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Ralph A. Tripp
Patricia A. Jorquera
Editors

Human Respiratory Syncytial Virus

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Edited by

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Preface

Respiratory syncytial virus (RSV) is the prototypic member of the *Pneumovirus* genus of the *Paramyxoviridae* family [1]. RSV has a negative-sense single-stranded RNA genome comprised of 15,222 nucleotides that encodes for 10 major species of mRNA and 11 proteins. The genome is transcribed sequentially from NS1 to L, with reduced expression levels along its length. Three proteins are found on the envelope surface, the heavily glycosylated G, fusion F, and small hydrophobic SH protein. The G and F proteins are the major antigens involved in virus neutralization. However, the G protein is highly variable, which allows for the classification of RSV strains into different clades based on its nucleotide sequence [2].

RSV is the leading cause of lower respiratory tract infections such as bronchiolitis and bronchopneumonia, as well as hospitalizations in infants, elderly, and immunocompromised individuals [3]. No effective licensed therapies are readily available, but existing and emerging point-of-care diagnostics and investigational RSV-specific antiviral inhibitors offer promising progress [4]. Prophylactic antibodies have been successfully developed against RSV [5]. However, their use is restricted to a small group of infants considered at high risk for developing severe RSV disease. No RSV vaccine has been licensed to date, but the availability of new tools to study the virus-host interaction, pathogenesis of RSV disease, and the ability to construct a wide variety of vaccines using different vaccine platforms suggests that an RSV vaccine should be feasible [5].

This book is intended to summarize the current techniques that have made this progress possible, ranging from protocols for virus growth, isolation, quantification, and generation of recombinant RSV virus to procedures for the efficient characterization of the host immune response to RSV infection. These techniques are used in numerous laboratories around the world and are, thus, the building blocks that support the majority of RSV virus research.

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References

1. Teng MN, Collins PL (1998) Identification of the respiratory syncytial virus proteins required for formation and passage of helper-dependent infectious particles. J Virol 72(7):5707–5716.
2. Anderson LJ et al (1985) Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis 151(4):626–633.
3. Leung TF et al (2014) Epidemiology and risk factors for severe respiratory syncytial virus infections requiring pediatric intensive care admission in Hong Kong children. Infection 42(2):343–350.
4. Simoes EA et al (2015) Challenges and opportunities in developing respiratory syncytial virus therapeutics. J Infect Dis 211(Suppl 1):S1–S20.
5. Broadbent L et al (2015) Respiratory syncytial virus, an ongoing medical dilemma: an expert commentary on respiratory syncytial virus prophylactic and therapeutic pharmaceuticals currently in clinical trials. Influenza Other Respir Viruses 9(4):169–178.

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Chapter 1

Human Respiratory Syncytial Virus: An Introduction

Patricia A. Jorquera, Lydia Anderson, and Ralph A. Tripp

Abstract

Human respiratory syncytial virus (RSV) is understood to be a significant human pathogen in infants, young children, and the elderly and the immunocompromised. Over the last decade many important mechanisms contributing to RSV infection, replication, and disease pathogenesis have been revealed; however, there is still insufficient knowledge which has in part hampered vaccine development. Considerable information is accumulating regarding how RSV proteins modulate molecular signaling and immune responses to infection. Understanding how RSV interacts with its host is crucial to facilitate the development of safe and effective vaccines and therapeutic treatments.

In this chapter, we provide a brief introduction into RSV replication, pathogenesis, and host immune response, and summarize the state of RSV vaccine and antiviral compounds in clinical stages of development. This chapter frames features of this book and the molecular methods used for understanding RSV interaction with the host.

Key words Respiratory syncytial virus, RSV proteins, Innate immune response, Adaptive immune response, Vaccine, Antiviral compounds

1 Introduction

1.1 Classification

Human respiratory syncytial virus (RSV) is a member of the *Paramyxoviridae* family that can cause severe respiratory infections throughout life [1]. RSV is a pleomorphic (80–350 nm in diameter) enveloped virus that belongs to the *Pneumovirinae* subfamily and is the type-species member of the *Pneumovirus* genus [2]. RSV was first isolated in 1956 as an agent causing coryza in chimpanzees; soon after the virus was isolated from children with bronchiolitis and pneumonia and was recognized as the most common cause of annual winter epidemics of lower respiratory tract infections globally. As RSV has a proclivity for type II respiratory epithelium in the respiratory tract and can form multinucleated giant cell syncytia in tissue culture, it was a respiratory syncytial virus in 1957 [2, 3]. RSV causes clinical signs of infection associated with bronchiolitis and pneumonia in young infants [3], and is a ubiquitous virus and the leading cause of lower respiratory tract

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disease in children in the USA resulting in over 100,000 hospitalizations and ~1000 deaths per year [2, 4, 5]. RSV is the primary cause of hospitalization for all respiratory tract illness in young children with infection rates approaching 70% in the first year of life [2]. In addition to young children, studies demonstrate that the elderly and immunocompromised are also at an increased risk for severe disease with RSV [5]. RSV isolates can be classified into two subgroups, designated A and B, that exhibit antigenic and genetic differences, and can be further segregated into virus lineages or clades. Although the sequences of the viral surface proteins differ between the two subgroups, the surface G glycoprotein shows the greatest ectodomain divergence, with only 53% amino acid identity between prototype A and B viruses [6]. RSV group A and B viruses can co-circulate during epidemics, although only one group typically predominates [7]. The impact of antigenic diversity on RSV epidemiology is not completely understood, but may in part explain the susceptibility to reinfection throughout life and the yearly variation in the severity of epidemics within communities [7].

1.2 Genome Organization and Viral Proteins

RSV is an enveloped virus having a single-strand negative-sense RNA genome approximately 15 kb. The genome encodes 11 proteins: the nucleocapsid protein (N), nucleocapsid-associated proteins (M2-1, P, L), an M2-2 protein (the second ORF of the M2 gene), one matrix protein (M), three transmembrane proteins (F, G, SH), and two non-structural proteins (NS1, NS2) [8, 9]. The viral RNA is tightly wrapped with the nuclear protein N forming the nucleocapsid. The nucleocapsid (N) protein has intrinsic RNA-binding properties, and it binds to the viral RNA in a non-specific manner to form an RNA-N protein complex [10]. It has been proposed that prior to nucleocapsid assembly, N binds to the P protein preventing the nonspecific binding to cellular RNAs [10]. The N protein, phosphoprotein (P), and RNA-dependent RNA polymerase (L) encapsulate the viral RNA to form a helical assembly termed the ribonucleoprotein (RNP) complex [9]. This structure protects the RNA and forms the minimal replication machinery [9].

The L protein functions as the RNA-dependent RNA polymerase to replicate the viral RNA genome and transcribe mRNAs, the capping enzyme to cap mRNA 5′ end, and the methylase to methylate cap [11]. To perform genome transcription and replication, the L protein engages with promoter sequences that lie at the 3′ ends of the genome and antigenome RNAs [12]. The phosphoprotein is a 241-amino acid-long protein capable of interacting with multiple partners [13]. It can form homotetramers by binding to L, N, and M2-1 [13, 14]. During the transcription and replication of the genome, P is believed to position on the RNA-nucleoprotein complex and to help translocate the polymerase

complex along the helical nucleocapsid [13, 14]. The M2-2 protein is dispensable for RSV replication; however, it has been suggested that it has a role in regulating the switch between the viral RNA transcription and replication process [15]. The M2-1 protein is a transcriptional processivity factor involved in RNA transcription, elongation, and switching RNA synthesis from protein transcription to genome replication [9]. M2-1 also has a structural function, in which it co-localizes with cytoplasmic inclusions contained within infected cells, associating with the RNP complex through P and the N-terminal domain of the matrix protein (M) [9]. The M protein is a non-glycosylated phosphorylated protein of 256 amino acids and a structural component of the RSV virion [9]. The M protein has a key role in virion assembly by inhibiting viral transcription and by forming a bridge between the viral RNP and envelope [16]. The polymerization of the matrix protein is important for driving RSV assembly and budding. M is essential for virus particle formation and studies of an M-null mutant indicate that the absence of M impairs the formation of long viral filaments [9]. M also interacts with the amino-terminal domain of the G protein and with the cytoplasmic tail of the F protein, and coordinates their recruitment to sites of assembly and budding [9].

RSV possesses three membrane proteins: small hydrophobic protein (SH), glycoprotein (G), and fusion protein (F). The SH protein is a type II integral membrane protein of 64–65 amino acids that forms a pentameric ion channel [9, 17, 18]. The role of SH protein is not well understood, since SH is not required for virus binding or for syncytia formation. Furthermore, RSV virus lacking of SH (RSV ΔSH) grows as well as the wild-type (WT) virus in cell culture. However, RSV ΔSH replicates ten times less efficiently in vivo compared to RSV WT in the upper respiratory tract, and is attenuated in vivo in mouse and chimpanzee models [18].

The G glycoprotein is a highly glycosylated protein that is expressed both as a secreted (Gs) and a membrane-anchored form (Gm), with the latter serving as a viral attachment protein [16, 17]. The RSV G glycoprotein has been implicated in the pathogenesis of disease after primary infection, and in formalin-inactivated RSV (FI-RSV) vaccine-enhanced disease. A central conserved region is found in the G glycoprotein which contains four evolutionarily conserved cysteines in a cysteine noose structure, within which lies a CX3C chemokine motif [19]. The C-terminal part of the central conserved region has structural homology with the fourth subdomain of the 55-kDa TNF receptor, suggesting that the cysteine noose may interfere with the antiviral and apoptotic effect of TNF [20]. Furthermore, the G protein CX3C motif can bind to the fractalkine receptor (CX3CR1) and it has been shown that this interaction between the CX3C motif and CX3CR1 alters pulmonary inflammation, RSV-specific T-cell responses, FI-RSV vaccine-

enhanced disease, and expression of the neurokinin substance P [19, 21–27]. Antibodies directed against the G protein CX3C motif prevent binding to CX3CR1, neutralize RSV A and B strains, and reduce pulmonary inflammation in mice [28–30].

The fusion (F) glycoprotein resembles the prototypic *paramyxovirus* fusion protein and is involved in viral penetration of the host cell during entry, and subsequently in formation of syncytia [17]. The F glycoprotein also appears to have a role in viral attachment where the host protein, nucleolin, was recently identified as a cellular receptor for the F glycoprotein [17]. The F glycoprotein is synthesized as a 67 kDa precursor that undergoes proteolytic cleavage to produce two disulfide-linked polypeptides, F1 and F2, from the C- and N-termini [4]. Adjacent to these two regions are two heptad repeat sequences, denoted HR-C and HR-N, that form a stable trimer of hairpin-like structures that undergo a conformational change to enable the viral and cell membranes to be opposed before viral entry [4]. The small GTP-binding RhoA, a member of the Ras superfamily, also binds RSV F glycoprotein and facilitates virus-induced syncytium formation in Hep-2 cells [4, 31]. RhoA is involved in actin mobilization and signal transduction, and infection with RSV upregulates surface expression of RhoA and stimulates RhoA-mediated signaling [4, 32]. In addition, the F glycoprotein has been shown to interact with, and subsequently signal through, components of the lipopolysaccharide (LPS) receptor system; in particular, the pattern recognition receptors, CD14 and Toll-like receptor 4 (TLR4), have been implicated [4, 33].

The two RSV non-structural proteins are small (NS1 and NS2 are 139 and 124 amino acids in length, respectively) and have no substantial sequence homology with any cellular protein in the database [34, 35]. NS1 and NS2 antagonize both the cellular antiviral response and the induction of IFN [35]. Together, NS1 and NS2 degrade or sequester multiple signaling proteins that affect both IFN induction and IFN effector functions [34]. While the mechanism of action of NS1 and NS2 is a subject of active research, their effect on adaptive immunity is also being recognized.

1.3 RSV Replication

The first step in virus replication is RSV attachment to the host cell surface, typically accomplished via electrostatic interaction between the heparin-binding domains on the G glycoprotein and the cell-surface glycosaminoglycans [36–38]. The G glycoprotein itself is not strictly required for attachment, as an RSV G glycoprotein mutant lacking the G glycoprotein gene (RSVΔG) can cause productive infections, albeit at much lower efficiency than wild-type RSV [26, 39–41]. Following attachment to the host cell, the pre-fusion form of the RSV F glycoprotein binds to nucleolin on the cell surface; this triggers a conformational change in the protein causing membrane fusion and entry of RSV particles [17, 42]. Following cell fusion and penetration, the nucleocapsid, containing the viral genome and the N, L, P, and M2-1 proteins, is released

into the cytoplasm [43–46] where the L protein initiates genome transcription and replication [47]. Transcription of the viral mRNA, 3′ to 5′, occurs using a single promoter near the 3′ end resulting in a series of subgenomic mRNAs [48–52]. The viral mRNA transcripts can be detected as early as 4 h post-infection (pi), with peak mRNA synthesis and protein expression occurring between 12 and 20 h pi. Importantly, the level of protein expression is related to mRNA abundance [49], meaning there are decreased levels of mRNA proportional to the gene distance from the promoter sequence. Virus assembly occurs at the plasma membrane where nucleocapsids localize with the cell-membrane-containing membrane viral glycoproteins. The polymerization of the matrix protein has a primary influence on RSV assembly and budding, and the interaction of M with the N-terminal domain of the G protein and with the cytoplasmic tail of the F protein coordinates their recruitment to sites of assembly and budding [9]. Finally, new virions mature in clusters at the apical surface in a filamentous form associated with caveolin-1, and extend from the plasma membrane [53].

1.4 RSV Pathogenesis

RSV is one of the most common respiratory viruses in children having bronchiolitis accounting for 60–80% of bronchiolitis cases in children younger than 1 year of age [54]. It has been estimated that RSV causes nearly 34 million episodes of acute lower respiratory tract infections yearly, with 3.4 million episodes severe enough to require hospitalization worldwide [55]. Regardless of RSV severity, several studies have demonstrated a long-term impact of RSV lower respiratory tract infections upon children leading to a higher risk of asthma development [56–59].

Although the vast majority of people infected with RSV develop mild upper respiratory tract infections and clinical symptoms, in some individuals RSV infections cause significant morbidity and some mortality. Even though a great deal of investigation in both humans and animal models has explained the timing and tropism of RSV infection, and the general principles by which the immune system responds to infection, at present we only partially understand the disparity in illness severity that can occur [60]. The disease pathogenesis mechanisms are not well understood, but the virus itself likely contributes to the level of pathogenesis and there is abundant evidence that the early innate host response to primary infection is important. Although RSV disease phenotypes vary in humans and among animal models, inflammatory mediators have been strongly implicated in RSV pathogenesis. For example, numerous studies have established that RSV can cause asthma exacerbations and bronchiolitis [58], and that these conditions are associated with enhanced CD4 T cell responses, inappropriate cytokine expression, inflammation, and reduced immune regulation [58, 59, 61].

1.5 Host Immune Response to RSV

RSV infection of host cells alters the expression patterns of various genes related to protein metabolism, cell growth and proliferation, cytoskeleton organization, regulation of nucleotides and nucleic acid synthesis, and cytokine/chemokine genes linked with inflammation [2]. While airway epithelium promotes gaseous exchange, it also functions as the boundary between the external environment and the host, thus acting as a first line of defense against pathogens [2]. To overcome the repertoire of immune defenses encountered following RSV infection and replication, RSV evokes a variety of immune modulatory and evasion strategies [2]. For example, RSV delays programmed cell death or apoptosis of epithelial cells to facilitate virus replication [2]. It has been shown that RSV-infected cells have increased expression of the anti-apoptosis gene *IEX-1L* and increased expression of several Bcl-2 family members including myeloid cell leukemia-1 and Bcl-XL [2, 62–65]. RSV also modulates the innate immune response generated by host cells via modulation of signals triggered from pattern recognition receptors (PRRs) and Toll-like receptors (TLRs). TLRs initiate a complex signaling cascade leading to the expression of a variety of genes and signaling through NF-κB [2]. Like LPS, the RSV F glycoprotein can interact with the shared activities of TLR4 and CD14 in human monocytes leading to the activation of NF-κB and the production of proinflammatory cytokines TNF-α, IL-6, and IL-12 [2, 33]. A previous study demonstrated that RSV promotes TNF-α, IL-6, MCP-1, and RANTES via interaction with TLR2 and TLR6 [66] and that RSV G glycoprotein can interact with TLR2 (ref) and interfere with the host antiviral cytokine response [2]. TLRs are broadly distributed along the airways by various cell types including respiratory epithelial cells, alveolar macrophages, and dendritic cells (DCs) [2]. Respiratory epithelial cells infected with RSV result in increased TLR4 expression on the cell surface within 24 h pi [2, 67, 68]. The upregulation of TLR4 is functional as it leads to increased sensitivity to LPS stimulation [65]. Likewise, the RSV G glycoprotein is involved in inhibition of IFN-β through the induction of suppressor of cytokine signaling (SOCS) 1 and SOCS 3 expression (ref). It is well known that type I IFNs have an important role in DC maturation, activation of NK cells, differentiation and function of T cells, as well as enhancing primary antibody responses. RSV-mediated inhibition of type I IFN expression by RSV NS1 and NS2 negatively impacts antiviral immunity and facilitates virus replication [2, 69, 70].

RSV infection induces antibody responses against several structural and nonstructural viral antigens; however, only two major surface glycoproteins, i.e., F and G glycoprotein, induce antibodies that have a major role in protection [2, 71]. It has been shown that both forms of F glycoprotein, i.e., the mature form found in virions and the immature-folded form, are able to induce antibody responses [2, 72]. However, the immature form of the F glycoprotein

does not contain all the neutralizing epitopes found on the mature form of the F glycoprotein. In comparison to the F glycoprotein, the G glycoprotein is the more divergent protein among RSV isolates [2]; therefore a small number of G glycoprotein-specific monoclonal antibodies are cross-reactive, while the majority of F glycoprotein-specific monoclonal antibodies are cross-reactive [2]. However, studies have shown that antibodies directed against the G glycoprotein central conserved region can cross-react with RSV strains from groups A and B, and protect from RSV disease pathogenesis [28, 73, 74].

Although antibody responses are vital for protection against RSV infection, cellular immune responses may have a greater role in virus clearance [2]. RSV-specific CD8+ cytotoxic T lymphocytes (CTLs) are found in the lungs and peripheral tissues after RSV infection, and have a vital role in viral clearance [2]. In humans, CD8+ CTLs recognize F, matrix, M2, and NS2 proteins, but there is little or no recognition of epitopes on G, P, or NS1 proteins [2, 75]. In contrast, in a BALB/c mouse model, $CD8^+$ CTLs primarily recognize M2, F, and N proteins, and the role of CTLs in the immune response to RSV is well illustrated by in vivo depletion studies [76–79]. In BALB/c mice, the F glycoprotein primes both $CD8^+$ and $CD4^+$ T cells toward a Th1-type-biased cytokine response, while the G glycoprotein primes $CD4^+$ T cells that are biased toward the Th2-type cytokine response [2, 80]. The Th1 and Th2 $CD4^+$ T cells elicited react to a single region comprising amino acids 183–197 of the G protein [2, 81]. The importance of CD4+ memory T cells to RSV infection has been investigated; however, the majority of studies have focused on the response to RSV G glycoprotein priming [2]. It has been shown that the memory $CD4^+$ T cell response to the RSV G glycoprotein in the lungs of primed BALB/c mice challenged with RSV is dominated by effector T cells expressing a single TCR Vβ chain, such as Vβ14 [82]. $CD4^+$ T cells expressing TCR Vβ14 preferentially proliferate and expand into activated T cells in the lungs rather than the lymph nodes, which drain into the site of infection [83]. Although this study is limited to a specific inbred strain of mice, these findings may be important for understanding the role RSV-specific $CD4^+$ memory T cells have in RSV-induced immunopathology, a feature linked to polarized Th2-type cytokine response and pulmonary eosinophilia [84]. These studies suggest that both $CD4^+$ and $CD8^+$ lymphocytes are important for clearing RSV and that both contribute to the inflammatory response associated with infection [77].

1.6 Control and Prevention of RSV Infection

Control, prevention, and treatment of RSV infection must become a global health priority. No efficacious treatment or vaccine is currently available, and passive immunoprophylaxis using monoclonal antibodies reactive to the RSV F glycoproteins (palivizumab; Synagis) is expensive and limited to those considered at high risk

for severe infection. Moreover, Synagis is inaccessible to children in developing countries and more than half the world's population does not have access to this life-saving treatment. Further, RSV resistance to palivizumab has been reported. Recently, another immunoprophylactic, motavizumab, was not approved by the US Food and Drug Administration and was withdrawn.

Progress is being slowly made on development of RSV vaccines and antiviral compounds. Currently there are several RSV vaccine candidates (RSV ΔNS2Δ1313/1314L [85], RSV LIDΔM2-2 [86], RSV cps2 [87], MEDI-534 [88–90], MEDI-7510, PanAd3-RSV/MVA-RSV and RSV F nanoparticles [91]) and several antiviral compounds (RSV604 [92], GS-5806 [93, 94], and ALN-RSV01 [95, 96]) in clinical stage of development. We now have tools to make and evaluate a wide range of promising prophylactics and therapeutics and the challenge is to use them wisely. In the next set of chapters, a compilation of the current techniques that have made this progress possible are discussed, ranging from protocols for virus isolation, growth, and subtyping to procedures for the efficient generation of recombinant RSV viruses.

References

1. Krzyzaniak MA et al (2013) Host cell entry of respiratory syncytial virus involves macropinocytosis followed by proteolytic activation of the F protein. PLoS Pathog 9(4):e1003309
2. Oshansky CM et al (2009) The host response and molecular pathogenesis associated with respiratory syncytial virus infection. Future Microbiol 4(3):279–297
3. Wright PF (2014) Progress in the prevention and treatment of RSV infection. N Engl J Med 371(8):776–777
4. Harris J, Werling D (2003) Binding and entry of respiratory syncytial virus into host cells and initiation of the innate immune response. Cell Microbiol 5(10):671–680
5. Castilow EM, Meyerholz DK, Varga SM (2008) IL-13 is required for eosinophil entry into the lung during respiratory syncytial virus vaccine-enhanced disease. J Immunol 180(4):2376–2384
6. Botosso VF et al (2009) Positive selection results in frequent reversible amino acid replacements in the G protein gene of human respiratory syncytial virus. PLoS Pathog 5(1):e1000254
7. Martinelli M et al (2014) Phylogeny and population dynamics of respiratory syncytial virus (Rsv) A and B. Virus Res 189:293–302
8. Maggon K, Barik S (2004) New drugs and treatment for respiratory syncytial virus. Rev Med Virol 14(3):149–168
9. Kiss G et al (2014) Structural analysis of respiratory syncytial virus reveals the position of M2-1 between the matrix protein and the ribonucleoprotein complex. J Virol 88(13): 7602–7617
10. Castagne N et al (2004) Biochemical characterization of the respiratory syncytial virus P-P and P-N protein complexes and localization of the P protein oligomerization domain. J Gen Virol 85(Pt 6):1643–1653
11. Tiong-Yip CL et al (2014) Characterization of a respiratory syncytial virus L protein inhibitor. Antimicrob Agents Chemother 58(7): 3867–3873
12. Noton SL et al (2012) The respiratory syncytial virus polymerase has multiple RNA synthesis activities at the promoter. PLoS Pathog 8(10):e1002980
13. Tran TL et al (2007) The nine C-terminal amino acids of the respiratory syncytial virus protein P are necessary and sufficient for binding to ribonucleoprotein complexes in which six ribonucleotides are contacted per N protein protomer. J Gen Virol 88(Pt 1):196–206
14. Galloux M et al (2015) Identification and characterization of the binding site of the respiratory syncytial virus phosphoprotein to RNA-free nucleoprotein. J Virol 89(7): 3484–3496
15. Cheng X et al (2005) Overexpression of the M2-2 protein of respiratory syncytial virus

inhibits viral replication. J Virol 79(22): 13943–13952
16. Mitra R et al (2012) The human respiratory syncytial virus matrix protein is required for maturation of viral filaments. J Virol 86(8): 4432–4443
17. Tayyari F et al (2011) Identification of nucleolin as a cellular receptor for human respiratory syncytial virus. Nat Med 17(9):1132–1135
18. Li Y et al (2014) Inhibition of the human respiratory syncytial virus small hydrophobic protein and structural variations in a bicelle environment. J Virol 88(20):11899–11914
19. Tripp RA et al (2001) CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nat Immunol 2(8):732–738
20. Langedijk JP et al (1998) Structural homology of the central conserved region of the attachment protein G of respiratory syncytial virus with the fourth subdomain of 55-kDa tumor necrosis factor receptor. Virology 243(2): 293–302
21. Haynes LM et al (2003) Enhanced disease and pulmonary eosinophilia associated with formalin-inactivated respiratory syncytial virus vaccination are linked to G glycoprotein CX3C-CX3CR1 interaction and expression of substance P. J Virol 77(18):9831–9844
22. Harcourt J et al (2006) Respiratory syncytial virus G protein and G protein CX3C motif adversely affect CX3CR1+ T cell responses. J Immunol 176(3):1600–1608
23. Kauvar LM et al (2010) Therapeutic targeting of respiratory syncytial virus G-protein. Immunotherapy 2(5):655–661
24. Tripp RA et al (2003) The G glycoprotein of respiratory syncytial virus depresses respiratory rates through the CX3C motif and substance P. J Virol 77(11):6580–6584
25. Tripp RA, Jones L, Anderson LJ (2000) Respiratory syncytial virus G and/or SH glycoproteins modify CC and CXC chemokine mRNA expression in the BALB/c mouse. J Virol 74(13):6227–6229
26. Tripp RA et al (1999) Respiratory syncytial virus G and/or SH protein alters Th1 cytokines, natural killer cells, and neutrophils responding to pulmonary infection in BALB/c mice. J Virol 73(9):7099–7107
27. Tripp RA et al (2000) Respiratory syncytial virus infection and G and/or SH protein expression contribute to substance P, which mediates inflammation and enhanced pulmonary disease in BALB/c mice. J Virol 74(4):1614–1622
28. Choi Y et al (2012) Antibodies to the central conserved region of respiratory syncytial virus (RSV) G protein block RSV G protein CX3C-CX3CR1 binding and cross-neutralize RSV A and B strains. Viral Immunol 25(3):193–203
29. Miao C et al (2009) Treatment with respiratory syncytial virus G glycoprotein monoclonal antibody or F(ab')2 components mediates reduced pulmonary inflammation in mice. J Gen Virol 90(Pt 5):1119–1123
30. Haynes LM et al (2009) Therapeutic monoclonal antibody treatment targeting respiratory syncytial virus (RSV) G protein mediates viral clearance and reduces the pathogenesis of RSV infection in BALB/c mice. J Infect Dis 200(3):439–447
31. Pastey MK, Crowe JE Jr, Graham BS (1999) RhoA interacts with the fusion glycoprotein of respiratory syncytial virus and facilitates virus-induced syncytium formation. J Virol 73(9):7262–7270
32. Gower TL et al (2001) RhoA is activated during respiratory syncytial virus infection. Virology 283(2):188–196
33. Kurt-Jones EA et al (2000) Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol 1(5):398–401
34. Barik S (2013) Respiratory syncytial virus mechanisms to interfere with type 1 interferons. Curr Top Microbiol Immunol 372: 173–191
35. Ling Z, Tran KC, Teng MN (2009) Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting with RIG-I. J Virol 83(8):3734–3742
36. Bourgeois C et al (1998) Heparin-like structures on respiratory syncytial virus are involved in its infectivity in vitro. J Virol 72(9): 7221–7227
37. Krusat T, Streckert HJ (1997) Heparin-dependent attachment of respiratory syncytial virus (RSV) to host cells. Arch Virol 142(6):1247–1254
38. Feldman SA, Hendry RM, Beeler JA (1999) Identification of a linear heparin binding domain for human respiratory syncytial virus attachment glycoprotein G. J Virol 73(8):6610–6617
39. Techaarpornkul S, Collins PL, Peeples ME (2002) Respiratory syncytial virus with the fusion protein as its only viral glycoprotein is less dependent on cellular glycosaminoglycans for attachment than complete virus. Virology 294(2):296–304
40. Karron RA et al (1997) Respiratory syncytial virus (RSV) SH and G proteins are not essential for viral replication in vitro: clinical evaluation

and molecular characterization of a cold-passaged, attenuated RSV subgroup B mutant. Proc Natl Acad Sci U S A 94(25): 13961–13966

