(2.5 U)
Water	23 4	
Total volume	100 µL	

*as supplied with *Taq* enzyme

2.4.4. Second Round PCR Mix

	µL	
First round reaction mixture	100	
10X PCR buffer*	1.0	
$MgCl_2$	0 6	(1.5 m*M* final)
dNTPs	6.0	(125 µ*M* final each)
Primer P1	0.5	(20 pmol)
Primer P2	0.5	(20 pmol)
Taq polymerase	0.5	(2.5 U)
Water	0.9	
Total volume	110 µL	

*as supplied with *Taq* enzyme.

2.5. Southern Blotting and Hybridization

This is carried out in the conventional way. Several manufacturers provide kits for the end-labeling of oligonucleotides with DIG, or the primer can be end-labeled during synthesis. Kits are also available for hybridization; the protocol described below is adapted from that provided by Boehringer-Mannheim (Milton Keynes, UK), and the blocking reagent, anti-DIG antibody, and Lumigen substrate are all included in the kit.

Specific materials are:

1. Large dish or trough to contain glass plate, supports, and gel-blotting absorbent material
2. 0.4 *M* NaOH.
3. Hybridization buffer: 5X SSC, 1% (final) blocking reagent (Boehringer Mannheim), 0.1% (w/v) *N*-lauroylsarkosine, 0.2% sodium dodecyl sulfate (SDS)
4. P2 primer end-labeled with DIG, stock concentration of 2.5 pmol/µL Working concentration is 50 pmol per mL hybridization buffer, and approx 2 5 mL solution is required /100 cm^2 membrane.
5. Wash solutions, both sterile: (1) 2X SSC + 0.1% SDS, (2) 0.1X SSC + 0 1% SDS.
6. Detection buffer 1: 100 m*M* maleic acid, 150 m*M* NaCl. Adjust pH to 7.5 with solid or concentrated (10 *M*) NaOH. Autoclave. Add Tween-20 to aliquots as required to a final concentration of 0.3% (v/v)
7. Detection buffer 2: Detection buffer 1 (without Tween) containing blocking reagent at 1% final concentration.
8. Detection buffer 3: 100 m*M* Tris-HCl, 100 m*M* NaCl, 50 m*M* $MgCl_2$. Adjust to pH 9.5 (exactly) with 10 *N* NaOH.
9. Saran wrap.
10. Nylon blotting membrane. It is easier if a membrane is used that does not require baking or UV exposure to fix the nucleic acid (e.g., Qiagen Nylon Plus, Crawley, UK)
11. Filter papers and paper towels.
12. Hybridization oven with appropriate sized tubes, or shaking water bath
13. Hybridization bags and sealing machine (if water bath used).

2.6. Cell Culture

BGM cells are a continuous monkey kidney line that have been shown to be susceptible to infection by many types of enteroviruses ***(9)*** and are widely used in water virology. BGM cells are most sensitive between passages 80 and 120, and may be split in a ratio of 1:8 or 1:10. Large numbers of cells may be produced using corrugated flasks, roller bottles, or a cell factory. A weekly routine of passage using trypsin/EDTA for cell disaggregation, 3–4 d on growth medium, followed by 3–4 d on maintenance medium produces the optimum number of cells per vessel.

1. Media for growth and maintenance of cultures: Eagles MEM containing fetal calf serum at 5% (growth) and 2% (maintenance), and buffered with sodium bicarbonate. L-glutamine, penicillin, streptomycin, and nystatin in distilled water are added as usual supplements.
2. Media for agar overlay. 50-mL quantities of double-strength Medium 199 containing 2% fetal calf serum, penicillin, streptomycin, nystatin, and gentamicin. Plaque visibility and enhancement is achieved by the addition of 0.5 mL 2% (w/v) $MgCl_2$ and 0 5 mL 0.1% (w/v) neutral red
3. 3% Nutrient agar (w/v) in 50 mL portions. One portion is sufficient for 10 Petri dishes

3. Methods

3.1. Virus Concentration

1 Place water sample (1 L–10 L) in pressure vessel (*see* **Note 6**).
2 Acidify to pH 3.5 with 1.0 *M* HCl.
3. Apply pressure from compressed air source or pump to force water through filter at a medium rate. Water is discarded
4 Release pressure and add 100–400 mL of 3% beef extract or 0.1% skimmed milk at pH 9 5 (using 1 *N* NaOH) to vessel (*see* **Note 5**)
5. Apply pressure to pass high-protein liquid slowly through filter Collect eluant
6 Carefully adjust pH to 3.5 for beef extract or 4 5 for skimmed milk until a flocculus forms (*see* **Notes 3 and 4**).
7. Mix well by shaking for 10 min.
8 Centrifuge at 7000*g* for 30 min (the use of a refrigerated centrifuge is advisable) (*see* **Note 9**).
9 Pour off liquid immediately and add 0.15 *M* Na_2HPO_4 at pH 7.0 to give a total volume of 10 mL Mix well to resuspend in buffer Concentrates are stored at –20°C or below pending cell culture assay or PCR analysis

3.2. RNA Extraction (see Note 1)

1. Thaw the 10-mL concentrate rapidly in a water bath at 37°C.
2 Add 40 µL silica suspension and shake by hand.
3 Adjust the pH to 3.5 with 2 *M* HCl. Since all reagents are sterile this is best done by aseptically taking a drop out of the suspension on a sealed Pasteur pipet and touching it on a piece of narrow-range pH paper. The milk proteins reflocculate and adsorb with the virus particles to the silica.
4 Centrifuge at 1500*g* at 4°C for 10 min. Aspirate off the supernatant with a Pasteur pipet and discard
5 Resuspend the protein flocculus/SiO_2 in 900 µL lysis buffer. Virus capsids are lysed and the released RNA adsorbs to the silica. The milk proteins are solubilized, so the universal contents will be slightly viscous at this stage No material is lost provided resuspension is carried out carefully, avoiding the creation of bubbles. The buffer conditions at this stage are protective of the RNA. It is most important to ensure complete resuspension of the silica particles at this stage.

6 Transfer the contents to a 1 5-mL Eppendorf tube.
7 Centrifuge 15 s at 13,000*g* in a microcentrifuge. Discard the supernatant.
8. Resuspend in 1.0 mL wash buffer.
9 Repeat the centrifugation and resuspension washing steps once more
10. Wash twice in 70% (v/v) ethyl alcohol and once in acetone, centrifuging each time at 13,000*g* to pellet the RNA. Dry the RNA in a heat block at 56°C (approx 5 min).
11 Take up the RNA pellet in 60 µL RNase-free water containing 30 U RNAsin and transfer to a fresh Eppendorf tube. At this stage it will be impossible to remove all the RNA without drawing up some silica, no matter how carefully the latter has been prepared, and this silica may interfere with the RT. It is therefore necessary to transfer the preparation twice to fresh Eppendorf tubes to ensure no silica is taken over into the transcription buffer
12 Centrifuge 15 s at 13,000*g* in a microcentrifuge
13 Transfer 50 µL RNA supernatant to a fresh Eppendorf tube.
14 Centrifuge 15 s at 13,000*g* in a microcentrifuge.
15 Transfer 45 µL RNA supernatant to a 0.5-µL Eppendorf tube ready for reverse transcription
16. RNA may be alcohol/sodium acetate-precipitated by conventional procedures (e.g , Maniatis) and taken up in 10 µL water if required In practice this is only needed for Southern blotting and hybridization

3.3. Reverse Transcription and PCR (see Note 2)

1 Add 2 drops mineral oil to the RNA solution from **step 15, Subheading 3.2.**
2 Incubate at 95°C for 5 min to denature RNA secondary structure. Transfer to ice.
3 Add reverse transcription mixture (18 µL, *see* **Subheading 2.4.2.**). Incubate at 42°C for 20 min.
4 Incubate at 95°C for 5 min to denature RT Transfer to ice.
5. First round PCR: Add PCR mixture (38 µL, *see* **Subheading 2.4.3.**). Run in PCR thermocycler at the following program:

95°C	3 min		Denatures (melts) RNA/cDNA hybrid
53°C	5 min		Anneals primers
72°C	2 min	repeat 30×	Extends primer sequence using one original sequence as template
95°C	1 min		Denatures all strands
53°C	5 min		Anneals primers
72°C	10 min		Final extension of primers

6. Second round PCR (seminested): Add second round PCR mixture (10 µL, *see* **Subheading 2.4.4.**) This mixture is added by piercing the oil layer with the tip of the pipet. Adding the new reaction mixture to the first round mix through the oil avoids the dangers associated with removing aliquots of the first round mixture into fresh tubes

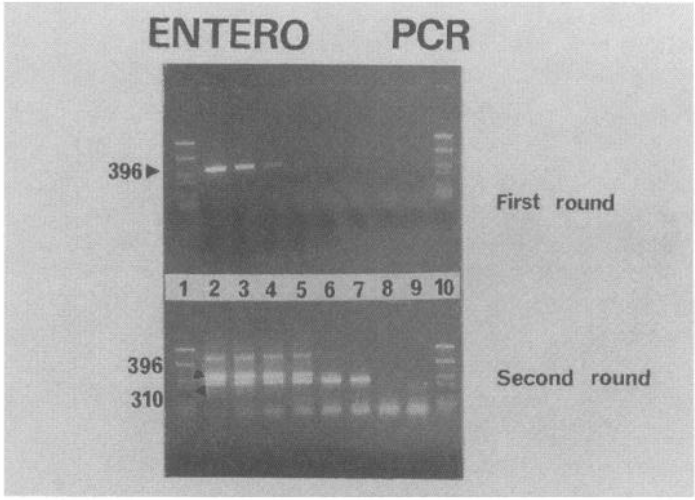

Fig. 2. This figure illustrates the sensitivity of the PCR and the increase in sensitivity obtained by performing a second round amplification. Both sets of samples have been run on a single gel to facilitate comparison.

(Upper set: first round PCR) Track 1, markers (plasmid pUC18 digested with *Sau3aI* restriction endonuclease); Tracks 2–8, dilutions of Coxsackie B5 virus from 10^4 to 10^{-2} pfu each seeded into 10 L water and processed as described in the text; Track 9, negative control. 200 mL water extracted as described in the text; Track 10, markers, bands in the expected region of 396bp can be clearly seen showing the presence of virus in seeded samples down to 10pfu (track 5). (Lower set: second round [semi-nested] PCR) Tracks are as in the upper set. Second round PCR mix (10 μL, **Subheading 2.3.**) was added to the total product of the first round, in the same tube to avoid the possibility of contamination. Amplification conditions were as for the first round. The expected product size in round 2 is 310 bp and bands of this size can be seen down to 0.1pfu (track 7), indicating a 100-fold increase in sensitivity. In the higher concentrations (tracks 2–5) there is still first round product present which is used up as the target sequence for the second round.

Run in thermocycler using the same program as the first round.

Ten microliters of the product may be removed from the tube for analysis by electrophoresis on 2% agarose gels.

Figure 2 shows PCR results of decreasing concentrations of Coxsackievirus B5, and **Fig. 3** shows analysis of environmental samples from two recreational water sources.

3.4. Southern Blotting and Hybridization

1. Precipitate the amplified DNA from the first round by normal ethanol/sodium acetate procedures. Run 10% of this in a 2% gel as in **Subheading 3.3.6.**

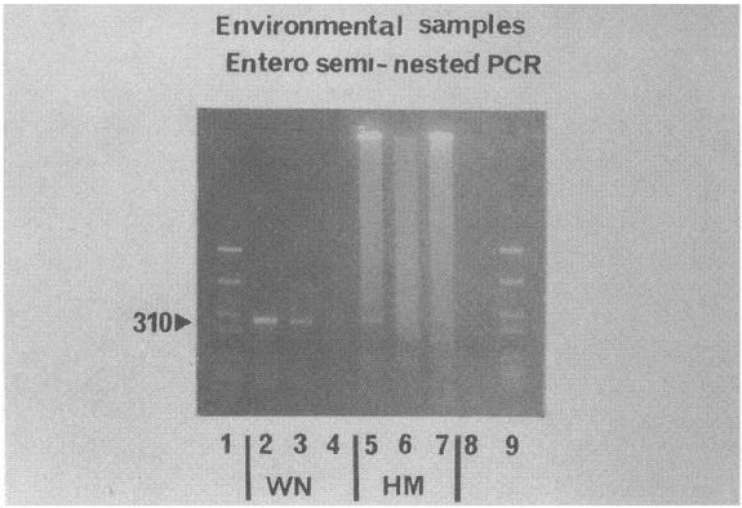

Fig. 3. This figure shows analysis by seminested PCR of three samples taken from each of two recreational water sites. All these samples were negative by cell culture analysis. Samples in tracks 2–4 (WN) are from an EU-designated beach; two of the samples are clearly positive. Samples in tracks 5-7 (HM) were taken from an estuary used for sailing and water-skiing as well as swimming. These samples contained greater quantities of contaminating material but positive bands can still be seen in tracks 5 and 7. Markers are as **Fig. 2**.

Markers should be DIG-labeled standards so their positions can be checked on the final X-ray film. Check the gel by UV-transillumination for satisfactory separation.

2. Transfer the gel, slots down, to a sheet of Whatman No. 1 filter paper supported on a glass plate. The ends of the paper should overhang the plate and dip into the reservoir of NaOH. Cut a piece of membrane to the exact size of the gel and gently smooth it to the back of the gel with the fingers, excluding air bubbles. Check the orientation of the gel/membrane (especially if more than one gel is being blotted); this is best done by cutting off one corner.
3. Surround the gel with Saran wrap to prevent NaOH being drawn into the absorbent layers by any route other than through the gel. Place five or six layers of filter paper over the membrane and add 10–15 paper towels on top of the filter paper. Place a weight (approx 500 g) on the towels. Allow the gel to blot overnight at room temperature.
4. Trim the membrane if required, using the bromophenol blue as a guide, and prehybridize at 68°C for at least 1 h, using at least 20 mL hybridization buffer per 100 cm^2 membrane. If bags are used ensure they are sealed without any air (this may require some practice).
5. Hybridize with P2-DIG. Probe containing 50 pmol per mL will require 2.5 mL solution per 100 cm^2. Hybridization should be carried out for 1–2 h at 45–50°C

(50°C gives a higher stringency). Wash solutions should be equilibrated to 50°C during this time

6 Discard the probe as toxic waste Remove the membrane from the hybridization bag (if used) and transfer to a shallow dish in a shaking water bath at 45–50°C. Add 50 mL 2X SSC + 0.1% SDS/100 cm^2 membrane and wash gently for 5 min. Replace the wash fluid for a further 5 min wash

7 Repeat the above wash procedures with 0 1X SSC + 0.1% SDS The membrane is then ready for detection.

8 Wash in detection buffer 1 for 2 min at room temperature on a rocking table Use 100 mL/100 cm^2 membrane

9 Wash in detection buffer 2 at room temperature for 30 min Discard.

10 Add 20 mL/100 cm^2 detection buffer 2 containing anti-DIG antibody at 1·10,000 dilution Incubate at room temperature for 30 min

11 Wash twice (15 min each) in detection buffer 1 at room temperature Use 100 mL per 100 cm^2 membrane

12 Wash in detection buffer 3 for 3 min (max) Use 20 mL per 100 cm^2 membrane. Discard

13 Incubate for 5 min (max) in detection buffer 3 containing Lumigen substrate at 1:100 dilution.

14 Edge-drain the membrane on a piece of filter paper but do not allow to dry. Wrap in Saran wrap and transfer to 37°C incubator for 10–15 min

15 Keep the membrane wrapped and transfer to an X-ray cassette loaded with film Trial and error must be used to determine the correct exposure time, but up to 2 5 h can be used without too great background interference Exposure times are much shorter than those required for radioisotope exposures

3.5. Cell Culture: Suspended Cell Plaque Assay

1 Melt agar and equilibrate in water bath at 44°C.

2 Prepare overlay medium, dispense in 5-mL portions into universals and place in water bath as in **Subheading 2.6.2.**

3. Trypsinize BGM stock cell cultures to prepare cell suspension at 1×10^7/mL

4. Add 5 mL of agar to 5 mL of overlay medium, retain at 44°C

5. Mix 2 mL cell suspension (to give final cell number per Petri dish of 2×10^7, *see* **Note 13**) with 2 mL sample concentrate, pour into the 10 mL agar/medium.