41. Teng MN, Whitehead SS, Collins PL (2001) Contribution of the respiratory syncytial virus G glycoprotein and its secreted and membrane-bound forms to virus replication in vitro and in vivo. Virology 289(2):283–296
42. Mastrangelo P, Hegele RG (2013) RSV fusion: time for a new model. Viruses 5(3):873–885
43. Levine S, Hamilton R (1969) Kinetics of the respiratory syncytial virus growth cycle in HeLa cells. Arch Gesamte Virusforsch 28(2):122–132
44. Hierholzer JC, Tannock GA (1986) Respiratory syncytial virus: a review of the virus, its epidemiology, immune response and laboratory diagnosis. Aust Paediatr J 22(2): 77–82
45. Arslanagic E et al (1996) Maturation of respiratory syncytial virus within HEp-2 cell cytoplasm. Acta Virol 40(4):209–214
46. Routledge EG et al (1987) Expression of the respiratory syncytial virus 22K protein on the surface of infected HeLa cells. J Gen Virol 68(Pt 4):1217–1222
47. Fearns R, Collins PL (1999) Model for polymerase access to the overlapped L gene of respiratory syncytial virus. J Virol 73(1):388–397
48. Huang YT, Collins PL, Wertz GW (1985) Characterization of the 10 proteins of human respiratory syncytial virus: identification of a fourth envelope-associated protein. Virus Res 2(2):157–173
49. Dickens LE, Collins PL, Wertz GW (1984) Transcriptional mapping of human respiratory syncytial virus. J Virol 52(2):364–369
50. Kuo L et al (1996) Effects of mutations in the gene-start and gene-end sequence motifs on transcription of monocistronic and dicistronic minigenomes of respiratory syncytial virus. J Virol 70(10):6892–6901
51. Harmon SB, Megaw AG, Wertz GW (2001) RNA sequences involved in transcriptional termination of respiratory syncytial virus. J Virol 75(1):36–44
52. Krempl C, Murphy BR, Collins PL (2002) Recombinant respiratory syncytial virus with the G and F genes shifted to the promoter-proximal positions. J Virol 76(23):11931–11942
53. Brown G et al (2002) Caveolin-1 is incorporated into mature respiratory syncytial virus particles during virus assembly on the surface of virus-infected cells. J Gen Virol 83 (Pt 3):611–621
54. Oymar K, Skjerven HO, Mikalsen IB (2014) Acute bronchiolitis in infants, a review. Scand J Trauma Resusc Emerg Med 22:23
55. Turner TL et al (2014) Respiratory syncytial virus: current and emerging treatment options. Clinicoecon Outcomes Res 6:217–225
56. Szabo SM et al (2013) Elevated risk of asthma after hospitalization for respiratory syncytial virus infection in infancy. Paediatr Respir Rev 13(Suppl 2):S9–S15
57. Tourdot S et al (2008) Respiratory syncytial virus infection provokes airway remodelling in allergen-exposed mice in absence of prior allergen sensitization. Clin Exp Allergy 38(6): 1016–1024
58. Sikkel MB et al (2008) Respiratory syncytial virus persistence in chronic obstructive pulmonary disease. Pediatr Infect Dis J 27(10 Suppl):S63–S70
59. Kallal LE, Lukacs NW (2008) The role of chemokines in virus-associated asthma exacerbations. Curr Allergy Asthma Rep 8(5):443–450
60. Peebles RS Jr, Graham BS (2005) Pathogenesis of respiratory syncytial virus infection in the murine model. Proc Am Thorac Soc 2(2): 110–115
61. Gern JE (2003) Mechanisms of virus-induced asthma. J Pediatr 142(Suppl 2):S9–S13, discussion S13–4
62. Domachowske JB et al (2000) Respiratory syncytial virus infection induces expression of the anti-apoptosis gene IEX-1L in human respiratory epithelial cells. J Infect Dis 181(3):824–830
63. Kotelkin A et al (2003) Respiratory syncytial virus infection sensitizes cells to apoptosis mediated by tumor necrosis factor-related apoptosis-inducing ligand. J Virol 77(17): 9156–9172
64. Lindemans CA et al (2006) Respiratory syncytial virus inhibits granulocyte apoptosis through a phosphatidylinositol 3-kinase and NF-kappaB-dependent mechanism. J Immunol 176(9):5529–5537
65. Monick MM et al (2005) Activation of the epidermal growth factor receptor by respiratory syncytial virus results in increased inflammation and delayed apoptosis. J Biol Chem 280(3):2147–2158
66. Murawski MR et al (2009) Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. J Virol 83(3):1492–1500
67. Xie XH et al (2009) Lipopolysaccharide induces IL-6 production in respiratory syncytial virus-infected airway epithelial cells through the toll-like receptor 4 signaling pathway. Pediatr Res 65(2):156–162

68. Monick MM et al (2003) Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. J Biol Chem 278(52):53035–53044
69. Becker Y (2006) Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy—a review. Virus Genes 33(2):235–252
70. Bruder D, Srikiatkhachorn A, Enelow RI (2006) Cellular immunity and lung injury in respiratory virus infection. Viral Immunol 19(2):147–155
71. Groothuis JR (1994) The role of RSV neutralizing antibodies in the treatment and prevention of respiratory syncytial virus infection in high-risk children. Antiviral Res 23(1):1–10
72. Sakurai H et al (1999) Human antibody responses to mature and immature forms of viral envelope in respiratory syncytial virus infection: significance for subunit vaccines. J Virol 73(4):2956–2962
73. Boyoglu-Barnum S et al (2013) A respiratory syncytial virus (RSV) anti-G protein F(ab')2 monoclonal antibody suppresses mucous production and breathing effort in RSV rA2-line19F-infected BALB/c mice. J Virol 87(20):10955–10967
74. Caidi H et al (2012) Combination therapy using monoclonal antibodies against respiratory syncytial virus (RSV) G glycoprotein protects from RSV disease in BALB/c mice. PLoS One 7(12):e51485
75. Cherrie AH et al (1992) Human cytotoxic T cells stimulated by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory syncytial virus. J Virol 66(4):2102–2110
76. Openshaw PJ (1995) Immunity and immunopathology to respiratory syncytial virus. The mouse model. Am J Respir Crit Care Med 152(4 Pt 2):S59–S62
77. Tripp RA, Anderson LJ (1998) Cytotoxic T-lymphocyte precursor frequencies in BALB/c mice after acute respiratory syncytial virus (RSV) infection or immunization with a formalin-inactivated RSV vaccine. J Virol 72(11):8971–8975
78. Connors M et al (1992) Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. J Virol 66(12):7444–7451
79. Graham BS et al (1991) Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 88(3):1026–1033
80. Johnson TR, Graham BS (1999) Secreted respiratory syncytial virus G glycoprotein induces interleukin-5 (IL-5), IL-13, and eosinophilia by an IL-4-independent mechanism. J Virol 73(10):8485–8495
81. Varga SM, Wissinger EL, Braciale TJ (2000) The attachment (G) glycoprotein of respiratory syncytial virus contains a single immunodominant epitope that elicits both Th1 and Th2 CD4+ T cell responses. J Immunol 165(11):6487–6495
82. Varga SM et al (2001) Immunopathology in RSV infection is mediated by a discrete oligoclonal subset of antigen-specific CD4(+) T cells. Immunity 15(4):637–646
83. Wissinger EL et al (2008) Proliferative expansion and acquisition of effector activity by memory CD4+ T cells in the lungs following pulmonary virus infection. J Immunol 180(5):2957–2966
84. Tripp RA (2004) Pathogenesis of respiratory syncytial virus infection. Viral Immunol 17(2):165–181
85. Luongo C et al (2013) Respiratory syncytial virus modified by deletions of the NS2 gene and amino acid S1313 of the L polymerase protein is a temperature-sensitive, live-attenuated vaccine candidate that is phenotypically stable at physiological temperature. J Virol 87(4):1985–1996
86. Cheng X et al (2001) Chimeric subgroup A respiratory syncytial virus with the glycoproteins substituted by those of subgroup B and RSV without the M2-2 gene are attenuated in African green monkeys. Virology 283(1):59–68
87. Luongo C et al (2012) Increased genetic and phenotypic stability of a promising live-attenuated respiratory syncytial virus vaccine candidate by reverse genetics. J Virol 86(19):10792–10804
88. Gomez M et al (2009) Phase-I study MEDI-534, of a live, attenuated intranasal vaccine against respiratory syncytial virus and parainfluenza-3 virus in seropositive children. Pediatr Infect Dis J 28(7):655–658
89. Nelson CL, Tang RS, Stillman EA (2013) Genetic stability of RSV-F expression and the restricted growth phenotype of a live attenuated PIV3 vectored RSV vaccine candidate (MEDI-534) following restrictive growth in human lung cells. Vaccine 31(36):3756–3762
90. Yang CF et al (2013) Implication of respiratory syncytial virus (RSV) F transgene sequence heterogeneity observed in phase 1 evaluation

of MEDI-534, a live attenuated parainfluenza type 3 vectored RSV vaccine. Vaccine 31(26): 2822–2827

91. Glenn GM et al (2013) Safety and immunogenicity of a Sf9 insect cell-derived respiratory syncytial virus fusion protein nanoparticle vaccine. Vaccine 31(3):524–532
92. Challa S et al (2015) Mechanism of action for respiratory syncytial virus inhibitor RSV604. Antimicrob Agents Chemother 59(2):1080–1087
93. DeVincenzo JP et al (2014) Oral GS-5806 activity in a respiratory syncytial virus challenge study. N Engl J Med 371(8): 711–722
94. Mackman RL et al (2015) Discovery of an oral respiratory syncytial virus (RSV) fusion inhibitor (GS-5806) and clinical proof of concept in a human RSV challenge study. J Med Chem 58(4):1630–1643
95. Alvarez R et al (2009) RNA interference-mediated silencing of the respiratory syncytial virus nucleocapsid defines a potent antiviral strategy. Antimicrob Agents Chemother 53(9):3952–3962
96. DeVincenzo J et al (2008) Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSV01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV). Antiviral Res 77(3):225–231

Chapter 2

RSV Growth and Quantification by Microtitration and qRT-PCR Assays

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Abstract

Defective interfering viral particles have been reported as important determinants of the course of viral infection, and they can markedly temper the virulence of the infection. Here, we describe a simple method, based on limiting dilution, for the removal of defective interfering particles from RSV. This method results in a high-titer viral preparation from both HEp-2 and Vero cell lines. We evaluated two concentrations of sucrose to stabilize the virus preparation, and demonstrate that RSV is stable when prepared and stored in 25 % sucrose at −152 °C. In addition, this chapter describes some commonly used methods of RSV titration, detection using microtitration and quantitative real-time RT-PCR, and the use of immunostaining for antigenic characterization.

Key words Respiratory syncytial virus, Limiting dilution, Immunostaining, Plaque assay, qRT-PCR

1 Introduction

Respiratory syncytial virus is commonly cultured in and isolated from epithelial cell lines. The commonly used cell lines include HEp-2 cells, a carcinoma cell line derived from human larynx tissue, and Vero cells, derived from African green monkey kidney epithelial cells. The differences in sensitivity to RSV infection that are exhibited by in vitro systems as well as in vivo systems may in part be due to differences in the attachment (RSV G) protein and its glycosylation that arise from the cell line in which stock RSV virus is produced [1]. HEp-2 cells predominantly produce virions containing heavily glycosylated, full-length RSV G protein with an apparent molecular weight of 90 kDa, whereas Vero cells predominantly produce RSV G protein with an apparent molecular weight of 55 kDa [1].

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The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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A high-titered and stable virus preparation is essential for vaccine development, quality control of vaccine preparations, induction of specific immune globulins, for serologic tests, and characterization and immunological studies [2, 3]. Defective interfering particles (DIP) are virus-like particles that arise during virus growth, fail to grow in the absence of virus, and replicate at the expense of virus during co-infections. The presence of DIP in a virus preparation used for in vitro or in vivo studies is not desirable as these particles may not possess protective epitopes and inhibit infection and replication of whole virions [3, 4]. The presence of the DIP impacts the reproducibility of tests based upon virus neutralization, since the non-infectious DIP compete with complete infectious virions for antibody binding, thereby falsely lowering neutralization titers [3, 4]. In vivo studies of RSV can be difficult due to variation in viral infection and disease severity in some animal models. Factors that may contribute to the variation are decreases in infectious viral titer due to DIP [3], virus preparation and storage, and method of virus administration [5, 6]. The interference of DIP is dependent on multiplicity of infection, i.e., the higher the MOI the higher the DIP accumulation, and can be eliminated by plaque reduction [7] or limiting dilution [3].

RSV is a heat-labile virus and preparations of RSV rapidly lose infectivity at room temperature and are easily inactivated by one freeze-thaw cycle [3, 8]. The addition of stabilizers, such as sugars (e.g., sucrose), salts (e.g., Mg_2SO_4), and polyols (e.g., glycerol) preserve viral infectivity and protein structure [3, 6, 8, 9]. We evaluated two concentrations of sucrose to stabilize the virus preparation, and demonstrate that RSV is stable after four freeze-thaws when prepared in 25% sucrose and stored at −152 °C (Table 1).

With regard to the antigenicity of RSV, the fusion (F) and attachment (G) proteins are considered to be the major antigenic determinants due to their ability to induce protective neutralizing antibodies in the host. Monoclonal antibodies against these surface proteins can be used for the identification and characterization of RSV strains, A and B [10–12]. The antigenic characterization of RSV subgroups can be made by enzyme-linked immunosorbent

Table 1
Stability of RSV virus stock in different concentrations of sucrose[a]

% Sucrose	Initial virus titer (PFU/mL)	Final virus titer (PFU/mL)
0	2.3×10^7	2.5×10^5
25	2.3×10^7	1.5×10^7
50	2.3×10^7	1.0×10^4

[a]RSV A2 strain was frozen (−152 °C) at varying sucrose concentrations (0%, 25%, 50%) to assess infectivity after four freeze-thaw cycles. The higher concentration of sugar was toxic to cells, consistent with previous findings [3]

assay (ELISA), immunofluorescence, or other serological methods using monoclonal antibodies directed against variable epitopes.

In this chapter, we describe a simple method of removal of DIPs from RSV to obtain a high titer and stable virus stock by using a modified limiting dilution method [2]. Real-time RT-PCR provides a rapid and sensitive tool for detection of RSV compared to conventional techniques and enables quantitation of viral load [13–15]. We describe a quantitative real-time RT–PCR (qRT-PCR) to quantitate RSV-specific RNA in samples and virus titration by ELISA-based microtitration method in 96-well flat-bottom plates. Viral antigen is quantitated by fixing the virus and then sequentially treating fixed virus-infected cells or cell lysate with anti-RSV fusion (F) and attachment (G) monoclonal antibodies, anti-mouse IgG horseradish peroxidase, and peroxide substrate [10]. This immunostaining method can be used to study the antigenic characteristics of RSV isolates.

2 Materials

2.1 Tissue Culture

1. Dulbecco's modified Eagle medium, DMEM: 25 mM Glucose, 4 mM l-glutamine, 0.03 mM phenol red serum-free (SF-DMEM). Store at 4–8 °C.
2. Fetal bovine serum, FBS: Endotoxin level: ≤5 EU/mL, hemoglobin level: ≤10 mg/dl. Triple filtered at 0.1 μm. Thaw frozen serum at 4–8 °C, room temperature, or rapidly at 37 °C (*see* **Note 1**). To heat inactivate the complement, incubate thawed serum at a thermostatically controlled temperature of 56 °C for 30 min. Store 50 mL aliquots of heat-inactivated serum −5 to −20 °C until use. Once thawed, do not store for more than 2–3 weeks at 4–8 °C.
3. DMEM-10: SF-DMEM, 10% FBS (v/v). Add 100 mL of heat-inactivated FBS to 900 mL of DMEM and mix thoroughly. To sterilize, filter through a 0.2 μm membrane filter. Store at 4–8 °C.
4. DMEM-5: SF-DMEM, 5% FBS (v/v). Add 50 mL of heat-inactivated FBS to 950 mL of DMEM and mix thoroughly. Sterile filter through 0.2 μm membrane filter and store at 4–8 °C.
5. Trypsin-EDTA: 0.05% Trypsin and ethylenediamine tetracetic acid, EDTA, 5.3 mM potassium chloride, 0.44 mM potassium phosphate monobasic, 4.16 mM sodium bicarbonate, 137 mM sodium chloride, 0.33 mM sodium phosphate dibasic, 5.5 mM glucose, 0.02 mM phenol red, pH 7.2–8.0. Store at −5 to −20 °C. Once thawed, store at 4–8 °C up to 2 weeks (*see* **Note 2**).
6. Infectious RSV stock: A2 strain and B1 strain (*see* **Note 3**).
7. Vero cells: ATCC CCL-81 (*see* **Notes 4** and **5**).
8. HEp-2 cells: ATCC CCL-23 (*see* **Notes 4** and **5**).

9. Hanks' balanced salt solution, HBSS: 1.26 mM Calcium chloride, 0.49 mM magnesium chloride, 0.4 mM magnesium sulfate, 5.3 mM potassium chloride, 0.44 mM potassium phosphate monobasic, 4.16 mM sodium bicarbonate, 137 mM sodium chloride, 0.33 mM sodium phosphate dibasic anhydrous, 5.5 mM glucose, 0.02 mM phenol red.
10. Dulbecco's PBS, D-PBS: 2.66 mM Potassium chloride, 1.47 mM potassium phosphate monobasic, 137 mM sodium chloride, 8.0 mM sodium phosphate dibasic, pH 7.0–7.2.
11. Sterile 25% (w/v) sucrose solution: Add 25 g sucrose to 100 mL sterile D-PBS and mix until sucrose is completely dissolved. Sterile-filter through 0.2 μm membrane filter and store at 4 °C.
12. Cell culture flasks: 25 cm^2, 75 cm^2, or 162 cm^2, vented caps.

2.2 Microtitration by Plaque Assay

1. SF-DMEM.
2. DMEM-10.
3. D-PBS.
4. Fixative: Cold 80% (v/v) acetone in D-PBS. Prepare and use 80% acetone in a biosafety cabinet with external venting or a chemical fume hood. Mix 80 mL acetone with 20 mL D-PBS. Store at 4–8 °C. After use, discard appropriately (*see* **Note 6**).
5. 96-Well flat-bottom tissue culture plates.

2.3 Immunostaining

1. D-PBS.
2. Blocking solution: 1% (w/v) skim milk in D-PBS. Dissolve 1 g powdered skim milk in 100 mL D-PBS and mix well until milk is thoroughly dissolved.
3. Primary antibody: Mouse anti-RSV monoclonal antibodies (Millipore, Cat #MAB858-4, MAB8599, MAB858-2KC, MAB858-3B), diluted to 1:200 (*see* **Note 7**).
4. Secondary antibody: Goat anti-mouse IgA + IgG + IgM, conjugated to horseradish peroxidase (HRP). Dilute in blocking solution at optimum concentration as determined by titration (*see* **Note 8**). Typically, antibody is diluted 1:2000 in blocking solution.
5. DAB/metal substrate concentrate: Store at −15 to −25 °C and dilute 1:10 in DAB peroxide buffer immediately before use. Discard unused working buffer.
6. DAB peroxide buffer: Thaw and store buffer at 2–8 °C.

2.4 Quantitative Real-time PCR (qRT-PCR)

1. RNA purification Kit: QIAamp® Viral RNA purification Mini Kit (QIAGEN) or equivalent.
2. Total RNA: Extracted from samples of interest using RNA purification kit. Store at −70 °C or below.

3. One-step RT-PCR kit: AgPath-ID One-Step RT-PCR (Life Technologies) or equivalent.
4. RSV-M forward primer: 5′-GGC AAA TAT GGA AAC ATA GCT GAA-3′. Dilute primer to a working concentration of 50 μM in sterile, RNase-free water.
5. RSV-M reverse primer: 5′-TCT TTT TCT AGG ACA TTG TAT TGA ACA G-3′. Dilute primer to a working concentration of 25 μM in sterile, RNase-free water.
6. TaqMan probe: 5′-GTG TGT ATG TGG AGC CTT CGT GAA GCT-3′, labeled at the 5′ end with the reporter molecule 6-carboxyfluorescein (FAM) and labeled with a Blackhole quencher-1 (BHQ1) at the 3′ end. Dilute primer to a working concentration of 5 μM in sterile, RNase-free water.

3 Methods

All procedures should be performed following good laboratory practices at the Biosafety Level 2 (BSL-2), as described in the Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition (http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf), unless otherwise indicated. Work with live RSV must be conducted within properly maintained biosafety cabinets using appropriate personal protective equipment.

3.1 Preparation of Cell Culture for Limiting Dilution

Prior to the passage of Vero or HEp-2 cells, the cell monolayer should be 90–95 % confluent. It is important that the cells do not overgrow and enter stationary phase. Cells must be in log-phase growth for optimal yield and virus infectivity (*see* **Note 5**).

1. Warm trypsin-EDTA and DMEM-10 in a 37 °C water bath.
2. To passage the Vero or HEp-2 cells grown in a 75 cm^2 flask, remove DMEM-10 medium and gently rinse monolayer with 5 mL of warm trypsin-EDTA. If using a 25 cm^2 flask, add 2 mL of warm trypsin-EDTA.
3. Decant trypsin-EDTA.
4. Add another 5 mL trypsin-EDTA (1 mL to a 25 cm^2 flask) to cover the cell monolayer.
5. Incubate at 37 °C in 5–7 % CO_2 and monitor by light microscopy until most of the monolayer detaches from flask, usually 3–5 min.
6. Gently tap the side of the flask to dislodge the remaining cells.
7. Add 5 mL warm DMEM-10 (5 mL to a 25 cm^2 flask) to trypsinized cells, bringing the total volume to 10 mL (6 mL in a 25 cm^2 flask).

8. Mix trypsinized cells by gently triturating cells up and down about five to ten times with a 5 or 10 mL serological pipette.
9. Determine the total number of viable cells per flask using trypan blue exclusion (*see* **Note 9**).
10. Seed two 25 cm^2 flasks with $\geq 4.0 \times 10^5$ of Vero or with $\geq 5.0 \times 10^5$ HEp-2 cells/mL in a total of 10 mL DMEM-10.
11. Incubate at 37 °C and 5–7% CO_2 for 24 h or until 80–85% confluence.

3.2 Preparation of Virus Seed Stocks by Limiting Dilution

1. Dilute RSV stock virus (A2 or B1) in 5 mL cold SF-DMEM to a final multiplicity of infection (MOI) of 0.2 (1 virion per every five cells), based on the total number of cells per flask determined in Subheading 2.1, **item 10.**
2. Decant the medium from a 25 cm^2 flask of cells (flask A).
3. Wash cells with 5 mL warmed SF-DMEM and decant medium.
4. Overlay monolayer of cells with 5 mL diluted RSV.
5. Incubate at 37 °C and 5% CO_2 for 1 h to allow for adsorption of virus.
6. During the 1h incubation, decant medium from a second 25 cm^2 flask of Vero or HEp-2 cells (flask B).
7. Rinse monolayer with 2 mL warmed trypsin-EDTA and remove trypsin wash.
8. Add 1 mL warmed trypsin-EDTA and monitor cells by light microscopy until all cells are detached from the flask.
9. Add 2 mL of SF-DMEM to the flask B and set flask aside.
10. At the end of the 1h incubation, gently wash the infected cell monolayer of the first 25 cm^2 flask (flask A) two times, with 5 mL warmed HBSS each wash. Trypsinize cells by adding 1 mL warmed trypsin-EDTA until all cells are detached from the flask. Add 2 mL of DMEM-5 to the flask. Determine the number of viable cells in flask A.
11. Adjust the cell concentration to 1.7×10^6 viable cells/flask for HEp-2 cells or 2.1×10^6 viable cells/flask for Vero cells.
12. Using these virus-infected cells (flask A), prepare eight tenfold serial dilutions, from 10^{-1} to 10^{-8}, in sterile 2 mL tubes containing SF-DMEM.
13. Add 100 µL of each serial dilution of infected cells to a sterile, flat-bottom 96-well plate, starting with 1:10 dilution in row A, from columns 3 to 12.
14. Add one dilution per row, for the remaining rows of the plate, from columns 3 to 12 (*see* Fig. 1).
15. Add 100 µL of serum-free DMEM to each well of columns 1 and 2, for the negative control for infection.

	1	2	3	4	5	6	7	8	9	10	11	12
A	No virus	No virus	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}
B	No virus	No virus	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}
C	No virus	No virus	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}
D	No virus	No virus	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
E	No virus	No virus	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}
F	No virus	No virus	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}
G	No virus	No virus	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}
H	No virus	No virus	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}

Fig. 1 Example of limiting dilution and microtitration plate layout. Example of template for plaque assay in 96-well plate format to determine the amount of infectious RSV in each sample. Each dilution of virus stock, from 1:10 to 1:10^8, is assayed in ten wells from column 3 through column 12. Columns 1 and 2 are for negative controls for infection

16. Adjust the cell concentration in the flask B to 2×10^5 Vero cells/mL or 3×10^4 HEp-2 cells/mL in DMEM-5.
17. Add 100 μL of uninfected cells to each well (total 2×10^4 Vero cells or 3×10^4 HEp-2 cells) in the plate.
18. Incubate the plates at 37 °C and 5 % CO_2 for 24–48 h for Vero cells, or 3–4 days for HEp-2 cells.
19. Monitor cells daily by visual microscopy for the development of single syncytium in each well. Syncytia are defined as the fused product of two or more cells, and appear as a single, multi-nucleated cell.
20. Select wells that have a single syncytium, and gently scrape cells from wells using a P-200 pipette.
21. Harvest the contents of the wells and transfer to a sterile 1.5 mL microcentrifuge tube.
22. To lyse the cells, freeze tube contents to −80 °C and quickly thaw cells at 37 °C until just thawed.
23. Transfer the pooled cells to a sterile 1.5 mL microcentrifuge tube and centrifuge at $500 \times g$ for 10 min at 4 °C, to remove cellular debris.
24. Transfer supernatant to a clean, sterile 1.5 mL microcentrifuge tube. The supernatant contains Passage #1 (P1) virus.
25. Determine the titer of P1 virus by plaque assay, as described in Subheading 3.3.

26. Repeat limiting dilution steps, as described in Subheadings 3.2, **items 1**–7, using P1 harvest to generate Passage 2 (P2).
27. Incubate the plates at 37 °C and 5 % CO_2 for 24–48 h for Vero, and 3–4 days for HEp-2.
28. Monitor cells daily by visual microscopy for the development of single syncytium in each well.
29. Select wells at the highest dilution factor that show the development of cytopathic effect (CPE), and gently scrape cells from wells using a P-200 pipette.
30. Harvest the contents of the wells and transfer to a sterile 1.5 mL microcentrifuge tube.
31. Freeze and quickly thaw to lyse the cells. Transfer the pooled cells to a sterile 1.5 mL microcentrifuge tube and centrifuge at $500 \times g$ for 10 min at 4 °C, to remove cellular debris.
32. Transfer supernatant to a clean, sterile 1.5 mL microcentrifuge tube. The supernatant contains Passage #2 (P2) virus.
33. Determine the titer of P2 virus by plaque assay, as described in Subheading 3.3.
34. Seed two 75 cm^2 flasks with 4×10^6 cells/mL Vero or 5×10^6 cells/mL HEp-2, plus one additional flask for each cell line for counting cells immediately prior to infection (two flasks per cell line; 5 mL total volume).
35. Eighteen to 24 h after seeding, trypsinize cells in one 75 cm^2 flask of each cell line and determine the number of viable cells.
36. Dilute the P2 virus stock in 5 mL SF-DMEM, to a final MOI of 0.1 (one virion per every ten cells).
37. Decant medium from one 75 cm^2 flask of cells per cell line.
38. Wash each flask of cells twice, with 10 mL of warmed SF-DMEM per wash.
39. Decant wash.
40. Overlay 5 mL diluted virus onto each flask of cells.
41. Incubate the flasks at 37 °C and 5 % CO_2 for 2 h, to allow for adsorption of virus. After 1 h, gently rock flasks.
42. Remove virus suspension from each flask and wash cell monolayers twice with 10 mL warmed HBSS.
43. Add 10 mL DMEM-5 to each flask and incubate the flasks at 37 °C and 5 % CO_2 for 48–72 h for Vero cells, and 3–4 days for HEp-2 cells. Monitor the flasks daily for the development of cytopathic effect (CPE).
44. When flasks demonstrate 85–90 % CPE, remove supernatant from flasks.
45. Add 5 mL SF-DMEM to each flask and loosely replace flask caps.

46. Transfer flasks to −80 °C until frozen, being sure that cell surface is covered with SF-DMEM while in the freezer (*see* **Note 10**).
47. Thaw cells at 4 °C until medium is just thawed.
48. Repeat freeze-thaw cycle one more time.
49. After second thaw, scrape down cell lysate in flask using a disposable scraper.
50. Transfer flask contents to sterile 50 mL conical tubes.
51. Centrifuge at 500 × *g* at 4 °C for 10 min, to remove cellular debris.
52. Prepare 0.2 mL aliquots of viral supernatants into 2.0 mL sterile, cryovials. This virus seed stock is Passage #3 (P3).
53. Determine the titer of P3 virus by plaque assay, as described in Subheading 3.3.

3.3 Preparation of RSV Working Stock

1. Seed 20–30 162 cm^2 tissue culture flasks with 30 mL of Vero cells at a concentration of 5×10^5 cells/mL, or HEp-2 cells at a concentration of 6×10^6 cells/mL, in DMEM-10. Incubate the flasks at 37 °C and 5 % CO_2 for 24 h.
2. Determine the number of viable cells in one 162 cm^2 flask of cells as described in **step 1**.
3. Dilute low-passage virus (P3) in 5 mL warmed SF-DMEM per flask to be infected at MOI of one virion per five cells (MOI=0.2).
4. Decant medium from cells in flasks. Rinse cells with 10 mL warmed SF-DMEM, and discard.
5. Add 5 mL virus diluted in SF-DMEM.
6. Rock flask gently to completely cover cells with virus.
7. Incubate the flasks at 37 °C and 5 % CO_2 for 2 h to allow adsorption of virus. Rock once after 1 h.
8. After incubation, add 6 mL DMEM-5 to each flask, without removing virus.
9. Return flasks to incubator, and incubate the flasks at 37 °C and 5 % CO_2 for 2–3 days for Vero, and 3–4 days for HEp-2, monitoring the development of CPE daily, until CPE ≥80 % of cell monolayers, but the monolayer is still intact and attached to flask bottom.
10. Remove supernatants from all flasks.
11. Add 5 mL cold 25 % (w/v) sterile-filtered sucrose to each flask (*see* **Note 11**).
12. Transfer flasks to −80 °C, being sure that cell surface is covered with sucrose solution while in the freezer. Thaw cells at 4 °C until just thawed. Repeat freeze-thaw cycle one more time.
13. After second thaw, scrape down cell lysates.

14. Transfer all lysates to sterile 50 mL conical tubes.
15. Remove the cellular debris by centrifugation at 500 × *g* and 4 °C for 10 min.
16. Pool supernatants and discard cell debris pellets. Alternate method: Virus may also be harvested by sonication (*see* **Notes 12** and **13**).
17. Aliquot supernatants into sterile cryovials and store vials at −152 °C for long-term storage. Sucrose in concentrations at 25% has a stabilizing effect and reduces loss of infectivity of this very labile virus (*see* Table 1) (*see* **Note 14**).
18. Virus titration can be performed in Vero or HEp-2 cells by immunostaining-based microtitration method in a 96-well plate format, as described in Subheading 3.4.

3.4 Microtitration of RSV by Plaque Assay

1. Prepare eight 1.5 mL tubes; each tube will contain 900 µL of SF-DMEM.
2. Add 100 µL of virus to the first containing 900 µL SF-DMEM (10^{-1} dilution). Mix the virus and SF-DMEM by pipetting.
3. To achieve a 10^{-2} dilution, add 100 µL of 10^{-1} virus mixture to a second tube containing 900 µL SF-DMEM and mix thoroughly.
4. Repeat the 1:10 dilution process through all eight tubes. The tubes will now have these effective dilutions of virus: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}.
5. To a sterile, 96-well flat-bottom tissue culture plate (*see* **Note 15**), add 100 µL of SF-DMEM to columns 1 and 2. These wells will serve as negative controls (*see* Fig. 2).
6. Add 100 µL of each virus dilution to one row of the 96-well plate (rows A–H), from columns 3 to 12. Add 2×10^4 Vero cells, or 3×10^4 HEp-2 cells, in 100 µL of DMEM-5 to each well of the tissue culture plate.
7. Incubate at 37 °C and 5% CO_2 for 48–72 h for Vero cells and 72–96 h for HEp-2 cells (*see* **Note 16**).
8. Monitor cells with an inverted microscope for plaque formation and monolayer integrity (*see* **Note 17**). After 72–96 h, gently aspirate the entire medium from each well.
9. Gently wash plate three times with 200 µL of D-PBS per well.
10. Completely air-dry plate within a biosafety cabinet (BSC).
11. Add 100 µL of cold fixative (80:20 (v/v)) acetone:PBS to each well.
12. Incubate at 4 °C for 10 min.
13. After this incubation, aspirate fixative from each well and wash plate three times with 200 µL of D-PBS per well D-PBS (*see* **Note 18**). RSV is now fixed and non-infectious, and the

remainder of the procedure may be performed outside of the BSC.