6 Pour into a 90 mm diameter Petri dish, mix to cover the whole dish

7 Place dark cover over Petri dishes until agar has set (*see* **Note 14**) Invert and place in damp container, incubate at 37°C in a 5% CO_2 incubator.

8. Score for pale plaques of cell death at 2, 3 and 4 d Each plaque is counted as a plaque-forming virus infectious unit All the sample concentrate (usually 10 mL) should be tested, the total number of plaques being the total virus infectious units present in the original sample.

9. Petri dish cultures should be autoclaved before discarding

4. Notes

1. Latex gloves must be worn for all nucleic acid manipulations, and automatic pipettes with aerosol-resistant tips are used for all volume measurements
2. It is to particularly important to separate virus handling, RNA extraction, and first and second round amplification stages. Extreme care in manipulation of materials is required to avoid first round product from one sample from contaminating other samples Rigorous controls (*see* **Subheading 1.**) are necessary, and it is vital that any second round is done in a laboratory physically separate from that where first rounds are set up, and to keep a set of automatic pipets, racks, and other minor items specifically for second round manipulations. Once tubes are sealed target sequences may be amplified in the same PCR machine. A cabinet with an effective UV source may be used for decontamination purposes but this should not be used in the place of separate facilities
3. A robust and precise pH meter is essential, ideally with a long electrode. All pH endpoints must be approached cautiously, taking care not to overshoot
4. All new batches of beef extract or skimmed milk must be checked to ensure a flocculus will form at the correct pH.
5. The addition of 5 mL of 1 *M* $AlCl_3 \cdot 6H_2O$/L of water may enhance virus adsorption to the fiberglass filter.
6. 10 L Sample is required for river or seawater One liter of sewage effluent may be filtered by diluting with 4 L of dechlorinated tap water
7. When river or seawater is filtered it may be necessary to use several layers of filter membranes: glass fiber pre-filter on the top, then 5, 1 2, and 0.45 μ on the lowest level. With the Balston cartridges a 25-μ filter followed by 8-μ filter in series may be required The two filter systems have a similar efficiency of virus recovery. The latter system involves less expensive capital outlay.
8. All reagents except acid/alkali should be sterile.
9. Remove samples from the centrifuge as soon as the run finishes and pour off the supernatant.
10. Two milliliters of chloroform may be added to 10 mL resuspended concentrate and shaken vigorously for 5 min. Centrifuge at 3000*g* for 10 min, pipet off supernatant into an open dish, and place in a warm, clean area for 20 min to allow residual chloroform to disperse. This will reduce microbial contamination of sample. Store frozen Dispose of contaminated solvent appropriately
11. Wash and sterilize equipment if appropriate between filtration of different samples.
12. Quality assurance should include: regularly filtering samples of virus from a stock of known concentration, and taking part in externally organized external quality assessment (EQA) scheme.
13. Count the cell harvest from stock culture flasks and dilute appropriately. (One 150-cm^2 flask should provide sufficient cells for one 90 mm Petri dish.)
14. Neutral red may inactivate virus in the presence of light Add virus/cell sample to agar/medium in reduced light and quickly cover to shield from light while agar sets.

15 Plaques may be seen more easily against a dark background with light source behind
16 It is essential to include a positive virus control of known count with each batch of plaque assays
17. It is assumed that normal microbiological and general safety precautions will be observed However it should be noted that GITC must be weighed out in a fume hood and solutions discarded into strong alkali before disposal

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Recombinant Antigens in Viral Diagnosis

John R. Stephenson and Alan Warnes

1. Introduction

1.1. The Limitations of Traditional Biological Assays

Traditionally, the accurate detection of viruses and the diagnosis of viral diseases has been difficult and expensive as viruses cannot be visualized by conventional light microscopy and need propagation in primary or continuous cell culture. The advent and increasing sophistication of electron microscopy (EM) has greatly facilitated the detection of those viruses with a defined and robust structures, e g., adenoviruses, polioviruses, rotaviruses and, more recently, Norwalk virus and "Norwalk-like agents" *(1)*. Diagnosis of diseases caused by other viruses, especially enveloped viruses such as influenza virus, measles virus, and yellow fever virus, has depended almost entirely on the detection of antibodies in sera, saliva, or cerebrospinal fluid (CSF). Initially, serological assays depended on detecting antibodies that inhibited biological functions of the virus, and such assays included plaque reduction neutralization tests (PRNT), hemagglutinin inhibition assays (HI), and complement fixation (CF) assays *(2)*. Although these assays can be specific and reliable in the hands of an experienced laboratory worker, they suffer from several significant disadvantages. HI and CF assays are frequently very specific, but are relatively insensitive, and require the preparation and at least partial purification of large amounts of viral antigen. Furthermore, these assays will not differentiate between recent and past infections. Because antigen production is nearly always dependent on virus growth in tissue culture, it can be expensive, laborious, and, in the case of human pathogens like yellow fever virus and rabies virus, potentially dangerous, requiring sophisticated containment facilities. Moreover, all these procedures require well-trained and competent technical experts to ensure the safe and reliable supply of high-quality reagents. PRNTs

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have significant advantages over other traditional tests in that they can be very sensitive and specific and do not require the preparation and purification of large amounts of antigen. They do, however, suffer from all the drawbacks of any tissue culture-based system and in addition necessitate the handling of live virus and live cells under sterile conditions throughout the assay procedure. These assays are very slow, requiring anything from 2 d to 3 wk to complete. In recent years the adaptability of laboratory assays to automation has been of increasing importance leading to the decreasing popularity of biological assays which are very difficult and expensive to automate.

1.2. The Development of Solid-Phase Assay Systems

Over the past two decades solid phase assays such as radioimmune assay (RIA) *(3)* and enzyme-linked immunosorbant assay (ELISA) *(4)* have become increasingly popular, both for their ease of operation, economic use of material, and ability to be readily and cheaply adapted to automated laboratory protocols. However, the sensitivity, reliability, and specificity of these assays relies heavily on the use of high-quality antigens, and, because these have to be prepared from infected material—nearly always produced in tissue culture—the quality of the data and the cost of the assay can be less than ideal.

Even so, the need for rapid tests led to the development of a range of simple assays, wherein whole virus particles were coated onto solid supports. Latex was a favored source of support, which, when coated, would rapidly produce agglutination in 1–3 min when mixed with sera containing the relevant antibodies, which would be indicative of disease. The system had the advantage of being able to be adapted by coating the latex particles with polyvalent sera raised against the virus, which would then detect the pathogen via agglutination *(5)*.

1.3. The Design and Production of Antigens with a Defined Structure

Viral proteins, or defined fragments of them, synthesized from genetically modified micro-organisms, could potentially overcome all the previously mentioned shortcomings of assays based on conventional antigens. With the considerable array of sophisticated techniques now available to the genetic engineer, genes coding for any protein, whether occurring naturally or "custom built," can be constructed with relative ease. Thus, the determination of the desired amino-acid sequence becomes the limiting factor in the production of antigen, not the ability of the gene to be cloned or of the protein to be purified. The designer of diagnostic antigens must therefore consider several key factors in constructing the gene (or genes) that will code for the antigen(s) to be used in the desired assay.

The first question that needs to addressed is whether the assay is to be used for the diagnosis of acute infections or for surveillance purposes. For acute disease detection, the assay must be rapid, because its purpose will be to determine which antiviral drugs should be used, or more commonly to rule out the use of expensive and potentially harmful antibiotics. Such an assay will probably rely on IgM detection, but must also use an antigen that is relatively specific for the disease in question, and antibodies to it must be known to be elicited very early in infection. Conversely, if immune surveillance is the prime use for the assay then an antigen must be chosen that elicits a long-lasting antibody response that can be distinguished from that produced by a recent infection. In most cases IgG-based assays, using a suitable viral antigen, will be system of choice.

The second factor to consider is that of specificity. With some viruses it may be necessary to choose a protein epitope that is heavily conserved on most, if not all, of the known viral isolates. This may need very careful consideration, because some epitopes demonstrate very rapid mutation rates, but it is essential that the diagnostic test detects all known isolates. A single consensus epitope may not exist on a naturally occurring epitope; however, the genetic engineer could design and construct a multivalent amino-acid sequence that would react with antibodies directed against all known variants of the virus. Alternatively, it may be necessary to determine a sequence that will only react with antibodies raised against a specific virus or virus isolate and exclude all others. This is particularly important where a population has been vaccinated with a traditional live attenuated or killed vaccine, and outbreaks of disease in the vaccinated population need to be evaluated to determine whether they are caused by ineffectual vaccination protocols or by new, antigenically distinct variants of the virus. These situations frequently arise with influenza virus outbreaks and more recently with outbreaks of measles and mumps ***(8)***. Another reason for using antigens with a narrow range of specificity is when a certain variant is pathogenic, but other variants or closely related viruses are not. Examples of these situations can be found with some of the arthropod-borne viruses. For example, certain variants of tick-borne encephalitis virus can have a very high mortality rate and cause severe neurological sequelae, whereas others appear completely nonpathogenic ***(9)***. Thus, if someone returns from an endemic area and is shown to have tick-borne encepahalitis-specific antibodies, it is important to know if they need to be given an intensive course of vaccine and/or immunoglobulin, or whether the infection was caused by a benign variant of the virus.

In addition to the accurate construction of defined epitopes, genetic engineering can assist in the production and purification of antigens. Vectors with a wide range of host cells can be used to assist in the choice of the optimum cell

line for industrial purposes. Signal sequences can be inserted to enable an internal antigen to be secreted from the cell and membrane-binding domains can be removed or inserted, depending on whether the antigen is required in a soluble form or bound to a cell surface *(10)*. Ligands such as histidine multimeres an also be attached to assist purification and tags added to enable easy detection.

Therefore, it is now possible to construct a gene coding for the precise antigen required, however, determining what amino-acid sequence is desirable can frequently only be possible after an extensive program of research.

In this chapter, we will review the general features, advantages, and disadvantages of the popular recombinant systems currently available to the scientific community. Two very widely used vector systems—those based on herpes viruses and adeno-associated viruses—will not, however, be discussed, because they have several limitations that make them unsuitable for diagnostic antigen production.

Synthetic peptides can be possible sources of pure, cheap, and highly specific antigens that can be readily adapted to automated laboratory systems. Many important viral epitopes are, however, conformational in nature *(6)*, and this property, along with the frequent inability of short peptides to maintain a stable three-dimensional conformation in aqueous solution *(7)*, has led to many disappointing performances from peptide-based diagnostic assays.

2. Comparison of Expression Systems

Recently, we have also evaluated a number of expression systems *(11)* which are well documented *(19,26)* and commercially available. We do not propose to detail the methods for the construction of these systems, which are readily available from the literature or from commercial companies (Invitrogen, Baculovirus; Microbix, Ontario, Canada, Adenovirus). However, we have simplified the major differences between the hosts when considering their use in the production of diagnostic proteins **(Table 1)**.

2.1. Diagnostic Antigens Produced in Prokaryotes

The use of *E. coli* as a host for the production of recombinant proteins is unparalleled, with numerous products being expressed since the advent of genetic engineering. However, their employment in generating eukaryotic proteins for use in vitro diagnostics has been less successful. This was partly owing to problems associated with the host system.

2.1.1. Disadvantages of E. coli

1. Initially only low levels of expression could be attained.
2. Prokaryotes do not have the ability to perform post-translational modification, which was a tremendous drawback in the synthesis of authentic eukaryotic glycosylated proteins.

Table 1
Comparison of Host Expression Systems for the Production of Recombinant Viral Proteins for Use in Diagnostic Assays

Expression parameters	*E coli*	Baculovirus	Yeast	Adenovirus
Detailed knowledge of host/vector system	Yes	Yes	No	No
Ease of construction	Yes	Yes	Yes	No
Post-translational modification	No	Partial[a]	Partial[b]	Yes
Isolation of recombinant proteins	Easy	Easy	Difficult	Easy
Background interference	Frequent	Rare	Rare	Rare
High-level expression[c]	Yes	Yes	Yes	Yes
Scale-up/cost	Easy/cheap	Difficult/costly	Difficult/costly	Difficult/costly

[a]Although insect cells afford glycosylation there are subtle differences from mammalian cells which can affect antigenicity

[b]Although yeast cells offer good glycosylation there can be problems with the quantities added, this has been helped by strains which confer low level glycosylation Further, yeast cells are difficult to disrupt and therefore isolation of the recombinant protein proved difficult, again strains easy to disrupt were defined which to some extent overcame initial problems.

[c]High level expression is possible in all these systems, but can never be guaranteed because it is frequently dependent upon the structure and post-translational modification of the protein and its synthetic pathway in each particular system

3 Background interference from contaminating bacterial proteins, although this may be overcome by purification of the recombinant protein, or redesigning the assay format with the use of antibody-capture assays or absorbing test sera with bacterial host-cell proteins.

Furthermore, the popularity of prokaryotic expression systems for the production of eukaryotic proteins was greatly diminished with the introduction of baculovirus-based systems, which heralded a new dimension in expression systems for virologists. The baculovirus system is described in **Subheading 2.2.**, and its potential for producing elevated levels of proteins with a high degree of post-translational modification, within a system that did not produce background interference, appeared to be the final epitaph for *E. coli* in the expression of eukaryotic proteins as diagnostic antigens *(11)*.

2.1.2. Advantages of E. coli

The obvious advantages of using *E. coli* include the use of familiar, well-tested expression systems, which are not only easy to construct but also to scale-up Recent advances in two main areas have also added to the potential of *E coli.*

2.1.2.1. Promoters

The introduction of a wide range of promoters in plasmid vectors has led to the maximization of expression, and the subsequent production of high yields of eukaryotic recombinant proteins in *E. coli*. Promoters can now be engineered using optimal DNA sequences to provide maximal expression ***(12)***. Indeed, work from our own laboratory has shown that eukaryotic proteins that form complex structures can also be produced in *E. coli* under the control of a hybrid *tac* promoter ***(13)***. Also, a wide range of native promoters have been evaluated that can produce high-level expression of eukaryotic proteins. Other workers have shown that using the T7 capsid promoter levels as high as 10% of a virus capsid protein can be produced in *E. coli* ***(14)***.

2 1.2.2. Fusion Proteins

When using *E. coli* as a host for expression, there still remains the problem of removing contaminating bacterial proteins, which could greatly influence background interference in an assay system. Obviously, conventional means can be used to purify the recombinant protein in question, although this can be laborious to perform and frequently does not provide a final product of sufficient purity. The introduction of fusion-expression systems was therefore, an opportunity to overcome these problems ***(15)***. The fusion-expression system works by cloning the recombinant DNA in line (at either the 5' or 3' end) with the fusion DNA, which could also include a peptide cleavage site. The final product can then be purified by use of an appropriate affinity column, and the recombinant protein released in a highly purified form after enzymic treatment at the cleavage site. However, early systems were beset by two fundamental problems. The first was that, depending on the recombinant protein being expressed, certain fusion systems produced inclusion bodies, which are highly concentrated forms of insoluble fusion protein. This caused many problems for process workers in resolublizing these protein complexes into authentic forms. The second problem was proteolysis, which could occur to the whole of the fusion protein. This could effect both the binding capacity of the fusion protein to the ligand and also drastically reduce the production of full-length native protein. However, the development of sophisticated fusion systems, e.g., histadine tagging ***(16)*** and cellulose-binding protein ***(17)***, has allowed the purification of recombinant proteins, which are relatively free from contaminating bacterial proteins, with comparative ease.

2.2. Baculovirus-Expression Systems

The advent of the baculovirus-expression system in the early 1980s enabled eukaryotic proteins to be expressed in a eukaryotic system, both at high levels

and with some degree of post-translational modification (albeit with incomplete glycosylation). A number of recombinant proteins derived from baculovirus-infected insect cells have now been used in indirect ELISA-based systems for the detection of antibodies to viral pathogens ***(18)***. Even so, the acceptance of recombinant proteins in diagnostic assays as commercial products was not automatic and much validation was required to satisfy regulatory authorities.