14. For immunodetection of plaques, add 200 µL of blocking solution to each well and incubate plate for 30 min to 1 h at room temperature.
15. Aspirate blocking solution and wash plate one time with 200 µL of D-PBS per well. Add 100 µL per well of primary anti-RSV antibody cocktail (anti-G and anti-F monoclonal antibodies), diluted in blocking solution, to each well.
16. Incubate plate at 37 °C for 1 h.
17. Aspirate primary antibody.
18. Wash plate three times with 200 µL of D-PBS per well. Add 100 µL per well of secondary antibody, HRP goat anti-mouse IgG+IgM+IgA, diluted in blocking solution, to each well.
19. Incubate plate at 37 °C for 1 h.
20. Aspirate secondary antibody.
21. Wash plate three times with 200 µL of D-PBS per well.
22. Add 100 µL 1× DAB substrate, diluted in peroxide buffer according to the manufacturer's directions, to each well.
23. Incubate plate at room temperature until infected plaques become visible. The plaques will appear as dark brown-black circular areas. This can take up to 10 min (*see* **Note 19**).
24. Stop development reaction by gently rinsing plate with tap water.
25. Remove excess water by tapping plate on an absorbent towel.
26. Allow plate to air-dry.
27. Determine whether each well of the plaque assay exhibits CPE (*see* **Note 20** and Fig. 2).
28. Determine the 50 % tissue culture infective dose ($TCID_{50}$ titer) per 0.1 mL by Reed-Muench method [16] (*see* Fig. 3).

3.5 Determination of the Viral Titer in RSV Stocks by qRT-PCR)

1. Extract total RNA extracted from 200 µL of RSV-infected cell suspensions (A2 or B1 strains) using viral RNA purification kit per the manufacturer's guidelines and store at −70 °C or below until use.
2. Prepare the RSV viral template control (VTC or Standard, *see* **Note 21**). Prepare a template for the qRT-PCR assay, to determine the number of reactions per assay (*see* Fig. 4).
3. Determine the number of reactions (N) to prepare for the assay, using the following guidelines:
   (a) For a number of samples (n), including the non-template control (NTC) and VTC ≤14, then $N = n + 1$
   (b) For a number of samples, n, including the NTC and VTC ≥15, then $N = n + 2$.

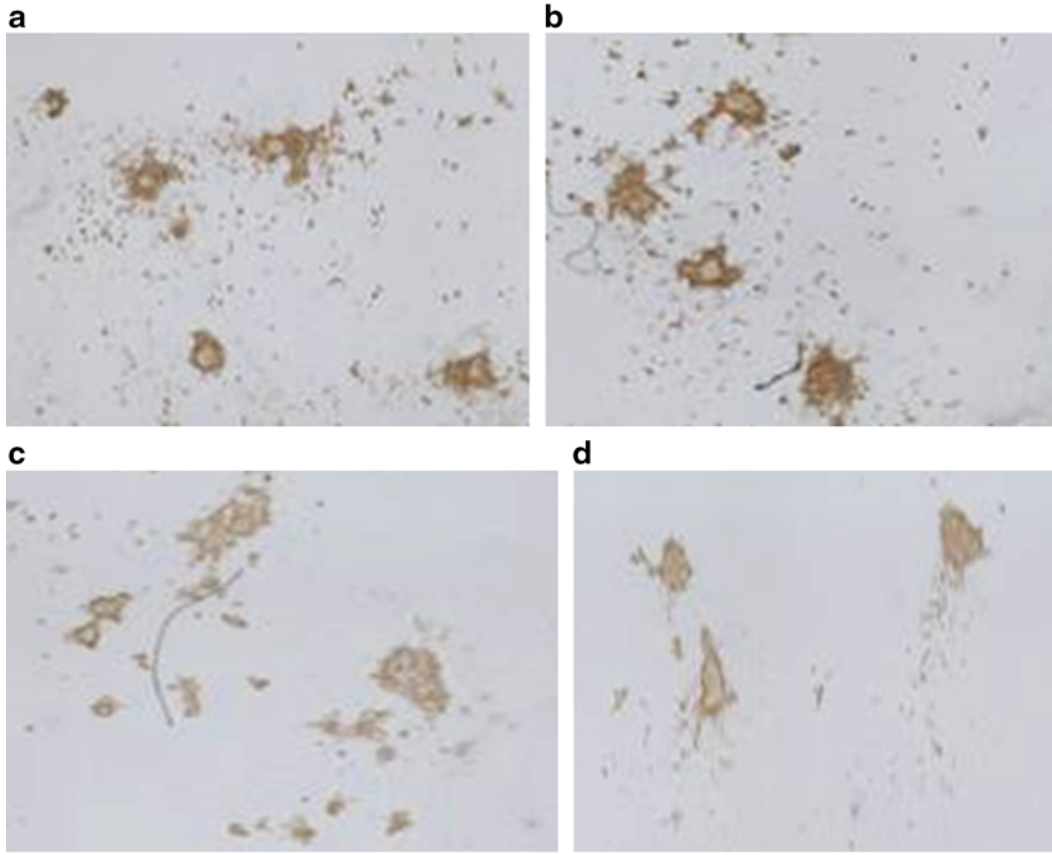

Fig. 2 Plaque detection using immunostaining to reveal RSV-infected cells. Infection with (**a**) RSV A2 in Vero cells, (**b**) RSV B1 in Vero cells, (**c**) RSV A2 in HEp-2 cells, and (**d**) RSV B1 in HEp-2 cells. At 3–4 days pi, cultures were immunostained using an antibody pool containing anti-RSV F, G, and N followed by secondary antibody of HRP-goat anti-mouse IgG + IgM + IgA at 1:2000 and stained with DAB. The plaques will appear as *dark brown-black* localized areas on the background of the cell monolayer

4. Calculate the amount of RT-PCR master mix to be prepared for the assay. If you are using the AgPath-ID™ One-Step RT-PCR Kit add the following amounts:
   (a) 2× RT-PCR reaction mix for probes = $N \times 12.5$ μL.
   (b) 25× AgPath-ID RT-PCR for One Step = $N \times 1$ μL.
   (c) Forward primer = $N \times 0.25$ μL.
   (d) Reverse primer = $N \times 0.25$ μL.
   (e) Probe = $N \times 0.25$ μL.
   (f) Nuclease-free water = $N \times 6.25$ μL.
   (g) Total volume = $N \times 20$ μL.
5. Under an RNase- and DNase-free BSL-2 environment, prepare the RT-PCR master mix as determined in **step 4**, in a sterile, RNase-free, DNase-free tube.

	1	2	3	4	5	6	7	8	9	10	11	12	% CPE-positive
10^{-1}	-	-	+	+	+	+	+	+	+	+	+	+	100
10^{-2}	-	-	+	+	+	+	+	+	+	+	+	+	100
10^{-3}	-	-	+	+	+	+	+	+	+	+	+	+	100
10^{-4}	-	-	+	+	+	-	+	+	+	+	+	+	90
10^{-5}	-	-	-	+	+	-	+	-	+	+	+	+	70
10^{-6}	-	-	-	-	+	-	+	-	-	+	-	+	40
10^{-7}	-	-	-	+	-	-	-	-	+	-	-	-	20
10^{-8}	-	-	-	-	-	-	-	-	-	-	-	-	10

Fig. 3 Calculation $TCID_{50}$ by the Reed and Muench method. An example of calculating $TCID_{50}$ and PFU/mL of virus preparations using the Reed and Muench method
Proportional distance (PD) formula:
[(%positive value > 50 % – 50 %)/(%positive value > 50 %) – (%positive value < 50 %)]
Example of $TCID_{50}$ calculation:
Well volume = 0.1 mL
[(% positive at 10^{-5} – 50 %)/(% positive at 10^{-5}) – (% positive at 10^{-6})] = (70 – 50)/(70 – 40) = 0.67
Log lower dilution: log(10^{-5}) = **–**5
PD + Log lower dilution: 0.67 + –5 = –4.33
Log $TCID_{50} = 10^{-4.33}$; $TCID_{50} = 2.13 \times 10^4$
$TCID_{50}$/mL = 2.13×10^4/0.1 mL = 2.13×10^5/mL
PFU/mL = $TCID_{50}$/mL × 0.67 (Poisson distribution constant) = 1.4×10^5/mL
+ = wells with CPE; – = wells without CPE

6. Thoroughly mix the reaction mixture by pipetting up and down.
7. Fit a clean 96-well PCR plate into a frozen cooling block to keep reaction mixture cold until prepared.
8. Add 20 μL of the master mix into each NTC well in column 1.
9. Add 20 μL of the master mix into the sample wells, then into the remaining NTC wells in column 10, and finally to the VTC wells.
10. Add 5 μL of nuclease-free water into NTC wells, and cap wells tightly with optical strip caps.
11. Loosely cover remaining wells to prevent contamination, and move the reaction plate to a designated BSL-2 nucleic acid-handling area.
12. Pipette 5 μL of sample RNA into designated sample wells, beginning with column 2 and working across the plate to column 10.
13. After each column is completed, cap tightly with an optical strip cap.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	S1	S1	S9	S9	S17	S17	S25	S25	NTC	NTC	NTC
B	NTC	S2	S2	S10	S10	S18	S18	S26	S26	NTC	NTC	NTC
C	NTC	S3	S3	S11	S11	S19	S19	S27	S27	NTC	VTC 10^{-6}	VTC 10^{-6}
D	NTC	S4	S4	S12	S12	S20	S20	S28	S28	NTC	VTC 10^{-5}	VTC 10^{-5}
E	NTC	S5	S5	S13	S13	S21	S21	S29	S29	NTC	VTC 10^{-4}	VTC 10^{-4}
F	NTC	S6	S6	S14	S14	S22	S22	S30	S30	NTC	VTC 10^{-3}	VTC 10^{-3}
G	NTC	S7	S7	S15	S15	S23	S23	S31	S31	NTC	VTC 10^{-2}	VTC 10^{-2}
H	NTC	S8	S8	S16	S16	S24	S24	S32	S32	NTC	VTC 10^{-1}	VTC 10^{-1}

Fig. 4 Example of template for real-time PCR relative quantitation of RSV. Each sample (S) is assayed in duplicate, including non-template (NTC) and viral template (VTC) controls

14. Add 5 µL to the remaining NTC wells, in column 10, and cap tightly.
15. Prepare tenfold serial dilutions of the VTC in nuclease-free water for standard curve.
16. Add 5 µL of each serial dilution to the VTC wells of the PCR plate, and cap tightly.
17. Centrifuge the PCR plate at 500 × *g* for 1 min at 4 °C to remove any air bubbles that may be present in the reaction mixtures.
18. Transfer the PCR plate to an RT-PCR machine, and amplify viral RNA with the following thermocycler conditions: 10 min at 45 °C, 10 min at 95 °C, then 45 cycles of 15 s at 95 °C, and 1 min at 55 °C.
19. Serial dilution of known PFU of RSV RNA is used to obtain a standard reference curve for quantitative real-time PCR [17]. Data are analyzed using real-time PCR analysis software and cycle threshold (*C*t) values are calculated. An example of a qRT-PCR standard curve for a RSV A2 stock (*see* Fig. 5).
20. Quantitative estimation of RSV RNA levels in positive samples is calculated using the standard reference curves generated and interpolation of specimen *C*t values (*see* **Note 22**). Since the concentration of RSV in the control sample is known as PFU/mL, the concentrations of the samples are in the PFU equivalents/mL (PFUe/mL) (*see* Table 2).

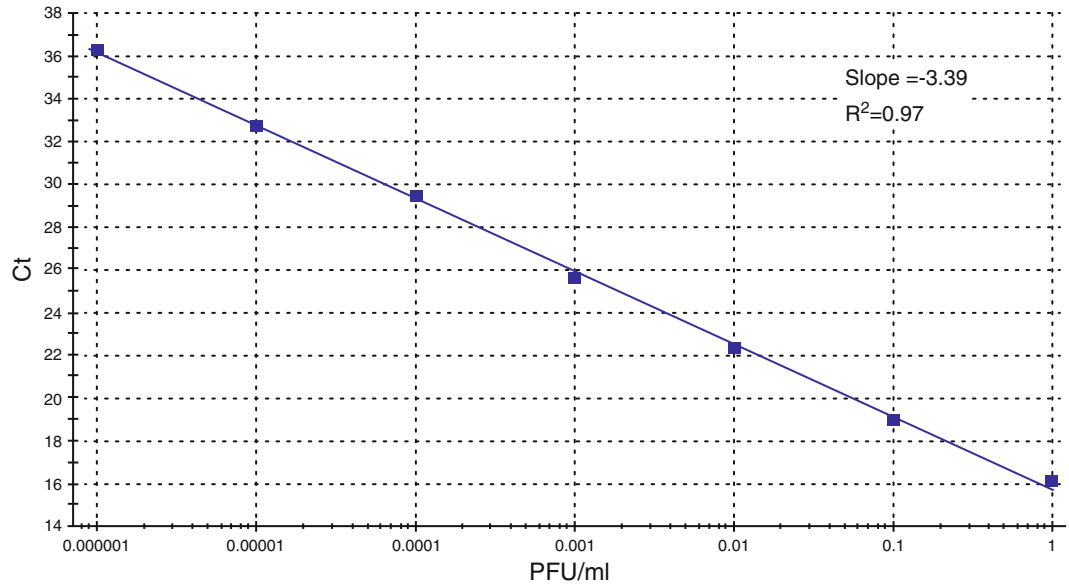

Fig. 5 qRT-PCR standard curve of RSV A2 stock. This standard curve has an efficiency of 96.9 % and curve slope of −3.399. A standard curve of known PFU/mL of RSV is used to determine the PFU equivalent of RSV stocks. *Ct* cycle threshold, PFU/mL = plaque-forming units

Table 2
Example of virus titers determined by two methods

	RSV A2[a]	RSV B1
Plaque assay[b]	10×10^7	5×10^7
qRT-PCR[c]	9×10^7	4×10^7
	RSV A2[d]	RSV B1
Plaque assay	4×10^7	3×10^7
qRT-PCR	6×10^7	2×10^7

[a]RSV virus strains grown in Vero cells and titrated after four limiting dilution passages
[b]Titer in PFU/mL
[c]Titer in relative genome level PFUe/mL
[d]RSV virus strains grown in HEp-2 cells and tittered after four limiting dilution passages

4 Notes

1. It is not recommended to incubate FBS at 37 °C for extended periods of time. FBS treated in this manner may appear cloudy. Under these conditions, the product's performance may be affected due to the liability of many serum components. It is not recommended to thaw and refreeze FBS more than once.

2. Length of trypsin-EDTA storage at 4–8 °C may vary depending on how often it is warmed up and used. It may also depend on how tightly the cells are attached, because there may be some loss of activity the longer the trypsin is stored at 4–8 °C.
3. RSV stock virus can be acquired through multiple sources, either as a gift or purchased from ATCC. Multiple strains are available. Before beginning to work with RSV, before expanding the working stock into an infectious stock and after the final infectious stock is generated, ensure that the stock is free of mycoplasma contamination.
4. HEp-2 and Vero cells should be tested for mycoplasma contamination before beginning work with infection, and before the infectious stock is expanded into an infecting stock. HEp-2 cells should also be karyotyped, to ensure lack of HeLa cross-contamination.
5. For the most consistent, reliable results, Vero and HEp-2 cells should be passaged at least twice after being thawed from frozen, and no more than 20 times before use in preparation of RSV seed stocks and infecting stocks, or for performing plaque assays.
6. Wear standard PPE which includes gloves, lab coat, and safety glasses. Prepare fixative the day before use and store at 4–8 °C.
7. Most anti-RSV monoclonal antibodies may be used for the immunostaining of RSV plaques; however, the antibody pool (MAB858-4) recommended here contains anti-RSV-fusion protein (RSV-F) monoclonal (133-1H), anti-RSV-attachment protein (RSV-G) monoclonal (131-2G), and anti-RSV-nucleocapsid (RSV-N) monoclonal (130-12H) antibodies, which will recognize multiple strains of RSV [17–19]. It is recommended that the anti-RSV antibody pool contain at a minimum anti-RSV-G and anti-RSV-F monoclonal antibodies. The optimal concentration of anti-RSV antibodies will need to be determined by the end user (*see* Table 3).
8. The optimal concentration of the secondary detection antibody will need to be determined by the end user. The optimal concentration may range from 1:2000 to 1:4000.
9. It is recommended that viability of Vero and HEp-2 cells be determined by trypan blue exclusion assay, but any method for determining viability is acceptable. To determine viability by trypan blue exclusion assay, gently mix an equal volume of trypsinized cells with a 0.4% solution of trypan blue solution by trituration. Score clear (viable) and blue (non-viable) cells counted on a hemacytometer. The percentage of viable cells is determined as [number of viable cells/(number of viable cells + number of blue cells) × 100]. Viability of cells should be ≥95% before proceeding with infections.

Table 3
Monoclonal antibodies reactive against RSV

MAb[a]	Protein specificity[b]	Immunizing strain	Reference	Catalog #[c]
131-2G	G	A2	[19]	M858-2KC
232-1F	G	A2	[19]	NA
130-6D	G	A2	[19]	NA
131-2A	F	A2	[11, 19]	MAB8599
133-1H	F	A2	[11, 19]	M8262-10K
102-10B	F	B18537	[11, 19]	M8582-KC
130-8F	F	A2	[11, 19]	MAB8598
143-6C	F	A2	[19]	MAB8594
92-11C	F	Long	[11, 19]	MAB8581
130-12H	N	A2	[11]	MAB858-3B

[a]*MAb* monoclonal antibody, some MAbs cross-react with A and B strains
[b]MAb are reactive against the RSV fusion (F), attachment (G), or nucleocapsid (N) proteins
[c]MAbs available through Millipore; *NA* not available through Millipore

10. Loosened caps prevent the flasks from cracking as gas expands during the freezing process. Preventing cracked flasks maintains virus sterility. Flasks should be thawed at 4 °C.
11. A 5 mL volume of 25% sucrose is recommended for optimal recovery of infectious virus with low concentrations of di particles. However, for higher titer recovery, the volume added to each flask may be reduced to as low as 3 mL, as long as the entire cell monolayer is covered with 25% sucrose when subjected to freeze-thaw lysis. Avoid concentrations of sucrose higher than 48%, as high concentrations have been shown to be toxic to cells [3].
12. Sonication is an alternate method that may be used to harvest RSV from infected cells. However, freeze-thaw lysis typically results in a higher recovery of infectious virus. To harvest RSV by sonication: Remove supernatants from flasks, and add 5 mL cold, sterile 25% (w/v) sucrose to each flask. Scrape the cells and collect all fluid into sterile 50 mL conical tubes. Place the tubes in an ice water bath and ensure that all fluid in the 50 mL tubes is below the ice/air interface of the ice water bath, not touching the sonicator probe. Sonicate the tubes for three cycles at output amplitude of 50 for 60 s. Rest the tubes in the ice water bath for 5 min between pulses. Remove cellular debris by centrifugation at 500 × *g* for 10 min at 4 °C. Pool the supernatants in a sterile 50 mL conical tube. Mix well, aliquot into sterile cryovials, and store at −152 °C for long-term storage.

13. An alternative virus preparation method includes sucrose purification of RSV virus. Briefly, virus lysate is harvested after one freeze-thaw cycle and the cell debris removed by centrifugation at 500 × *g* for 7 min at 4 °C. The supernatants are purified by centrifugation through a 20% sucrose cushion at 16,000 × *g* for 4 h. Remove the supernatant and resuspend the pellet in SF-DMEM and store at −80 °C or below [18].
14. To minimize loss of virus infectivity, stocks should be stored below 80 °C, preferably at −152 °C for long-term storage.
15. The plaque assay performed in a 96-well plate format is particularly suitable for high-throughput virus titrations. The plaque size may be increased with using standard liquid culture media; however, distinctive plaque formation is still observed. In 96-well culture plates, media overlays can be easier to use than methylcellulose overlays. Formation of smaller, localized plaques may be observed when using diluted methylcellulose overlays.
16. Disrupting plaque assay plates will result in the generation of plaques with satellite staining, making it more difficult to score the plaques at the end of the assay. Plates should be placed in an incubator where they will remain undisturbed except to monitor the development of CPE.
17. Beginning on day 3 post-infection (pi), monitor CPE development by light microscopy, handling the plates as gently as possible. RSV infection will result in the formation of CPE that begins as a fusion of two cells, and will slowly progress as the MOI increases, and as the time pi increases. The optimal time for fixing and scoring the plaque assay is between 3 and 5 days pi, when the aggregation of multiple nuclei in the milieu of fused cells is evident, but infected cells have not yet lysed.
18. After fixation, the cell monolayers in each well should remain intact on the plate, with minimal or no loss of cells. The plaque assay development may proceed immediately after fixation, or up to 5 days after fixation. To store fixed plaque assay plates, add 100 μL sterile D-PBS to each well, and store at 4 °C for up to 5 days before developing. To develop, remove D-PBS, rinse each well one time with D-PBS, and proceed with staining as described in protocol. Alternatively, the plate may also be completely air-dried, stored covered and inverted at 4 °C, and held for up to 5 days before proceeding with the immunostaining.
19. For antigenic characterization: HEp-2 or Vero cell monolayers are infected with the RSV strains (96-well plate format) and fixed with 80% acetone when 80–85% CPE is evident. Monolayers are stained with appropriate monoclonal antibody, then conjugate antibody, and finally incubated ABTS peroxidase substrate. Stop reaction using 1× ABTS peroxidase

stop solution. Place the plate on ELISA reader. Read at 414 (or 405 or 410) nm and 490 nm dual-wavelength mode, if available. The absorbance minus background absorbance (mock-infected cells) will give the specific absorbance value.

20. When scoring plaque assays, it is important that no plaques are detectable in the negative control wells.
21. To reduce contamination of samples, primers, probes, and reagents, it is critical that tissue culture, RNA extraction, and nucleic acids are handled in a separate area from the RT-PCR reaction reagents.
22. Quantification data and C_t values, indicative of the quantity of target, were calculated using the second derivative maximum method. These data were used to generate standard reference curves for the quantification of RSV titer in virus samples by interpolation of sample C_t values. The lowest viral titer at which RSV is detectable is assigned the detection limit.

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References

1. Kwilas S et al (2009) Respiratory syncytial virus grown in Vero cells contains a truncated attachment protein that alters its infectivity and dependence on glycosaminoglycans. J Virol 83:10710–10718
2. Bs G (2011) Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. Immunol Rev 239: 149–166
3. Gupta CK, Leszczynski J, Gupta RK et al (1996) Stabilization of respiratory syncytial virus (RSV) against thermal inactivation and freeze-thaw cycles for development and control of RSV vaccines and immune globulin. Vaccine 14:1417–1420
4. Huang AS, Baltimore D (1970) Defective viral particles and viral disease processes. Nature 226:325–327
5. Huang AS (1973) Defective interfering viruses. Ann Rev Microbiol 27:101–117
6. Grosz D, van Geelen A, Gallup J et al (2014) Sucrose stabilization of respiratory syncytial virus (RSV) during nebulization and experimental infection. BMC Res Notes 18:158–162
7. Treuhaft M (1982) Defective interfering particles of respiratory syncytial virus. Infect Immun 37:439–444
8. Ausar S, Espina M, Brock J et al (2007) High-throughput screening of stabilizes for respiratory syncytial virus: identification of stabilizers and their effects on the conformational thermostability of viral particles. Human Vaccinesv 3:68–72
9. Fernie B (1980) The stabilization and purification of respiratory syncytial virus using $MgSO_4$. Virology 106:141–144
10. Anderson L, Hierholzer J, Tsou C et al (1985) Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis 151:626–633
11. García-Barreno B, Palomo C, Peñas C (1989) Marked differences in the antigenic structure of human respiratory syncytial virus F and G glycoproteins. J Virol 63:925–932

12. Valdivia A, Gonzalez G, Chacon D et al (1999) Antigenic and genetic characterization of twenty-six strains of human respiratory syncytial virus (subgroup A) isolated during three consecutive outbreaks in Havana City, Cuba. Mem Inst Oswaldo Cruz 94:469–475
13. Borg I, Rohde G, Loseke S, Bittscheidt J et al (2003) Evaluation of a quantitative real-time PCR for the detection of respiratory syncytial virus in pulmonary diseases. Eur Respir J 21:944–951
14. Falsey A, Formica M, Treanor J, Walsh E (2003) Comparison of quantitative reverse transcription PCR to viral culture for assessment of respiratory syncytial virus shedding. J Clin Microbiol 41:4160–4165
15. Hu A, Colella M, Tam J, Rappaport R et al (2003) Simultaneous detection, subgrouping, and quantitation of respiratory syncytial virus A and B by real-time PCR. J Clin Microbiol 41:149–154
16. Reed LJ, Muench H (1938) A simple method of estimating fifty per cent endpoints. Am J Hyg 27:493–497
17. Caidi H, Tripp R, Anderson L et al (2012) Combination therapy using monoclonal antibodies against respiratory syncytial virus (RSV) G glycoprotein protects from RSV disease in BALB/c mice. PLoS One. doi:10.1371/journal.pone.0051485
18. Boyoglu-Barnum S, Gaston KA, Todd SO et al (2013) A respiratory syncytial virus (RSV) anti-G protein F(ab′)2 monoclonal antibody suppresses mucous production and breathing effort in RSV rA2-line19F-infected BALB/c mice. J Virol 87:10955–10967
19. Anderson L, Bingham P, Hierholzer J (1988) Neutralization of respiratory syncytial virus by individual and mixtures of F and G protein monoclonal antibodies. J Virol 62: 4232–4238

Chapter 3

Quantification of RSV Infectious Particles by Plaque Assay and Immunostaining Assay

Patricia A. Jorquera and Ralph A. Tripp

Abstract

One of the most commonly used approaches for determining the quantity of infectious RSV particles in a given sample is the plaque assay. RSV infectious particles can be quantified by various direct and indirect methods. Here, we explain two simple methods for RSV titration: plaque assay and immunostaining assay.

Key words RSV, F protein, G protein, Plaque assay, Immunostaining, HEp-2 cells, VERO cells

1 Introduction

One of the most important procedures in virology is measuring the virus titer. There are multiple direct and indirect methods that can be used to quantify the amount of virus in a given sample. One of the most commonly used methods for determining the quantity of infectious viral particles in a sample, as plaque-forming units per mL (PFU/mL), is the plaque assay. The RSV plaque assay can be used to measure infectious virus in—cell culture—biofluids, and homogenized tissue samples.

RSV infection of VERO and HEp-2 cells in regular DMEM or MEM medium induces syncytia formation that can be detected under microscope observation, with very small plaques or no plaque formation. Therefore, immunostaining must be performed to detect and quantify RSV plaques using either polyclonal serum or monoclonal antibodies against the fusion (F) [1] and/or the glycoprotein (G) [2, 3] followed by a conjugated anti-species antibody and substrate. Then, plaques need to be viewed microscopically for counting.

By performing the plaque assay in DMEM/F12 medium (for HEp-2 cells) and Leibovitz-L15 medium (for VERO cells), RSV produces plaques which are sufficiently large enough to be visualized by formalin fixation and neutral red staining. Under optimized

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conditions RSV A and B viruses tend to produce large plaques in HEp-2 cells. In contrast, in VERO cells RSV A strains produce small plaques (by 5–7 days) and RSV B strains form pinpoint plaques which usually take longer (7–9 days) to develop than RSV A strains [4].

Several parameters can influence the quality of the plaque assay, including the RSV strain, cell type, seeding density, and volume and type of overlay medium. VERO cells can show variations in morphology from laboratory to laboratory, which can explain differences in plaquing ability. Aged VERO cells have a swirling appearance, and plaque RSV better than those with an even mosaic-like morphology [4].

In this book chapter we explain how to quantify RSV infectious particles in cell culture, fluids, and/or homogenized tissue sample by plaque assay and immunostaining assay.

2 Materials

2.1 RSV Immunostaining Assay

1. Biosafety level II cabinet.
2. 37 °C Incubator with 5 % CO_2.
3. Vero E6 cells (ATCC® CRL-1586).
4. 24-Well tissue culture plate (flat bottom).
5. Sample containing RSV: Cell culture supernatant, cell lysate, blood, homogenized tissue sample.
6. FBS: Fetal bovine serum, heat inactivated at 56 °C for 30 min.
7. Serum-free Dulbecco's modified Eagle medium (SF-DMEM)-high glucose: 4.5 g/L Glucose, 4 mM L-glutamine, 0.03 mM phenol red. Store at 4–8 °C.
8. DMEM-5: SF-DMEM supplemented with 5 % (v/v) FBS. Store at 4–8 °C.
9. Overlay medium (1 % methylcellulose): 5 g Methylcellulose, 100 mL Hanks' balanced salt solution (HBSS), 5 mL 100× antibiotic/antimycotic solution, 10 mL FBS, and 400 mL DMEM (*see* **Note 1**). Store at 4–8 °C.
10. Sterile phosphate buffer saline (PBS) pH 7.4, cell culture grade.
11. 15 and 50 mL conical tubes.
12. PBS 10×: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, and pH 7.4.
13. PBS-T: 100 mL of PBS 10×, 0.5 mL of Tween-20, and distillated water to 1 L.
14. Fixative solution: 60 % (v/v) Acetone and 40 % (v/v) methanol. 4 % Paraformaldehyde (PFA) can also be used for fixation (*see* **Note 2**).

15. Blocking solution: PBS-T+ 5% (w/v) skim milk.
16. Primary antibody: Mouse monoclonal antibody anti-RSV F (clone 131-2A), anti-RSV G (clone 130-2G or 131-2G), anti-RSV polyclonal serum.
17. Secondary antibody: Goat anti-mouse IgG-alkaline phosphatase conjugated or goat anti-mouse IgG-HRP conjugated.
18. Colorimetric alkaline phosphatase substrate: 1-step Nitroblue Tetrazolium (NBT)/5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP). Alternatively, 3, 3-diaminobenzidine (DAB) HRP substrate can be employed if preferring to use an HRP secondary antibody.