2.2.1. Construction of Recombinant Baculoviruses

Construction of suitable viruses involves placing the gene of interest under the control of either the Polyhedron or P10 promoters, which have been excised along with baculovirus flanking regions in a transfer vector ***(19)***. A terminator sequence is also present to prevent readthrough from these strong promoters. A *lacZ* gene has also been incorporated that can be used to detect recombinants and determine efficiency of transfection. Once the gene has been cloned into the transfer vector, purified plasmid DNA can then be co-transfected with baculovirus double-stranded DNA (which has the corresponding promoter sequence deleted) into an appropriate *S. frugiperda* cell line (Sf 9 or High 5 cells can be used). After uptake, recombination can occur, resulting in the generation of a recombinant baculovirus containing the cloned gene at an efficiency of 0.1–5.0%. The resultant plaques containing the recombinant viruses are then selected and plaque-purified three times to ensure that no minor population has broken through. Once the recombinant virus has been purified, then protein expression should be characterized to ensure the correct gene has been cloned.

Because this system has proved very popular, it is constantly under development, and numerous improvements, modifications, and adaptations are regularly announced by specialist companies.

2.2.2. Advantages of Baculoviruses

Baculovirus-expression systems offer the potential to produce large quantities of viral recombinant proteins to levels greater than 50% total protein ***(20)***, and in our laboratory we have obtained 40% expression for measles virus nucleoprotein (MVNP) ***(21)***. Further, the MVNP has been shown to produce structures similar to those produced in a natural infection Insect cells are also easy to grow and scale-up can easily be achieved, thus maximizing expression. New vectors have subsequently been developed that contain essential genes, thus preventing any relegated viral DNA forming infectious progeny, which ensures that 100% recombinants are produced upon co-transfection. In addition, post-translation modification occurs that includes acetylation, phosphorylation, amidation, and O- and N-linked glycosylation.

2.2.3. Disadvantages of Baculoviruses

In many cases, the sites for cloning are limited to a *Bam*HI insertion site because only two promoters can be used within the baculovirus. Thus, a great deal of care and consideration is required when considering cloning strategies However, the number of sites available for cloning has increased with the development of the new Baculogold vectors. Although insect cells confer post-translational modification, one difference is in the synthesis of glycoproteins, in that they do not produce complex carbohydrate side chains (e.g., only have carbohydrates of the short or long mannose form). In some cases, this can result in the production of proteins that are not of an authentic form, this has to be ascertained on a trial and error basis and depends totally on the recombinant protein being expressed

2.3. Yeast-Expression Systems

Although yeast-expression systems have been used for the production of recombinant proteins, there are few examples that depict their use as diagnostic reagents for the detection of viruses. Those that have been used with some success are the nucleocapsid and nonstuctural proteins NS3-NS5 of hepatitis C virus *(22)* and the gp120 protein of HIV, expressed in Saccharomyces cerevisiae. Fundamental problems with cell breakage and excessive glycosylation, however, have limited the use of Saccharomyces cerevisiae as a successful expression system. Although the yeast-expression systems have not been used to any great extent, new systems have been developed *(23,24)* that are thought to overcome the major problem of excessive glycosylation. However, more research will have to be undertaken before these expression systems can be considered to be suitable for the production of diagnostic antigens.

2.4. Expression of Diagnostic Antigens from Recombinant Adenoviruses

The possibility of using recombinant adenoviruses as vaccines has been explored for several years because they have many features that make them attractive vectors to deliver a wide variety of important viral immuogens. Conventional adenoviruses, based on serotypes 4 and 7, have been used for many years to vaccinate US military recruits. These vaccines are administered orally, yet can successfully induce a mucosal immune response at a remote site that is capable of protecting against a respiratory infection. Several million adults have now received these vaccines, with little or no reported adverse reactions. Human clinical trials have also been carried out with serotype 1, 2, and 5 viruses (reviewed in **ref. *25***). More recently, adenoviruses have been used to generate diagnostic antigens because they can overcome many of the shortcomings of prokaryotic or insect-based systems.

2.4.1. Construction of Recombinant Adenoviruses

Most recombinant adenoviruses have been derived from serotype 5 viruses, which have a double-stranded linear genome of about 36 kilobases in length. If deletions are made in the E3 region alone, the virus can grow to high titers in many cell lines If deletions are made in E1a region also, more foreign genetic material can be incorporated (0.5–7kb), but the recombinant viruses will only grow in cell lines, such as 293 cells, which will supply the gene products encoded by this region (review, **ref. *26***). Construction is straightforward using standard recombinant DNA techniques. First, the gene of choice is inserted into a subgenomic viral DNA fragment propagated on a bacterial plasmid. We have found that best results are obtained if the gene of choice is first placed under the control of a powerful constitutive eukaryotic promoter (such as the IE promoter from cytomegalovirus [CMV]) along with the appropriate termination and polyadenylation signals. The resulting construct can then be "rescued" by cotransfection into permissive mammalian cells with a second plasmid containing the complete viral genome, along with a "stuffer fragment" of noncoding, unrelated DNA. The strict packaging requirements of the adenovirus virion ensures that only recombinant viruses will be rescued, and the "stuffer fragment" ensures no "wild-type" virus particles are packaged into infectious virions. Although recombination rates in adenoviruses are comparatively low, these powerful positive selection mechanisms make their generation a relatively efficient process. Infectious virus can then be cloned in 293 cells and their genetic structure determined by conventional restriction enzyme analysis.

2.4.2. Advantages of Using Recombinant Defective Adenoviruses

Although developed initially for vaccine research, defective recombinant adenoviruses can be readily adapted as tools to produce high quality diagnostic antigens as they exhibit several useful features. The most valuable of these for the development of novel high-quality viral antigens is their ability to grow in a wide variety of eukaryotic cell types, thus ensuring the correct post-translational modifications are performed. This feature is of particular importance for the production of diagnostic assays from enveloped viruses because most of their important antigens are membrane-bound molecules whose three-dimensional structure is dependent upon them being synthesized into the lumen of the endoplasmic reticulum (ER) by membrane-bound ribosomes. In addition, these proteins frequently need to be correctly glycosylated and/or cleaved from precursors to ensure they maintain maximum activity and stability ***(27)***. Thus, adenoviruses (and other mammalian viruses) may be the only systems suitable for the manufacture of antigens from many important viruses

(e.g., influenza virus, MV, and yellow fever virus), because prokaryotic cells and insect cells do not fulfill these stringent criteria.

In addition to their ability to produce authentic viral antigens, adenovirus-based vectors have a number of practical advantages. Because their genome is comprised of double-stranded DNA, they are much more genetically stable than viruses with RNA genomes. High levels of expression can be achieved by putting the desired gene under the control of powerful constitutive promoters, such as that from the IE region of the CMV genome, and up to 4kb of foreign genetic material can be inserted into the adenovirus genome, more than enough to code for all the suitable antigens from most viruses. Moreover, positive selection of the required viruses is easily achieved owing to the tight packaging constraints of the virion particle. Growth of the recombinant viruses is greatly facilitated because they do not lyse the host cell until late in infection, allowing titers as high as high as 10^5 virions per infected cell. Because infectious virus can only be made in helper cell lines, such as 293 cells, these viruses are very safe to handle, with no danger of infecting other cell types or colonizing laboratory workers.

2.4.3. Disadvantages of Defective Recombinant Adenoviruses

Although these viruses have many advantages over other recombinant DNA vectors, they may not be suitable for every application. Packaging constraints in the virion mean that whole genomes, even of small viruses such as poliovirus, cannot be incorporated into a single vector A more serious problem is that most adults and many children have encountered an adenovirus infection and would have antibodies against many of the common serotypes. Because "breakthrough" of some adenovirus proteins has been observed in some cases *(28)*, well-chosen control antigens or some form of antigen purification may have to be included in some experimental protocols. If this proves to be a major problem, the researcher could switch to the less common serotypes, of which there are at least 40, in which to produce the antigen of choice.

2.5. Pox Viruses as Genetic Vectors

The simultaneous announcement by Moss *(29)* and Paoletti *(30)* and their colleagues that vaccinia virus could be used to express foreign viral genes was greeted with enthusiasm and excitement, as researchers worldwide realized these experimental systems could be used to express and study eukaryotic genes and their products without the limitations eukaryotic biologists had experienced when employing prokaryotic DNA-manipulation systems. Although hailed initially as a revolutionary method for producing new vaccines, based on its recent success in eradicating smallpox, genetically engineered vaccinia viruses rap-

idly grew in popularity as powerful tools to study the expression, control, structure, and immune response to both viral and eukaryotic cellular proteins.

Cloning procedures resemble those used for adenoviruses in that transfer vectors are constructed containing the genes of interest, and these are transferred into cells co-infected with a vaccinia virus containing a selection marker. Selection of recombinants is, therefore, achieved by genetic markers and not by packaging constraints, as with the adenovirus system.

2.5.1. Advantages of Using Poxviruses to Produce Diagnostic Antigens

The genome of vaccinia virus is a linear, double-stranded DNA molecule, which is genetically stable and resistant to the ubiquitous RNases, which hamper genetic manipulation with systems based on RNA viruses. In addition to their genetic stability, these viruses are very resistant to environmental stresses, largely owing to their complex membrane structure. Pox viruses are very self-sufficient and, unlike several other DNA viruses, replicate in the cytoplasm of the host cell, because they contain genes coding for the enzymes for nucleic-acid metabolism, both RNA and DNA polymerases and enzymes to cap, methylate and polyadenylate messenger RNA ***(31)***. Vaccinia virus does, however, have one shortcoming in this area: it does not contain machinery for mRNA splicing. Although this is a severe impediment to the study of the biology of mammalian genes, it is not normally a significant disadvantage to the production of diagnostic antigens because genes containing unspliced messengers can be artificially constructed. Thus, because poxviruses contain a virtually complete DNA replication and transcription package, they are less susceptible to the limitations sometimes encountered with other DNA virus-based recombinant systems (such as those based on adenoviruses), arising from their dependence on the host cell to provide essential components such as transcription factors, which can be in short supply in some cell types.

Not only do these viruses contain many components of their own replication machinery, they also contain several genes that code for immune modulators. Although these are important for the virus's life cycle *in vivo*, they are unnecessary in vitro and can therefore be deleted, enabling large lengths of foreign genetic material to be incorporated in their place.

A further major advantage of using poxviruses as gene vectors is that as they replicate in the cytoplasm of eukaryotic cells, foreign-messenger RNAs transcribed from them can be translated in an authentic manner, using the cells' full range of protein synthetic and modification machinery. This is especially important for many viral diagnostic antigens that are membrane-bound, glycosylated, secreted, phosphorylated, cleaved from a precursor molecule, or required to undergo some other post-translational modification to be fully functional.

2.5.2. Disadvantages of Poxvirus-Based Vectors

Although vaccinia virus-based vectors have proved a powerful research tool and have become very popular over the decade and a half since their introduction, they display some properties that may make them unattractive for the large-scale production of diagnostic antigens. The main problem lies in the relative reactogenicity of vaccinia itself and the consequent need, at least until recently, for laboratory and process workers to be vaccinated. In the absence of smallpox and the availability of other vector systems, vaccinia-based vectors have not proved as popular in the commercial world as they have in the laboratory. The safety issue is further complicated because these viruses have a very large genome, with most of its genes not yet having functions assigned to them. This uncertainty in understanding vaccinia's basic structure may make some manufacturers more comfortable with viruses with a smaller number of genes, all with a known function. However, recent research has given rise to attenuated strains of vaccinia and to the use of animal poxviruses as gene vectors *(32)*. These recent developments may therefore lead to a resurgence in interest in using these popular vectors for commercial purposes.

The second most frequently encountered problem is that although these systems more often than not produce a good-quality product, the quantity produced can sometimes be too small for commercial purposes. Another problem is a practical one associated with the production of the mutant viruses. Because the genome is so large recombinants can only be produced by random recombination events in *vivo*, and because this is a rare event, the number of desired new viruses produced is small. Because the packaging requirements of the virus particle are not as stringent as other DNA viruses (e.g., adenoviruses), an artificial selection mechanism must be introduced. This is normally achieved by inserting the chosen foreign genetic material in the thymidine kinase (tk) gene thus creating a tk(-) mutant, which then needs to be selected in the presence of 5-bromodeoxyuridine. If other sites for genetic insertion are required, then a separate selection mechanism must be derived for each one.

2.6. Retrovirus Vectors as Vectors for Diagnostic Antigen Production

Although retrovirus-based vectors have been developed primarily as tools for gene therapy, several features of their biology make them attractive as sources of diagnostic antigens. The implication of these viruses in cancer and the more recent burgeoning interest in lentiviruses such as HIV has resulted in a deeper knowledge of the structure, replication, and pathogenesis of this group of viruses than of almost any other virus family. Ironically, in spite of intense effort, none of the diseases associated with retrovirus infection have been brought under control.

2.6.1. Advantages of Retrovirus Vectors

Because retroviruses need no *de novo* gene expression for infection, reverse transcription, integration, and mRNA transcription to occur, all the genetic elements coding for proteins can be deleted from the viral genome, allowing up to 9kb of foreign DNA to be inserted into the viral vector. These artificial virus genomes can, of course, only be replicated in helper cell lines (also known as packaging cell lines), which provide the proteins for viral replication and virion structure, i.e., gag, pol, and env. The second useful feature of these vectors is that they integrate into the chromosome of the host cell from which mRNA coding from the foreign proteins can transcribed. Thus, a stable cell line, expressing the desired diagnostic antigen, can been generated and cultured *ad infinitum* without the necessity of initiating a virus infection every time a fresh batch of antigen is needed. Moreover, because the foreign gene is transcribed in the nucleus of the host cell, both mRNA and the proteins it encodes can be subjected to all the normal cellular-processing machinery available to a eukaryotic viral protein. Thus, an authentic antigen can be produced that has undergone the post-translational modifications such as glycosylation and phosphorylation, which are frequently needed for maximum activity and stability (reviewed in **ref.** *33*).

2.6.2. Limitations of Retroviral Vectors

The main difficulty in using retroviral vectors is the unpredictability of the chromosome integration event, without which transcription and antigen production cannot occur. Because the chromosomal location of the integration site seems to be essentially a random event, the recombinant cell line thus produced can be unstable, or grows very slowly. In addition, the cells may have lost important cellular growth functions, which may make them difficult to adapt to large-scale culture. In most cells, integration of the viral genome occurs only once and thus with only a single copy of the foreign gene in each cell, levels of antigen production can be low. A second limitation of these vectors is that although they can accommodate up to 9kb of inserted nucleic acid—significantly more than other vectors, such as those based on adenoviruses—they can still be too small to accommodate exceptionally large genes or particularly long regulatory or promoter elements. In practice, the upper limit for the size of genetic material that can be accommodated is significantly less than this, because a reduction in virus titer is frequently observed as the upper packaging limit of the virion is reached. Other aspects of the biology of these viruses also makes them unsuitable for some purposes. Virus titers in helper cell lines rarely exceed 10^6 pfu/mL, which compares unfavorably with adenoviruses that can produce 10^8–10^9 pfu/mL. Low viral titers are frequently the result of the instability of

virions produced in the helper cell lines, although significant improvement has been observed using "pseudotypes" from a rhabdovirus, vesicular stomatitis virus *(34)*. Safety issues have been raised over the use of helper cell lines, mainly concerning the possibility of recombination events giving rise to replication-competent virus *(35)*. However, recent helper cell lines with different constructs for different proteins need three separate recombination events to occur to support virus replication, and the statistical probably of this happening is calculated to be too low to cause a major hazard.

3. Conclusions

The recent development of high-throughput robotic systems has meant that viral diagnosis is entering an exciting new phase of rapid and cheap assays The development of carefully defined and robust analytical reagents, through one or more of the recombinant DNA technologies reviewed in this chapter, will provide materials well-suited to these powerful analytical tools. Recombinant DNA technology has now reached a level where any protein sequence can be synthesized in a variety of in vitro systems to give a reagent with the desired characteristics. The challenge still remains, however, to discover which amino acid sequence, with which post-translational modifications, will give the best analytical result. In addition, the wide genetic variation and rapid mutation observed in many virus types, especially those with RNA genomes, will keep researchers busy for many years to ensure the patient is given the appropriate treatment. And when we think we understand those viruses currently with us, new ones will emerge (currently at a rate two or three a year) to challenge us.