2.2 RSV Plaque Assay

1. 37 °C Incubator with 5% CO_2.
2. HEp-2 cells (ATCC® CCL-23) or Vero E6 cells (ATCC® CRL-1586).
3. 6-Well tissue culture plate (flat bottom).
4. FBS.
5. Dulbecco's modified Eagle medium/nutrient mixture F-12 with phenol red (DMEM/F12): 2.5 mM L-Glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, and 1200 mg/L sodium bicarbonate. Store at 4–8 °C.
6. DMEM/F12-5% FBS: DMEM/F-12 supplemented with 5% (v/v) FBS. Store at 4–8 °C.
7. Leibovitz's L-15 Medium: 2 mM L-Glutamine and no sodium bicarbonate.
8. SF-DMEM.
9. 0.6% (w/v) Agarose: Suspend 0.6 g of immunodiffusion agarose in a total volume of 100 mL of distilled water (molecular biology grade) in a glass bottle. Autoclave for 20–30 min (*see* **Note 3**).
10. 10% FBS-L-15 Medium: Leibovitz's L-15 Medium supplemented with 10% (v/v) FBS.
11. VERO cell overlay medium: Mix equal volumes of 10% FBS-L-15 Medium and 0.6% (w/v) agarose immediately before overlaying (*see* **Note 4**).
12. HEp-2 cell overlay medium: Mix equal volumes of DMEM/F12 and 0.6% (w/v) agarose immediately before overlaying (*see* **Note 4**).
13. 1%)PFA fixative solution: Mix 10 mL of 4%)PFA (*see* **Note 2**) with 30 mL of PBS.
14. Neutral red working stock (0.05%): To prepare 10× neutral red stock (0.5% w/v), add 0.5 g of neutral red to 100 mL of water. Dilution of 0.5% neutral red with water prevents

precipitation. Fixed cells stained with 0.5% neutral red can be stored indefinitely. A working stock of 1× can be diluted in water from 10× neutral red stock.

3 Methods

3.1 RSV Immunostaining Assay

1. One day prior to infection, seed 1–2× 10^5 VERO E6 per well of a 24-well plate in a volume of 1 mL of DMEM-5 (*see* **Note 5**). Prepare one plate for every sample to analyze.
2. Dilute the virus sample by mixing 100 µL of sample with 900 µL of cold SF-DMEM (dilution 10^{-1}), and perform tenfold serial dilutions until achieving dilution 10^{-6}. Keep the virus dilutions on ice. A total of 800 µL of a particular dilution will be needed in order to generate quadruplicates (*see* **Note 6**). SF-DMEM should be used as mock control.
3. Remove the media from the plates and rinse once with PBS.
4. Add 200 µL of diluted virus inoculum per well in quadruplicates (*see* Fig. 1a and **Note 7**).
5. Make sure that there is a row of mock-infected cells as a control on each plate.
6. Incubate at 37 °C for 2 h to allow virus adsorption.
7. While incubating put the 1% methylcellulose (MC) in a 37 °C water bath.
8. After 2 h, overlay with 1 mL of 1% MC per well and incubate for 5–7 days at 37 °C with 5% CO_2 (*see* **Note 8**).
9. Carefully aspirate off the MC from the plates, add 300 µL/well of ice-cold 60:40 acetone:methanol, and incubate for 20 min at room temperature.

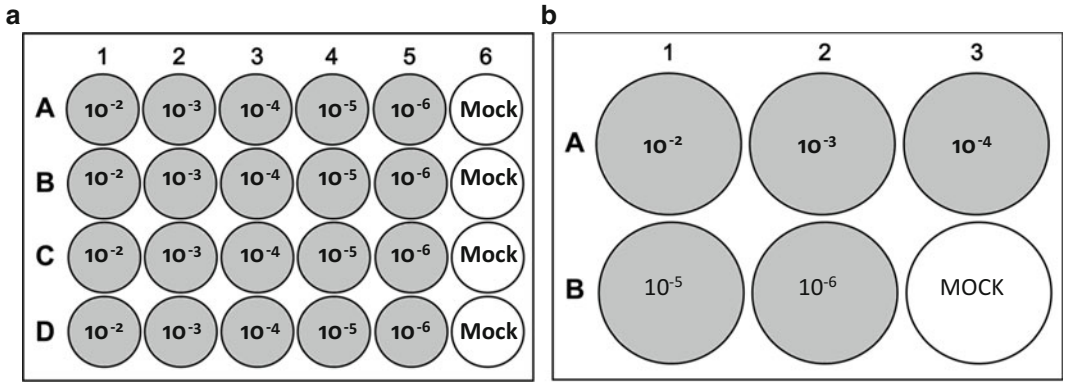

Fig. 1 Plate layout for RSV plaque assay and RSV immunostaining. (**a**) 24-Well plate layout showing quadruplicates of sample dilution; and (**b**) 6-well plate layout showing a single sample dilution

10. Remove acetone:methanol and let the plate air-dry (*see* **Note 9**).
11. Rinse the plates with PBS-T and shake gently to avoid disrupting the monolayer.
12. Block cells with 300 μL/well of blocking solution for 30 min at room temperature placing the plates on an orbital shaker.
13. Remove the blocking solution, and add 250 μL/well of anti-RSV antibody diluted in blocking solution. Incubate for 1 h at room temperature placing the plates on an orbital shaker.
14. Rinse the cells with 300 μL of PBS-T per well, shake for 5 min, and repeat this step two more times.
15. Add 250 μL/well of goat anti-mouse IgG-alkaline phosphatase (AP) secondary antibody or goat anti-mouse IgG-HRP secondary antibody diluted in blocking solution. Incubate for 1 h at room temperature while shaking.
16. Rinse the cells with 300 μL of PBS-T per well, shake for 5 min, and repeat this step two more times.
17. Add 250 μL/well of 1-step NBT/BCIP substrate per well. If using an HRP-conjugated secondary antibody use DAB substrate instead. Develop until sufficient color is observed (*see* **Note 10**).
18. Stop the reaction by rinsing the wells with water.
19. Count the syncytia and determine PFU/mL using the following formula:

$$\left[\left(\text{average \# plaques}\right) / \left(\text{dilution factor} \times \text{volume of virus added to the well}\right) = \text{PFU / mL}\right]$$

(*see* **Note 11** for an example).

3.2 RSV Plaque Assay

1. Seed 1.5×10^6 HEp-2 cells per well or 8×10^5 VERO E6 cells per well of a 6-well plate in a volume of 3 mL of DMEM/F12-5% FBS and incubate overnight at 37 °C (*see* **Note 5**). Prepare four plates for every sample to analyze.
2. After overnight incubation cells should be confluent; do not use if there are holes in monolayer.
3. Dilute the virus sample by mixing 0.5 mL of sample with 4.5 mL of cold SF-DMEM (dilution 10^{-1}), and perform ten-fold serial dilutions until achieving dilution 10^{-6} (*see* **Note 7**). Keep the virus dilutions on ice. A total of 4 mL of a particular dilution will be needed in order to generate quadruplicates. SF-DMEM should be used as mock control.
4. Remove growth medium and wash once with 1 mL of sterile PBS per well.
5. Add 1 mL of diluted virus per well in quadruplicate plates (*see* Fig. 1b) and incubate at 37 °C for 2 h.

6. Carefully remove virus inoculum and slowly add 3 mL of overlay to the edge of each well by placing the pipette tip against the wall of each well. Use VERO cell overlay medium for VERO E6 cells and HEp-2 cell overlay medium for HEp-2 cells (*see* **Note 12**). Incubate for 7 days at 37 °C with 5 % CO_2.
7. Allow the overlay to solidify for approximately 10 min at room temperature before placing plates upright into the tissue culture incubator. Incubate plates for 6–7 days at 37 °C in 5 % CO_2.
8. Add 2 mL of 1 % PFA to cells and allow fixative to penetrate agarose overnight.
9. Flick off agarose and wash the edge of the plate gently with running tap water to remove remaining agarose.
10. Stain monolayers with 2–3 mL of 0.05 % neutral red and incubate cells at room temperature for 1–2 h.
11. Remove neutral red, wash gently with running tap water, and allow plates to dry with lids off.
12. Determine the number of plaques and calculate the PFU/mL (*see* **Note 11**).

4 Notes

1. To prepare 500 mL of 1 % methylcellulose (MC) bring 100 mL of HBSS to boil and slowly add 5 g of MC while stirring; once the methylcellulose is dissolved sterilize it by autoclaving (the solution will have a "milky" appearance). Mix 400 mL of DMEM with 10 mL of FBS and 5 mL of antibiotic/antimycotic solution (100×) and incubate at 37 °C in a water bath. Once the MC has been autoclaved, let it cool down while stirring and monitor the temperature by touching the bottle. When the bottle is approximately 40–42 °C add the medium containing FBS and antibiotics and keep stirring until the solution appears completely clear. The solution normally clears out within 3–5 h. We recommend to keep stirring overnight at 4 °C. Store at 4 °C for up to 6 months. If methylcellulose is not available a solution of agarose (low melting point) in DMEM-5 medium can be used instead (*see* Subheading 2.2, **item 9**, for protocol).
2. For 500 mL of 4 % paraformaldehyde), add 400 mL of PBS to a glass beaker on a stir plate in a ventilated hood. Heat while stirring to approximately 60 °C (do not boil the solution) and add 20 g of paraformaldehyde) powder. Slowly raise the pH by adding 1 M NaOH dropwise until the solution clears. Once the paraformaldehyde) is dissolved, the solution should be cooled and filtered. Adjust the volume of the solution to

500 mL with PBS. Check the pH of the solution and adjust it to 6.9 with small amounts of HCl if needed. Store at 4 °C. To produce 1% paraformaldehyde), dilute 1:4 in PBS 1×.

3. 0.6% Agarose can be prepared beforehand and re-melted by microwaving before use. It is important to equilibrate the agarose to 42 °C in a water bath before using, because if the agarose is too hot, it will kill the cells. Make sure that water level is equal to or above the level of the agarose to avoid undesired solidification. Various brands of agarose can be used including low melting temperature, molecular biology, and immunodiffusion grade. However efficiency and size of plaques are better with ICN immunodiffusion-grade agarose, with larger plaques obtained under lower agarose concentrations.
4. Calculate the amount of overlay medium required for the total volume of plates 1 h before virus adsorption is complete. The volume needed is 2 mL/well or 12 mL/6-well plate. Mix 0.6% agarose (*see* **Note 3**) with medium in a 1:1 ratio in a sterile bottle. Medium should be pre-warmed at 37 °C before mixing with the agarose; if cold medium is used the agarose will solidify in the bottle.
5. Cells must be seeded to obtain 80–90% confluency. The cells need to be confluent at infection for optimal plaquing. If cells are not confluent do not use them and incubate for additional 12–24 h before infection.
6. You will need 200 μL of diluted virus per well and 800 μL total to generate quadruplicates. SF-DMEM should be used as mock control and included in every plate.
7. If you are testing RSV stocks grown in cell culture dilutions = 10^{-2}–10^{6} or 10^{-3}–10^{7} should be used, but if you are testing biofluids or homogenized tissue sample the RSV titer will be lower and dilutions 10^{-1}–10^{5} should be used. Homogenized tissue sample containing cell lysates should be clarified by centrifugation before testing.
8. Syncytia formation will start to become visible at day 3 post-infection. Incubation times may vary depending on the RSV strain used and the type of sample. Five days is the standard incubation time, but longer incubation time will render larger plaques.
9. After this point plates can be air-dried and kept at room temperature for 1–2 days before immunostaining is performed.
10. The infected wells should develop dark purple spots where the syncytia is present, while mock-infected wells should have no dark purple spots and should look homogeneously stained (light violet color).

11. For example if 40 plaques was the average number of plaques (or syncytia) in the dilution 10^{-5}:
    - RSV titer = 40 plaques observed/[(10^{-5} dilution factor) × (0.2 mL virus added)].
    - RSV titer = 2×10^7 PFU/mL.
12. Both HEp-2 and VERO cells require different overlay medium. For HEp-2 cells, use DMEM/F12 and 0.3% agarose (no serum required), while for VERO E6 cells use Leibovitz L15, 0.3% agarose, and 5% FBS. Plaquing in HEp-2 cells is better in the absence of serum, whereas Vero cells needed serum. The volume of overlay must not be less than 3 mL to prevent poor plaque formation and cell mortality.

References

1. Power UF et al (1997) Induction of protective immunity in rodents by vaccination with a prokaryotically expressed recombinant fusion protein containing a respiratory syncytial virus G protein fragment. Virology 230(2): 155–166
2. Boyoglu-Barnum S et al (2015) An anti-G protein monoclonal antibody treats RSV disease more effectively than an anti-F monoclonal antibody in BALB/c mice. Virology 483:117–125
3. Haynes LM et al (2009) Therapeutic monoclonal antibody treatment targeting respiratory syncytial virus (RSV) G protein mediates viral clearance and reduces the pathogenesis of RSV infection in BALB/c mice. J Infect Dis 200(3): 439–447
4. McKimm-Breschkin JL (2004) A simplified plaque assay for respiratory syncytial virus--direct visualization of plaques without immunostaining. J Virol Methods 120(1):113–117

Chapter 4

Detection of RSV Antibodies in Human Plasma by Enzyme Immunoassays

Samadhan J. Jadhao and Larry J. Anderson

Abstract

Enzyme immunoassays (EIAs) to detect and quantify antibodies against respiratory syncytial virus (RSV) and RSV proteins in human plasma or sera are described. The first EIA uses RSV lysate antigens produced in HEp-2 cell line. The second EIA uses RSV F or G gene-expressed antigen in HEp-2 cells. The third EIA uses 30-amino acid synthetic peptides from central conserved region of G protein of RSV A2 or RSV B1 virus and a peptide from the SARS CoV nucleoprotein as a negative control peptide. All three EIAs have been evaluated for detecting and quantifying the respective antibodies in human sera or plasma.

Key words Respiratory syncytial virus, Enzyme immunoassay, Peptide, RSV F gene, RSV G gene

1 Introduction

Antibodies against RSV proteins play an important role in preventing disease with RSV by various mechanisms including virus neutralization, antibody-dependent cellular cytotoxicity (ADCC), and complement-mediated neutralization. The RSV attachment (G) and fusion (F) proteins are the major targets of RSV-specific neutralizing antibodies but antibodies against other virus proteins are also induced by infection and can contribute to serological diagnosis of infection. EIA detects both neutralizing and non-neutralizing antibodies and use of RSV lysate antigen in EIA provides a way to detect antibodies against multiple RSV proteins. Use of F or G gene-expressed antigens, e.g., in a HEp-2 cell line, can be used to determine protein-specific antibody response. The 30-amino acid synthetic peptides are from a region of the G protein that is immune active and antibodies to this region can prevent some aspects of RSV disease.

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2 Materials

2.1 Production of RSV Virus and HEp-2 Lysate Antigens

1. Class II biosafety laminar flow cabinet.
2. 175 cm^2 Vented cell culture flasks.
3. HEp-2 cells.
4. RSV virus stock.
5. 37 °C Incubator with 5 % CO_2.
6. SF-MEM: Serum-free minimum essential medium (MEM).
7. 5 % Fetal bovine serum-minimum essential medium (FBS-MEM): MEM, 5 % v/v FBS, 1× penicillin/streptomycin, 1× glutamine.
8. –80 °C Freezer.
9. 15 mL Conical tubes.
10. 50 mL Conical tubes.
11. 2 mL Cryovials.
12. Liquid nitrogen.
13. Benchtop centrifuge with rotor for 50 mL conical tubes.

2.2 Determining the Dilution of RSV Lysate Antigen to be Used in the RSV Lysate EIA

1. Class II biosafety laminar flow cabinet.
2. 96-Well EIA plates.
3. 200 µL Multichannel pipette.
4. Micropipettes.
5. Pipette tips.
6. Fluid reservoirs.
7. RSV lysate antigen.
8. HEp-2 lysate antigen.
9. 10× PBS: 1.37 M NaCl, 27 mM KCL, 100 mM Na_2HPO_4, 10 mM KH_2PO_4. Dissolve the reagents in water, adjust pH to 7.4 with HCl, and sterilize by autoclaving.
10. PBS: To prepare 1× PBS dilute 10× PBS 1:10 in sterile distillated water.
11. EIA coating buffer (carbonate-bicarbonate buffer 0.05 M, pH 9.6): 1.6 g of Na_2CO_3, 2.94 g of $NaHCO_3$, 0.2 g of NaN_3, distillated water to 1 L. Check for pH 9.4–9.6; usually there will be no need to adjust pH. Store in screw-capped bottle at room temperature.
12. EIA washing buffer: 200 mL of 10× PBS, 1 mL of Tween-20, and 1800 mL of water. Add 10× PBS, distilled H_2O and Tween 20 in a 2 L measuring cylinder and stir for 10 min using a magnetic stirrer at 200 rpm. Store at room temperature.
13. EIA blocking buffer: 1× PBS, 1 % w/v cold water fish skin gelatin, 1 % w/v skim milk, and 0.05 % Tween-20.

14. Goat anti-RSV polyclonal serum (AB1128, Millipore Inc., USA).
15. Anti-goat IgG-HRP-labeled secondary antibody of choice.
16. 0.15 M Citric acid: 7.74 g of $C_5H_7COOH{\cdot}1H_2O$, 17.93 g Na_2HPO_4, distillated water to 1 L. Adjust pH 6.0 if needed. Store at room temperature.
17. OPD substrate: 25 mL of 0.15 M citric acid, 15 mg O-phenylenediamine (OPD), 15 μL H_2O_2. Prepare in a 50 mL tube wrapped in aluminum foil, screw cap the tube, and mix by vigorous shaking for 45 s. Prepare freshly, store at room temperature in the dark, and use within 10–20 min.
18. 4 N H_2SO_4: 444.5 mL distillated water, 55.5 mL of 18 M H_2SO_4. In 1 L glass bottle, add H_2SO_4 to water in chemical full exhaust hood, allow to cool, then screw cap the bottle, and store in screw-capped bottle at room temperature.
19. ELISA plate reader.

2.3 Detection of Anti-RSV Antibodies in Human Plasma or Serum Using RSV Lysate EIA

20. 96-Well EIA plates.
21. 50 mL Conical tubes.
22. 200 μL Multichannel pipette.
23. Micropipettes.
24. Pipettes tips.
25. Fluid reservoirs.
26. EIA coating buffer.
27. EIA washing buffer.
28. EIA blocking buffer.
29. Human reference antiserum to RSV (available from NIH BEI Resources, USA, Cat # NR-4020).
30. Goat-anti-human IgG—HRP-labeled antibody of choice.
31. OPD substrate.
32. 4 N H_2SO_4.
33. ELISA plate reader.

2.4 Detection of Anti-RSV G or F Protein Antibodies in Human Plasma or Serum

1. 96-Well culture plates.
2. HEp-2 cells.
3. Mammalian expression plasmid containing the RSV F or G gene (pCDNA3.1+ or similar).
4. DNA transfection reagent of choice.
5. Complete medium: MEM, 10% v/v FBS, 1× glutamine.
6. Opti-MEM® I Reduced serum medium or regular high-glucose DMEM without serum.
7. Vortex.

8. 15 mL Conical tubes.
9. 96-Well EIA plates.
10. 200 µL Multichannel pipette.
11. Micropipettes.
12. Pipettes tips.
13. Fluid reservoirs.
14. PBS.
15. 4% PFA: 4 g of paraformaldehyde, distillated water to 100 mL, pH 7.4 (*see* **Note 1**).
16. EIA coating buffer.
17. EIA washing buffer.
18. EIA blocking buffer.
19. Goat-anti-human IgG—HRP-labeled antibody.
20. OPD substrate.
21. 4 N H_2SO_4.
22. ELISA plate reader.

2.5 Detection of Anti-RSV Antibodies in Human Plasma or Serum Using RSV Lysate EIA

1. 96-Well EIA plates.
2. 200 µL Multichannel pipette.
3. 200 µL pipette tips.
4. EIA coating buffer.
5. 50 mL Conical tubes.
6. RSV G protein and SARS CoV N protein peptides (*see* Table 1).
7. Fluid reservoirs.

3 Methods

The RSV lysate antigen-based enzyme immunoassay (EIA) detects antibodies against RSV proteins present in preparation including anti-F, anti-G, and anti-N protein antibodies. The EIA can be

Table 1
Peptides used for quantification of human anti-RSV group A and B antibodies to the central conserved region of the G protein

Peptide ID	Amino acid location	Amino acid sequence
RSV A2-glycoprotein	161–190	NDFHFEVFNF VPCSICSNNP TCWIACKRIP
RSV B1-glycoprotein	161–190	DDYHFEVFNF VPCSICGNNQ LCKSICKTIP
SARS CoV-nucleoprotein[a]	1–30	MDLFMRFFTL GSITAQPVKI DNASPASTVH

[a]The SARS CoV nucleoprotein peptide 1–30 amino acid (30 mer) is used as a negative control peptide

performed using just an RSV group A or RSV group B strain or by combining lysates from each group. The EIA's sensitivity for detecting past infection can be improved, e.g., in young children, by using both RSV A and RSV B group antigens.

All the procedures involving handling and production of infectious RSV virus in cell culture must be performed in a Class II biosafety laminar flow cabinet adhering to the biosafety guidelines.

3.1 Production of RSV Virus and HEp-2 Lysate Antigens

1. Seed 175 cm^2 vented cell culture flasks with 3×10^6 HEp-2 cells in 30 mL of 5% FBS-MEM. Seed six flasks per every RSV virus (e.g., RSV A2, RSV B1) and HEp-2 lysate antigen control. Incubate the flask at 37 °C in 5% CO_2 for 24–36 h (*see* **Note 2**).
2. Remove the medium and wash cells with 5 mL of SF-MEM three times.
3. Thaw the RSV virus stock to use on ice. Once thawed, dilute virus in SF-MEM to achieve 1×10^5 pfu/mL (MOI = 0.1).
4. Infect the HEp-2 cells with 3 mL of diluted virus and incubate at 37 °C in 5% CO_2 for 2 h by rocking every 15 min to spread the virus inoculum on the cell monolayer. For the flasks that will be used to produce HEp-2 lysate antigen control use 3 mL of SF-MEM instead (mock infection).
5. Remove the inoculums and wash cells thrice with 5 mL of SF-MEM medium.
6. Add 40 mL 5% FBS-MEM and incubate flasks at 37 °C in 5% CO_2 for 72 h.
7. On day 3 (72 h) post-infection, when viral cytopathic effects (CPE) are first becoming evident, remove culture medium and wash the flask thrice with 5 mL of SF-MEM (*see* **Note 3**).
8. Add 30 mL fresh SF-MEM and incubate the flask at 37 °C/5% CO_2 for further 24–48 h.
9. On days 4–5 post-infection, when 90% viral)CPE is observed, freeze the 175 cm^2 flasks at −80 °C.
10. Thaw the flasks, remove 2 mL of the virus fluid from each flask, and pool in a 15 mL tube. Subdivide into 0.5 mL aliquots in cryovials, snap freeze using liquid nitrogen, and then store at −80 °C for 24 h. Refreeze the 175 cm^2 flasks at −80 °C until determination of virus titer.
11. Take out one cryovial that has been frozen at −80 °C for 24 h and perform a plaque assay (*see* Chapter 3) to determine virus titer.
12. Thaw, freeze, and thaw the 175 cm^2 flask to lyse the cells, and then immediately after the third thaw transfer the cell lysate to 50 mL conical tubes. Centrifuge the cell lysate at $1880 \times g$ for 20 min at 4 °C. Aliquot the supernatant in 1.5 mL into cryovials and store at −80 °C.

3.2 Determining the Dilution of RSV Virus Lysate Antigen to be Used in the RSV Lysate EIA

1. Perform serial twofold dilutions (1:2 to 1:256) of the RSV lysate EIA and HEp-2 cell lysate in EIA coating buffer.
2. Dispense 100 μL of diluted antigen solution in each column of 96-well EIA plate using multichannel pipette. Seal the plate with adhesive plastic sheet and incubate 37 °C for 2 h and then at 4 °C for 16 h.
3. Remove the lysate antigen and wash five times with 300 μL of EIA washing buffer using an automated plate washer or by hand pipetting up and down.
4. Dry the plates by blotting on paper towel at the end of washing but not between washes.
5. Dispense 200 μL of EIA blocking buffer per well, seal the plates with adhesive plastic sheet, and incubate at 37 °C for 1 h.
6. Wash five times with 300 μL of EIA washing buffer.
7. Dilute goat anti-RSV polyclonal serum in EIA blocking buffer, add 100 μL per well, and incubate for 1 h at 37 °C.
8. Wash five times with 300 μL of EIA washing buffer.
9. Dilute anti-goat IgG-HRP-labeled secondary antibody in EIA blocking buffer. Add 100 μL per well and incubate at 37 °C for 1 h.
10. Wash five times with 300 μL of EIA washing buffer and blot the plates on paper towel at the end of washing.
11. Add 100 μL of OPD substrate per well and incubate the plate for 30 min in the dark at room temperature.
12. Stop the reaction by adding 50 μL of 4 N H_2SO_4 and gently rock the plate to mix it.
13. Read absorbance at 490 nm within 10–15 min of stopping the EIA reaction.
14. Data analysis can be performed on Microsoft Excel or similar software.
15. Report the highest dilution of RSV lysate antigen that gives corrected (RSV antigen—HEp-2 control antigen) absorbance >1.5 at 490 nm to be used in the subsequent assays.

3.3 Detection of Anti-RSV Antibodies in Human Plasma or Serum Using RSV Lysate EIA

1. Using sterile 50 mL conical tubes, dilute the HEp-2 lysate antigen and RSV lysate antigen (according to dilution determined in Subheading 3.2) in EIA coating buffer.
2. Dispense 100 μL of diluted antigen solution in each column of 96-well EIA plate using multichannel pipette as required in the plate layout. Use new tips to dispense RSV and HEp-2 lysate antigens.
3. Seal the plate with adhesive plastic sheet and incubate 37 °C for 2 h and then at 4 °C for 16 h.

4. Remove RSV lysate antigen or HEp-2 lysate antigen liquid from the wells and add 200 μL of EIA washing buffer. Make sure that the micropipette tips do not touch the surface of the wells and cross contaminate the wells.
5. Wash five times with 300 μL of EIA washing buffer using an automated plate washer or by hand pipetting up and down.
6. Dry the plates by blotting on paper towels at the end of washing but not between washes.
7. Dispense 200 μL of EIA blocking buffer per well, seal the plates with adhesive plastic sheet, and incubate at 37 °C for 1 h.
8. Wash five times with 300 μL of EIA washing buffer and dry the plates by blotting on paper towels at the end of washing but not between washes.
9. Dilute human plasma samples in EIA blocking buffer to 1:200 dilution in triplicate, dilute the human reference antiserum to 1:200, and perform serial fourfold dilutions (Fig. 1).
10. Add 100 μL of diluted samples per well and incubate for 1 h at 37 °C.
11. Wash five times with 300 μL of EIA washing buffer and dry the plates by blotting on paper towel at the end of washing but not between washes.
12. Dilute the goat-anti-human IgG-HRP-labeled secondary antibody in EIA blocking buffer. Add 100 μL per well and incubate at 37 °C for 1 h (*see* **Note 4**).
13. Wash five times with 300 μL of EIA washing buffer and dry the plates by blotting on paper towels.
14. Add 100 μL of OPD substrate per well and incubate the plate for 30 min in the dark at room temperature.
15. Stop the reaction by adding 50 μL of 4 N H_2SO_4 and gently rock the plate to mix it.

	Human reference antiserum to RSV									Plasma sample # 1		
Plasma dilution:	HEp2 lysate			RSV A2 lysate			RSV B1 lysate			Hep2 lysate	A2 lysate	B1 lysate
200	0.212	0.215	0.234	1.878	1.852	1.889	1.729	1.787	1.769	0.063	1.705	1.345
800	0.067	0.064	0.066	0.814	0.814	0.809	0.727	0.714	0.716	0.06	1.668	1.343
3200	0.022	0.022	0.022	0.249	0.245	0.247	0.206	0.211	0.201	0.065	1.493	1.426
12800	0.009	0.008	0.009	0.067	0.065	0.065	0.057	0.055	0.057	0.004	0.004	0.004
51200	0.006	0.007	0.007	0.021	0.019	0.019	0.018	0.018	0.017	0.091	0.869	0.508
204800	0.006	0.005	0.005	0.008	0.009	0.009	0.008	0.034	0.007	0.083	0.854	0.508
819200	0.006	0.006	0.006	0.007	0.006	0.006	0.005	0.005	0.005	0.087	0.856	0.49
No primary Ab	0.006	0.006	0.006	0.005	0.006	0.005	0.004	0.004	0.004	0.004	0.003	0.003
										Plasma sample # 2		

Fig. 1 Example result of an RSV lysate EIA assay for quantification of antibodies in human plasma

16. Read optical density/absorbance at 490 nm within 10–15 min of stopping the EIA reaction.
17. Data analysis can be performed on Microsoft Excel or similar software (*see* **Note 5**).

3.4 Detection of Anti-RSV G or F Protein Antibodies in Human Plasma or Serum

1. In a 96-well culture plate seed 2×10^4 HEp-2 cells/well in 100 µL of complete medium and incubate at 37 °C in 5 % CO_2 for 18–24 h.
2. Prepare the transfection complexes by mixing the mammalian expression plasmid containing the RSV F or G gene and the transfection reagent of choice. Avalanche®-Omni transfection reagent will be used as an example for this protocol.
3. In a 15 mL conical tube add 11 µg of DNA into 1250 µL of Opti-MEM® Reduced-Serum medium or regular high-glucose DMEM without serum (*see* **Note 6**). Mix by vortexing.
4. Briefly vortex Avalanche®-Omni and add 11 µL into the 15 mL conical tube containing the DNA. Mix by inversion 8–10 times and incubate for 15 min at room temperature.
5. Add 9.75 mL of complete medium to the 15 mL conical tube containing the transfection complexes and mix by inversion 8–10 times.
6. Add 100 µL per well of the plasmid-Avalanche®-Omni lipid polymer complexes to the 96-well culture plate. Incubate at 37 °C in 5 % CO_2 for 36–48 h.
7. Remove the medium from the 96-well culture plate by aspiration and wash wells four times with 250 µL of PBS.
8. Fix the cells with 100 µL of 4 % PFA for 20 min.
9. Remove PFA and wash the wells four times with PBS (*see* **Note 7**).
10. Dispense 200 µL of EIA blocking solution per well, seal the plate with adhesive plastic sheet, and incubate at 37 °C 1 h.
13. Wash five times with 300 µL of EIA washing buffer using an automated plate washer or by hand pipetting up and down. Blot the plates on paper towel at the end of washing.
14. In triplicate wells, add 100 µL of test plasma or serum diluted in EIA blocking buffer at a final dilution of 1:200, and incubate the plate at 37 °C for 1 h.
15. Wash five times with 300 µL of EIA washing buffer.
16. Dilute the goat-anti-human IgG-HRP-labeled secondary antibody in EIA blocking buffer, dispense 100 µL per well, and incubate at 37° for 1 h.
17. Wash five times with 300 µL of EIA washing buffer.
18. Add 100 µL of OPD substrate to each well and incubate the plate for 20 min in the dark at room temperature.

	HEp2 cells untransfected						HEp2 cells Transfected with:					
Human Plasma 1094	**No primary Ab**			**Added primary Ab**			**RSV A2/G Plasmid**			**RSV B/G Plasmid**		
1:200	0.105	0.112	0.132	0.303	0.300	0.294	1.256	1.166	1.105	0.363	0.323	0.302
1:400	0.113	0.132	0.112	0.225	0.216	0.225	0.884	0.903	0.844	0.268	0.259	0.226
1:800	0.106	0.100	0.111	0.180	0.181	0.189	0.526	0.543	0.517	0.210	0.197	0.196
No primary Ab	0.091	0.106	0.115	0.113	0.114	0.111	0.097	0.090	0.088	0.081	0.079	0.100
Human Plasma 1094	**No primary Ab**			**Added primary Ab**			**RSV A2/ F Plasmid**			**pCDNA 3.1+ Empty vector**		
1:200	0.101	0.108	0.114	0.265	0.262	0.272	1.073	1.121	1.156	0.250	0.254	0.246
1:400	0.088	0.096	0.113	0.208	0.203	0.210	0.923	0.940	0.917	0.210	0.194	0.182
1:800	0.098	0.108	0.108	0.170	0.190	0.180	0.719	0.726	0.759	0.143	0.142	0.138
No primary Ab	0.11	0.109	0.121	0.096	0.125	0.106	0.098	0.091	0.103	0.077	0.071	0.088

Fig. 2 Example result of an RSV F and G protein EIA assay for quantification of antibodies in human plasma

	Standard curve anti-RSV A2 peptide Antibody											
	Human reference antiserum to RSV						Plasma sample # 1			Plasma sample # 2		
Plasma Dilution	**SARS Peptide**			**A2 peptide**			**SARS**	**A2**	**B1**	**SARS**	**A2**	**B1**
200	0.158	0.148	0.158	2.082	2.119	2.127	0.146	0.152	0.191	0.108	0.194	1.406
400	0.112	0.108	0.108	1.577	1.497	1.535	0.148	0.147	0.19	0.098	0.209	1.385
800	0.08	0.077	0.078	1.046	1.041	1.049	0.151	0.150	0.186	0.106	0.197	1.319
1600	0.061	0.062	0.063	0.650	0.611	0.584	0.041	0.042	0.043	0.044	0.042	0.043
3200	0.056	0.052	0.051	0.382	0.364	0.353	0.111	1.416	0.323	0.069	0.627	0.716
6400	0.048	0.049	0.048	0.217	0.216	0.204	0.121	1.456	0.313	0.070	0.622	0.759
12800	0.046	0.045	0.044	0.133	0.133	0.127	0.11	1.452	0.32	0.069	0.613	0.758
No primary Ab	0.045	0.056	0.042	0.042	0.044	0.042	0.043	0.042	0.041	0.042	0.042	0.041
							Positive Control Plasma RSV A2 (1:400)			Positive Control Plasma RSV B1 (1:400)		

Fig. 3 Example result of a G protein central conserved region peptide EIA assay

19. Stop reaction by adding 50 µL of 4 N H_2SO_4 and gently rock the plate to mix it.
20. Read optical density/absorbance at 490 nm within 10–15 min of stopping the EIA reaction.
21. Data analysis can be performed on Microsoft Excel or similar software (*see* Fig. 2 and **Note 8**).

3.5 Detection of Antibodies to Central Conserved Region of the RSV G Protein (see Note 9)

1. Using sterile 50 mL conical tubes dilute RSV A2, RSV B1, and SARS peptide to 5000 ng/mL in EIA coating buffer. Mix five times by vortexing with 5-min intervals in between each vortex until completely dissolved.
2. Transfer peptides to separate fluid reservoirs.
3. Dispense 100 µL of diluted peptide solution in each column of 96-well EIA plate using a multichannel pipette as per the assay design plate layout (*see* Fig. 3 and **Note 10**)

4. After adding peptides, seal the plate with adhesive plastic sheet and incubate at 37 °C for 1 h or at 4 °C overnight.
5. Aspirate the liquid from the wells and wash five times with PBS + 0.05 % Tween 20.
6. Dispense 200 μL of EIA blocking solution per well, seal the plate with adhesive plastic sheet, and incubate at 37 °C for 1 h.
7. Wash five times with 300 μL of EIA washing buffer using an automated plate washer or by hand pipetting up and down. Blot the plates on paper towel at the end of washing.
8. In triplicate wells, add 100 μL of test plasma or serum diluted in EIA blocking buffer at a final dilution of 1:200 and incubate the plate at 37 °C for 1 h.
9. Wash five times with 300 μL of EIA washing buffer using an automated plate washer or by hand pipetting up and down. Blot the plates on paper towel at the end of washing.
10. Dilute the goat-anti-human IgG-HRP-labeled secondary antibody in EIA blocking buffer, dispense 100 μL per well, and incubate at 37 °C for 1 h.
11. Wash five times with 300 μL of EIA washing buffer. Blot the plates on paper towel at the end of washing.
12. Add 100 μL of OPD substrate to each well and incubate the plate for 20 min in the dark at room temperature.
13. Stop reaction by adding 50 μL of 4 N H_2SO_4 and gently rock the plate to mix it.
14. Read optical density/absorbance at 490 nm within 10–15 min of stopping the EIA reaction.
15. Data analysis can be performed on Microsoft Excel or similar software (*see* **Note 11**).

4 Notes

1. To dissolve paraformaldehyde in water add 1 M NaOH and stir gently on a heating block at 60 °C until the PFA is dissolved. Add 10 mL of 10× PBS and allow the mixture to cool to room temperature. Adjust the pH to 7.4 with 1 M HCl (~1 mL), and then adjust the final volume to 100 mL with H_2O. Filter the solution through a 0.45 μm membrane filter to remove any particulate matter
2. The HEp-2 cell culture is grown in 175 cm^2 vented cell culture flask and should be processed in a separate hood for mock infection. The HEp-2 cell lysate should be prepared as described for RSV lysate antigen production and 5 % FBS-MEM replaced by SF-MEM at comparable times.

3. The RSV virus CPE is characterized by formation of syncytia, formation of giant cells, and rounding and sloughing of HEp-2 cells. The CPE can be observed using an inverted microscope at 40–100× magnification. The uninfected HEp-2 cells that will be used for HEp-2 lysate antigen control should not present any CPE.
4. Follow the manufacturer's recommendation to determine the dilution of secondary antibody. Approximately 10 mL of diluted conjugate is used for one plate. Scale up or down according to the number of plates being tested.
5. Data analysis can be performed using a standard curve generated for serial twofold dilutions of a human reference antiserum to RSV (available from NIH BEI Resources, USA, Cat # NR-4020) and predict the end point antibody titers in test plasma/serum samples.
6. As a negative control we recommend to use the empty vector lacking of the RSV F and G gene.
7. After this step the plates can be stored for up to 2 weeks in ziplock bags at 4 °C for further use. Before storage, the individual plates should be sealed with adhesive plastic sheet.
8. Final data can be presented as positive or negative for RSV antibodies (absorbance against F- or G-transfected cells (P) significantly above those for plasmid control (N)) and antibody titer for positive specimens. The titer of RSV antibody can be estimated by comparing corrected absorbance values (P-N) for the specimen to those for serial twofold dilutions of the human reference antiserum to RSV (available from NIH BEI Resources Cat # NR-4020, USA) or an RSV antibody high positive plasma from a donor.
9. This assay is developed to detect human anti-RSV group A and B antibodies to the central conserved region of glycoprotein. Peptides of 30 amino acid length are custom synthesized at 75 % or higher purity (*see* Table 1). A standard high-titer serum specimen with reactivity to RSV G peptides should be used to monitor consistency and quality of results. Seven plasma specimens can be tested at one dilution (dilution 1:200) against RSV G peptide and control peptide in one ELISA plate. The SARS CoV nucleoprotein peptide 1–30 amino acid (30 mer) is used as a negative control peptide.
10. Two plate layouts are used, one that includes serial twofold dilutions of the serum standard to generate a standard curve and the other plates that allows testing seven plasma/serum specimens at a 1:200 dilution. Each dilution or specimen is tested in triplicate wells for RSV peptide and SARS control peptide.

11. Data analysis can be performed by plotting standard curve for the twofold dilution of standard reference positive plasma or serum sample using Microsoft Excel. The prediction of the antibody titers in test plasma/serum samples can be determined using the standard curve.

Chapter 5

Secretory Expression and Purification of Respiratory Syncytial Virus G and F Proteins in Human Cells

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Abstract

Respiratory syncytial virus (RSV) is one of the leading causes of range of symptoms from mild upper to serious lower respiratory virus infections in infants, immunocompromised individuals, and the elderly. Despite many decades of research and development, a licensed RSV vaccine is not available for use in human. Since the RSV F and G proteins induce neutralizing antibodies and confer protection from infection, they are important for understanding disease and for developing vaccines and access to purified, expressed proteins is important to RSV research and diagnostics. We describe methods to produce recombinant RSV F and G proteins in human cells and purify these proteins using Ni Sepharose affinity chromatography.

Key words Respiratory syncytial virus, Expression, Human cell, F protein, G protein, Western blot

1 Introduction

RSV encodes for 11 distinct proteins and three of these proteins, the attachment glycoprotein G, the fusion protein F, and the small hydrophobic protein SH, are expressed at the surface of infected cells [1]. Two of these, F and G proteins, are responsible for inducing RSV-neutralizing antibodies. A humanized anti-F protein-neutralizing mAb is used to prevent RSV disease in high-risk young infants [2]. In addition to inducing antibodies that neutralize virus in vitro, F and G proteins may also induce antibodies that neutralize virus in vivo through Fc-mediated mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated neutralization [3, 4]. Their role in protective immunity makes measuring anti-G and anti-F protein antibodies important for understanding RSV immune responses. The full-length G protein is of variable size around 298 amino acid residues and a heavily glycosylated type II membrane protein. The G protein mediates attachment to the cell by binding to heparin-like motifs [5] and the chemokine receptor, CX3CR1 [6]. The predicted mass of G protein is about

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32 kDa but heavy glycosylation with N-linked and O-linked sugars increases its actual mass to about 90 kDa [7–9]. The fusion protein F is 574 amino acids long, is involved in cell fusion and binding to the cells, and consists of a dimer connected by disulfide bonds. The predicted molecular mass is about 70 kDa, but under nonreducing conditions often forms homodimers of about 140 kDa [7]. F protein in contrast to G protein has only N-linked glycosylation [10, 11].

Two groups of RSV, A and B, have been identified with monoclonal antibodies [12–14] and later by sequence studies [15, 16]. The F protein is less variable than the G protein and induces cross-reacting and cross protective antibodies while the G protein induces antibodies that tend to be group specific. Thus, a sensitive anti-G protein antibody EIA should include a G protein from both a group A and group B strain while anti-F protein antibodies can reliably be detected with a protein from a group A strain [12–14].

A variety of methods have been used to obtain purified RSV F and G proteins including immune affinity, immune precipitation, and preparative SDS-PAGE gel electrophoresis [6, 7, 10, 17, 18]. The techniques have been associated with loss of some antigenicity of the RSV-G protein [17]. A milder, one buffer system has been used for simultaneous chromatographic purification of RSV F and G protein from RSV-infected HEp2 cells [19]. This method uses a milder one-buffer system that does not denaturate the protein and may maintain antigenicity. We describe expression and metal affinity column purification of recombinant RSV G and F proteins from human cells using serum-free medium.

2 Materials

2.1 Transfection of 293F Human Cells for Secretion of RSV F and G Proteins

1. Suspension FreeStyle™ 293-F cells (Thermo Fisher Scientific).
2. Endotoxin-free purified pcDNA-RSV G-His and pcDNA-RSV F-His plasmids.
3. 293fectin™ (Life Technologies) or similar transfection reagent.
4. Opti-MEM® I Reduced Serum Medium.
5. FreeStyle™ 293 Expression Medium (Life Technologies) or similar.
6. 125 ml Polycarbonate, disposable, sterile Erlenmeyer flasks with vented filter caps.
7. Orbital shaker in 37 °C incubator with a humidified atmosphere of 8% CO_2.
8. Room-temperature tabletop centrifuge.
9. Sterile conical centrifuge 15 and 50 ml tubes.

10. Hemocytometer.
11. Trypan blue.
12. Sterile, disposable, polycarbonate 5 ml snap-cap tubes.
13. Vortex mixer.
14. 0.45 μm Sterile media filter unit.

2.2 Purification of RSV G or F Protein

1. Ni Sepharose excel.
2. Nanopure sterile water.
3. High-purity imidazole.
4. Binding buffer: 20 mM Sodium phosphate, 0.5 M NaCl, pH 7.4, 30 mM imidazole, pH 7.4, nanopure water. Filter sterilize through 0.45 μm filter.
5. Wash buffer: 20 mM Sodium phosphate, 0.5 M NaCl, 30 mM imidazole, pH 7.4, nanopure water. Filter sterilize through 0.45 μm filter.
6. Elution buffer: 20 mM Sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4, nanopure water. Filter sterilize through 0.45 μm filter.

2.3 Detection of RSV G and F Proteins by Western Blotting

1. 2× Laemmli sample buffer: 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris–HCl. Adjust pH to 6.8 if necessary.
2. Running buffer: Tris/glycine/SDS, 25 mM Tris, 190 mM glycine, 0.1% SDS. Adjust pH to 8.3 if necessary.
3. 1× Transfer buffer: 25 mM Tris, 190 mM glycine, 20% methanol. Adjust pH to 8.3 if necessary. For proteins larger than 80 kDa add SDS to a final concentration of 0.1%.
4. Tris-buffered saline with Tween 20 (TBST) buffer: 20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20.
5. Blocking buffer: 5% Skim milk powder in TBST.

3 Methods

3.1 Transfection of FreeStyle 293-F Cells with pcDNA-G-His and pcDNA-F-His

1. Seed 30 ml of FreeStyle™ 293-F cells at a density of 0.7×10^6 viable cells/ml in 125 ml volume sterile Erlenmeyer flask and incubate at 37 °C with orbital shaking for 24 hours.
2. On day 2, vigorously vortex cells in the flask for 45 s to break up cell clumps. Determine viability of cells using the trypan blue dye exclusion and a hemacytometer. Viability of cells must be over 90%.
3. For each 30 ml transfection, prepare lipid-DNA complexes by diluting 30 μg of plasmid DNA in Opti-MEM® I to a total volume of 1 ml and mix gently (*see* **Note 1**).

4. Dilute 60 µl of 293fectin™ in Opti-MEM® I to a total volume of 1 ml and incubate for no more than 5–6 min at room temperature. Subsequent to the incubation, add the diluted 1 ml DNA to the diluted 1 ml 293fectin™ to obtain a total volume of 2 ml and mix gently.
5. Incubate the DNA-293fectin™ at room temperature for 20–30 min to allow formation of DNA-293fectin™ complexes.
6. Transfer a total of 3×10^7 FreeStyle™ 293-F cells into a sterile disposable 125 ml Erlenmeyer flask and add pre-warmed FreeStyle™ 293 Expression Medium up to a total volume of 28 ml.
7. Once DNA-293fectin™ complex incubation is complete, add the 2 ml of DNA-293fectin™ complex to each flask and incubate the cells at 37 °C in a humidified atmosphere of 8% CO_2 in air on an orbital shaker rotating at 125 rpm (*see* **Note 2**).
8. At 48 h post-transfection harvest cell supernatant by centrifuging the cell culture at $400 \times g$ at 4 °C.
9. Filter cell supernatant through a 0.45 µm filter to remove cell debris and other particulate material.
10. Adjust the cell supernatant to the composition and pH of the binding buffer by adding sodium phosphate, sodium chloride, and imidazole from concentrated stock solutions.
11. Chill the medium containing secreted RSV G or RSV F proteins on ice.
12. Proceed to the purification of secreted recombinant RSV A2 virus F or G protein by nickel affinity chromatography (*see* **Notes 3** and **4**).

3.2 Ni Sepharose Column Packing

1. Allow the Ni Sepharose excel medium slurry to settle/sediment for 3 h in the container provided by the manufacturer (*see* **Note 5**).
2. Using a wide-bore 10 ml sterile disposable pipette remove 3 ml of Ni Sepharose excel medium slurry sediment in a 15 ml sterile disposable tube. Add 3 ml of overlayed 20% ethanol from the same container and mix to achieve a 1:1 ratio of settled medium and 20% ethanol (*see* **Note 6**).
3. Assemble the column and packing reservoir if necessary.
4. Rinse the column with 20% ethanol to make sure that no air will be trapped under the column bed support. Close the column outlet leaving the bed support covered with 20% ethanol.
5. Resuspend the Ni Sepharose excel medium slurry medium in 15 ml sterile disposable tube and pour into the column in a

single continuous motion with column held in slightly slanted angle to minimize the introduction of air bubbles.

6. Add 20% ethanol to completely fill the column. Mount the column to the adapter and avoid shaking and trapping air bubbles.
7. Allow the medium slurry to sediment for at least 3 h. Fill the reservoir with 50 ml of 20% ethanol and connect the tubing to the top of the column.
8. Open the bottom outlet of the column and set the flow rate to 1 ml/min and at the same time set the similar filling rate of 20% ethanol into the column.
9. Maintain packing flow rate of 1 ml/min for at least 4 column volumes after the constant bed height is reached and then stop the packing of the column.
10. Connect the column using a capillary tube to the reservoir containing wash buffer and start the equilibration.
11. Equilibrate the column in a 4 °C room with at least 5 column volumes of chilled 4 °C wash buffer. The flow rate should be kept at 1 ml/min. This procedure will result in a well-packed and well-equilibrated column. Fill the column with equilibration buffer and keep at 4 °C temperature in the chromatography stand.

3.3 Purification of RSV G or F Protein

1. Use the equilibrated column to load the chilled cell culture medium that contains secreted RSV G or RSV F protein conditioned to the composition and pH of the binding buffer.
2. Load the sample at a flow rate of 1 ml/min.
3. Wash with 20 column volumes of wash buffer at a flow rate of 1 ml/min. To prevent nonspecific binding of the unwanted cellular proteins, the concentration of imidazole in wash buffer could be increased to 45 mM imidazole, pH 7.4.
4. Elute with elution buffer using 5 column volumes of elution buffer.
5. Collect 2 ml elution fractions (*see* **Note 7**).
6. Remove a small volume (10–20 μl) of purified protein to perform a protein assay. Determine the protein concentration for each eluate.
7. If necessary, aliquot the protein samples for long-term storage at –20 °C (*see* **Note 8**).

3.4 Detection of RSV G and F Proteins by Western Blotting

1. Take 20 μg of each sample and add an equal volume of 2× Laemmli sample buffer.
2. Boil each sample at 95 °C for 5 min and centrifuge at 16,000 × *g* for 1 min.

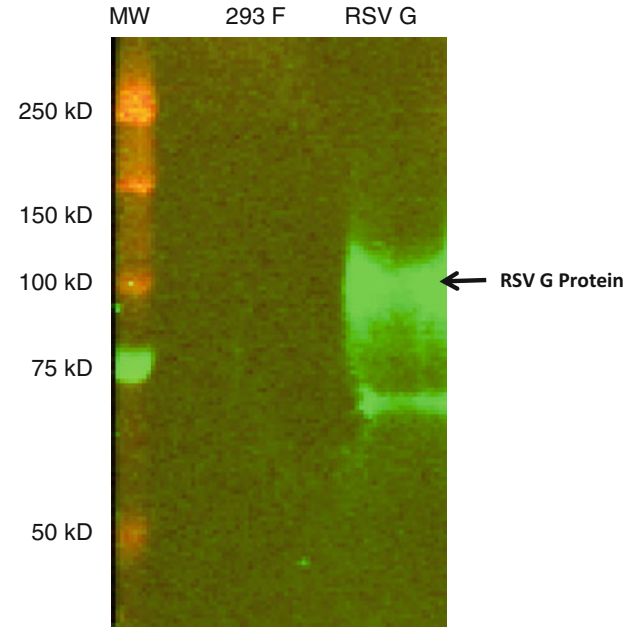

Fig. 1 Western blot reactivity of recombinant full-length secreted RSV A2 virus G protein from 293F human cell culture supernatant (36 h post-transfection) with anti-RSV G 131-2G mouse monoclonal antibody

3. Load equal amounts of purified RSV F or RSV G protein (5–20 μg) into the wells of a mini (8.6×6.7 cm) or midi (13.3×8.7 cm) format SDS-PAGE gel, along with molecular weight markers.
4. Run the gel for 5 min at 50 V and then increase the voltage to 100 V for 1 h (*see* **Note 9**).
5. Place the gel in 1× transfer buffer for 10–15 min. Use appropriate gel size nitrocellulose membrane.
6. Assemble the transfer sandwich and make sure that no air bubbles are trapped in the sandwich. The blot should be on the cathode and the gel on the anode.
7. Place the cassette in the transfer tank and place an ice block in the tank.
8. Transfer at 100 V for 1 h.
9. Rinse the blot in TBST for 5 min.
10. Block in blocking buffer for 1 h at room temperature.
11. Incubate with goat anti-RSV polyclonal serum diluted in blocking buffer for 1 h at room temperature or 4 °C for overnight (*see* **Note 10**).
12. Rinse the blot 3–5 times for 5 min with TBST.

Fig. 2 Schematic diagram of a cytomegalovirus promoter-driven plasmid construct encompassing full-length RSV G gene open reading frame flanked upstream by a mammalian secretory signal sequence and downstream by 6× histidine tag with transcription termination signal. The transfection of plasmid construct in 293F human cells results in secretion of RSV G protein in cell culture supernatant as shown in Fig. 1

13. Incubate with donkey anti-goat IR 800-conjugated secondary antibody diluted in blocking buffer for 1 h at room temperature (*see* **Note 11**).
14. Rinse the blot 3–5 times for 5 min with TBST.
15. Scan the blot using Li-Cor imaging system and perform qualitative analysis of the western blot for RSV F and G protein band sizes (*see* Fig. 1).

4 Notes

1. In the RSV protein expression constructs pcDNA-G-His and pcDNA-F-His, the full-length RSV F and RSV G genes were cloned in pcDNA 3.1+ vectors in Bam HI and Hind III cloning sites. In these two plasmids, the carboxyl terminal end of the F and G gene was modified by inserting a series of 6-histidine codons followed by the TAG termination codon to enable nickel or cobalt affinity column purification of the histidine-tagged protein. The amino terminal end of the F and G genes was modified by inserting the self-cleaving mammalian secretory signal sequence. The schematic representation of the gene cloning is shown for RSV G gene in Fig. 2. *E. coli* clones are grown in bulk culture for preparation of large amount of high-quality transfection-grade plasmids by using high-speed MaxiPrep method.
2. To the negative control flask containing 28 ml medium with 3×10^7 FreeStyle™ 293-F cell, add 2 ml of Opti-MEM® I instead of DNA-293fectin™ complex. The final FreeStyle™ 293-F cell density of approximately 1×10^6 viable cells/ml is desirable. The numbers of cells required for transfection experiment are cultured and it is ensured that more than 90% cells are viable before performing the transfection. The FreeStyle™ 293-F cell line is derived from the 293 cell line of human embryonic epithelial cells [20, 21]. FreeStyle™ 293-F cells are adapted to suspension culture in FreeStyle™ 293 serum- and protein-free defined expression medium.

3. You can take an aliquot of 0.2 ml medium from RSV A2 F and G plasmid-transfected and empty plasmid-transfected control cell medium at approximately 24, 36, and 48 h post-transfection and assay for recombinant protein expression by enzyme immune assay using goat-anti RSV polyclonal antibody.
4. The RSV G and F proteins that carry 6× histidine amino acid tags at the carboxyl-terminus are best purified by using columns of immobilized metal (nickel, cobalt or copper) affinity chromatography. The immobilized metal affinity chromatography-based reagents and kits for purification of 6× His-tagged proteins are available in traditional gravity columns, automated high-pressure purification columns, and centrifuge column purification formats from many commercial sources. The method described here uses Ni Sepharose excel affinity column purification that uses simple gravity flow as a means for draining the buffer and elution of proteins from the column and provides the ability for rapid purification of proteins without the need of expensive purification devices.
5. Ni Sepharose excel is made up of highly cross-linked spherical agarose beads of about 90 μm size to which a chelating ligand has pre-coupled. The ligand is also pre-charged with nickel ions which are very strongly bound. Therefore Ni Sepharose excel allows direct loading of cell culture medium in which target protein with histidine tag is secreted. Use of Ni Sepharose excel allows loading of large sample volumes of up to 1 liter that are generally required because of low concentrations of secreted target protein in the medium. The binding capacity of Ni Sepharose excel is 10 mg 6× histidine-tagged protein/ml of Ni Sepharose excel sedimented medium. The purification of histidine-tagged proteins on Ni Sepharose excel can be divided into five stages: column packing, equilibration, sample application, washing, and elution of target protein. Protein purifications can be performed under either native or denaturing conditions.
6. Ni Sepharose excel is provided pre-swollen in 20% ethanol and can be packed in suitable gravity flow column of 20–40 ml or higher bed volume depending on requirement. Use of short and wide columns, typically of 5–15 cm bed height, is recommended for rapid purification.
7. Imidazole can be removed from the eluted protein fractions by using disposable G25 desalting column and recommended procedure. Desalting is advantageous than dialysis in terms of convenience and time requirement.
8. Repeated freeze and thaw cycles cause protein degradation and should be avoided.
9. A 4–20% gradient gel separates proteins of all sizes very well.

10. An anti-RSV G protein 131-2G mouse monoclonal antibody and anti-RSV F protein 143-C6 mouse monoclonal antibody can also be used.
11. If mouse monoclonal antibodies are used as primary antibody, anti-mouse secondary antibodies conjugated with IR dye need to be used.

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References

1. Collins PL, Melero JA (2011) Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years. Virus Res 162:80–99
2. Geevarghese B, Simoes EA. (2012) Antibodies for prevention and treatment of respiratory syncytial virus infections in children. Antivir Ther 17:201-211
3. Miao C, Radu GU, Caidi H, Tripp RA, Anderson LJ, Haynes LM (2009) Treatment with respiratory syncytial virus G glycoprotein monoclonal antibody or F(ab')2 components mediates reduced pulmonary inflammation in mice. J Gen Virol 90:1119–1123
4. Hiatt A, Bohorova N, Bohorov O, Goodman C, Kim D, Pauly MH, Velasco J, Whaley KJ, Piedra PA, Gilbert BE, Zeitlin L (2014) Glycan variants of a respiratory syncytial virus antibody with enhanced effector function and in vivo efficacy. Proc Natl Acad Sci U S A 111:5992–5997
5. Krusat T, Streckert HJ (1997) Heparin-dependent attachment of respiratory syncytial virus (RSV) to host cells. Arch Virol 142:1247–1254
6. Tripp R, Jones LP, Haynes LM, Zheng H, Murphy PM, Anderson LJ (2001) CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nat Immunol 2:732–738
7. Walsh EE, Brandriss MW, Schlesinger JJ (1985) Purification and characterization of the respiratory syncytial virus fusion protein. J Gen Virol 66:409–415
8. Wertz GW, Krieger M, Ball LA (1989) Structure and cell surface maturation of the attachment glycoprotein of human respiratory syncytial virus in a cell line deficient in O glycosylation. J Virol 63:4767–4776
9. Collins PL, Mottet G (1992) Oligomerization and post-translational processing of glycoprotein G of human respiratory syncytial virus: altered O-glycosylation in the presence of brefeldin A. J Gen Virol 73:849–863
10. Lambert DM (1988) Role of oligosaccharides in the structure and function of respiratory syncytial virus glycoproteins. Virology 164:458–466
11. Pastey MK, Samal SK (1997) Analysis of the bovine respiratory syncytial virus fusion protein (F) using monoclonal antibodies. Vet Microbiol 58:175–185
12. Anderson LJ, Hierholzer JC, Tsou C, Hendry RM, Fernie BF, Stone Y, McIntosh K (1985) Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis 151:626–633
13. Mufson MA, Orvell C, Rafnar B, Norrby E (1985) Two distinct subtypes of human respiratory syncytial virus. J Gen Virol 66:2111–2124
14. Gimenez HB, Hardman N, Keir HM, Cash P (1986) Antigenic variation between human respiratory syncytial virus isolates. J Gen Virol 67:863–870
15. Peret TC, Hall CB, Schnabel KC, Golub JA, Anderson LJ (1998) Circulation patterns of genetically distinct group A and B strains of

human respiratory syncytial virus in a community. J Gen Virol 79:2221–2229

16. Collins PL, Melero JA (2011) Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years. Virus Res 162:80–99
17. Routledge EG, Willcocks MM, Samson AC, Morgan L, Scott R, Anderson JJ, Toms GL (1988) The purification of four respiratory syncytial virus proteins and their evaluation as protective agents against experimental infection in BALB/c mice. J Gen Virol 69:293–303
18. Roberts SR, Lichtenstein D, Ball LA, Wertz GW (1994) The membrane-associated and secreted forms of the respiratory syncytial virus attachment glycoprotein G are synthesized from alternative initiation codons. J Virol 68:4538–4546
19. Roder C, Krusat T, Reimers K, Werchau H (2000) Purification of respiratory syncytial virus F and G proteins. J Chromatogr B Biomed Sci Appl 737:97–106
20. Graham FL, Smiley J, Russell WC, Nairn R (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 36:59–74
21. Harrison T, Graham F, Williams J (1977) Host-range mutants of adenovirus type 5 defective for growth in HeLa cells. Virology 77:319–329

Chapter 6

Development of Human Monoclonal Antibodies Against Respiratory Syncytial Virus Using a High Efficiency Human Hybridoma Technique

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Abstract

Human monoclonal antibodies against RSV have high potential for use as prophylaxis or therapeutic molecules, and they also can be used to define the structure of protective epitopes for rational vaccine design. In the past, however, isolation of human monoclonal antibodies was difficult and inefficient. Here, we describe contemporary methods for activation and proliferation of primary human memory B cells followed by cytofusion to non-secreting myeloma cells by dielectrophoresis to generate human hybridomas secreting RSV-specific monoclonal antibodies. We also provide experimental methods for screening human B cell lines to obtain RSV-specific lines, especially lines secreting neutralizing antibodies.