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NASBA

A Method for Nucleic Acid Diagnostics

Dianne van Strijp and Pierre van Aarle

1. Introduction

Nucleic acid sequence-based amplification (NASBA) is a primer dependent, homogeneous, isothermal amplification process for the detection of RNA *(1)*. It can result in 10^9- fold amplification of a specific RNA sequence and offers the unique possibility to amplify RNA in a background of genomic DNA. Detection of RNA can be used to monitor gene expression or cell viability or can be a prognostic indication for virus replication and production. Amplification of RNA beyond the detection limit is easily achieved because of the often high copy numbers of RNA targets in a cell. NASBA already has been used for the detection of human immunodeficiency virus (HIV) *(2–11)* hepatitis C virus (HCV) *(12,13)* and cytomegalovirus (CMV).

The entire process using the NASBA amplification method basically consists of three parts:

1. Nucleic Acid Isolation (**Subheading 1.1.**).
2. Nucleic Acid Amplification (**Subheading 1.2.**).
3 Detection of Amplification product (**Subheading 1.3.**).

1.1. Nucleic Acid Isolation

For nucleic acid isolation a procedure *(14)* is used in which cells are lysed in a buffer containing guanidinium isothiocyanate (GuSCN). Simultaneously, released nucleic acid (DNA and RNA) is bound to activated silicon dioxide particles, and RNase, DNase, and other proteins are denatured by this chaotropic reagent. Subsequently, the silica particles are washed several times in specific washing buffers. After drying the silica particles, bound nucleic acid

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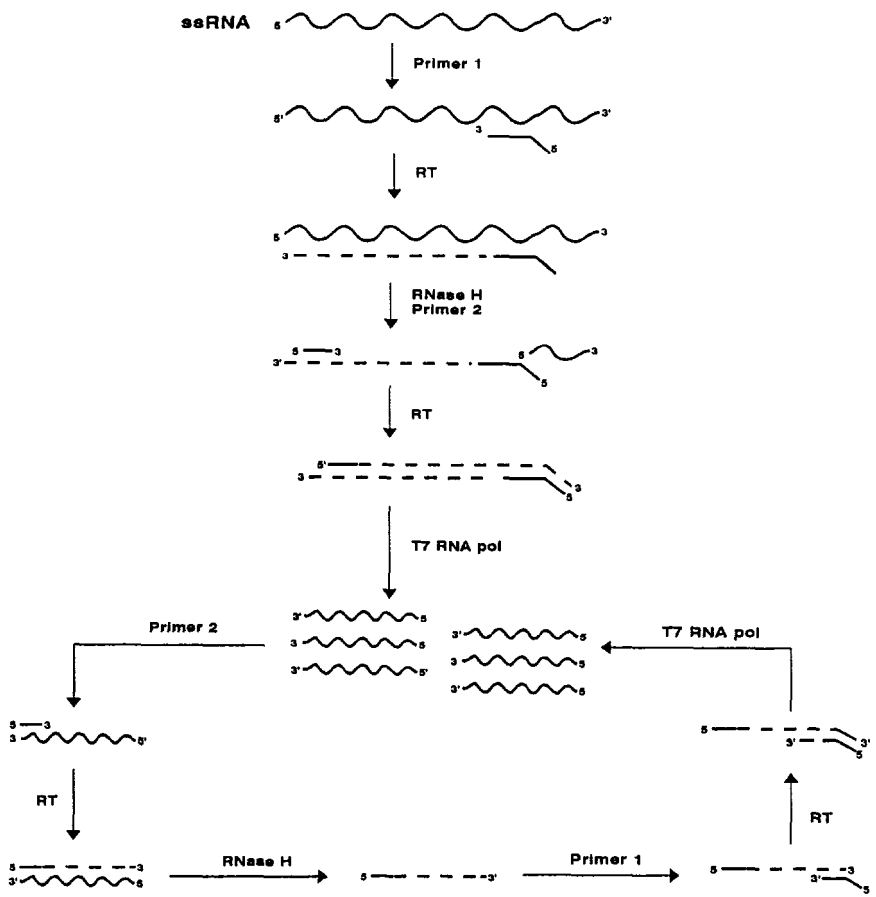

Fig. 1 The amplification principle of NASBA. Broken lines represent newly synthesized DNA and wavy lines represent RNA. RT, Reverse Transcriptase; and T7 RNA pol, T7 RNA polymerase For further explanation *see* **Subheading 1.2.**

is eluted in RNase-, DNase- and protease-free water. A part of this nucleic acid solution can be used directly for amplification.

1.2. Nucleic Acid Amplification

The amplification principle of NASBA is shown schematically in **Fig. 1.** The reaction mixture contains three enzymes–avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H and T7 RNA polymerase–and two target specific primers. Primer 1 consists of a 3' terminal sequence that is target specific and a 5' terminal T7 promoter sequence that can be recognized by T7 RNA polymerase. Primer 2 is entirely a target specific sequence.

The NASBA process starts with the annealing of primer 1 to the target RNA followed by extension of primer 1 by AMV-RT, creating a copy DNA (cDNA) of the RNA template and forming an RNA:cDNA hybrid. The RNA in this hybrid is degraded by RNase H, which allows annealing of primer 2 to the single-stranded cDNA. Primer 2 is elongated by AMV-RT thereby forming a DNA molecule with a functional double-stranded T7 promoter. T7 RNA polymerase recognizes the T7 promoter and generates multiple copies of antisense RNA (-RNA). One double-stranded DNA molecule can give rise to 100–1000 RNA molecules. Now the amplification reaction enters the cyclic phase. Each new antisense RNA molecule can in its turn again be converted to a cDNA containing a functional, double stranded, T7 promoter in a similar way, except that primer annealing and extension occur in reverse order because the newly generated RNA template is opposite in orientation to the original target. Again, many copies are generated from each RNA target that reenters the reaction resulting in exponential amplification. All enzymes in this process work simultaneously at 41°C.

1.3. Detection of Amplification Product

Amplified products can in principle be detected on ethidium bromide-stained agarose gels, but often background amplification of nonspecific products and poor resolution of short RNA fragments hampers interpretation of the results. However, since the main amplification product is single-stranded RNA (-RNA), it can readily be diagnosed by hybridization with sequence-specific probes.

2. Materials

2.1. Nucleic Acid Isolation

1. Silica (silica suspension in 0.1 *N* HCl): Suspend 60 g silica (SiO_2 Sigma, St. Louis, MO); S5631; MW 60.08; type: ± 80% particle size 1–5 µm) in double-distilled H_2O (dd H_2O) and adjust the volume to 500 mL in a cylinder having a diameter of 5 cm (the height of the aqueous column is approx 26 cm). After 1*g* sedimentation for 25 h at room temperature remove the supernatant until 70 mL are left. Add dd H_2O up to 500 mL again and resuspend the particles by shaking the cylinder. After 1*g* sedimentation for 5 h remove supernatant until 60 mL are left. After addition of 600 µL 32% (v/v) HCl resuspend the particles by vortexing. Autoclave the suspension at 121°C for 20 min. Store aliquots at room temperature.
2. 0.2 *M* Ethylenediaminetetraacetic acid (EDTA), pH 8.0· Dissolve 37.2 g EDTA and 4 g NaOH in 450 mL dd H_2O. Adjust the pH to 8.0 with NaOH and adjust the final volume to 500 mL with dd H_2O. Autoclave the solution at 121°C for 20 min.
3. L2 buffer: Dissolve 12.1 g Tris in 800 mL dd H_2O. Adjust the pH to 6.4 with HCl (37%) Adjust the final volume to 1 L with dd H_2O. Autoclave the solution at 121 °C for 20 min..
4. Wash buffer: Add 120 g GuSCN (Fluka no. 50990, MW 118.16, Fluka Chemie, AG., Buchs, Switzerland) to 100 mL L2 buffer (total volume about 200 mL). Dissolve at 60°C. Store at room temperature and protect from excessive light.

5. Lysis buffer: Add 120 g GuSCN to 100 mL L2 buffer Dissolve at 60°C Add 22 mL 0.2 *M* EDTA (pH 8.0) and 2.6 g Triton X-100. pH should be 7–7.4.
6. 70% Ethanol.
7 Acetone.
8. Sterile water (DNase-, RNase-free).

2.2. Nucleic Acid Amplification

1. Sterile water (RNase-, DNase-free).
2. 1 *M* Tris-HCl, pH 8.5 (Sigma, Trizma T-8524).
3 1 *M* $MgCl_2$ (Sigma, M-1028, solution).
4. 4 *M* KCl (Sigma P-9541).
5 1 *M* Dithiothreitol (DTT) (Sigma, D-9779).
6 100 m*M* Inosine-5'-triphosphate (ITP) (Boehringer Mannheim, Mannheim, Germany, 106 747).
7. 100 m*M* dNTP and rNTP solutions (Pharmacia, Uppsala, Sweden).

dATP (cat. no. 27-2050-01)	ATP (cat. no 27-2056-01)
dCTP (cat. no. 27-2060-01)	CTP (cat. no. 27-2066-01)
dGTP (cat no. 27-2070-01)	GTP (cat. no 27-2076-01)
dTTP (cat. no. 27-2080-01)	UTP (cat. no. 27-2086-01)

8. AMV-RT (Seikagaku, Rockville, MD, 120248, or Life Sciences, St. Petersburg, FL, LME704)
9. T7 RNA polymerase (Pharmacia, 27-0801-02).
10 RNase H (Pharmacia, 27-0894-02)
11. 20 mg/mL Bovine serum albumin (BSA) (Boehringer, 711 454, solution).
12. Sorbitol (Sigma S-6021).
13 Dimethyl sulfoxide (DMSO) (Sigma, D-5879).
14. 5X NASBA buffer.

Ingredient	Volume, µL	Final conc. in NASBA (m*M*)/ (1X NASBA buffer)
Water	27 5	
Tris-HCl (1 *M*), pH 8.5	200	40
$MgCl_2$ (1 *M*)	60	12
KCl (4 *M*)	87 5	70
DTT (1 *M*)	25	5
dATP,dCTP,dGTP,dTTP (100 m*M*) (50 µL each)	4 × 50	1
ATP,UTP,CTP (100 m*M*) (100 µL each)	3 × 100	2
GTP (100 m*M*)	75	1 5
ITP (100 m*M*)	25	0.5
Total		1000

Remark. final volume is 1 mL.
Store at –20°C in aliquots (100–200 µL).

15. 5X Primer mix(*see* **Note 20**): 75% DMSO final concentration in NASBA is 15%), 1 μ*M* purified primer 1 and primer 2 (final concentration in NASBA is 0.2 μ*M*). Use sterile water to adjust the final volume.
16. 4X Enzyme mixture (*see* **Notes 1–6**):

Water	485.2 μL	
Sorbitol (4.5 *M*)	333.3 μL	(375 m*M*/reaction)
BSA (20 mg/mL)	21 μL	(2.1 μg/reaction)
RNase H (0.87 U/μL)	18.4 μL	(0 08 U/reaction)
T7 RNA polymerase (70 U/μL)	91.5 μL	(32 0 U/reaction)
AMV-RT (25 3 U/μL)	50.6 μL	(6.4 U/reaction)
Total	1000 μL	

2.3. Detection of Amplification Product

1. [γ^{32}P]- or [γ^{33}P]-labeled probes:

Water	13 μL
10X One-Phor-All Buffer (Pharmacia)	2 μL
Probe (10 μ*M* stock solution)	1 μL
T4 Polynucleotide kinase (Pharmacia; ± 10 U/μL)	1 μL
[γ^{32}P]- or [γ^{33}P]-ATP (Amersham, Buckinghamshire, UK; 10 μCi/μL)	3 μL
Total	20 μL

   Incubation: 15–30 min at 37°C.
2. 50X TAE buffer: 2 *M* Tris-acetate, 0.05 *M* EDTA pH 8.0.
3. Pronarose (Hispanagar SA, Burgos, Spain) or Nusieve agarose (Nusieve Agarose 3.1 (FMS, Rockland, ME).
4. 10 mg/mL Ethidium bromide solution.
5. 0 1 *M* Sodium phosphate buffer (NaP_i), pH 6.0–7.0.
6. Layermix:

50% Glycerol	5.0 mL (25%)
0 1 *M* NaP_i, pH 6–7 (solution 5)	1.0 mL (10 m*M*)
Saturated bromophenol-blue solution (Sigma, B-5525)	0 025 mL
Saturated xylenecyanol/FF solution (Sigma, X-4126)	0.025 mL
Sterile water	3.95 mL

7. 20X SSC: 3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0–8.0.
8. 2X SSC (10X diluted solution 7).
9. 100X Denhardt's: 20 g/L Polyvinylpyrolidone (PVP), 20 g/L BSA, 20 g/L Ficoll (type 400).
10. 20% Sodium dodecyl sulfate (SDS).
11. Hybridization mixture. 5X SSC, 7% SDS, 20 m*M* NaP_i, pH 6.0–7.0, 10X Denhardt's
12. Wash buffer: 3X SSC, 1% SDS
13. Zèta-Probe: nucleic acid blotting membrane (Bio-Rad Laboratories, Hercules, CA; 162-0156).

3. Methods

Precautions *(see* **Notes 7–13**).

3.1. Nucleic Acid Isolation

1 Mix in a 1.5-mL Eppendorf tube 900 µL lysis buffer and 100-µL sample (plasma, blood, serum, cells, etc.), vortex *(see* **Note 14**)
2 Add 70 µL silica suspension and vortex.
3 Incubate 10 min at room temperature and vortex regularly, e.g , every 2 min.
4 Spin down the silica for at least 15 s at maximum speed *(see* **Note 15**) and remove the supernatant.
5 Add 1 mL wash buffer and dissolve the silica pellet by vortexing Washing procedure Wash the silica pellet by adding 1 mL washing solution and vortex until the pellet is resuspended Centrifuge for 15 s at maximum speed and remove the supernatant
6. Spin down the silica and wash the pellet once again with wash buffer
7 Wash the silica subsequently with 70% ethanol (twice) and acetone (once).
8 Dry the pellet in a heating block for 10 min at 56°C *(see* **Note 16**)
9 To elute the nucleic acid, add 100 µL water, vortex, and incubate for 10 min at 56°C. Vortex once after 5 min
10 Centrifuge 2 min at maximum speed and transfer about 80 µL nucleic acid solution to a clean Eppendorf tube
11. Centrifuge the nucleic acid again for 2 min at maximum speed (to remove all silica) and transfer about 70 µL nucleic acid solution to a new Eppendorf tube *(see* **Note 17**). Store at –70°C

3.2. Nucleic Acid Amplification

1. Prepare a premix for a number of reactions *(see* **Note 18**).

Reagent	Volume/reaction, µL
Water	2
5X NASBA buffer *(see* **Note 19**)	4
5X Primer mix *(see* **Notes 20** and **21**)	4
Total volume	10

2 Mix the premix by vortexing.
3. Aliquot the premix into Eppendorf tubes in portions of 10 µL.
4 Add desired nucleic acid sample volume (in this case 5 µL)
5 Mix by tapping or by pipeting.
6 Incubate at 65°C (±1°C) for 5 min *(see* **Note 22**)
7 Cool to 41°C (±0.5°C) for 5 min *(see* **Note 22**).
8. Add 5 µL enzyme mixture and immediately put the tube back at 41°C **Caution:** The enzyme mixture may not be vortexed!
9 Mix by gently tapping the tubes

10. Incubate at 41°C for 5 min (*see* **Note 22**).
11 Centrifuge to collect the sample in the bottom of the tube.
12 Incubate at 41°C for 90 min (*see* **Note 22**).
13 Store the amplification products at –20°C.