Key words Respiratory syncytial virus, Monoclonal antibodies, Hybridoma, Virus neutralization

1 Introduction

Hybridoma technology is a remarkable method for the efficient isolation and production of human monoclonal antibodies. Human B lymphocytes can be isolated from peripheral blood mononuclear cells (PBMCs) by Ficoll density gradient centrifugation and transformed by Epstein-Barr virus (EBV) to induce B cell proliferation of B lymphoblastic cell lines. Epstein-Barr virus can be obtained from the cultured supernatant of the B95-8 cell line. These EBV-transformed B cells then can be fused to a non-secreting human–mouse myeloma analog (HMMA 2.5) cell line to produce stable hybridomas.

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2 Materials

2.1 Expression of RSV F_{ECTO} and G_{ECTO}

1. cDNA encoding full-length RSV F or G protein (*see* **Note 1**).
2. pcDNA™3.1/*myc*-His B mammalian expression vector.
3. *Escherichia coli* strain DH5α competent cells.
4. Miniprep plasmid purification kit.
5. FreeStyle™ 293-F Cells: this cell line is derived from the 293 cell line and it can be obtained from Life Technologies.
6. FreeStyle™ 293 Expression Medium (Life Technologies) or equivalent.
7. 0.2 μm filter unit.
8. HisTrap HP Ni Sepharose column.
9. 1× phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM of Na_2HPO_4, 2 mM of KH_2PO_4. Adjust pH to 7.4 with HCl and sterilize by autoclaving.
10. Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-30 membrane.
11. NanoDrop Spectrophotometer 2000.

2.2 Generation of Epstein-Barr Virus (EBV)

1. One aliquot of approximately 1×10^6 cells of B95-8 cell line.
2. 50 mL conical tubes.
3. Medium A: ClonaCell™-HY Medium A (STEMCELL Technologies) or equivalent pre-fusion medium containing DMEM, serum, gentamycin, and supplements.
4. T-75 flask (75 cm^2 flask).
5. T-225 flasks (225 cm^2 flask).
6. 0.45 μm filter unit.
7. 0.2 μm filter unit.
8. 5 mL cryovials.
9. Swinging bucket tabletop centrifuge.

2.3 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

1. Sodium-heparin green top blood collection tubes.
2. Serum red top blood collection tubes with clot activator.
3. 1× sterile D-PBS.
4. 50 mL conical tubes.
5. Ficoll 1077 (Histopaque-1077).
6. Medium A (*see* Subheading 2.2).
7. Trypan blue.
8. Dimethyl sulfoxide (DMSO).

9. Cryovials.
10. Mr. Frosty™ Freezing Container.
11. Hemocytometer.

2.4 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) for Feeder Layers

12. Subject PBMCs—4×10^6 cells per plate.
13. Medium A (*see* Subheading 2.2).
    (a) CpG stock: 2.5 mg/mL oligonucleotide ZOEZOEZZZZZOEEZOEZZZT dissolved in nuclease-free water (stored at −20 °C).
14. Chk2i stock: 10 mM 2-(4-(4-chlorophenoxy)phenyl)-1H-benzimidazole-5-carboxamide hydrate (Chk2 inhibitor II) dissolved in DMSO (stored at −20 °C).
15. 96-well cell culture plates.
16. Cesium irradiator.

2.5 EBV Transformation of B Cells from RSV-Immune Subject PBMCs

1. Subject PBMCs—4×10^6 cells per plate.
2. Medium A (*see* Subheading 2.2).
3. Trypan blue.
4. CpG stock.
5. Chk2i stock.
6. CSA stock: 1 mg/mL cyclosporin A in DMSO.
7. EBV stock: EBV-containing supernatant from cultured B95-8 cells.
8. EBV medium (EBV transformed B-cell growth medium): for one plate combine 16 mL warmed Medium A, 50 µL CpG stock (2.5 mg/mL), 20 µL CSA stock (1 mg/mL), 15 µL Chk2i stock (10 mM), and 4.5 mL EBV stock.
9. 384-well plates.
10. Electronic multichannel pipette 1250 µL.
11. Pipette tips.
12. 96-well cell culture plates.

2.6 Growth and Maintenance of HMMA 2.5 Cell Line

1. HMMA2.5 cells.
2. 50 mL conical tubes.
3. Medium A (*see* Subheading 2.2).
4. Canted-neck tissue culture flasks: T-25, T-75, T-150, and T-225.
5. Cell scraper.

2.7 Cytofusion of EBV Transformed B Cells and HMMA 2.5 Cells

1. HMMA 2.5 cells.
2. BTX cytofusion medium: 300 mM sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 1.0 mg/mL BSA. Filter-sterilize and store at 4 °C.
3. BTX cytofusion cuvettes (BTX620: 2 mm gap width; 400 μL)
4. Modified cytofusion device: BTX cuvette holder (BTX Safety Stand, Model 630B) and CytoPulse Sciences Generator (CytoPulse Sciences, Model PA-4000).
5. 384-well cell culture plates.
6. 50× HAT (Hypoxanthine–aminopterin–thymidine media supplement 50×): 5 mM hypoxathine, 20 μM aminopterin, and 800 μM thymidine.
7. Medium A (*see* Subheading 2.3).
8. Medium E: ClonaCell™-HY Medium E (STEMCELL Technologies) or equivalent growth medium containing DMEM, serum, HT, gentamycin, and supplements.
9. Ouabain stock: 1 mg/mL Ouabain in water.
10. 50× HT (Hypoxanthine–thymidine media supplement 50×): 5 mM hypoxathine and 800 μM thymidine.
11. HAT medium: 400 mL Medium A, 100 mL Medium E, 10mL 50× HAT and 150 μL Ouabain stock.
12. HT medium: 400 mL Medium A, 100 mL Medium E, 10 mL 50× HT, 150 μL Ouabain stock.
13. Electronic multichannel pipette 1250 μL.
14. Pipette tips.

2.8 Subcloning Hybridomas by Flow Cytometry

1. Medium E (*see* Subheading 2.7).
2. Flow cytometry tubes.
3. Cell sorter.
4. 48-well cell culture plates.
5. 96-well cell culture plates—U-bottom.
6. Hybridoma culture growing in a 384-well plate.
7. Propidium iodide.

2.9 Hybridoma Expansion

1. 12-well cell culture plates.
2. Medium E (*see* Subheading 2.7).
3. T-75 Flasks.
4. T-225 Flasks.
5. Hybridoma serum free medium (Gibco or equivalent).
6. Dimethyl sulfoxide (DMSO).
7. Cryovials.

8. Cell scrapers.
9. 0.45 μm filter unit.

2.10 Plaque Reduction Assay

1. HEp-2 cells.
2. RSV strain A2 stock virus suspension.
3. OptiMEM I medium with 0.5% gentamicin and 1% amphotericin B (Fungizone).
4. Guinea pig complement (if performing complement enhanced neutralization assay).
5. Overlay medium: 3.75 g of methylcellulose-autoclaved and dissolved in 500 mL OptiMEM I medium. Stirred at room temperature for 2–3 h and then transferred to 4 °C to be stirred overnight.
6. Primary (RSV detecting) antibodies (a wide variety of antibodies from many species could be used for this step, as long as the monoclonal or polyclonal antibodies detect RSV antigens in fixed cells. We use a cocktail of three purified anti-F murine monoclonal antibodies) (*see* **Note 2**).
7. Secondary antibodies (conjugate). Goat Anti-Mouse IgG (H + L) Horseradish Peroxidase (HRP) Conjugate.
8. Colorimetric reagent: 1:1 ratio of Peroxidase Substrate Solution B and 4CN Peroxidase Substrate.
9. 24-well standard tissue culture plate.
10. 80% cold methanol with 20% distilled water.

3 Methods

3.1 Cloning, Expression, and Purification of RSV F_{ECTO} and G_{ECTO}

1. Using the synthesized cDNA encoding full-length RSV F or G genes amplify the ectodomain (F*ECTO* or G*ECTO*) to remove the transmembrane domain and cytoplasmic tail regions and allowing cloning in-helix with the trimerization domain (for full-length protein expression and purification *see* Chapter 5).
2. Clone the PCR product directionally into pcDNA™3.1/*myc*-His B expression vector at restriction sites 5′-BamHI-EcoRI-3′ (*see* **Note 3**).
3. Transform ligated product into *E. coli* strain DH5α competent cells.
4. Purify plasmids using any plasmid purification kit.
5. Confirm in-frame cloning of all plasmids by DNA Sanger sequencing.
6. Transiently transfect confirmed pcDNA3.1-F or G*ECTO*-*myc*/His plasmid into suspension 293-F cells, according to the manufacturer's protocol.

7. Transfected cells are incubated for 4 days at 37 °C with 5% CO_2 on a shaker.
8. Centrifuge cells for 10 min at 100 × *g* at 4 °C.
9. Filter supernatant through a 0.2-μm filter prior to purification and store at 4 °C or purify directly.
10. Purify 6× His-tagged RSV F or G_{ECTO} protein by immobilized affinity chromatography using prepacked 5mL HisTrap HP Ni-Sepharose columns according to the manufacturer's protocol.
11. Concentrate protein using Amicon Ultra centrifugal filters.
12. Determine recombinant RSV F protein concentration using NanoDrop device.
13. Confirm status of purified protein by denaturing, non-reducing SDS-PAGE analysis. There should be a single band migrating at ~ 60-kDa.
14. Purified RSV F or G protein can be stored for several weeks at 4 °C until analyzed, if the preparation is free of proteases, but is best kept in single-use aliquots stored at –80 °C.

3.2 Generation of Epstein-Barr Virus (EBV) Stock for Transformation

1. Thaw an aliquot of the B95-8 cell line rapidly in a water bath at 37 °C. Remove stock from the water bath as soon as it has thawed (*see* **Note 4**).
2. Gently transfer the cells to a 15 mL conical tube.
3. Dropwise, add 1 mL of warmed Medium A to the cells.
4. Resuspend the cells in 10 mL of warmed Medium A.
5. Centrifuge cells in a swinging bucket tabletop centrifuge at 150 × *g* for 8 min.
6. Discard the supernatant and resuspend the cells in 1 mL of warmed Medium A.
7. Add 29 mL of Medium A to a T-75 flask. Place 1 mL of cells into the T-75 flask and gently rock the flask. Incubate at 37 °C with 5% CO_2.
8. Grow cells for 3–5 days, until the medium develops a yellow color and adherent cells are greater than 50% confluent.
9. Once the medium is yellow and cells are greater than 50% confluent, gently scrape the cells off the bottom of the flask and transfer 30 mL of cells to five T-225 flasks, splitting the cells evenly amongst the flasks. Fill each flask up to 55 mL with warmed Medium A. There should be 6 mL of cells and 49 mL fresh Medium A per flask.
10. Incubate flasks at 37 °C with 5% CO_2 for 5–7 days, until the medium develops an orange (not yet yellow) color and adherent cells are greater than 50% confluent.

11. Harvest the medium and centrifuge in a swinging bucket tabletop centrifuge at 800 × *g* for 10 min. EBV will be in the supernatant.
12. Filter the supernatant through a 0.45 μm filter unit, followed by a 0.2 μm filter unit.
13. Aliquot 4.5 mL of the filtrate into 5 mL cryovials and store for future use at −80 °C.

3.3 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Respiratory Syncytial Virus (RSV) Immune Subject Blood Samples

1. Obtain peripheral blood from the subject by venipuncture. Have blood drawn into a sodium-heparin green top tube (*see* **Note 5**).
2. Add 15 mL of warmed 1× D-PBS to a 50 mL conical tube (*see* **Note 6**).
3. Add 10 mL of blood to each 50 mL conical tube containing 1× D-PBS.
4. Underlay the 25 mL of blood and D-PBS with 14 mL of warmed Ficoll.
5. Centrifuge in a swinging bucket tabletop centrifuge for 25 min at 800 × *g* with the brake and acceleration set to zero, or as low as possible.
6. Remove and discard most of the plasma on top, down to about 2–3 mm from the white buffy coat layer, which contains the cells (*see* **Note 7**).
7. Remove buffy coat by tilting the tube and gently aspirating cells until the middle of liquid in the tube starts to clear, then pipette the material into a new 50 mL conical tube. Be sure to move the pipette around the sides of the tube in order to collect all PBMCs.
8. Add up to 50 mL of warmed Medium A to the receiving tube containing the transferred buffy coat layers.
9. Centrifuge at 400 × *g* for 18 min in a swinging bucket tabletop centrifuge.
10. Remove supernatant and resuspend cells in 2 mL of warmed Medium A for every initial 10 mL of blood.
11. Count cells and assess viability with trypan blue staining.
12. PBMCs can be used directly for EBV transformation (*see* Subheading 3.4 EBV transformation of B cells from RSV-immune subject PBMCs) or can be frozen for future use.
13. To freeze PBMCs, resuspend cells at 5.5×10^6 cells per 900 μL in Medium A, and then add 1/10 final volume of DMSO.
14. Freeze PBMCs in 1 mL aliquots in cryovials.

15. Place tubes in a Mr. Frosty freezing container and put in the −80 °C freezer for at least 100 min (for estimated 1° cooling per minute).
16. Move samples to the vapor phase of liquid nitrogen for storage.

3.4 Preparation of Peripheral Blood Mononuclear Cells (PBMCs) from Human Blood for Feeder Layers

1. When using a frozen stock of subject PBMCs, thaw samples rapidly in 37 °C water bath. Remove stock from the water bath as soon as it has thawed. When using freshly isolated PBMCs, skip **steps 1–5**.
2. Gently transfer the cells to a 15 mL conical tube.
3. Dropwise, add 1 mL of warmed Medium A to the cells.
4. Resuspend the cells in 10 mL warmed Medium A.
5. Centrifuge the cell suspension at $150 \times g$ for 8 min.
6. Discard the supernatant and resuspend cells in 20 mL warmed Medium A. Use one vial of frozen PBMCs containing 1×10^7 cells for every 20 mL of Medium A (after thawing, there should be about five to eight million viable cells per vial).
7. Irradiate cells for 15 min using a Cs source (9000 rad).
8. Following irradiation, add 20 μL CpG stock and 20 μL Chk2i stock.
9. Dispense 200 μL/well of mixture into each well of a 96-well plate. This procedure should be performed about an hour before the feeder layer is used to support cell growth.

3.5 EBV Transformation of B Cells from RSV-Immune Subject PBMCs

1. When using a frozen stock of subject PBMCs, thaw samples rapidly in 37 °C water bath. Remove stock from the water bath as soon as it has thawed. When using freshly isolated PBMCs, skip **steps 1–5**.
2. Gently transfer the cells to a 15 mL conical tube.
3. Dropwise, add 1 mL of warmed Medium A to the cells.
4. Resuspend the cells in 10 mL warmed Medium A.
5. Centrifuge the cell suspension at $150 \times g$ for 8 min.
6. Discard the supernatant and resuspend cells in 1 mL warmed Medium A.
7. Count cells and assess viability with trypan blue staining.
8. Rapidly thaw a 4.5 mL vial of EBV stock in 37 °C water bath.
9. Prepare EBV medium by combining 16 mL warmed Medium A, 50 μL CpG stock, 20 μL CSA stock, 15 μL Chk2i stock, and 4.5 mL of EBV stock into a 50 mL conical tube.
10. Add the cells to EBV medium.

11. Using a multichannel pipette, dispense 50 μL/well of mixture containing PBMCs and EBV into a 384-well plate. There should be one plate for every four million viable cells.
12. Centrifuge plates at 40×*g* for 5 min in a swinging bucket tabletop centrifuge to settle the culture medium.
13. Incubate plates at 37 °C with 5% CO_2 for 10–12 days. Check plates daily for growth of lymphoblastoid cells.
14. After 10–12 days of incubation, screen plates by enzyme-linked immunosorbent assay (ELISA) using an appropriate RSV protein antigen.
15. Prepare 96-well plates with a feeder layer of cells (*see* Subheading 3.3 Preparation of Peripheral Blood Mononuclear Cells (PBMCs) from human blood for feeder layer).
16. Transfer wells that were determined by ELISA to be producing desired antibodies to a 96-well plate.
17. Incubate plates at 37 °C with 5% CO_2 for 4 days.
18. Screen by ELISA using appropriate RSV antigen.
19. Incubate plates for an additional 3-4 days at 37 °C with 5% CO_2 prior to fusion of transformed B cells with HMMA 2.5 myeloma cells.

3.6 Growth and Maintenance of HMMA 2.5 Cells

1. If starting with a frozen stock of HMMA 2.5 cells, thaw an aliquot of the cells rapidly at 37 °C. Remove the stock from the water bath as soon as it has thawed.
2. Gently transfer the cells to a 15 mL conical tube.
3. Dropwise, add 1 mL of warmed Medium A to the cells.
4. Resuspend the cells in 10 mL of warmed Medium A.
5. Centrifuge the cells for 8 min a 150×*g* in a swinging bucket centrifuge.
6. Discard the supernatant.
7. Add 25 mL Medium A to a T-75 flask.
8. Resuspend the cells in 1 mL of warmed Medium A and transfer to the T-75 flask.
9. Incubate at 37 °C with 5% CO_2.
10. At 80% confluence, cells can be split and expanded. Split the cells at least 3 days prior to performing fusions.

3.7 Fusion of EBV-Transformed B Cells with HMMA 2.5 Cells

1. Count HMMA 2.5 cells.
2. Add 1×10^7 cells to 1 mL of warmed BTX cytofusion medium in a 1.5 mL microcentrifuge tube. You will need 120 μL of 1×10^7 cells/mL for each fusion.

3. Gently resuspend the contents of the well containing an EBV transformed B cell line secreting RSV antigen-specific antibodies, as determined by ELISA, and transfer them to a 1.5 mL microcentrifuge tube that contains 1 mL of warmed BTX cytofusion medium.
4. Centrifuge the microcentrifuge tubes containing the HMMA 2.5 cells and the microcentrifuge tubes containing the positive EBV-transformed B cells at $1100 \times g$ for 4 min in a tabletop centrifuge.
5. Decant the supernatant.
6. Resuspend each cell pellet in 1 mL of warmed BTX cytofusion medium.
7. Repeat the centrifugation, dispose of the supernatant, and resuspend the pellet in cytofusion medium two times (resulting in a total of 3 centrifugations). After the last centrifugation, DO NOT resuspend the positive EBV-transformed B cells in 1 mL of BTX medium. Simply decant the supernatant and wait until **step 8** to resuspend the cells.
8. Resuspend the HMMA 2.5 cell pellet in 1 mL of BTX cytofusion medium. The concentration should remain 1×10^7 cells/mL.
9. Use 120 μL of the HMMA 2.5 cell solution at 1×10^7 cells/mL to resuspend the positive EBV-transformed B cells in each microcentrifuge tube prior to transfer to a cytofusion cuvette.
10. Transfer the mixture of HMMA 2.5 cells and EBV-transformed B cells, volume should be approximately 200–250 μl, to a cytofusion cuvette.
11. Place the cuvette(s) (device holds one or two cuvettes) into the modified cytofusion device, using a BTX cuvette holder and Cyto-Pulse Sciences generator. Use the following settings:
    (a) Pre: 70 V × 40 s, AC current.
    (b) Pulse: 300 V × 0.04 ms DC current.
    (c) Post: 20 V × 30 s, AC current.
12. After the fusion, incubate the cuvettes at 37 °C with 5 % CO_2 for 30 min.
13. Add the contents of cuvettes to 20 mL of HAT medium (*see* **Note 8**).
14. Use an electronic multichannel pipette to plate the fusion products at 50 μL/well in 384-well cell culture plates.
15. Incubate the plates at 37 °C with 5 % CO_2 for 7 days.
16. Refeed cells with 25 μL/well of Medium E and incubate at 37 °C with 5 % CO_2 for 7 more days.
17. Once again, screen by ELISA using appropriate RSV antigen.
18. Expand positive hybridoma cells to a 48-well plate.

3.8 Subcloning Hybridoma Cell Lines by Flow Cytometric Sorting

1. Gently resuspend the cells from wells with desired reactivity, as determined by ELISA, from a 384-well plate and put 1 drop into a flow cytometry tube containing 1 mL of Medium E.
2. Put the rest of the cells into one well of a 48-well plate containing 750 μL of Medium E.
3. Repeat for up to six cell lines desired for sorting; add the single drop of cells to the same tube for each cell lines and make individual cultures in the 48-well plate.
4. Pick one well from the 384-well plate that was not positive, but had cells growing, and use this for the stained and unstained controls.
5. Dispense 50 μL/well of Medium E onto one 384-well plate per hybridoma.
6. Add 1 μL of propidium iodide to each tube and bring this, the 384-well target plate, and the controls to the sorting flow cytometer.
7. Using a sterile sorting flow cytometer, distribute 1 hybridoma cell per well in each well of a 384-well plate.
8. Incubate the plates at 37 °C with 5 % CO_2 for 10–15 days.
9. Screen the supernatants collected from 48-well plate to 384-well plates by ELISA (see Chapter 4) or functional assay.
10. If no reactive supernatants are found on the 384-well plate, repeat the limiting dilution and plating of a 384-well plate, assuming one or more of the 48-well culture supernatant contain antibody with desired activity.

3.9 Expanding Hybridoma Clones

1. Select cultures derived from single cell sorting that secret antibodies with desired activity in the 384-well plate clonal hybridoma ELISA and transfer each to a well of a 48-well plate containing 750 μL/well of Medium E. Incubate at 37 °C with 5 % CO_2 until cells are 25 % confluent.
2. Check antibody production in the supernatant by ELISA.
3. Gently resuspend cells and take an aliquot of cells for freezing.
4. Add remainder of the cells to a 12-well plate containing 2 mL/well of Medium E.
5. Grow 12-well plates in an incubator at 37 °C with 5 % CO_2 until cells are 25 % confluent.
6. Once again, check antibody production in supernatant by ELISA.
7. Cryopreserve an aliquot of cells that represents 25 % of the culture.
8. Transfer the remainder of the cells in the 12-well plate to a T-75 flask and fill up with Medium E to 20 mL.

9. Every 3 or 4 days, feed the cells by aspirating off the old medium and adding back fresh, warm medium. Feed the cells every 3 or 4 days until the cells are 80% confluent.
10. Add 250 mL/flask of hybridoma serum free medium to four Corning T-225 flasks.
11. Remove medium from the 80% confluent T-75 flask and add 11 mL of hybridoma serum free medium.
12. Scrape cells off of the bottom of the T-75 flask using a cell scraper.
13. Add 2.5 mL of cell suspension to each T-225 flask.
14. Add 30 mL Medium E back to T-75 flask and grown to 70–80% confluence.
15. Once confluent, harvest cells from T-75 flask for RNA extraction and to make frozen stocks of monoclonal hybridoma cells.
16. Grow hybridoma cells in T-225 in an incubator at 37 °C with 5% CO_2 for mAb production.
17. Grow until cells are 10% viable.
18. Filter cell suspension medium through a 0.45 μM filter and then harvest the antibodies from the supernatant by FPLC or affinity chromatography. Before purifying, perform an ELISA on the supernatant, and only purify those supernatants that are positive in an appropriate assay.
19. Characterize the antibody neutralization by performing a plaque reduction assay.

3.10 Characterization of Antibody Neutralization by Plaque Reduction Assay (See Note 9)

1. Plate HEp-2 cells on a 24-well tissue culture plate so that they are 90% confluent on the day of the plaque reduction assay.
2. Prepare serial dilutions of antibody. For instance, in a 96-well plate set up serial fourfold dilutions of 1:10, 1:40, 1:160, and 1:640. For diluent use OptiMEM I medium with 0.5% gentamicin and 1% amphotericin B.
3. Quickly thaw a vial of RSV strain A2 virus and dilute with OptiMEM I diluent. Dilute so that 50 μL of virus can generate 50 plaques.
4. Antibodies can be tested in the presence or absence of 10% guinea pig complement. To test with complement, add 10% by volume of guinea pig complement to the diluted virus.
5. Combine diluted monoclonal antibody with the RSV strain A2 virus with complement at a 1:1 ratio.
6. Incubate mixture at 37 °C with 5% CO_2 for 1 h.
7. Replace medium in the 24-well plate containing monolayer of HEp-2 cells with 100 μL of RSV-A2/antibody/complement mixture.

8. Incubate plate at 37 °C with 5 % CO_2 for 1 h.
9. Aspirate infection mixture with a sterile pipette tip.
10. Quickly, to avoid cell desiccation, overlay each well with 1 mL of overlay medium.
11. Incubate cells for 4 days at 37 °C with 5 % CO_2.
12. Discard overlay medium and fix cells with 80 % cold methanol and place at −20 °C for 1 h.
13. Pour off methanol (save as chemical waste) and freeze 24-well plate or stain immediately.
14. To stain, wash wells with 1× PBS.
15. To minimize nonspecific binding of antibodies, add 5 % milk in PBS and incubate at room temperature for 30 min.
16. Remove milk and 200 μL of primary RSV F antibody cocktail at appropriate dilution in 5 % milk.
17. Remove the primary antibody and wash with 5 % milk.
18. Add 200 μL of secondary antibody (goat anti-mouse HRP if using mouse primary antibodies), at appropriate dilution in 5 % milk and incubate at 37 °C with 5 % CO_2 for 1 h.
19. Remove secondary antibody and wash wells three times with PBS. Then discard PBS.
20. Add 200 μL of colorimetric reagent.
21. Incubate at room temperature for 20 min.
22. Remove substrate and count RSV plaques.

4 Notes

1. Some considerations about RSV proteins: The choice of RSV protein gene sequences for expression is an important one, and considerations for optimal protein expression strategies should take into account the desired strain, form of protein (monomeric or trimeric and pre- or post-fusion conformation), and post-translational modifications. The user should keep in mind that RSV F protein is a Type I integral membrane protein, while RSV G is a Type II protein. This protocol produces oligomeric proteins in mammalian cells that glycosylate the proteins in a similar fashion to virus infection of the same cells, and yields post-fusion conformation F protein.
2. A wide variety of antibodies from many species could be used for this step, as long as the monoclonal or polyclonal antibodies detect RSV antigens in fixed cells. We use a cocktail of three purified anti-F murine monoclonal antibodies).

3. We have inserted a structural protein of bacteriophage T4, the C-terminal domain of T4 fibritin (foldon), as a trimerization domain into the vector to accomplish trimerization of the recombinant protein.
4. EBV is a Risk Group 2 agent and should be used with biosafety level two practices in a BSL-2 facility, and only after proper training. Laboratory scientists should never transform their own PBMCs in vitro, nor work with EBV-transformed B cells derived from their own PBMCs.
5. If desired, have another aliquot drawn into a red top tube in order to freeze away an aliquot of subject serum (you may also choose to save subject plasma in **step 6**). The approximate yield of peripheral blood mononuclear cells (PBMCs) is $1–2 \times 10^6$ cells/mL of peripheral blood.
6. One conical tube is needed for every 10 mL of blood drawn.
7. Save 1 mL plasma for testing, if desired (freeze plasma at −80 °C). Alternatively, blood can be collected into a red top tube for separation and storage of serum. Serum typically performs better than plasma in most serologic tests for antibodies.
8. The amount of HAT medium may need to be adjusted, depending on which electronic multichannel pipette is used. There should be enough medium for one 384-well plate.
9. Another version of the antibody neutralization assay in a 96-well plate format can be found in Chapter 7.

Chapter 7

Respiratory Syncytial Virus (RSV): Neutralizing Antibody, a Correlate of Immune Protection

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Abstract

Assays that measure RSV-specific neutralizing antibody activity are very useful for evaluating vaccine candidates, performing seroprevalence studies, and detecting infection. Neutralizing antibody activity is normally measured by a plaque reduction neutralization assay or by a microneutralization assay with or without complement. These assays measure the functional capacity of serum (or other fluids) to neutralize virus infectivity in cells as compared to ELISA assays that only measure the binding capacity against an antigen. This chapter discusses important elements in standardization of the RSV-specific microneutralization assay for use in the laboratory.

Key words Neutralization assay, Microneutralization assay, Neutralizing antibodies

1 Introduction

RSV is a negative-sense, single-stranded RNA virus in the genus *Pneumovirus*, subfamily *Pneumovirinae*, and family *Paramyxoviridae*. It consists of two major antigenic subgroups, RSV/A and RSV/B viruses. RSV/A accounts for the subgroup most frequently found in the community [1]. During an outbreak it is common to find multiple genotypes of RSV cocirculating in the community, with one or two of the genotypes being the dominant isolates [2, 3]. The antigenic diversity is primarily found in the attachment (G) glycoprotein [2, 3]. The analysis of the nucleotide sequence on the second hypervariable region of the G gene has led to classification of RSV genotypes within RSV/A and RSV/B subgroups [2]. For RSV/A, at least ten genotypes (GA1–GA7 [2], SAA1 [4], NA1 and NA2 [5]) have been described. Recently, a novel RSV/A genotype (Ontario or ON1) with a 72-nucleotide G gene duplication has been reported in Canada [6]. ON1 is becoming the dominant RSV/A genotype reported in molecular epidemiologic studies [7]. Similar to the ON1 genotype, a 60 nt-duplication in the G gene of HRSV/B has been identified in

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1998 in Buenos Aires. This novel RSV/B subgroup is named BA genotype [8], and has spread worldwide within a 7-year period, replacing the majority of other prevailing RSV/B genotypes by 2005 [9]. It is remarkable that neither ON1 nor BA genotypes have been associated with a different virulence profile, even though major changes occurred in the distal fragment of the G gene. The G glycoprotein is capable of inducing a virus-specific serum neutralizing antibody response; however, the large antigenic diversity found in the G protein diminishes its potential as an antigen in ELISA assays for immune correlates of protection.

The other surface glycoprotein is the fusion (F) glycoprotein, which is a major target of the host's immune response. The F protein undergoes major conformational changes (pre-fusion to post-fusion) to cause membrane fusion [10, 11]. The post-fusion F is relatively conserved within the RSV subgroups; however, the recently described antigenic site Ø in the pre-fusion F appears to be variable, especially among contemporary RSV/A strains to the prototype GA1 viruses [3]. A significant component of RSV-specific serum antibodies is directed to the pre-fusion form [12]. There are three major antigenic sites on the post-fusion form and they remain relatively conserved [3]. Antibodies directed to sites II and IV recognize both the pre-fusion and the post-fusion F form because they bind to sites whose structure does not change as the F protein undergoes conformational changes during membrane fusion.