3.3. Detection of Amplification Product

3.3.1. Pronarose or Nusieve Gel Electrophoresis and Blotting

1. Analyze 5 µL of the amplification product (with 1 µL layermix) on a 2% Pronarose gel or 3% Nusieve gel (with 0.5 µg/mL ethidium bromide in the gel) in 1X TAE (*see* **Note 23**).
2. Electrophorese about 20 min at 150 V and analyze the gel using UV light (*see* **Note 24**)
3. Blot the gel in 2X SSC on a vacuum-blotting system for 1 h on Zèta-probe blotting material.
4 After blotting, crosslink the amplification product on the membrane, using UV light for 2 min

3.3.2. Radioactive Hybridization

1 Prewarm the hybridization mixture in a water bath at 50°C
2 Place the blot in a tray and add the warm hybridization mixture.
3. Add 10–20 µL of the [γ^{32}P]- or [γ^{33}P]-labeled probe; mix gently (*see* **Note 25**)
4 Incubate overnight in a shaking water bath at 50°C
5 Wash the blot 3 times for 5–10 min at 50°C with wash buffer.
6 Dry the blot between two tissues and pack the blot in foil
7 Make an autoradiogram Exposure is dependent on the amount of radioactive probe on the blot (3 h at room temperature, or overnight at –70°C).

4. Notes

1. The concentration of enzymes in the stock solutions can vary Adjust the volumes added to the enzyme mixture to achieve the final concentrations stated.
2. Prepare this enzyme mixture on ice, mix by tapping and spin down briefly
3 **Caution:** The enzyme mixture must not be vortexed!
4. Store the enzyme mixture at –70°C in aliquots (for example, 50–60 µL, about 10 reactions) in Eppendorf tubes.
5. After thawing a tube of enzyme mixture do not freeze any remainders for reuse
6. It is also possible for each series of reactions to prepare fresh enzyme mixture (make excess enzyme mixture for two extra reactions!)
7. NASBA can give 10-fold RNA amplification, enabling detection of low copy numbers of RNA (10–100 molecules). Therefore, care must be taken to prevent contamination
8 To avoid contamination, perform nucleic acid isolation, amplification and detection in separate laboratories/fume hoods. There should be no transport of materials/buffers from detection to isolation and amplification laboratories Amplification

setup should be performed under laminar flow, down flow or in a fume hood. The amplification reaction itself should not be incubated in the same lab as the reaction setup area but preferably in the detection room. Air from the detection area must not be allowed to enter the two other labs.

9. Reagents and buffers for nucleic acid isolation and amplification should be prepared in a nucleic acid-free room.
10. Use pipets with aerosol-resistant tips for nucleic acid isolation and amplification
11 During preparation all tubes should be kept closed when not in use
12 While working with clinical materials during nucleic acid isolation, gloves should be used; frequently change gloves
13 Use new pipet tips for each handling
14. Lysate can be stored at –70°C. Use maximally 5×10^5–10^6 cells per isolation.
15. Maximum speed for an Eppendorf centrifuge usually is 10,000*g*
16 Acetone can disturb the amplification reaction. Therefore, be sure to dry the pellet completely before eluting the nucleic acid. Otherwise, dry longer. To remove the acetone, for example, first use a 1-mL pipet tip and then a 100-µL pipet tip.
17. Silica can also disturb the amplification reaction Therefore, be sure that the sample for amplification does not contain silica. When there is still a little silica left in the sample, centrifuge again just before taking a sample.
18 If the nucleic acid sample volume is decreased (<5 µL), adjust the water volume accordingly, such that the total volume remains 15 µL when the nucleic acid is added (in this example 5 µL sample is used).
19. Before use be sure the 5X NASBA buffer is completely thawed and vortex until the buffer is clear.
20 Primers should be purified so that only full-length primers are used for amplification. For example, purify the primers on a 20% polyacrylamide/7 *M* urea gel. Purified primers can also be obtained commercially (e.g., PAGE-purified) For choosing NASBA primers (*see* **Note 21**)
21. Choosing NASBA primers (*see* also **Fig. 2**): A characteristic of the NASBA primer 1 that distinguishes it from the primers used in other amplification processes, such as, the polymerase chain reaction (PCR), is the presence of a T7 promoter sequence This region consists of a highly conserved 25-nt sequence (5'AATTCTAATACGACTCACTATAGGG-3') positioned at the 5' end of primer 1. The hybridizing sequence of primer 1 is antisense After the 3 Gs of the T7 promoter it is important to have a purine-rich region (A or G) in the (antisense) target sequence (about 4 nt) Cs or Ts give a higher risk for abortive transcription. When there are no Gs or As available in the sequence, insertion is possible after the three Gs of the T7 promoter (e.g., AGAG). Primer 2 is a sense primer The hybridizing part of primers 1 and 2 should be 20–25 nt. Primers that have an A at their 3' ends give best results. A G/C content of 40–60% is recommended but tracts of the same nucleotide should be avoided. Prevent internal structures/loops in primers. Primers (1 and 2) with more than 2 complementary nucleotides at

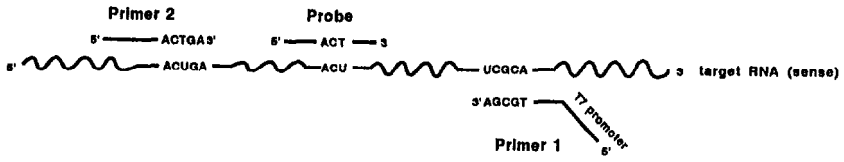

Fig. 2. Choosing NASBA primer 1 (antisense hybridizing sequence), primer 2 (sense sequence) and probe (sense sequence). For further explanation *see* **Note 21**.

their 3' ends may result in primer dimerization. For optimal amplification, the total length of the product should be between 120–250 nt. It is recommended to select and test more than one primer pair for each target to find one that gives the desired performance.

22. For incubation at 41°C and 65°C, heating blocks are used To start the amplification reaction the 41°C heating block can be used. After a minimum of 5 min incubation the tubes can be transferred to a 41°C water bath in the detection room.
23. Pronarose or Nusieve agarose gels give a better resolution for small RNA products than normal agarose gels.
24. Although the main amplification product is single-stranded RNA, the amount is so high that detection with ethidium bromide is possible.
25. Choosing a probe (*see also* **Fig. 2**): A probe for radioactive detection should be 20–25 nt long (melting temperature above 50°C). The probe (sense) should hybridize to the main amplification product (antisense RNA) Choosing the probe in the center of the product or near primer 2, ensures detection of full-length products as much as possible.

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26

Oligonucleotides

Stina Syrjänen

1. Introduction

During the past 30 years, several strategies have been used to synthesize DNA. Moreover, it was not possible to utilize automated DNA synthesis until the phosphate triester method using phosphoramidite reagents was introduced *(1,2)*. Since then, chemically synthesized oligonucleotides have been powerful tools in the molecular biologist's repertoire. The availability of high-quality oligonucleotides of defined sequence has had a significant impact on the techniques and applicability of molecular biological methods even in routine diagnosis of infectious diseases. Currently, most institutions have access to automated DNA synthesis facilities. Oligonucleotides with or without modifications are also commercially available from several companies with a reasonable price worldwide. Oligonucleotides can be used as probes in different hybridization methods or as primers, linkers, adaptors, gene synthons, and so on.

In diagnostic virology, the majority of oligonucleotides are used either as probes or as primers in polymerase chain reaction (PCR) methods. However, for the synthesis of oligonucleotides, a series of steps must be followed, with detailed instructions being provided by the manufacturer of the synthesizer. In this chapter, a short introduction to the chemistry of oligonucleotide synthesis is given. The chapter includes protocols outlining the steps involved in synthesizing oligonucleotides, deprotecting synthesized DNA, and purification of oligonucleotides. This chapter also covers the use of oligonucleotides for the localization of viral DNA or mRNA transcripts by *in situ* hybridization (ISH), while the use of oligonucleotides for the analysis of mRNA levels by Northern blot is described elsewhere *(3,4)*. In ISH, labeled oligonucleotide probes are applied to tissue sections where they hybridize with intracellular complementary target messenger RNA or DNA sequences that have been preserved during the tissue preparation. The hybrids formed, e.g., at the sites of viral DNA or

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viral gene expression are localized in the tissue by the probe label. Also covered are the factors involved in the formation and melting of oligonucleotide/target DNA or RNA duplexes. Another important aspect for routine diagnosis is that oligonucleotides confirm a high degree of consistency to a given set of experiments, as the product of one synthetic batch provides numerous aliquotes of probe, which are equivalent in concentration and specific activity.

1.1. Introduction to DNA Synthesis Chemistry

Individual nucleotides are the monomeric building blocks for chemically synthesized oligonucleotides. First, nucleotides have to be protected to allow the step-wise, sequential 3'–5' addition of each monomer (**Fig. 1**). Subsequently, these protecting groups are removed so that the synthesized and deprotected oligonucleotide is composed of the desired natural nucleotides. Nucleophilic amino functions on the bases are protected with either isobutyryl or benzoyl groups. These groups are removed at the completion of synthesis by ammoniolysis. The 5' primary hydroxyl of the deoxyribose sugar is protected with an ether moiety, which is removed by protic acids at the beginning of each coupling cycle. These trityl groups are chromophores and they can be utilized in monitoring each of the coupling steps. The 3' secondary hydroxyl function of the deoxyribose sugar is derivatized with a phosphoramidite group. The phosphate oxygen of this moiety is masked by either a methoxy or β-cyanoethoxy protecting group. The chemistry of automated DNA synthesis can be simplified in terms of the consecutive removal and addition of sugar protecting groups *(1,2)*. *Current Protocols in Molecular Biology (5)* are recommended for further reading on DNA and RNA synthesis chemistry.

2. Materials

2.1. DNA Synthesis

All reagents are provided by the suppliers of the DNA synthesizers (e.g., Amersham Pharmacia Biotech, Uppsala, Sweden; Applied Biosystems, Foster City, CA).

1. Acetonitrile (special dry)
2. Ammonium hydroxide: Purchase ammonium in 0 5–1.0-L bottles. The concentration of ammonia will decrease on repeated opening Keep at 4 °C.
3 Oxidizer: Prepare oxidizer solution with 0.1 *M* iodine in 7:2 1 (v/v/v) tetrahydrofuran/pyridine/ water Use resublimed iodine.
4. Phosporamidites. Dissolve phoshoramidites to give equal molarities of the four bases. Extremely dry acetonitrile is required to dissolve the phosporamidities
5 Synthesis columns. DNA synthesis takes place on controlled pore glass (CPG), which is porous particle, 125–177 μm in diameter. For oligonucleotides up to 50 bases use CPG with 500-Å pores, for longer oligonucleotides CPG with 100-Å pores.

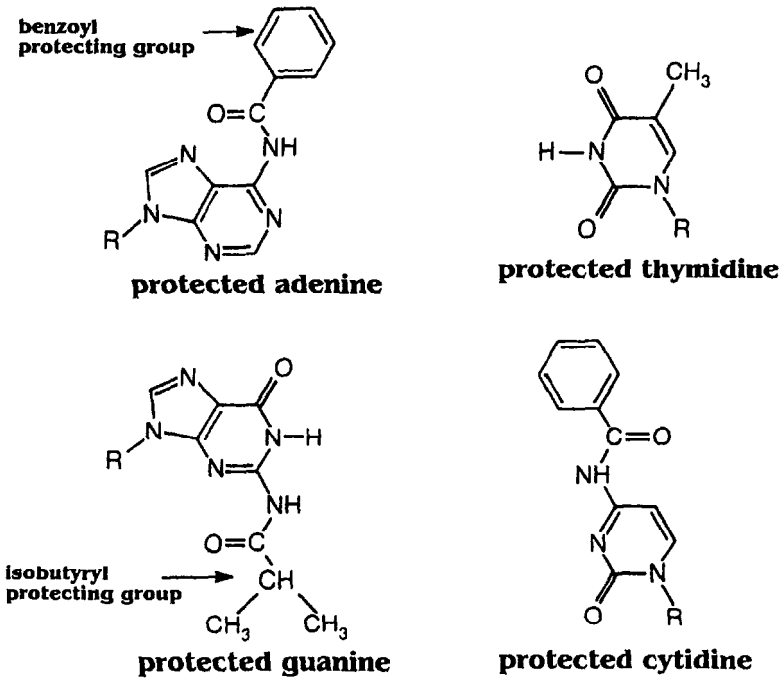

Fig. 1. Protected nucleotides as building blocks of oligonucleotides.

2.2. Deprotection of Oligonucleotides

1. Concentrated ammonium hydroxide (Amersham Pharmacia Biotech)
2. Triethylamine (Amersham Pharmacia Biotech)

2.3. Oligonucleotide Purification

2.3.1. Sizing Columns

1. Sephadex G-25 (for oligomers less than 25 bases)
2. Sephadex G-50 (for longer oligonucleotides).
3. MicroSpin G-25 Column (Amersham Pharmacia Biotech).
4. MicroSpin G-50 Column (Amersham Pharmacia Biotech)
5. ProbeQuant G-50 Micro Column (Amersham Pharmacia Biotech)

2.3.2. Polyacrylamide Gel Electrophoresis (PAGE) Stock Solutions

All stock solutions should be made up with distilled water and filtered using a 0.45-μm filter prior to use.

1. 10X TBE, pH 8.3 0.9 *M* Tris base, 0.9 *M* boric acid, 20 m*M* ethylenediaminetetraacetic acid (EDTA)

2. 40% Acrylamide stock: 38% (w/v) acrylamide, 2% (w/v) bisacryl-amide.
3. TEMED (N,N,N', N'-tetramethylethylenediamine).
4. 10% Ammonium persulfate (w/v) in water, freshly prepared.
5. Deionized formamide. perform deionization with mixed-bed ion exchange resin (3 g resin/100 mL formamide).
6. Urea (electrophoresis grade).
7. Dye mixture: 1 m*M* EDTA, 10 m*M* NaOH, 80% (v/v) formamide, deionized, 0.1% (w/v) xylene cyanol blue, 0.1% (w/v) bromophenol blue.
8. Elution buffer. 0.5 *M* ammonium acetate, 0.01 *M* magnesium acetate, 0.1% sodium dodecyl sulfate (SDS), 0.1 *M* EDTA

2.4. Labeling of the Oligonucleotides

2.4.1. 5'-End-Labeling with T4 Polynucleotide Kinase

1. [(γ-^{32}P)] ATP, [(α-^{35}S)]dATP (Amersham, Buckinghamshire, UK).
2. 10X Denaturation buffer: 0.2 *M* Tris-HCl, pH 9.5, 10 m*M* spermidine, 1 m*M* EDTA
3. 10X Kinase buffer: 0.5 *M* Tris-HCl, pH 9.5, 0.1 *M* $MgCl_2$, 50 m*M* dithiothreitol (DTT), 50% glycerol (Amersham Pharmacia Biotech)
4. Polynucleotide kinase (PNK), 10 U/mL (Amersham Pharmacia Biotech).
5. PNK dilution buffer: 50 m*M* Tris-HCl, pH 7.5, 10 m*M* 2-mercaptoethanol, 0.05% DNase-free bovine serum albumin (BSA) (Amersham Pharmacia Biotech)

2.4.2. 3'-Labeling with Terminal Deoxynucleotidyltransferase (with [α-^{32} P]) dATP)

1. [(α-^{32}P)]dATP and [(α-^{32}P)]dCTP (Amersham)
2. Terminal deoxynucleotidyltransferase (TdT) 10 U/mL (Amersham).
3. 10X Tailing buffer: 1 *M* potassium cacodylate, 250 m*M* Tris-base, pH 7.6, 10 m*M* $CoCl_2$, 2 m*M* DDT.

2.4.3. Oligonucleotide 3'-End-Labeling with Digoxigenin (DIG)-ddUTP, Biotin-ddUTP, or Fluorescein-ddUTP, or DIG-dUTP, Biotin-dUTP, or Fluorescein-dUTP

1. DIG-dUTP, biotin-dUTP, DIG-ddlUTP, or biotin-dUTP (Boehringer Mannheim, Mannheim, Germany).
2. 5X Reaction buffer: 1 *M* potassium cacodylate, 0.125 *M* Tris-HCl, 1.25 mg/mL BSA, pH 6.6 (25°C)
3. Transferase buffer: 200 m*M* potassium cacodylate, 1 m*M* EDTA, 200 m*M* KCl, 4 m*M* 2-mercaptoethanol, 50% (v/v) glycerol, pH 6.5 (4°C)

2.5. Purification of the Labeled Probe

1. Sephadex G-25 DNA Grade (Amersham Pharmacia Biotech)
2. Sephadex G-50 DNA Grade (Amersham Pharmacia Biotech).
3. Sephadex G-25 column buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 0.1 *M* NaCl.
4. tRNA (St. Louis, MO).

2.6. In Situ *Hybridization*

2.6.1. Pretreatment of Slides

1. Poly-D-lysine (Sigma 7886).
2. Organosilane (γ-aminopropyltriethoxy-silane, Sigma #3648).
3. Acetone.

2.6.2. Fixation of Samples

1. Liquid nitrogen.
2. Isopentane C_5H_{12} (Fluka 59075, Bucks, Switzerland).
3. Embedding medium for frozen tissue specimens (OCT Compound) (Sakura Finetetz, Torrance, CA)
4. 4% Paraformaldehyde: Stock solution A: 0.2 *M* NaH_2PO_4; Stock solution B: 0.2 *M* Na_2HPO_4. To make 0.2 *M* phosphate buffer, pH 7.4, add 60 mL stock solution A, 250 mL stock solution B, and adjust to 600 mL with distilled H_2O. Prepare buffered 4% paraformaldehyde by mixing 12 g paraformaldehyde and 300 mL 0.2 *M* phosphate buffer, pH 7.4. Dissolve at 70°C by using a magnetic stirrer. The fixative can be used for 2 wk, when stored at 4°C.
5. Carnoy's fixative: 60% chloroform, 30% ethanol, 10% acetic acid.

2.6.3. Pretreatment of the Sections

1. HCl.
2. Proteinase K (Boehringer Mannheim, GmbH, Mannheim, Germany).
3. Triton-X-100 (BDH, Poole, Dorset, UK).

2.6.4. Hybridization

1. Hybridization mixture (radioactive probes): 600 m*M* sodium chloride, 50 m*M* sodium phosphate buffer, pH 7.0, 5.0 m*M* EDTA, 0.02% Ficoll, 0.02% BSA, 0.02% poly (vinylpyrrolidone), 0.1% herring sperm DNA, and 40% formamide (deionized by adding 3 g resin to 100 mL formamide, incubate, and filter). Reagents are dissolved in distilled water with gentle heating, the solution is then filtered, and the formamide added
2. Hybridization mixture (nonradioactive probes): 25% formamide, 4X SSC, 50 m*M* Na_2PO_4/Na_2HPO_4, pH 7.0, 1 m*M* EDTA, carrier DNA/RNA (1 μg/μL each), probe (1 ng/μL), 5X Denhardt's
3. 4X SSC (standard saline-citrate). 0.6 *M* sodium chloride, 0.06 *M* sodium citrate in distilled water.
4. 50X Denhardt's: 1% polyvinylchloride, 1% pyrrolidone, 2% BSA

2.6.5. Liquid Emulsion Autoradiography

1. Plastic slide holders (e.g., peel-a-way slide holder 48436-107, Algol, Finland).
2. A light-tight box for slide holders
3. Kodak nuclear track emulsion (Kodak No. 165-4441).
4. 0.6 *M* Ammonium acetate.

2.6.6. Detection of Biotinylated Hybrids

1. Streptavidin-alkaline phosphatase (Amersham).
2. SSC: 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7 0. Make a 20X stock solution
3. Buffer A. 0 1 *M* NaCl, 0.1 *M* Tris-HCl, pH 7.5, 0 05% Triton X-100
4. Buffer B: 0 1 *M* NaCl, 0.1 *M* Tris-HCl, pH 9.5
5. 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) (Sigma)
6. Nitroblue tetrazolium (NBT) (Sigma).
7. Dimethyl-formamide (Sigma).

2.6.7. Counterstaining

1. Gill's hematoxylin (Sigma).
2. Carbol fuchsin: 2.5 mL phenol, 5.0 mL absolute ethanol, 0 5 g fuchsin (basic), 50 mL distilled H_2O. Filter before use and dilute to 1 200

3. Methods

3.1. Synthesis of Oligonucleotides

Presently, synthesis of oligonucleotides or their several analogs is being carried out on commercially available automated DNA synthesizers using phosphoramidite or H-phosphonate chemistry, using CPG as a solid support *(6,7,8)*. Conditions have been optimized to carry out oligonucleotide synthesis at quantities up to 600 µmol. Currently, the size limit of synthesizing usable amounts of an oligonucleotide is 150 bases. Progress in the field of antisense oligonucleotides has created the need for scale-up of oligonucleotide synthesis from milligram to multigram quantities. Recently, Padmapriya et al. *(9)*, described a protocol where 12 g of purified 25-mer oligodeoxyribonucleotide could be synthesized

Detailed protocols to synthesize oligonucleotides are provided by manufacturers of the automated synthesizers. The reagents needed include synthesis columns, nucleoside phoshoramidities, tetrazole, capping reagents and ancillary reagents. The coupling efficiency of nucleoside phosphoramidite during the synthesis is automatically monitored by trityl assay. A trityl cation is released from the 5'-end of the growing oligonucleotide during each synthesis cycle. The amount of dimethoxytrityl (DMT) released is then measured spectrometrically *(5–7)*. Low coupling efficiency increases the percentage of impurities and makes purification more difficult. Usually, > 98% coupling efficiency (0.2–1.3 µmol scale) is considered a successful synthesis, and will not effect the choice of the purification technique. The yield of oligonucleotide is basically determined by choosing the synthesis scale. The length of the oligonucleotide plays an important role, since longer sequences mean less of the final product, more contaminants and more purification steps, which results in a lower yield. The postsynthetic work comprises detritylation, deprotection, cleavage of the synthesized oligonucleotide and purification of the final product from its byproducts (**Fig. 2**).

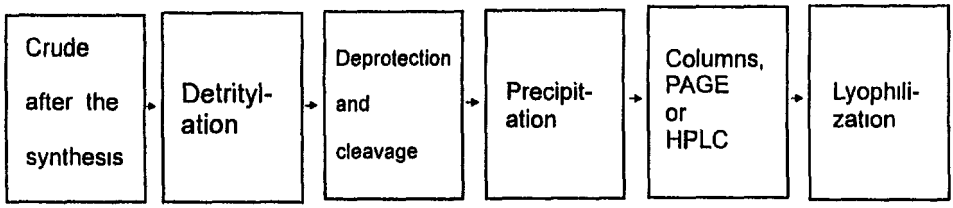

Fig. 2. Purification scheme of oligonucleotides.

3.2. Detritylation

This step includes the removal of dimethoxytrityl group (DMTr) from the 5'-hydroxyl terminal. It is usually done as the final step in the synthesis, but removal can be carried out manually at any stage in the purification procedure as well.

3.3. Deprotection and Cleavage

Deprotection is the removal of the protecting groups from the bases (amides) and phosphorous (β-cyanoethyl) using ammonia. Ammonia will also release the oligonucleotide from the support by breaking the succinate ester bridge between the 3'-hydroxyl group on the first nucleoside and the support. The following procedure removes the protecting groups completely from DNA.

1 Remove the oligonucleotide-support cassette from the column reactor Open the cassette by removing the inner frit. Transfer the support resin to a glass vial.
2 Add 25–35% ammonic solution to bring samples to 1.5–2 mL.
3 Place the sample in a heat block or oven >16 h at 55–60°C, sealing the vial tightly with parafilm Treat the eluated product with concentrated ammonia at 50–60°C (>16 h). To speed up the deprotecting reaction, raise the temperature (>5 h at 70°C). Phosphoramidites with more labile protecting groups are commercially available and deprotection can be done within 30–60 min at 70°C
4. Spin the sample for a few seconds to pool the ammonia collected in the cap.
5 Let the vial cool to room temperature before opening it. Then remove the solvent by lyophilization to dryness in Speedvac evaporator.
6 If a yellowish liquid remains or the lyophilized powder is not white, resuspend the pellet in 0.5 mL distilled water and repeat the lyophilization **(step 4)**

Depending on the quality of oligonucleotides and their applications commercially available columns (MicroSpin G-25 or NAP Amerisham Pharmacia Biotech) can be used for rapid desalting of oligonucleotides followed by deprotection in ammonia.

3.4. Oligonucleotide Purification

There are several methods to isolate synthesized DNA. The simplest method is precipitation. For more thorough purification of the crude mixture, either chromatography or electrophoresis can be chosen. The separation is based either on charge (ion exchange chromatography), on hydrophobicity (reverse phase chromatography), or on size (electrophoresis). Electrophoresis can be used to purify both short and long oligonucleotides with good results The purity needed for oligonucleotide depends on the application. Choice of the method also depends on the availability of resources and time. The length of the sequence has affects on impurities; the longer the sequence, the higher concentration of impurities and salts. For long (>75 bases) sequences, a one-step chromatographic procedure might not give satisfactory results In such cases, purification can be repeated twice.

3.4.1. Precipitation

After deprotection and cleavage, the redissolved mixture contains impurities like ammonium hydroxide, some ammonium salts, and oligonucleotides of different sizes. The simplest method for oligonucleotide purification is precipitation from redissolved lyophilized material as follows:

1. Resuspend the pellet in sterile distilled H_2O
2. Add $MgCl_2$ to a final concentration of 10 m*M* and mix
3. Add ethanol (5X vol), and freeze the sample at –70°C for 2 h
4. Centrifuge at 10,000*g* for 5 min and resuspend the sample in 95% ethanol, dry, and resuspend in distilled H_2O to make a 50 ng/µL stock. Precipitated oligonucleotides can be used for sequencing or cloning and also in PCR reactions. However, the amplification is much less in every cycle than with gel-purified oligonucleotides. More extensive purification is needed if DNA is phosphorylated while ammonium ions inhibit T4 polynucleotide kinase

3.4.2. Columns

This method is used in conjunction with some other methods described. It is useful as a final purification method, especially if problems occur with otherwise pure oligonucleotides. Columns will separate precipitated low- molecular-weight materials from oligonucleotides, and columns are also useful to clean oligonucleotides from residual phenol and urea after purification with PAGE. Columns containing Sephadex G-25 of DNA-grade are suitable for 25 mers or less, while Sephadex G-50 is, more suitable for longer sequences. There are also commercially available prepacked disposable columns (MicroSpin G-25 columns and NAP columns) that are both rapid and convenient in the purification of oligonucleotides (>10 mers).

Table 1
Length of Oligonucleotides that Comigrate with Marker Dyes in Denaturing Polyacrylamide Gels

% Acrylamide	Bromophenol blue[a]	Xylene cyanol blue[a]
5	35	130
8	19	75
10	12	55
12	10	46
16	9	35
20	8	28

[a]The numbers represent the approximate length of oligonucleotides with which the dyes comigrate

3.4.3. Reverse-Phase Cartridges

Several companies provide columns specifically designed for the purification of trityl from oligonucleotides. Sequences which lack trityl groups are washed away while full-length oligonucleotides are retained. In this method, sequences lacking trityl groups that have been capped will be washed off. The oligonucleotides are cleaved *in situ* from the attached trityl, eluted in acetonitrile, and dried down. This procedure can be performed in a few hours, the yield from these columns is >80% of the applied sample. Oligonucleotides can also be purified by reverse-phase chromatography using a silica gel. The oligonucleotides absorb to the column when the polarity of the solvent is high (e.g., aqueous buffers) and elute from it when the polarity of the solvent is reduced (e.g., mixture of methanol and H_2O). A detailed protocol is given in *Molecular Cloning, Laboratory Manual (5)*

3.4.4. Polyacrylamide Gel Electrophoresis (PAGE)

The most convenient method for routine purification is PAGE, which is the method of choice for full-length oligonucleotides. When purifying oligonucleotides in a 10–100 nucleotide range, gel concentration of 10–20% acrylamide will be suitable (**Table 1**). For analytical purposes, a gel length of 10–15 cm will be sufficient to obtain an estimation of the purity of the sample. The thickness of the gel is not critical but best analytical results are obtained on relative thin gels, preferably 0.2–0.9 mm.

3.4.4.1. Preparation of the Gel

The stock solutions for PAGE are given in the Materials Section.

1 Determine the gel concentration and calculate the gel volume. Prepare the gel solution. For every 100-mL gel solution combine the following 10 mL TBE 10X, 42 g urea, 100 µl TEMED. The stock solutions are given in **Subheading 2.3.2.**

2. Add an appropriate amount of acrylamide stock solution with distilled water to a final vol of 100 mL Make sure that everything is dissolved and degas carefully (oxygen inhibits the polymerization). Add 250 µL ammonium persulphate solution This initiates the polymerization and at this point there are only a few minutes to cast the gel

3.4.4.2. Running the Gel

The running buffer is TBE. It is preferable to perform the electrophoresis at an elevated temperature (50–60°C) to minimize the risk of problems due to sample molecules interacting. This can be done either with a thermostatic device or by applying enough voltage to heat up the gel.

1 The gel has to be pre-electrophoresed (400 V) for at least 1 h to remove acrylic acid and to heat the gel uniformly
2 Mix the sample with at least 50% formamide (sample can be lyophilized if the volume is too large)
3 Denature the sample by heating it at 90°C for 3 min, then rapidly cool on ice
4 Load the sample on the gel. It is recommended to run an aliquot of the dye mixture in one slot beside the sample as reference It is not recommended to mix the sample with dye mixture since this can interfere with the detection of DNA bands by UV-shadowing technique A 30-mer oligonucleotide (10% gel, 0.8-mm thick, 400 V) runs to approximately half way between the bromophenol blue and xylene cyanol dye, and the gel is electrophoresed until the fast dye (bromophenol blue) has reached the bottom of the gel

3.4.4 3. Detection and Recovery

1 To visualize the bands on the gel, the gel plates are pried apart and a fluorescent-thin-layer chromatography (TLC) plate is placed under the gel wrapped in Saran wrap.
2. Under UV light, (254 nm), the TLC plate will fluoresce whereas the oligonucleotides, which absorb UV light, appear as dark shadows against the fluorescent background The product is usually the slowest moving major band A standard of known oligonucleotide length should always be run on the adjacent track for comparison
3 For recovery, the areas containing the oligonucleotide of correct size are excised with a razor blade without undue delay
4. The gel is then crushed and soaked in eluting buffer overnight according to the diffusing method of Smith et al ***(10)*** at 37°C. The eluted oligonucleotide is ready for use after purification on a Sephadex G-25 column There are also commercially available, prepacked MicroSpin G-25 columns (oligos less than 20 bases) and ProbeQuant G-50 or MicroSpin G-50 columns (oligos longer than 20 bases) (Amersham Pharmacia Biotech)

3.4.5. High-Pressure Liquid Chromatography (HPLC)

Using HPLC, oligonucleotides can be purified with the trityl group on or off. Although this method has the advantage of speed it is only applicable for oligonucleotides <40–50 bases in size. When compared with PAGE, the oligo-

nucleotides can be loaded at 2–10 times less than the amount in a single lane. Secondary structures or sequence heterogeneity are also more crucial in the detection than with PAGE. The yield is approximately 70% of the loaded sample but several runs might be needed to purify the oligonucleotide *(5)*.

3.5. Measurement of Oligonucleotide Concentration

Concentrations of nucleic acids in solutions are measured with UV absorbance. Although there are well-established specific absorption coefficients for double-stranded DNA or RNA, there is confusion in a number of commonly used practical manuals and texts about the specific absorption coefficient for oligonucleotides as recently indicated by Rodger and Manchester *(11)*. Maniatis et al. *(12)* and more recently Berger *(13)* give a value of 20 $\mu g/A_{260\ unit}$ (50 $A_{260\ units}$/mg). Ausubel et al. *(14)*, by contrast, give a value of 25 A_{260}/mg. Based on the assumption that the specific absorption coefficient of an oligonucleotide is the sum of the contribution of constituent mononucleotides, the specific absorption coefficient can fluctuate between 24 and 46 $A_{260\ units}$/mg. The concentration of an oligonucleotide of known sequence can be calculated based on the known absorption coefficient (in a 1-cm path-length cuvet) as follows: dGTP = 11.7 mL/μmol, dCTP = 7.3 mL/μmol, dATP = 15.4 mL/μmol, and dTTP = 8.8 mL/μmol. The concentration *(C)* can be calculated from the equation optical density *(OD)* = $\alpha \cdot C$, where α is the absorption coefficient for the entire oligonucleotide based on the values for individual nucleotides. However, a general figure of 30 $A_{260\ units}$/mg is commonly quoted *(15)*. The fluctuation of specific absorption coefficients might be particularly significant when comparing the efficacy of hybridization methods or PCR reactions.