The small hydrophobic (SH) protein of RSV is also a surface protein that tends to cluster on the cell surface of infected cells. There is low genetic variability but sufficient to allow phylogenetic clustering between RSV/A and RSV/B isolates [3, 13]. During natural infection a weak immune response is generated to the SH protein. The SH protein (extracellular domain) as a vaccine candidate can elicit antibody-dependent cell-mediated cytotoxicity and or antibody-dependent cellular phagocytosis but not serum neutralizing antibody activity [14].

RSV-specific microneutralization assay has been used to establish a correlate of immunity against severe RSV disease in children, evaluate RSV vaccines in preclinical and clinical phases of development, and relate neutralizing antibody titers to different concentrations of palivizumab (Synagis®), the only approved monoclonal antibody for prevention of severe RSV disease. It has been shown that for every log2 increase in neutralizing antibody titer there is an approximate 20% reduction in the likelihood of RSV-related hospitalization [15, 16]. Additionally, an RSV/A-specific, serum neutralizing antibody titer of ≥6 log2 (≥1:64) is associated with an approximately 70% likelihood of not having an RSV-related hospitalization [15]. Of interest, RSV/A-specific, serum neutralizing antibody titer of 6 log2 is comparable to 40 μg/ml of palivizumab which is the nadir concentration achieved in serum with the

recommended immunoprophylactic dose of 15 mg/kg of palivizumab in infants [17]. It has been demonstrated that for every log2 increase in RSV-specific, serum neutralizing antibody titer there is a doubling of the palivizumab concentration required to achieve the same level of virus neutralization activity [17]. For example, a 7 log2 serum neutralizing antibody titer is comparable to the neutralizing activity of 80 μg/ml of palivizumab and 8 log2 is similar to 160 μg/ml of palivizumab.

Assays that measure RSV-specific, neutralizing antibody activity are very useful for evaluating vaccine candidates, performing seroprevalence studies, and for detecting infection. Neutralizing antibody activity is normally measured by a plaque reduction neutralization assay or by a microneutralization assay with or without complement. These assays measure the functional capacity of serum (or other fluids) to neutralize virus infectivity in cells as compared to ELISA assays that only measure the binding capacity against an antigen. Measuring functional activity is very important for the preclinical and clinical evaluation of vaccines and monoclonal antibodies. A vaccine that induces a low level of neutralizing antibody with a high level of ELISA binding antibody is unlikely to move forward in preclinical development.

1.1 Key Elements in the Microneutralization Assay

(a) *The virus.* We use RSV/A/Tracy and RSV/B/18537 for the microneutralization assay. RSV/A/Tracy was isolated in 1989 from a child hospitalized with RSV bronchiolitis. In phylogenetic analysis RSV/A/Tracy clusters with other GA1 viruses like RSV/A/Long, RSV/A/Bernett, and RSV/A/A2. These are considered prototypical RSV/A viruses and are often used in assays and vaccine development. The GA1 viruses are infrequently found in current molecular epidemiology studies. The RSV/B/18537 was provided to our laboratory by Dr. Robert M. Chanock in the early 1980s. It was isolated in 1962 and clusters with other GB1 viruses. RSV/B/18537 is a prototypical RSV/B virus; it is often used in assays and is rarely detected in molecular epidemiology studies.

(b) *Virus inoculum.* It is very important that the amount of virus used in an assay is repeatable over time. Changes in the virus inoculum can have a significant change in the neutralizing antibody titer. In general, a low virus inoculum will result in a higher neutralizing antibody titer and greater variability from assay run to assay run, while a high virus inoculum will result in a lower neutralizing antibody titer and less variability per assay run. It is not uncommon to see four- to eightfold differences in an individual titer if a low versus high inoculum is used. To mitigate assay variability from the virus inoculum effect, we place major emphasis in selecting and standardizing the virus inoculum with each new virus working pool we prepare.

The target virus inoculum we recommend to use should be 5.5 log3 TCID50 per 50 μl. This virus concentration should kill all the cells up to 3 log3 to 4 log3 (TCID100) dilutions in the TCID50 CONTROL PLATE (Fig. 1), which measures

a

POS/ NEG Control Plate	VIRUS	Date
Before+ Positive Control wells		After + Positive Control wells
Negative control		

b

TCID50 Control Plate	Virus								Date				
0.5X in Duplicates													
1X in Duplicates													
2X in Duplicates													
4X in Duplicates													

Fig. 1 96-well plate configuration diagram. (**a**) POS/NEG control plate, (**b**) TCID50 control plate, (**c**) serum control plate (internal serum control), (**d**) dilutions (IVIG) control plate, (**e**) IVIG control plate and (**f**) test plate

c

Control Plate	Virus	Date
0.5X in Duplicates		
1X in Duplicates		
2X in Duplicates		
4X in Duplicates		

d

Dilutions of IVIG Control Plate	Virus	Date
IVIG 5 Undil. in Duplicates		
IVIG 5 1:4 in Duplicates		
IVIG 5 1:16 in Duplicates		
IVIG 5 1:64 in Duplicates		

Fig. 1 (continued)

e

IVIG Control Plate	Virus	Date
IVIG 5 in Duplicates		
IVIG 2 in Duplicates		
IVIG 3 in Duplicates		
IVIG 4 in Duplicates		

f

Plate A	Virus	Date
A1 in Duplicates		
A2 in Duplicates		
A3 in Duplicates		
A4 in Duplicates		

Fig. 1 (continued)

the back titration of the virus inoculum used in the assay. To obtain this value consistently over time, the working virus pool is first titered to determine the TCID100 concentration of the working pool. Then several microneutralization assays using the internal standards and controls are performed to fine tune the dilution of the virus working pool to ensure that the back titration results in 3 log3 to 4 log3 TCID100 and approximately 5.0 log3 to 6 log3 TCID50 per 50 µl. To improve the consistency of the results, the volume used to generate the virus inoculum is always the same.

For improved consistency, each of the viruses used in the microneutralization assay should have a master seed and a working seed. The working seed should be used to produce the working pool that is used in the microneutralization assay. This ensures that the viruses used in the assay always contain the same number of cell passages. The master seed, working seed and working pool should be stabilized 1:1 in 15% glycerol Iscove's media, snap-frozen, and maintained at –80 °C in an ultralow freezer. Also all working pools prior to use should be demonstrated to be free of bacteria, fungi, and mycoplasma.

(c) *Cell concentration.* Our preferred cell line for the microneutralization assay is the HEp-2 cell, originally derived from a human laryngeal carcinoma. Like the viruses, you should developed a master seed, working seed and working pools of HEp-2 cells that are maintained in liquid nitrogen. Our working pool of HEp-2 cells starts at a passage number of 383. For the microneutralization assay we recommend to use cells that are 3–5 days old with a passage history up to 410. All the working pools prior to use should be demonstrated to be free of bacteria, fungi and mycoplasma.

The optimal cell concentration for the microneutralization assay is 1.8×10^5 to 2×10^5 trypsinized cells per ml. The cell concentration depends on the virus inoculum, age of the cells, and passage of cells. A low virus inoculum will generally have a lower cell count of about 1.8×10^5 cells per ml compared to a high viral inoculum which will have a cell count of about 2×10^5 trypsinized cells per ml. When performing a TCID100 we recommend to use a count of 2×10^5 cells per ml so that one can adjust the number of cells when fine tuning or standardizing the assay.

(d) *Neutralizing antibody titer determination.* The last serum dilution at which a 50% reduction in viral cytopathic effect (CPE) is observed is defined as the neutralizing antibody titer. The neutralizing antibody titers assigned are categorical log numbers in multiples of 0.5 log2 (2.5, 3.0, 3.5, 4.0, etc). The lowest detectable neutralizing antibody titer is 2.5 log2.

Samples with nondetectable neutralizing antibody titers are assigned a value of 2 log2.

(e) *Laboratory standards and controls.* Standards and controls should be used to ensure that the microneutralization assay is working within specification of the assay. The standards are used to determine if an assay meets the criteria for acceptance while the controls are used within the assay to ensure the assay produces results consistent throughout the assay. Any serum sample with ≥8-fold difference between duplicate tests should not be accepted and that serum sample should be repeated. Table 1 contains the criteria for determining if a microneutralization assay meets acceptance criteria and Table 2 lists the median, 10th and 90th percentile for the standards and internal controls used in the microneutralization assays.

2 Materials

2.1 Hardware

1. 96-well plates, flat bottom with lid.
2. Fine tip permanent marker.
3. 20–200 µl multipipettor.
4. 5–20 µl pipettor.
5. Pipette tips.
6. Sterile boat
7. Incubator (37 °C, 85% humidity).
8. Biosafety level II cabinet.
9. 50 ml conical tubes.
10. 15 ml conical tube.
11. 25 ml pipettes.
12. 5 ml pipette.
13. 1000 ml beaker.
14. Mechanical pipette.
15. Water bath set at 56 °C.

2.2 Tissue Culture

1. HEp-2 cells (ATCC® CCL-23).
2. Infectious RSV A and B stocks.
3. Fetal bovine serum, FBS: Endotoxin level: ≤5 EU/ml, Hemoglobin level: ≤10 mg/dl. Triple filtered at 0.1 µm. Thaw frozen serum at 4–8 °C, room temperature or rapidly at 37 °C. To heat-inactivate the complement, incubate thawed serum at a thermostatically controlled temperature of 56 °C for 30 min. Store 50 mL aliquots of heat-inactivated serum –5

Table 1
Criteria for accepting a standardize RSV-specific microneutralization assay

	Preclinical study		Clinical study	
Variable	RSV/A/Tracy	RSV/B/18537	RSV/A/Tracy	RSV/B/18537
Duration of assay	6–7 Days	7–8 Days	6–7 Days	7–8 Days
$TCID_{100}$	3^2–$3^{4.5}$	3^2–$3^{4.5}$	3^2–$3^{4.5}$	3^2–$3^{4.5}$
Virus positive controls	100% Destruction of cell monolayer	100% Destruction of cell monolayer	100% Destruction of cell monolayer	100% Destruction of cell monolayer
Virus negative controls	Complete cell monolayer	Complete cell monolayer	Complete cell monolayer	Complete monolayer
At least 7 of 9 standards within 10th to 90th percentile	Not applicable	Not applicable	Must be met	Must be met
Percent of individual tests with ≥4-fold difference between duplicate samples	<10%	<10%	<10%	<10%

Table 2
Median, 10th, and 90th percentile for the standards and internal controls used in the microneutralization assays

	RSV/A/Tracy			RSV/B/18537		
Standard	**Median**	**~10th percentile**	**~90th percentile**	**Median**	**~10th percentile**	**~90th percentile**
IVIG[a]-1	9.5	8.5	10.5	11	9.0	12.0
IVIG-2	10.0	9.0	11.5	11.5	10.0	13.0
IVIG-3	11.0	9.0	12.5	12.5	10.5	13.5
IVIG-4	10.5	9.0	12.0	12.0	10.0	13.5
1× IVIG-1 or 5	9.5	8.0	10.5	11.0	9.5	11.5
1:4× IVIG-1 or 5	7.0	5.5	8.5	8.5	7.0	9.5
1:16× IVIG-1 or 5	5.0	4.0	6.0	6.0	5.0	7.5
1:64× IVIG-1 or 5	3.0	2.0	4.0	4.0	2.5	5.0
1× PW	6.0	5.0	7.0	10.5	9.0	11.5
Internal control						
PW[b]	6.25	5.5	7.5	10.5	9.0	11.5
PAP[b]	8.0	7.0	9.0	9.5	8.0	10.0

[a]Intravenous immunoglobulin
[b]Sample initials for internal control sera

to –20 °C until use. Once thawed, do not store for more than 2–3 weeks at 4–8 °C.

4. Serum-free medium (SF-MEM): Minimum essential media (MEM), 10 units/ml penicillin G sodium, 10 μg/ml streptomycin sulfate, 0.025 mg/ml amphotericin B, and 0.2 mM L-glutamine
5. Dulbecco's PBS, D-PBS: 2.66 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 137 mM sodium chloride, 8.0 mM sodium phosphate dibasic, pH 7.0–7.2.
6. 2% FBS/MEM: MEM, 10 units/ml penicillin G sodium, 10 μg/ml streptomycin sulfate, 0.025 mg/ml amphotericin B, 0.2 mM L-glutamine, and 2 % FBS.
7. 10% FBS/MEM: MEM, 10 units/ml penicillin G sodium, 10 μg/ml streptomycin sulfate, 0.025 mg/ml amphotericin B, 0.2 mM L-glutamine, and 10% FBS.
8. 2% FBS/MEM with protamine sulfate: 2% FBS/MEM and 200 μg/ml protamine sulfate (*see* Note **1**)

9. Trypsin–EDTA: 0.05% trypsin and ethylenediamine tetracetic acid (EDTA), 5.3 mM potassium chloride, 0.44 mM potassium phosphate monobasic, 4.16 mM sodium bicarbonate, 137 mM sodium chloride, 0.33 mM sodium phosphate dibasic, 5.5 mM glucose, 0.02 mM phenol red, pH 7.2–8.0. Store at −5 to −20 °C. Once thawed, store at 4–8 °C for up to 2 weeks
10. Cell culture flasks: 25 cm^2, 75 cm^2, or 150 cm^2, vented caps.

2.3 Solutions and Reagents

1. Intravenous immunoglobulin (IVIG) control (high-titer human polyclonal RSV immunoglobulin).
2. Internal serum control (RSV-neutralizing serum).
3. Test samples (serum samples, secretions including breast milk, animal samples such as nasal washes and lung lavages, etc.).
4. 10% Neutral Buffered Formalin/0.01% Crystal Violet solution: 1000 ml of 10% buffered formalin solution and 1 g of crystal violet powder. Close bottle cap and shake until the solution turns a deep purple.

3 Methods

3.1 RSV Microneutralization Assay (See Note 2)

The purpose of this assay is to quantify, the amount of RSV neutralizing antibody contained within a given sample. The samples can be serum samples, secretions including breast milk, animal samples such as nasal washes and lung lavages, or any other sample thought to contain RSV specific neutralizing antibody.

1. Thaw frozen samples at room temperature.
2. Heat-inactivate serum samples by incubating at 56 °C for 30 min.
3. After 30 min of incubation, remove sera from the 56 °C water bath and allow to cool to room temperature.
4. Label each 96-well plate with the following information: (a) plate code (A-Z) for test samples or the name of the standard, (b) virus, (c) current date, (d) sample number, and (e) the dilution scheme. *See* Fig. 1 for an illustration of how to label plates.
5. Add 2% FBS/MEM to the plates (*see* **Note 3**, Fig. 1):
   (a) POS/NEG control plate: Add 100 µl of 2% FBS/MEM to the negative control portion and 50 µl of 2% FBS/MEM to the positive portion of the plate (Fig. 1a).
   (b) TCID50 control plate: Add 100 µl of 2% FBS/MEM to all of the wells of the plate (Fig. 1b).
   (c) Serum control plate: Add 50 µl of 2% FBS/MEM to all wells except for the wells in the first column of plate (Fig. 1c).

(d) DILUTIONS control plate: Add 50 µl of 2% FBS/MEM to all wells except for the wells in the first column of the plate (Fig. 1c).

(e) IVIG control plate: Add 50 µl of 2% FBS/MEM to all wells except for the wells in the first column of plate (Fig. 1e).

(f) Test plate(s): Add 50 µl of 2% FBS/MEM to all wells except for the wells in the first column of plate (Fig. 1f, *see* **Note 4**).

6. For a 1:8 dilution, add 87.5 µl of 2% FBS/MEM to the first column of the plates labeled with Serum control plate, DILUTIONS control plate, IVIG control plate, and all test plates (Fig. 1).

7. Add 12.5 µl of sera to first column of the plates according to the labels of the plate:

(a) Serum Control Plate: add 12.5 µl of positive control serum to each well in duplicate and dilute twofold up the plate by transferring 50 µl to the next column of wells. Mix solution by pipetting content three to five times and continue the twofold dilution process until mixing of the final column of wells and dispense of the final 50 µl of content. Final volume of all wells is 50 µl.

(b) DILUTIONS (IVIG) Control Plate: add 12.5 µl of the IVIG dilutions (labeled IVIG at undiluted, 1:4, 1:16, and 1:64) and dilute twofold up the plate by transferring contents as previously described.

(c) IVIG Control Plate: add 12.5 µl of the current IVIG utilized for the experiment and dilute twofold up the plate by transferring contents as previously described.

(d) Test Plates: add 12.5 µl of the appropriate sample to each well in column 1 in duplicate. After addition of samples to the test plates perform serial twofold dilutions (50 µl transfer) to all of the plates.

8. Prepare the virus inoculum by diluting the RSV stock in 2% FBS/DMEM (*see* **Note 5**). Prepare a 4× concentrated solution (1600 PFU/ml) and dilute twofold to 2× (800 PFU/ml), 1× (400 PFU/ml), and finally 0.5× (200 PFU/ml). For 20 plates you will need 90 ml of 1× concentrated solution:

(a) 4× conical tube: 30 ml of 2% FBS/MEM + 4.8×10^4 PFU of RSV stock virus.

(b) 2× conical tube: 25 ml of 2% FBS/MEM + 25 ml 4× (a).

(c) 1× conical tube: 45 ml of 2% FBS/MEM + 45 ml 2× (b).

(d) 0.5× conical tube: 1 ml of 2% FBS/MEM + 1 ml 1× (c).

9. Add 1× viral dilution to all of the test plates and control plates (*see* Fig. 1):
   (a) POS/NEG control plate: Add 50 µl of the 1× virus to each well labeled positive control before + (virus viability at the start of dispensing virus into test plates). After adding the 1× viral sample to all other plates, add 50 µl of the 1× viral sample to each well labeled positive control after + (virus viability at the end of dispensing virus into test plates). DO NOT ADD VIRUS TO WELLS LABELED NEGATIVE CONTROL.
   (b) TCID control plate: Add 50 µl of viral inoculum 4×, 2×, 1×, and 0.5× to appropriate wells and make threefold dilutions by transferring 50 µl from one well to the subsequent well. Dispense 50 µl for the last wells so that the final volume in all wells is 100 µl (*see* **Note 6**)
   (c) Serum control plate: Add 50 µl of viral inoculum 4×, 2×, 1×, and 0.5× according to scheme for the plate. For example, 50 µl of 0.5× viral inoculum are dispensed into each well of rows A + B. Then 50 µl of 1× viral inoculum are dispensed into each well of rows C + D and so on.
   (d) Test plates and the rest of the control plates: Add 50 µl of 1× virus to all wells of the test plates, DILUTIONS (IVIG) control plate, and IVIG control plate.
10. After addition of virus to the plates, place the plates in the incubator for 90 min at 36 °C and 5 % CO_2 with 80–90 % humidity.
11. 30 min before the completion of the incubation period, trypsinize HEp-2 cells and prepare a cell suspension in 10 % FBS/MEM to a final density of $1.8–2.5 \times 10^5$ cells/ml.
12. Add 100 µl of the HEp-2 cell suspension to the wells (negative controls first, then positive, etc.) of all plates and place the plates in incubator.
13. Observe the plates daily for virus-specific CPE. Once the positive controls reach 100 % CPE, incubate for an additional 24 h before fixing and staining the cells (*see* **Note 7**).
14. Gently remove the medium–inoculum–serum mixture and gently overlay the cells with 120 µl of a 10 % Neutral Buffered Formalin/0.01 % Crystal Violet solution. Let it stain for 24–48 h.
15. Decant the stain into a chemical waste container and rinse the plates (VERY GENTLY) with water. Allow the plates to dry upside down until they are ready.
16. Determine the neutralization titers of the samples and express the values $\log_2$ units. The neutralization titer is defined as the last serum dilution with 50 % or greater intact HEp-2 (*see* **Note 8**).

4 Notes

1. Protamine sulfate is included ONLY in the 2% FBS/MEM solution used for making PLASMA dilution. Protamine Sulfate is used to counteract the anti-RSV activity of heparin when plasma is collected in heparinized Vacutainer tubes.
2. In accordance with laboratory standard operating procedures, personal protective equipment such as gloves and lab coat are required. Respiratory syncytial virus is a Class 2 agent requiring Biosafety Level 2 practices. All procedures to occur using sterile laboratory practices should be done in a BSL II-A2 certified hood. Biosafety level 2 practices should be used in accordance with the stated guidelines; Department of Health and Human Services CDC Biosafety in Microbiological and Biomedical Laboratories: 4th Edition, 1999. (Section II, Section III, Section VII-F, Appendix A: CDC/NIH Primary Containment for Biohazards: Selection Installation and Use of Biological Safety Cabinets.
3. Make enough 2% FBS/MEM for the assay; estimate about 5.5 ml per plate (calculations for the amount of 2% FBS/MEM should be done prior to performance of the assay.
4. If you are testing plasma, add 50 μl of 2% FBS/MEM to the heparin/protamine control of all wells but the wells in the first column. The first well will contain 50 μl of heparinized PBS (6 ml PBS in 10 ml green-top tube) and dilute twofold up the plate.
5. To prepare the virus inoculum calculations should be done prior to performing the experiment, using the $V1 = (V2 \times C2)/C1$ equation in order to calculate the amount of virus needed:
   - $V1$ = volume of viral sample desired for 4× (variable).
   - $V2$ = volume of virus that needs to be added to make the 4× viral inoculum (variable).
   - $C1$ = estimated final viral titer 4× = (4) (3^3 TCID100/0.05) = ~1600 PFU/ml.
   - $C2$ = titer of virus stock pool (known from the plaque assay).

   The viral inoculum is made by first preparing a 4× concentrated solution. This 4× viral sample is diluted twofold to 2×, 1×, and finally 0.5×. The 1× viral dilution is added to all of the test plates and control plates. Prepare the 4× viral sample so that the final concentration of 1× virus contains 400 PFU/ml).
6. Between each transfer of diluted virus, the tips need to be changed to avoid carryover of virus. Mix contents in each well by pipetting three to five times. Be careful not to generate bubbles.

7. RSV/A/Tracy typically takes about 6–7 days until the plates are ready to be fixed while RSV/B 18537 takes about 7–8 days.
8. The lower limit of detection is 2.5 log and for samples with a titer less than 2.5 assign a value of 2.0. If it is not possible to determine the end titer of a given sample, repeat the assay at a lower dilution.

References

1. Mansbach JM, Piedra PA, Teach SJ, Sullivan AF, Forgey T, Clark S, Espinola JA, Camargo CA Jr (2012) Prospective, multicenter study of viral etiology and hospital length-of-stay in children with severe bronchiolitis. Arch Pediatr Adolesc Med 166:700–706
2. Peret TC, Hall CB, Hammond GW, Piedra PA, Storch GA, Sullender WM, Tsou C, Anderson LJ (2000) Circulation patterns of group A and B human respiratory syncytial virus genotypes in 5 communities in North America. J Infect Dis 181:1891–1896
3. Tapia LI, Shaw CA, Aideyan LO, Jewell AM, Dawson BC, Haq TR, Piedra PA (2014) Gene sequence variability of the three surface proteins of human respiratory syncytial virus (hRSV) in Texas. PloS One 9(3):e90786
4. Agenbach E, Tiemessen CT, Venter M (2005) Amino acid variation within the fusion protein of respiratory syncytial virus subtype A and B strains during annual epidemics in South Africa. Virus Genes 30:267–278
5. Shobugawa Y, Saito R, Sano Y, Zaraket H, Suzuki Y et al (2009) Emerging genotypes of human respiratory syncytial virus subgroup A among patients in Japan. J Clin Microbiol 47:2475–2482
6. Eshaghi A, Duvvuri VR, Lai R, Nadarajah JT, Li A et al (2012) Genetic variability of human respiratory syncytial virus a strains circulating in Ontario: a novel genotype with a 72 nucleotide g gene duplication. PLoS One 7:e32807
7. Avadhanula V, Chemaly RF, Shah DP, Ghantoji SS, Azzi JM, Aideyan LO, Mei M, Piedra PA (2015) Infection with novel respiratory syncytial virus genotype Ontario (ON1) in adult hematopoietic cell transplant recipients, Texas, 2011-2013. J Infect Dis 211(4):582–589
8. Trento A, Galiano M, Videla C, Carballal G, Garcia-Barreno B et al (2003) Major changes in the G protein of human respiratory syncytial virus isolates introduced by a duplication of 60 nucleotides. J Gen Virol 84:3115–3120
9. Trento A, Viegas M, Videla C, Carballal G, Mistchenko AS (2006) Natural history of human respiratory syncytial virus inferred from phylogenetic analysis of the attachment (G) glycoprotein with a 60-nucleotide duplication. J Virol 80:975–984
10. Swanson KA, Settembre EC, Shaw CA, Dey AK, Rappuoli R et al (2011) Structural basis for immunization with postfusion respiratory syncytial virus fusion F glycoprotein (RSV F) to elicit high neutralizing antibody titers. Proc Natl Acad Sci U S A 108:9619–9624
11. McLellan JS, Chen M, Leung S, Graepel KW, Du X et al (2013) Structure of RSV fusion glycoprotein trimer bound to a prefusion-specific neutralizing antibody. Science 340:1113–1117
12. Magro M, Mas V, Chappell K, Vazquez M, Cano O et al (2012) Neutralizing antibodies against the preactive form of respiratory syncytial virus fusion protein offer unique possibilities for clinical intervention. Proc Natl Acad Sci U S A 109:3089–3094
13. Lima HN, Botosso V, Oliveira DBL, Almeida Campos AC, Leal AL et al (2012) Molecular epidemiology of the SH (small hydrophobic) gene of human respiratory syncytial virus (HRSV), over 2 consecutive years. Virus Res 163:82–86
14. Schepens B, Sedeyn K, De Baets S, Schotsaert M, Roose K et al (2014) Protection and mechanism of action of a novel human respiratory syncytial virus vaccine candidate based on the extracellular domain of small hydrophobic protein. EMBO Mol Med 6(11):1436–1454
15. Piedra PA, Jewell AM, Cron SG, Atmar RL, Glezen WP (2003) Correlates of immunity to respiratory syncytial virus (RSV) associated-hospitalization: establishment of minimum protective threshold levels of serum neutralizing antibodies. Vaccine 21:3479–3482
16. Luchsinger V, Piedra PA, Ruiz M, Zunino E, Martínez MA, Machado C et al (2012) Role of neutralizing antibodies in adults with community-acquired pneumonia by respiratory syncytial virus. Clin Infect Dis 54:905–912
17. Glenn GM, Smith G, Fries L, Raghunandan R, Lu H, Zhou B, Thomas DN, Hickman SP, Kpamegan E, Boddapati S, Piedra PA (2013) Safety and immunogenicity of a Sf9 insect cell-derived respiratory syncytial virus fusion protein nanoparticle. Vaccine 31(3):524–532

Chapter 8

Host Factors Modulating RSV Infection: Use of Small Interfering RNAs to Probe Functional Importance

Leon Caly, Hong-mei Li, and David Jans

Abstract

Although respiratory syncytial virus (RSV) is the leading cause of bronchiolitis and pneumonia in infants and the elderly worldwide [1], the protein–protein interactions between the host cell and virus remain poorly understood. We have used a focused small interfering RNA (siRNA) approach to knock-down and examine the role(s) of various host cell proteins. Here, we describe approaches for casein kinase 2α (CK2α) as a key example. We show how to study the effect of host gene (CK2α) knockdown using siRNA on cell-associated and released virus titers, using both quantitative RT-PCR, which measures the level of viral RNA, and plaque assay, which measures infectious virus directly. Both assays identified reduced viral titers with CK2α gene knock-down, indicating that it is likely required for efficient viral assembly and/or release. Effects were confirmed in RSV infected cells using the specific CK2α inhibitor 4,5,6,7-tetrabromobenzotriazole, revealing a similar reduction in viral titers as CK2α specific siRNA. This demonstrates that siRNA can be used to characterize critical host cell–RSV protein–protein interactions, and establishes CK2α as a future druggable target.

Key words Respiratory syncytial virus, RSV, siRNA, Focussed screen, qRT-PCR, Plaque assay, Casein kinase 2α

1 Introduction

Respiratory Syncytial virus (RSV) is the leading cause of bronchiolitis and pneumonia in infants and the elderly worldwide [1, 2], but there is no commercially available vaccine, nor is viable drug treatment available [3]. Despite RSV having such a high prevalence in society, much remains unknown regarding the viral life cycle. A key area where information is sorely lacking is the interplay between proteins of the newly infected host cell and RSV itself. In recent years there have been many studies that have utilized techniques including cDNA microarray, chip and mass-spectrometry/proteome analyses which have built up a database of genes that are either upregulated or downregulated during RSV infection [4–9]. This information, although useful in providing a global map of host cellular changes and responses to RSV infection, does not

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provide detailed information with respect to direct interaction between host and virus at the protein-protein interaction level. In order to identify new potential targets for further development of specific anti-RSV therapeutics, such as small molecular inhibitors, detail at the protein–protein interaction level between host and virus needs to be elucidated.

A tool that can be used to characterize and identify key host cell proteins playing roles in RSV replication is small interfering RNA (siRNA), which is able to specifically target any gene desired. siRNAs are small double-stranded (5′ antisense and 3′ passenger strand) RNA molecules which are ~25 bp long and possess phosphorylated 5′ and hydroxylated 3′ ends. When introduced into the cell by transfection, the siRNA 5′ antisense strand which is complimentary to the target gene mRNA is loaded into the Argonaute protein containing RNA Induced Silencing Complex (RISC) which is then activated. Activated RISC then binds to the target mRNA resulting in gene silencing through mRNA degradation [10].

High-throughput siRNA screening at a genome-wide level has been utilized by many groups on numerous viruses in the past in an attempt to characterize their host-cell interaction partners [11–15]. Although entirely valid, this approach is prone to missing candidate hits (false negatives) as the sheer number of genes involved (10,000+) precludes validating each and every siRNA oligonucleotide for knockdown efficiency and target gene specificity. Genome-wide screens also generate vast quantities of data which must be "sifted" through to determine the best candidate genes/targets for further validation by other means.