3.6. Oligonucleotide Labeling

There are two alternative methods using enzyme-catalyzed reactions that are commonly used for radioactive labeling of oligonucleotides. The first is where T4 polynucleotide kinase catalyzes the phosphorylation of the 5'-end of an oligonucleotide using [(γ–^{32}P)]ATP as the radioactive phosphate donor. As chemically synthesized oligonucleotides lack a 5'-phosphate, prior dephosphorylation with alkaline phosphatase is unnecessary. This method incorporates one radioactive phosphate per molecule of the oligonucleotide *(5,12)*. The second method utilizes terminal deoxynucleotidyltransferase that catalyzes the repetitive addition of mononucleotides from a dNTP to the 3'-hydroxyl-group of a DNA initiator accompanied by a release of inorganic phosphate. Various labeled deoxynucleoside triphosphates [(α-^{35}S)], [(α-^{32}P)], or [(^{3}H)]dNTP, or nonradioactively labeled triphosphates (DIG-dUTP, biotinylated-dUTP, or fluorescein-dUTP) can be incorporated using this tailing method.

3.6 1. 5'-End-Labeling with T4 Polynucleotide Kinase

1. Add the following reagents into an Eppendorf tube·
   a. The appropriate volume of oligonucleotide solution (from a 50 ng/mL stock), and
   b. 4 µL 10X denaturation buffer (*see* **Subheading 2.4.1.**): add distilled H_2O up to 40 mL
2. Heat the tube at 70°C for 5 min and then immediately chill on ice.
3. Add 5 µl of 10X kinase buffer (*see* **Subheading 2.4.1.**)
4. Add 1 µL T4 polynucleotide kinase (10 U/mL) (PNK) If a number of oligonucleotides are to be labeled in one session, then the commercial stock of PNK can be diluted immediately before use with a dilution buffer (*see* **Subheading 2.**) to a level of 5 U/µL, and 1 µL of this solution is used.
5 Mix the tube by gently flicking it with a finger.
6 Add two molar equivalents of label, i e , 5 µL (50 µCi) of a 10 mCi/mL solution of [γ-^{32}P]ATP (5000 Ci/mmol) for 50 ng of 30-mer oligonucleotide
7 Centrifuge briefly and incubate at 37°C for 1 h

3.6.2. 3'-Labeling with Terminal Deoxynucleotidyltransferase (with [a^{32}P])dATP and ([a-^{32}P])dCTP)

1 A typical tailing reaction is set up as follows.
   a 1 µL of 50 ng/µL oligonucleotide stock solution, and
   b 2.5 µL each of ([α-^{32}P]) dATP and ([α-^{32}P]) dCTP, add distilled H_2O up to 50 µL. The cold nucleotides TTP and dGTP can be added to a final concentration of 1 µ*M* each to minimize background
2 Mix gently.
3 Add 5 µL of 10X tailing buffer (*see* **Subheadings 2.4.2.** and **4.1.**)
4 Add 1 µL of terminal deoxynucleotidyltransferase (TdT)
5 Mix the tube by a quick spin in the centrifuge and incubate at 37°C for 1 h.
6. Purify the tailed probes in spin column and precipitate with ethanol as for the kinase end-label probes
7 The resulting tail length from the TdT-labeling reaction is influenced by the concentration of the enzyme, duration of the incubation period, and the relative amount of dNTPs to oligonucleotide. Pyrophosphate, a product of the transferase reaction, forms an insoluble precipitate with the cobalt cofactor. Thus, depletion of the cofactor can stop the reaction at high substrate concentrations. The choice of radioisotope used for incorporation into the oligonucleotide depends on the experimental requirements.

3.6.3. Oligonucleotide 3' End-Labeling with DIG-ddUTP, Biotin-ddUTP, or Fluorescein-ddUTP

This protocol is based on the procedure described in Boehringer Mannheim Manual *(17)*.

1 Use HPLC- or gel-purified oligonucleotides (14–100 bases).
2 Dissolve the oligonucleotide in sterile water

3. Mix the following reaction:
   a. 4 µL 5X reaction buffer.
   b. 4 µL 25 m*M* $CoCl_2$ solution.
   c. 100 pmol Oligonucleotide.
   d. 1 µL 1 m*M* DIG-, biotin- or fluorescein-ddUTP solution, in redistilled water.
   e. 1 µL (50 U) Terminal transferase, in transferase buffer (*see* **Subheading 2.4.3.**)
   f Make up to a final vol of 20 µL with redistilled water
4. Incubate at 37°C for 15 min, then place on ice
5 Mix 1 µL glycogen solution (20 mg/mL in redistilled water) with 200 µL 0 2 *M* EDTA solution, pH 8.0; add 2 µL of this mixture to the reaction mixture to stop the reaction. Do not use phenol/chloroform to stop the reaction since this treatment leads to partitioning of labeled oligonucleotide into the organic layer
6. Precipitate the labeled oligonucleotide with 2 5 µL 2 m*M* LiCl and 75 µL prechilled (–20°C) absolute ethanol. Mix well
7 Leave for at least 30 min at –70°C or 2 h at –20°C.
8. Centrifuge at 12,000*g* for 5 min, wash the pellet with 70% (v/v), cold ethanol, dry under vacuum, and dissolve in an appropriate volume of sterile, redistilled water.
9 Store labeled oligonucleotide at –20°C if not used immediately.

3.6.4. Oligonucleotide 3'-Tailing with DIG-dUTP, Biotin-dUTP, or Fluorescein-dUTP

Using the procedure described here the range of tail length is 10–100 (average: 50) nucleotides, and the average number of hapten molecules added is 5 ***(17)***. However, it is also possible to add only 2–3 nucleotides that consist of DIG-dUTP only. The protocol used is that described in the Boehringer Mannheim Manual ***(17)***

1 Dissolve the oligonucleotide (HPLC- or gel-purified oligonucleotides from 14–100 nucleotides in length) in sterile water
2. Mix the following in a microcentrifuge tube on ice·
   a. 4 µL 5X Reaction buffer (*see* **Subheading 2.4.3.**).
   b 4 µL 25 m*M* $CoCl_2$ solution
   c 100 p*M* oligonucleotide
   d. 1 µL m*M* DIG-, biotin- or fluorescein-dUTP-solution, in redistilled water.
   e 1 µL 10 m*M* dATP solution, in Tris buffer, pH 7.5 (25°C)
   f 1 µL (50 U) Terminal transferase buffer.
   g Make up to a final vol of 20 mL with redistilled water
3. Incubate at 37°C for 15 min, then place on ice.
4. Mix 1 µL glycogen solution (20 mg/mL in redistilled water) with 200 µL 0.2 *M* EDTA solution, pH 8 0, add 2 µl of the dilution to the reaction mixture to stop the reaction Do not use phenol/chloroform to stop the reaction since this treatment leads to partitioning of tailed oligonucleotides into the organic phase.
5. Precipitate the tailed oligonucleotide with 2.5 µL 4 *M* LiCl, and 75 µL prechilled (–20°C) absolute ethanol Mix well.

6. Leave for at least 30 min at –70°C or 2 h at –20°C.
7 Centrifuge at 12,000g for 5 min, wash the pellet with 50 mL 70% (v/v) cold ethanol, dry under vacuum, and dissolve in an appropriate volume of sterile, redistilled water Store the tailed oligonucleotides at –20°C if not used immediately
8. Estimate the amount of labeled oligonucleotide by comparing it to unlabeled oligonucleotide in polyacrylamide gel electrophoresis and silver staining Labeling of oligonucleotides results in a heterogeneous shift to higher molecular weights and is detected as a smear in polyacrylamide gels. The control oligonucleotide labeled in the standard reaction is completely transferred to the labeled form *(17)*

3.7. Purification of the Labeled Probe

To purify the labeled oligonucleotide probe from free label, the sample can be either run through the conventional Sephadex G-25 gel filtration column or purified using a Sephadex G-25 spin column. Spin columns are useful because they are more convenient to handle with a large number of samples. Spin column can be prepared as follows:

1 Swell Sephadex G-25 DNA grade (6 g) in 40 mL of column buffer (*see* **Subheading 2.5.**) for a minimum of 3 h.
2. Place sterile glass wool on the bottom of a 1-mL disposable syringe, place in an uncapped 10-mL plastic tube.
3. Pipet the Sephadex slurry in the syringe barrel Add a sufficient quantity of Sephadex so that it will bed down to the 0.9-mL mark of the syringe after centrifugation at 735*g* for 4 min
4. Just before use, add 190 µL of column buffer to the column and centrifuge as before to equilibrate the column, transfer to a clean 10-mL tube
5 Place the tube in a disposable/50-mL screw-cap tube. This outer tube is used to contain the radioactive material in the event of a spillage.
6 Make up a reaction mixture (after incubation) to purify the labeled oligonucleotide using a total volume of 190 µL with column buffer. Apply to the top of the spin column, then spin again for 4 min at 735*g*
7 The free label remains in the column and the oligonucleotide is eluted
8. Transfer the eluted oligonucleotide to an Eppendorf tube and precipitate with ethanol overnight at –20°C by adding 5 mL of 10 µg/mL tRNA, 0.1 vol of 3 *M* sodium acetate, and 2 5 vol absolute ethanol
9. Centrifuge the sample in an Eppendorf centrifuge 1500 rpm for 15 min and decant the supernatant.
10. Dilute the oligonucleotide in the pellet in the appropriate concentration and use to make hybridization buffer for probing the samples (final concentration of the labeled probe in hybridization mixture is 40 n*M* (400 ng/mL) for a 30-mer oligonucleotide A 1-µL sample from oligonucleotide stock solution should be taken at this stage for the analysis on a 10% denaturing polyacrylamide gel.

3.8. In Situ *Hybridization (ISH) with Oligonucleotides*

Viral DNA or mRNA transcripts can be localized in tissue samples with *in situ* hybridization using labeled oligonucleotides. In this method labeled oligonucleotide probes are applied to 22 tissue sections where they hybridize with intracellular complementary target messenger RNA or DNA sequences that have been preserved during the tissue preparation. Depending on the target the use of oligonucleotides in *in situ* hybridization may result in decreased sensitivity when compared to that of conventional double-stranded DNA or RNA probes, which is related to the smaller amount of label per probe used. Although the 3'- and 5'-labeling methods of oligonucleotides have been described earlier (*see* **Subheading 3.6.**), they can also be labeled by using an amino-linking group. Biotin, fluorochromes, and alkaline phosphatase can all be crosslinked to a single modified base that carries a linker arm. This modified base is incorporated directly during the automated oligonucleotide synthesis carried out on a DNA synthesizer. Then, labels can be crosslinked to the primary amine group on the linker arm using an amine-reactive bifunctional crosslinker ***(18)***. In our hands the use of biotinylated or fluorochrome-labeled oligonucleotides prepared with this method results in significantly lower signals by ISH than those labeled with end-labeling methods. The signal intensity can be increased by simultaneously using several oligonucleotides (up to 30) covering different areas of the target DNA.

3.8.1. Pretreatment of the Slides for ISH

Detachment of sections from glass slides for ISH was a major problem earlier. However, when using poly-L-lysine care should be taken to ensure that the molecular weight of the preparation is more than 150,000, otherwise adhesion is minimal. The most preferable method is, however, the organosilane method that we have modified as follows:

1. Wash the slides with 1 *M* HCl for 20 min, then with running tap water for 30 min.
2. Dip the slides into sterile water and allow to dry.
3. Put the slides into 2% organosilane (γ-aminopropyltriethoxy-silane) in acetone. This solution is stable only for 1 d, although 500 slides can be processed
4 Incubate for 30 min at room temperature
5. Wash the slides with running tap water for 30 min and dip them into three changes of sterile water.
6. Keep the slides in an incubator adjusted to 100°C for 1 h.
7 Store the slides at room temperature in a dust-free place. The slides are usable for a minimum of 1 yr.

3.8.2. Samples

ISH can be performed on cytological smears, cells derived from cultures, or on biopsy samples either snap-frozen or formalin-fixed. The optimal proce-

dure for fixation and tissue preparation should preserve the maximum level of cellular target DNA or RNA while maintaining optimal morphological detail and allowing sufficient accessibility. Highly crosslinking fixatives provide good morphology, but they may limit probe penetration For proper localization of mRNA, tissue should be fixed or frozen as soon as possible after surgical excision

3.8.2.1. Cells

According to our experience a superior preservation of cell morphology is achieved by using Carnoy's fixative (*see* **Subheading 2.6.2.**). Acetone at 4°C for 1 min can also be used as a rapid method of fixation for cells derived from cultures.

3.8.2.2. Cryostat Sections

For the localization of mRNA, we prefer fixing tissues in buffered 4% paraformaldehyde.

1. Fix tissues in buffered 4% paraformaldehyde (*see* **Subheading 2.6.2.**) prior to freezing to decrease artifacts
2. Store samples in liquid nitrogen until cryocutting. (Alternatively, the biopsy can be snap-frozen immediately after excision, and stored in liquid nitrogen covered by OCT; *see* **Subheading 2.6.2.**)
3. Cut the frozen sections at 6–10 µm, with a conventional cryomicrotome at –20°C and mount onto the pretreated slides (*see* **Subheading 3.8.1.**).
4. Fix the sections in 4% paraformaldehyde (*see* **Subheading 2.6.2.**) for 10 min, air dry, and store at –80°C prior to hybridization

3.8.2.3. Formalin-Fixed, Paraffin-Embedded Material

In surgical pathology, most of the tissues are fixed in formalin. In our laboratory, formalin-fixed, and paraffin-embedded material is routinely used to detect viral DNA (for human papillomavirus [HPV], Epstein-Barr virus [EBV], cytomegalovirus [CMV], and herpes simplex virus [HSV]). The optimal fixation time depends on the size of the biopsy, but should not exceed 24 h, Bouin's fixative results in substantially decreased hybridization signals

3.8.3. Prehybridization

A number of procedures are commonly employed to treat specimens after fixation to both increase accessibility for the probe and reduce the nonspecific binding during hybridization.

1. Paraffin sections are dewaxed to remove the solvents and paraffin, using fresh xylene.
2. Samples can then be treated similarly to frozen sections.
3. The sections are permeabilized to allow the entry of the probe to the tissues without opening the intracellular targets. These steps are additive and have to be optimized in combination where HCl is used at a concentration of 0.02–0.1 *M*, Triton

X-100 between 0.01% and 0.3% (v/v), and proteinase K at 1–1000 µg/mL with an incubation time of 5–30 min, *see* **Subheading 2.6.3.** ***(19)***

HCl treatment deproteinizes the tissues. However, in our hands, omission of HCl treatment gives increased signal intensity for viral detection. The proteolytic digestion of the sections is the most critical step of the entire procedure. Extreme care is required to guarantee optimal treatment. If the treatment is too short, the probe will not penetrate to its intracellular targets. If it is too long, several artifacts may result; cell structure may be destroyed and interpretation becomes difficult, or nucleic acid targets may lose their anchor and be washed out.

3.8.4. Oligonucleotide Hybridization

The advantages of *in situ* hybridization include their small size (good penetration properties) and the use of single strands (no probe reannealing). The small size, however, is also a disadvantage because it covers less target. The following formula can be used to calculate the melting temperature (T_m) of an oligonucleotide probe:

$$T_m = 16\ 6 \log M + 0.41\ (\%\ G + C) + 81.5 - 820/L - 1.2\ (100 - h)$$

where M is the monovalent cation concentration (molarity), % $G + C$ refers to the base composition of the oligonucleotide, L is the probe length in nucleotides, and h is percentage identity between the probe and the target sequence. Hybridizations should be performed between 5 and 25°C below T_m and high stringency washing is carried out between 0 and 5°C below T_m ***(3,20,21)***.

3.9. In Situ *Hybridization Protocols*

There are several protocols available to perform ISH with oligonucleotides labeled either radioactively or nonradioactively. This chapter describes the ISH protocols used in our laboratory ***(19)***.

3.9.1. In Situ *DNA Hybridization of Paraffin Sections with ^{35}S-Labeled Oligonucleotide Probes*

Use only organosilane-coated slides (**Subheading 3.8.1.**) and place the sections on the third of the slide distal to the frosted end.

3.9.1.1. Pretreatment of Slides

Use racks for 20–48 slides.

1. Bake the sections overnight at 60°C or for a minimum of 2 h
2. Deparaffinize the sections using the following sequence (always use fresh xylene).
   a. Xylene: two changes, 10 min each.
   b. Absolute ethanol: two changes, 5 min each.

c. 95% ethanol· two changes, 5 mın each.
d 70% ethanol. two changes, 5 min each.
3 Aır dry for approx 10 mın
4. Pipet 100–200 µL proteinase K solution (0 5–1.0 mg/mL) on to each sectıon and ıncubate in a humıdıfied chamber at 37°C for 15 mın (ıf the workıng tıme ıs 2 mın keep at 37°C for 13 mın).
5 Rinse in two changes of PBS, then 70% and 95% ethanol for 2 mın each

3.9.1.2. Prehybridization

1. Put slıdes ın hybrıdızatıon buffer at 40°C for 4 h.
2 Rinse ın three changes of absolute ethanol and allow to dry
3. Then hybrıdıze ın hybrıdızatıon buffer (**Subheading 2.6.4.**).

3.9.1.3. Hybridization

The labeled probe is diluted in hybridization buffer (*see* **Subheading 2.6.4.**).

1. Apply the appropnate amount of probe solutıon (15–30 µL) directly on to the sectıons and place a coverslıp ımmedıately onto the sectıon (22 × 22 mm, enough for 20 µL)
2. Seal the slıdes ın a plastic box and ıncubate at 110–120°C in an ıncubator for approx 6 min (to denature the probe and target), until the slides have reached a temperature of 93°C Use a temperature indıcator to control the temperature After ıncubation, transfer the box to room temperature
3 Seal the edges with rubber cement (or nail polish) pushed through a syringe without the needle
4 Place the slides in a sealed box humidified with the same salt concentration as the hybrıdization mıxture at 40°C for DNA probes larger than 25 nucleotides An overnight incubatıon is sufficient for detectıng, an abundant target, however, up to 3 d ıs recommended to achıeve greater sensitivity
5. Remove the coverslıps by inserting a thin hypodermic needle under the raised edge to loosen and lift in one motion Place the slide immediately ın the first wash as given ın **Subheading 3.9.1.4.**

3 9.1.4 Washes

1. After hybrıdizatıon, slides are placed vertically in a beaker of 4X SSC (*see* **Subheading 2.6.5.**) until the coverslips dıslodge.
2 The slides are transferred to 2X SSC at room temperature, then soaked ın 1X SSC for 45 mın at 40°C wıth occasional agitation
3 Dehydrate the slides ın 70% ethanol for 5 min, then 95% ethanol for a further 5 mın. The ethanol solutıons should contain 0 3 *M* ammonıum acetate
4 Air-dry the slıdes

3.9.1.5. Liquid Emulsion Autoradiography

1. Load the slides into plastic slide holders (e.g., Peel-a-way slıde holder)
2. Continue work in the darkroom as given in **Subheading 2.**

3 Turn the light off, remove the emulsion from the protective box, and place it in a 50°C water bath to melt.
4. Turn the red No. 2 safe-light on, fill a 50-mL beaker containing ammonium acetate to the top with emulsion, and mix with a spatula.
5. Pour this mixture through a Buchner funnel with gauze (to remove bubbles), into a second beaker.
6. Dip the racks of slides into the emulsion for 1 s and blot the ends on a paper towel before transferring them to the light box. Close the box and allow the slides to dry overnight.
7. Next morning, add silica gel to the bottom of the box and continue the exposure for 3–7 d at 4°C (depending on the specific activity of the probe and the amount of target DNA).
8 Develop the slides (in the dark) by dipping them into the following solutions:
   a Developer (D-19, Kodak) for 5 min at 15°C.
   b. Stop bath for a few seconds.
   c. Rapid fix (Eastman Kodak, Rochester, NY) for 5 min
   d. Water rinse for 5 min.
   e. Turn the lights on at this point
9 Dip the slides in Gill's hematoxylin for 1–2 min.
10 Place the slides in tap water substitute (200 mL water containing 4 g $NaHCO_3$ and 40g $MgSO_4 \cdot 7H_2O$) for 2 min.
11 Rinse in tap water
12. Dehydrate through ethanols (three changes each of 70%, 95%, and absolute ethanol) for 3 min each change, then in two changes of xylene for 5 min each, and mount in mounting medium (e.g., Permount [Fischer Scientific]).

3.9.2. ISH for Cytological Samples when Excellent Morphology Is Needed

1. Take the slides from the freezer and allow them to warm to room temperature Use racks for 20–48 slides
2. Incubate the slides in 2X SSC for 30 min at 70°C.
3 Transfer the slides into 70% ethanol at room temperature and incubate in fresh ethanol twice for 10 min
4. Incubate in 95% ethanol for 5 min. Air dry the slides.
5 Put the slides into 0.1 *M* triethanolamine, mix vigorously, and add acetic anhydride (3 µL/300 mL vol). Incubate for 10 min at room temperature.
6. Wash the slides in 2X SSC for 5 min at room temperature.
7. Dehydrate in 70% ethanol twice for 10 min and 95% ethanol twice for 5 min. Air dry the slides.
8 Place the slides in 70 m*M* NaOH for 3 min
9. Dehydrate in three changes of 70% ethanol for 10 min each and in two changes of 95% ethanol twice for 2 min each. Air dry the sections.
10 Continue the protocol with prehybridization step as given in **Subheading 3.9.1.2.**

3.9.3. ISH with Nonradioactive Oligonucleotide Probes

For nonradioactive ISH the procedure is mostly the same when using radioactive ISH, although the hybridization mixture and detection step are different. The oligonucleotide probe can be labeled as described earlier in this chapter (**Subheading 3.6**). The probe should be labeled either at the 3'- or the 5'- end, as the T_m may be affected if internal modifications are used. The optimization of hybridization conditions can be started by using hybridization conditions described below. The reaction is based on the assumption that a 20-mer oligonucleotide of 40–60% GC content is used.

1. Perform the hybridization at room temperature for 2–16 h (the hybridization mixture is given in **Subheading 2.5.4.**).
2. Perform posthybridization washes as described in **Subheading 3.9.1.4.**, but no dehydration is needed and the final wash is in 1X SSC at room temperature.

3.9.3.1. Detection of Biotinylated Hybrids

Although the detection system depends on the nonradioactive label used, we have included our protocol to detect biotinylated hybrids.

1. Pipet streptavidin-alkaline phosphatase conjugate on the slides and incubate in a humidified chamber for 30 min at 37°C We use the conjugate from Amersham diluted in buffer A containing 0.05 % Tween-20 (*see* **Subheading 2.6.6.**).
2 Wash the slides in three changes of buffer A for 3 min each
3 Wash the slides in three changes of buffer B for 3 min each (*see* **Subheading 2.6.6.**).
4. Incubate in NTB and BCIP solution for 30–60 min at 37°C.
5 Stop the reaction by washing in three changes of water for 3 min each.
6 Counterstain with carbol fuchsin (*see* **Subheading 2.6.7.**) if required
7. Dehydrate in ethanols by sequential incubation in a single change each of 70%, 95% and absolute ethanol for 3 min and then in xylene twice.
8. Mount the slides in an aqueous mountant

3.9.4. Hybridization to RNA in Tissue Sections

Hybridization to RNA can be performed with both cryostat and paraffin sections. However, RNA is extremely sensitive to fixation and improper handling of biopsies during formalin fixation can totally destroy the RNA. As a consequence, we prefer the use of cryostat sections for RNA hybridization. The same protocol can be applied as described in **Subheading 3**. However, to avoid contamination wear gloves to handle slides, and use diethylpyrocarbonate (DEPC) (0.2 mL DEPC to 100 mL of solution, shake vigorously, autoclave)-treated water and sterile stock reagents at room temperature until the posthybridization washes. For frozen sections use a milder Proteinase K treatment (1–10 μg/mL in PBS, 15 min at 37°C). Sections are not to be denatured by heat because RNA is the target.

4. Notes

4.1. Labeling of Oligonucleotides

1. It is recommended not to increase the amount of oligonucleotide in the standard labeling reaction. Larger amounts may be labeled by increasing the reaction volume and components proportionally.
2. Care must be taken in preparing the 10X tailing buffer for 3'- labeling with terminal deoxynucleotidyltransferase, as cobalt ions can easily be precipitated. Buffer described by Roychoudhury and Wu *(16)* is recommended. Cacodylic acid (1 38 g) and 0.3 g Tris base are suspended in 3.5 mL distilled water The pH is adjusted to pH 7.6 by addition of solid KOH with constant mixing The solution is then made up to 8.8 mL with distilled water and chilled on ice; 200 µL of 0.1 *M* DTT is mixed in and then 1 mL of 0.1 *M* $CoCl_2$ is added dropwise with constant mixing
3 When oligonucleotides are labeled with fluorescent nucleotides the crucial thing in the labeling reaction is to have a correct molar ratio of oligonucleotide to labeled nucleotide. A routine assay would have 100 pmol oligonucleotide, a 20–50 fold molar excess fluorescent nucleotide, and around 30 U labeling enzyme (terminal transferase). Under these conditions the labeling reaction will yield a tail length around 4–10 bases This has proved to be the optimum size range for the best sensitivity without risking nonspecific binding and affecting stringency control

4.2. In Situ Hybridization

4.2.1. Probes and Labels

4. A selection of labels largely depends on the desired results ^{32}P-labeling is applicable to all probe types at high specific activity and permits rapid results on X-ray film and on liquid emulsion, but has poor resolution. ^{35}S is similarly applicable, with a higher resolution than ^{32}P, but requiring longer exposure times ^{3}H enables higher resolution of signals but with longer exposure than the other isotopes. Nonradioactive probe labels seem to be as sensitive as radioactive ones, and even single cell resolutions can be achieved.
5. The major advantage to using oligonucleotide probes is the fact that short probes hybridize 5–15 times faster than long probes. These probes, once synthesized, are also stable for years, providing a reliable and reproducible source of reagents amenable to use in the clinical laboratory. The major disadvantage to short probes is the limited number of reported molecules that can be carried or attached Although linker arms can be used to label oligonucleotide probes with several enzyme molecules, such as, alkaline phosphatase, long probes are still cited as 10–100 times more sensitive than short probes. The signal intensity can be increased by using simultaneously several oligonucleotides (up to 30) covering different areas of target DNA

4.2.2. Denaturation

6. Denaturation of target DNA is one of the most critical steps of the ISH procedure, especially when working with paraffin sections. The denaturation tempera-

ture must be strictly controlled by a temperature indicator We use a temperature indicator within the range of 82–93°C to control the denaturation temperature on the slide. Denaturation is performed in an incubator adjusted to 100°C The incubation time for frozen sections should be 3–5 min and for paraffin sections 6–10 min Overheating (i e., above 100°C) should be avoided, because this will compromise the sample preservation Denaturation times longer than 10 min should also be avoided to prevent drying of the sections. Drying at this step will invariably result in heavy background signals.

4.2.3. Hybridization Temperature

7. The temperature for hybridization with oligonucleotides is related to the probe length, specificity of the probe for the target mRNA/DNA, formamide, and salt concentration of the hybridization buffer Raised temperature, formamide, and lowered salt concentration are inhibitory to hybridization, thus lower temperatures (and/or formamide) are necessary for hybridization of short sequences *(22,23)*. This, however, reduces specificity, thereby generating an increase in background. It is preferred that only temperature is changed according to the probe length and specificity, while maintaining a constant salt concentration at 0 6*M* and formamide at 40% The following guideline temperatures can be given room temperature for 15–18-mer, 30°C for 19–25-mer, 40°C for probes longer than 25-mer If cross-hybridization is a potential problem, higher temperatures may be preferable. If lower temperatures are used, (for nonhomologous systems), multiple samples should be hybridized at different temperatures.

4.2.4. Posthybridization Washes

8 During hybridization, duplexes are formed between the perfectly matched sequences and also between less homologous sequences To remove the probe hybridized to the latter, the sections must be washed with the desired stringency. The stringency of the washes can be manipulated by varying the salt concentration, formamide concentration, and temperature For ISH employing radiolabeled probes, washing is usually extensive, whereas sections hybridized with biotinylated probes require only a short washing procedure *(5,11)* RNase treatment of the sections after hybridization with antisense RNA probes appears to be a reliable means of digestion of nonhybridized probe Hybrids formed *in situ* are extremely stable to low-salt /high temperature washes, and nonspecific interaction that may have occurred during the hybridization remains through the most stringent of washes. It is essential to minimize background and to have probes that are not contaminated by shorter sequences or unincorporated label.

4.2.5. Detection Systems

9 Radioactive probes: Signal detection can be achieved by autoradiography employing liquid emulsions The exposure time for autoradiography depends on.
   a. the isotope used for labeling,
   b. the specific activity of the probe;

c. the copy number of target DNA or RNA;
d. the efficiency of hybridization; and
e the sensitivity of the detection system (liquid emulsion).

Two methods of autoradiographic detection are used routinely to visualize the hybrids. One is a film-based autoradiography method in which a sheet of autoradiography film is placed in direct contact with the hybridized slides, exposed for an optimal time, and developed. The second method is emulsion autoradiography in which a hybridized section is coated with a layer of nuclear emulsion, exposed in the dark, and developed. The emulsion technique is mostly used in the detection of microbes (viruses and bacteria) in clinical samples, whereas the film method is used to localize mRNAs. Autoradiography should follow the protocols provided by the manufacturers of the emulsion, developer, and photographic fixer

10 Detection of nonradioactive hybrids: For detection of biotinylated probes, conjugated avidin or streptavidin have been used. Streptavidin as a detector is superior to avidin owing to its lower background levels. An alternative approach is indirect immunohistochemical detection using antibiotin antibodies Multistep detection systems are designed to increase the sensitivity, reduce nonspecific binding, and allow the use of labels not directly linked to the probe. In the author's experience, best results are achieved by a streptavidin-alkaline phosphatase complex *(24)* The commercial streptavidin-alkaline phosphatase complexes differ considerably in their sensitivity. This is partly due to the differences in enzyme activity (U/mL) in different complexes. Most commercial products work well with a concentration of 4–10 U/mL. However, the different products should be individually tested in the laboratory to find out the most appropriate concentration. Thus, by using the optimal complex, the sensitivity of ISH can be dramatically improved. Furthermore, streptavidin-alkaline phosphatase detection complexes give results superior to those obtained using standard horseradish peroxidase. Simultaneous detection of two different viral genomes after double-labeled ISH can also be performed.

4.2.6. Control

11. Several controls can be included for each hybridization reaction. If mRNA is detected, there should be controls containing abundant target mRNA and samples with no mRNA that should be hybridized along with the tissues of interest. Also control probes of the same type and length as the test probes but without any specificity should be prepared at the same specific activity and concentration as the test probe. With ISH, by hybridizing control and test probes of identical sets of sections, the sites of nonspecific binding in tissue can be identified. Another specificity control is pretreatment of sections with a ribonuclease A, which should abolish or greatly reduce the hybridization signal, the degree of reduction depending on the abundance of mRNA in the tissue and the extent of the treatment The protocol is as follows: The sections are fixed and then rinsed three times in 4X SSC and incubated at 37°C for 30 min in 20 μg/mL ribonuclease A in 0.1 *M*

phosphate buffer at pH 7.2. A control set of slides is incubated without ribonuclease Following the incubations, the slides are rinsed at 37°C in one change of 0.1 *M* phosphate buffer, pH 7.2, and two changes of 4X SSC and then prehybridized and hybridized according to the method

4.2.7. Quantitation

12. Relative levels of mRNA or DNA in tissue section may be determined by densitometry of autoradiographs using an automated image analyzer. Manual grain counts of liquid emulsion autoradiographs may also provide similar data with a higher resolution. However, the quantity of mRNA present in a tissue may be the result of high-level transcription in a small number of cells or lower level transcription in a large group of cells Both of these instances provide the same net result. Measurements of extracted mRNA by dot-blot, Northern blot, or solution hybridization can be undertaken in parallel with hybridization histochemistry to measure relative tissue mRNA levels.

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