The alternative approach to a genome-wide screen is a smaller "focused" siRNA screen whereby candidate host-cell genes are selected for analysis based on their previous identification and interaction with viral protein(s) by other methods, which may include microfluidics, mass spectrometry and SILAC labeling, yeast 2-hybrid or immunoprecipitation assays [7, 8, 16, 17]. We have used this technique to assess numerous proteins and identify candidates that appear to be key host factors that contribute efficient and rapid viral replication. Here, we use siRNA approaches to characterize one such protein, casein kinase 2α (CK2α) which has previously been shown to phosphorylate the RSV matrix protein [17]. We document reduced viral titer in cells treated with siRNA specific to CK2α, using both quantitative RT-PCR (qRT-PCR) and viral plaque assays. In addition we corroborate our results using a specific CK2α inhibitor 4, 5, 6, 7-tetrabromobenzotriazole (TBB) [18]. Through the use of siRNA and chemical inhibitors, in combination with quantitative RT-PCR and infectious plaque assays, we show that CK2α activity is critical for RSV virus production, and thus represents an exciting target for anti-RSV therapeutics.

2 Materials

2.1 Growth of A549 Cells

1. Cells of the human lung cell carcinoma cells line (A549; ATCC® CCL-185).
2. F-12K complete medium: Ham's F-12K (Kaighn's modification) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM l-glutamine, 50 U/L penicillin, 0.05 mg/L streptomycin.
3. Humidified incubator with 5% CO_2 atmosphere at 37 °C.
4. 25 and 175 cm^2 sterile cell culture flasks.
5. 1× PBS: 8 g NaCl, 0.2 g KCl, 1.44 g of Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L of water, adjust pH to 7.4 and autoclave.
6. 0.05% trypsin.
7. 12-well cell culture plates.
8. 24-well cell culture plates.
9. Hemocytometer.

2.2 Preparation of siRNA Oligos and Transfection

1. SMARTPool siRNA oligos (Dharmacon) or equivalent.
2. DNAse/RNAse free water.
3. 5× siRNA buffer (supplied by Dharmacon).
4. DharmaFECT1 siRNA transfection reagent (Dharmacon) or equivalent.
5. F-12K serum free medium: Ham's F-12K (Kaighn's modification) medium, without FBS and antibiotic.
6. F-12K antibiotic free medium: Ham's F-12K medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine.
7. Sterile, autoclaved 1.5 mL microcentrifuge tubes.
8. 12-well cell culture plates containing A549 cells at 70% confluency.

2.3 Bradford Assay

1. 96-well clear plate.
2. Bradford protein reagent.
3. 0.2 mg/mL bovine serum albumin (BSA).
4. Milli-Q H_2O.
5. 1.5 mL microcentrifuge tubes.
6. Multichannel pipette (able to dispense 160 μL).
7. Disposable pipetting reservoirs.
8. BMG ClarioStar plate reader (or equivalent that can read absorbance at 595 nm).

9. User generated protein samples to assay.
10. SDS lysis buffer (6×): 0.05 % w/v bromophenol blue, 7.5 % v/v glycerol, 2.5 % w/v SDS, 0.35 M Tris, pH 6.8, 600 mM DTT.

2.4 SDS Polyacrylamide Gel

1. Running gel buffer: 1.5 M Tris–HCl; adjust pH 8.8 with HCl. Store 4 °C.
2. Stacking gel buffer: 0.5 M Tris–HCl; adjust pH 6.8 with HCl. Store 4 °C.
3. Glycerol.
4. Milli-Q H_2O.
5. 40 % acrylamide–bis solution 37.5:1.
6. 10 % SDS solution in H_2O.
7. APS: 10 % (w/v) ammonium persulfate in H_2O.
8. TEMED: *N,N,N,N′*-tetramethyl-ethylenediamine.
9. SDS-PAGE running buffer; 25 mM Tris, pH 8.3, 0.19 M glycine, 0.1 % SDS w/v.
10. SDS lysis buffer (6×): 0.05 % w/v bromophenol blue, 7.5 % v/v glycerol, 2.5 % w/v SDS, 0.35 M Tris, pH 6.8, 600 mM DTT.
11. Pre-stained molecular weight markers.
12. Microcentrifuge.
13. Mini PROTEAN II System 1.5 mm glass plates (Bio-Rad) or equivalent.
14. Mini-PROTEAN electrophoresis system (Bio-Rad) or equivalent.
15. PowerPac basic power supply (Bio-Rad) or equivalent.

2.5 Western Blotting

1. Nitrocellulose membrane.
2. Transfer buffer: 25 mM Tris, 192 nM glycine, 5 % isopropanol in H_2O.
3. Filter paper.
4. Mini-PROTEAN electrophoresis system (Bio-Rad) or equivalent.
5. PowerPac basic power supply
6. Radioimmunoprecipitation assay (RIPA) lysis buffer: 50 mM Tris (pH 7.6) 150 mM NaCl, 0.1 % (w/v) SDS, 0.5 % (w/v) sodium deoxycholate, 1 % (v/v) Triton X-100, protease inhibitor cocktail EDTA free (Roche) added fresh before use.
7. Blocking buffer: 5 % (w/v) skim milk in PBS.
8. Wash buffer: PBS and 0.05 % (v/v) Tween 20.
9. Primary antibody to protein of interest.
10. HRP-conjugated secondary antibody raised against species of primary antibody.

11. ECL detection reagent.
12. Fuji X-Ray film.
13. Film developer.

2.6 Quantitative RT-PCR (qRT-PCR)

1. pSFV1-RSV-N: Plasmid encoding RSV N (available from Dr. Leon Caly upon request).
2. Spectrophotometer/Nanodrop or equivalent.
3. RNA purification kit.
4. RNA samples isolated from A549 cells infected with RSV.
5. TaqMan Fast Virus 1-step Master Mix (Applied Biosystems) or equivalent qRT-PCR kit.
6. RNase-free pipettes.
7. RNase-free pipette tips.
8. DNase/RNase Away spray.
9. Nuclease free H_2O.
10. Forward RSV-A2 N primer (50 μM): 5′-CTC AAT TTC CTC ACT TCT CCA GTG T-3′.
11. Reverse RSV-A2 N primer (50 μM): 5′-CTT GAT TCC TCG GTG TAC CTC TGT-3′.
12. TaqMan probe directed towards a region between forward and reverse primers (5′-TCC CAT TAT GCC TAG GCC AGC AGC A-3′), labeled fluorescently at 5′ end with 6-carboxyfluorescein (FAM) as the reporter dye and a 3′ quencher dye 6-carboxytetramethylrhodamine (TAMRA).
13. 96-well PCR plate.
14. Transparent optical adhesive plate seal (RT-PCR grade).
15. Benchtop centrifuge and rotor capable of holding 96-well PCR plate.
16. Real-time PCR machine (Eppendorf Realplex 4 Mastercycler Ep Gradient S, or equivalent).

2.7 Treating RSV Infected Cells with the CK2α Inhibitor TBB

1. 12-Well tissue culture plate.
2. A549 cells.
3. F-12K complete medium.
4. F-12K 2% FBS medium: Ham's F-12K medium supplemented with 2% heat-inactivated fetal bovine serum (FBS), 2 mM l-glutamine, 50 U/L penicillin, 0.05 mg/L streptomycin.
5. TBB: 25 mM 4,5,6,7-tetrabromobenzotriazole.
6. Dimethylsulfoxide (DMSO).
7. Humidified incubator with 5% CO_2 atmosphere at 37 °C.

8. SPGA: 218 mM sucrose, 7.1 mM K_2HPO_4, 3.76 mM KH_2PO_4, 4.9 mM sodium glutamate, 1% (w/v) bovine serum albumin (BSA).
9. RSV A2 viral stock.
10. Sterile 1.5 mL microcentrifuge tubes.
11. 1× PBS: (standard 1× PBS contains 8 g NaCl, 0.2 g KCl, 1.44 g of Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L of water, adjust pH to 7.4).

3 Methods

3.1 Maintenance and Preparation of A549 Lung Epithelial Cells

A549 cells are cultured in F-12K complete medium to 90% confluency by passaging at a ratio of 1:5–1:10 every 3 or 4 days, respectively, in 25 cm^2 sterile tissue culture flasks.

1. Once cells have grown to 90% confluency in a 25 cm^2 flask, remove media and wash cells gently three times with 2 mL sterile PBS that has been pre-warmed to 37 °C.
2. Remove PBS and add 300 μL room temperature 0.05% Trypsin. Place flask into 37 °C incubator with 5% CO_2 for 2 min. Rock flask gently and observe if cells have detached from flask surface.
3. Once cells have been released, add 2 mL F-12K complete medium into the flask and pipette mixture up and down gently to ensure single cell suspension of cells.
4. Label a new 25 cm^2 flask with name, date, and passage number and add 5 mL pre-warmed (37 °C) F-12K complete medium.
5. For a 1:5 split, add 460 μL of resuspended cells to newly labeled flask. For 1:10 split, add 230 μL cells to new flash.
6. Rock flask gently back and forth, side to side to ensure an even distribution of cells within the flask.
7. Return flask to humidified 37 °C, 5% CO_2 incubator to grow.

3.2 Preparation of A549 Cells for siRNA Transfection

1. Grow enough A549 cells for the number of samples to be assayed. Each sample requires 1×10^5 cells/well.
2. Place 1 mL F12-K complete medium in each well of a 12-well tissue culture plate and return to 37 °C, 5% CO_2 humidified incubator.
3. Trypsinze A549 cells from pre-prepared flasks and count using a hemocytometer.
4. Seed cells at 1×10^5 cells/well and incubate overnight in humidified 37 °C with 5% CO_2 incubator.
5. The next morning confirm that cells are at the required density (~70%). If cells are over- or under-confluent, adjust seeding density accordingly to give 70%.

3.3 Preparation of siRNAs for Your Gene(s) of Interest

1. Design and synthetize the siRNA for the gene you want to target (*see* **Note 1**). In addition to your gene(s) of interest, a scrambled nucleotide (negative) siRNA control which does not target any known genome sequence should be included in all assays.
2. Reconstitute the lyophilized siRNA at the desired stock concentration (standardly 50 μM), using 5× siRNA buffer (supplied by Dharmacon but purchased separately) taking care to minimize RNAse contamination (*see* **Note 2**)
3. Aliquot siRNA into 5 μL stocks to minimize freeze–thaw cycles and store at −80 °C.

3.4 Transfection of siRNA Using DharmaFECT1

1. On Day 1, seed a 12-well tissue culture plate with A549 cells to be 70% confluent for transfection.
2. On Day 2, prepare a 5 μM working stock of siRNA from the frozen 50 μM stock (*see* **Note 3**). To do this, add 45 μL of 1× siRNA buffer to 5 μL of 50 μM stock (1:10 dilution).
3. In separate 1.5 mL microcentrifuge tubes dilute siRNA to your desired final concentration (Tube 1) and DharmaFECT transfection reagent (Tube 2) with serum free media (*see* pipetting guide in Table 1).
4. Gently mix the contents of each tube by pipetting up and down carefully, then incubate for 5 min at room temperature.
5. Add the 100 μL contents of Tube 1 to Tube 2 for a total volume of 200 μL.
6. Mix by pipetting up and down and incubate at room temperature for 20 min.
7. Add 800 μL of antibiotic-free (*see* **Note 4**) complete media to the mix in **step 5** for a final volume of 1 mL.
8. Remove the culture media from the cells and replace with 1 mL of siRNA/transfection media from **step 7**.

Table 1
siRNA pipetting schema

siRNA final concentration (nM)	Tube (1) volume of 5 μM siRNA stock (μL)	Tube (1) serum free media (μL)	Tube (2) DharmaFECT1 (μL)	Tube (2) serum-free media (μL)
2	0.4	99.6	2	98
5	1	99	2	98
10	2	98	2	98
20	4	96	2	98
40	8	92	2	98
50	10	90	2	98

9. Return plate to 37 °C, 5% CO_2 humidified incubator for 48–72 h.

3.5 Optimizing siRNA Transfection (See Note 5)

1. Pre-label 2× sets of 12-well plates (48 and 72 h) for the gene(s) of interest to be tested and seed with cells as per Subheading 3.2. For the purpose of this book chapter, KPNA2 (importin α1) will be used as an example, together with a mouse anti- human Karyopherin α antibody.
2. Each gene(s) requires 4 wells of A549 cells (1 well for scrambled control and 3 for testing different concentrations of siRNA) in duplicate 12-well plates (8 wells total).
3. As a starting point, use Table 1 to transfect siRNA at 5, 10 and 20 nM concentrations for each gene of interest in duplicate 12-well plates (48 and 72 h).
4. Return plates to 37 °C with 5% CO_2 incubator and leave for 48 and 72 h respectively (*see* Fig. 1a).
5. At 48 and 72 h respectively, remove plates from incubator and aspirate off media.
6. Add 200 μL ice-cold RIPA buffer (with protease inhibitor included) to each well to lyse cells on ice (*see* **Note 6**).
7. Aspirate the lysed cell mixture and place into pre-labeled 1.5 mL microcentrifuge tubes on ice.
8. Pipette lysate up and down briefly every 10 min for 30 min to ensure even sample lysis.
9. Centrifuge lysate for 10 min at maximum rpm at 4 °C to pellet any remaining cellular debris.
10. Transfer supernatant to fresh 1.5 mL microcentrifuge tube and perform a Bradford assay to determine the protein concentration of each sample (*see* Subheading 3.6).

3.6 Determining Protein Concentration Using Bradford Assay

When performing a Bradford protein assay in a 96-well plate, the total protein sample volume is 40 μL.

1. Prepare BSA standards (negative control, 0.5–8 mg) as per Table 2 on ice.
2. Pipette 40 μL of each standard in duplicate within the designated wells of the 96-well plate (*see* Fig. 2a for example plate layout).
3. For each protein sample/lysate to be assayed, prepare 4 wells of the 96-well plate with 39.5, 39.0, 38.5, and 38 μL Milli-Q water.
4. The total protein assay volume is 40 μL, so add 0.5, 1.0, 1.5, and 2.0 μL of each unknown protein sample to the respective well containing Milli-Q water to give a total volume of 40 μL.
5. Prepare enough diluted Bradford Protein reagent stock 1:5 with Milli-Q water for the assay (160 μL per well required) and pour into buffer dam.

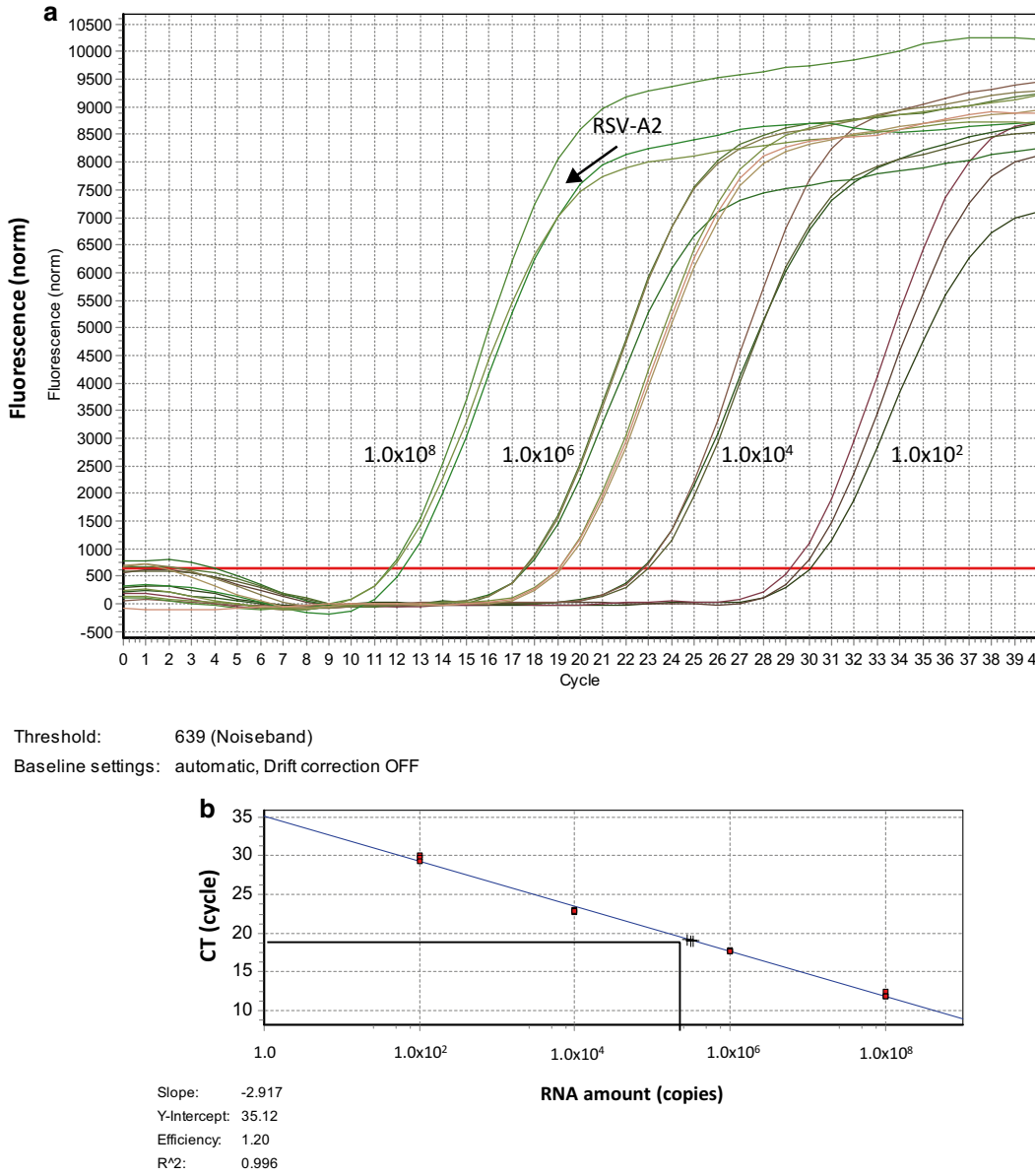

Fig. 1 Optimising siRNA concentration using Western blot analysis. **(a)** Timeline outlining steps required for optimization of siRNA knockdown. **(b)** Timeline depicting steps required for infection of siRNA treated cells.

6. Once all protein samples and standards are loaded onto the plate pipette 160 µL of diluted Bradford reagent into each well using a multichannel pipette and allow reaction to proceed for 5 min.
7. Place plate into plate reader and read absorbance at 595 nm.
8. Plot BSA amounts (0.5–8 mg) on *x*-axis against absorbance (595 nm) on *y*-axis using preferred graphing software (*see* Fig. 2b).

Table 2

BSA standard curve pipetting schema for Bradford protein assay

BSA amount (mg)	0	0.5	1	2	3	4	5	6	7	8
Volume 0.2 mg/ml BSA (μL)	0	5	10	20	30	40	50	60	70	80
Volume H_2O (μL)	80	75	70	60	50	40	30	20	10	0

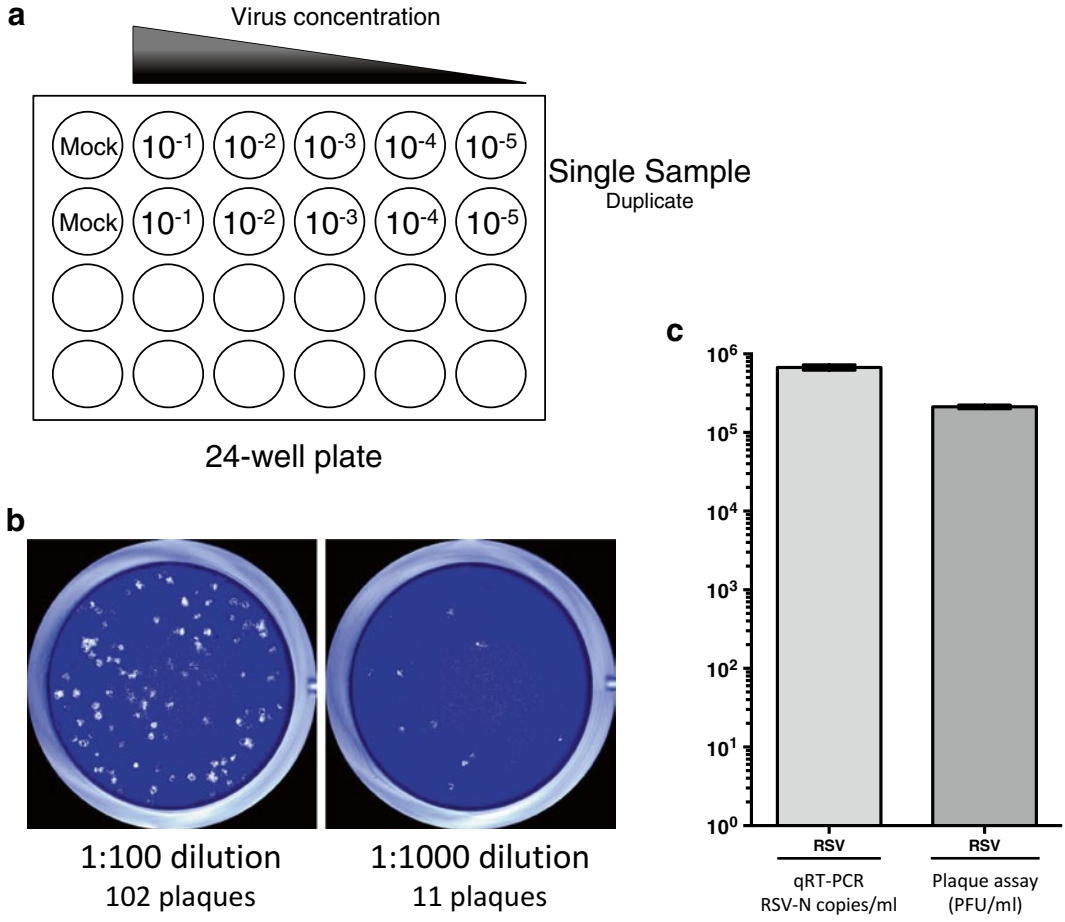

Fig. 2 Determining cell lysate concentration using Bradford protein reagent assay. Determination of the protein concentration from cell lysates harvested from CK2α siRNA knockdown experiments was performed using the Bradford assay. **(a)** Example 96-well plate layout for assay; B = Blank, Standard curve (0.5–8 mg BSA) indicated in *red*, Sample(s) (0.5–2 μL) indicated in *green*, additional samples can be loaded in *blue* areas. **(b)** Typical standard curve generated by plotting concentration of BSA (mg/mL) along *x*-axis with protein absorbance at 595 nm on *y*-axis. R^2 value of 0.98 indicates a good curve fit. **(c)** Typical linear regression plots of lysate samples generated by plotting volume of lysate measured (μL) on *x*-axis against protein absorbance at 595 nm on *y*-axis

9. Plot volume of protein samples assayed (0.5–2 μL) on *x*-axis against absorbance (595 nm) on *y*-axis (*see* Fig. 2c)
10. Create a linear trend line for both the standards and the protein samples and calculate the slope (m) and *y*-intercept (b) of the line ($y = mx + b$). Make sure to select the "show equation on graph" and R^2 values (*see* **Note 7**).
11. To determine the concentration of the unknown samples use the following equation:

    Concentration of unknown (mg/mL) = slope of unknown trend line/slope of standard curve;

    e.g., Scrambled = 0.2198/0.0972 = 2.26 mg/mL.
12. Once lysate concentrations have been determined, add 40 μL of 6× SDS lysis buffer to each sample and boil at 100 °C for 10 min before placing in −20 °C for storage or loading onto SDS-PAGE gel for analysis.

3.7 Using SDS-PAGE Gel Separation and Western Analysis to Confirm siRNA Gene Knockdown (See Note 8)

1. Select a final acrylamide concentration suitable for your protein of interest (*see* Table 3). For most proteins between 25 and 130 kDa, a 12% final concentration gives adequate separation to see them clearly. For proteins below 25 kDa, use a 15% acrylamide gel. For those greater than 130 kDa, use a 10% acrylamide gel.
2. Combine a 1.5 mm spacer with a short plate in a Bio-Rad Mini Protean III gel pouring apparatus and confirm that there are no leaks from the glass plates by filling them with ethanol.
3. Following Table 3 (12% acrylamide), make a running gel mixture by combining 1 mL Glycerol, 2.5 mL Milli-Q H_2O, 2 mL 1.5 M Tris (pH 8.8), 2.4 mL 40% acrylamide, 80 μL SDS, 40 μL APS, and 8 μL TEMED in a 50 mL conical tube. The quantities provided are for a single 1.5 mm gel only. If multiple gels are required, scale reagents up accordingly.
4. Before pouring gel(s), place the comb into the gel apparatus and mark the outer glass plate surface with a pen approximately 7 mm below the comb. This line will mark the level to which the running gel is poured.
5. Pour off any residual ethanol from **step 2** and immediately pipette in the running gel mixture (*see* **Note 9**) to the marked line (approximately 7 mL of gel).
6. Gently overlap the running gel with 80% (v/v) ethanol to ensure that the top surface of the gel is level.
7. Return any remaining Running buffer from the pipette to the conical tube and observe when it sets. This will indicate that the poured gel has set.

Table 3

SDS-PAGE gel pipetting schema

Running gel			
All these volumes in mL			
Final acrylamide concentration (%)	10%	12%	15%
Final volume (mL)	8	8	8
Glycerol	1	1	1
Milli-Q H_2O	2.8	2.5	1.9
1.5 M Tris (pH 8.8) lower gel	2	2	2
Acrylamide (40%)	2	2.4	3
All these volumes in µL: add these last in this order			
10% SDS	80	80	80
10% APS	40	40	40
TEMED	8	8	8

Stacking gel		
All these volumes in mL		
Final acrylamide concentration (%)	4	6
Final volume (mL)	4	4
Milli-Q H_2O	2.6	2.4
0.5 M Tris (pH 6.8) upper gel	1	1
Acrylamide (40%)	0.4	0.59
All these volumes in µL: add these last in this order		
10% SDS	40	40
10% APS	20	20
TEMED	4	4

8. Prepare stacking gel by combining 2.4 mL Milli-Q H_2O, 1 mL 0.5 M Tris (pH 6.8), 590 µL 40% acrylamide, 40 µL 10% (w/v) SDS, 20 µL 10% (w/v) APS, and 4 µL TEMED in a 50 mL conical tube.
9. Remove ethanol from the top of the running gel by inverting, then pipette in stacking gel.
10. Gently lower comb taking care not to introduce bubbles.
11. Remove protein samples from –20 °C freezer and heat at 95 °C for 10 min.
12. Centrifuge samples at 16,000 × *g* for 1 min after heating to spin down condensate on the lid and pellet any debris.
13. Upon stacking gel setting, remove comb and assemble Mini PROTEAN III electrophoresis system.
14. Load 5 µL of pre-stained protein benchmark in the first lane of each gel, then 20 µg of each protein lysate to be assayed. Load siRNA samples with scrambled (negative) control first, then

increasing amounts of siRNA (e.g., 5, 10, 20 nM) for the 48 h samples, then 72 h.

15. Run gel at 80 V for 10 min to allow samples to enter stacking gel before increasing voltage to 150 V until loading dye front is run off the bottom of the gel (*see* **Note 10**).
16. Following electrophoresis, separate the gel plates with a plastic spatula and rinse the gel briefly with Milli-Q H_2O before transferring to a plastic container containing Transfer buffer. Use spatula to remove stacking gel from running gel.
17. Cut a nitrocellulose membrane to the approximate size of the gel and place in container of transfer buffer with gel.
18. Assemble the Bio-Rad Mini PROTEAN TransBlot apparatus in the following order taking care to remove any bubbles between layers.
    (a) Open Western blot transfer cassette and submerge in a container containing transfer buffer with the black side down.
    (b) Foam pad soaked in transfer buffer.
    (c) Mini Trans-Blot Filter paper.
    (d) Sample containing SDS-gel.
    (e) Nitrocellulose membrane.
    (f) Mini Trans-Blot Filter paper.
    (g) Foam pad soaked in transfer buffer.
    (h) Close Western blot transfer cassette and secure closed with integrated clip.
19. Place assembled cassette into TransBlot tank and fill with chilled transfer buffer.
20. Put lid on gel tank and place within a large polystyrene box and surround tank with ice (*see* **Note 11**).
21. Set transfer to run at a constant 100 mA per membrane (apparatus can hold 2) for 3 h.
22. Upon completion of transfer take cassette apart and place nitrocellulose in blocking buffer at 4 °C overnight. Confirm that transfer was complete by the presence of all bands of the pre-stained protein benchmark on the membrane.
23. Wash membrane three times with wash buffer for 5 min in a plastic container.
24. Prepare primary antibody against target protein according to manufacturers instructions and incubate with membrane at 4 °C overnight within a heat-sealed plastic membrane.
25. Wash membrane three times with wash buffer for 5 min.

26. Prepare HRP-conjugated secondary antibody and incubate with membrane for 1 h at 4 °C within a heat sealed plastic membrane.
27. Wash membrane three times with wash buffer for 5 min in a plastic container.
28. Incubate membrane with ECL reagent of choice according to manufacturer's instructions.
29. Place membrane within heat sealed plastic membrane and seal up three sides.
30. Place bag on paper towel and using a tissue slowly push out and remove excess ECL reagent from the unsealed side.
31. Seal up the remaining side and place within an X-ray cassette, using sticky tape to hold it down, taking care not to apply tape over the membrane.
32. Within a dark room expose films to membrane and develop according to the signal intensity of the blot (10 s up to 15 min can be required).
33. Examine blot for a decrease in band intensity of targeted protein relative to increasing amounts of siRNA (*see* Fig. 1).
34. The lowest concentration of siRNA that gives the greatest target protein knockdown at 48 and 72 h post transfection should be used for further experiments.

In the example shown in Fig. 3, the Western blot analysis depicts that for KPNA2 a final siRNA concentration of 10 nM is sufficient to give ~>90% knockdown at 72 h post transfection, and thus this concentration should be used for any further experiments.

3.8 Infecting siRNA Transfected Cells

Once the optimal concentration of siRNA has been determined for the target gene(s) of interest, their specific role in viral infection is then elucidated by determining the effect of knockdown on RSV viral titer (Fig. 1b).

1. On Day 1, for each gene of interest (including negative control), prepare duplicate wells of a 12-well plate with A549 cells as per Subheading 3.2.
2. On Day 2 transfect cells with DharmaFECT1 and siRNA as per Subheading 3.4 using the optimal concentration of siRNA determined from Subheading 3.5.
3. Incubate cells in 37 °C, 5% CO_2 incubator for 48 h.
4. After 48 h, thaw enough RSV viral stock (see Chapter 2) on ice to infect the transfected wells at an MOI of 1.0 (*see* **Note 12**)
5. Determine the total amount of virus required to infect all of the wells within the experiment and make up a virus master mix (*see* **Note 13**).

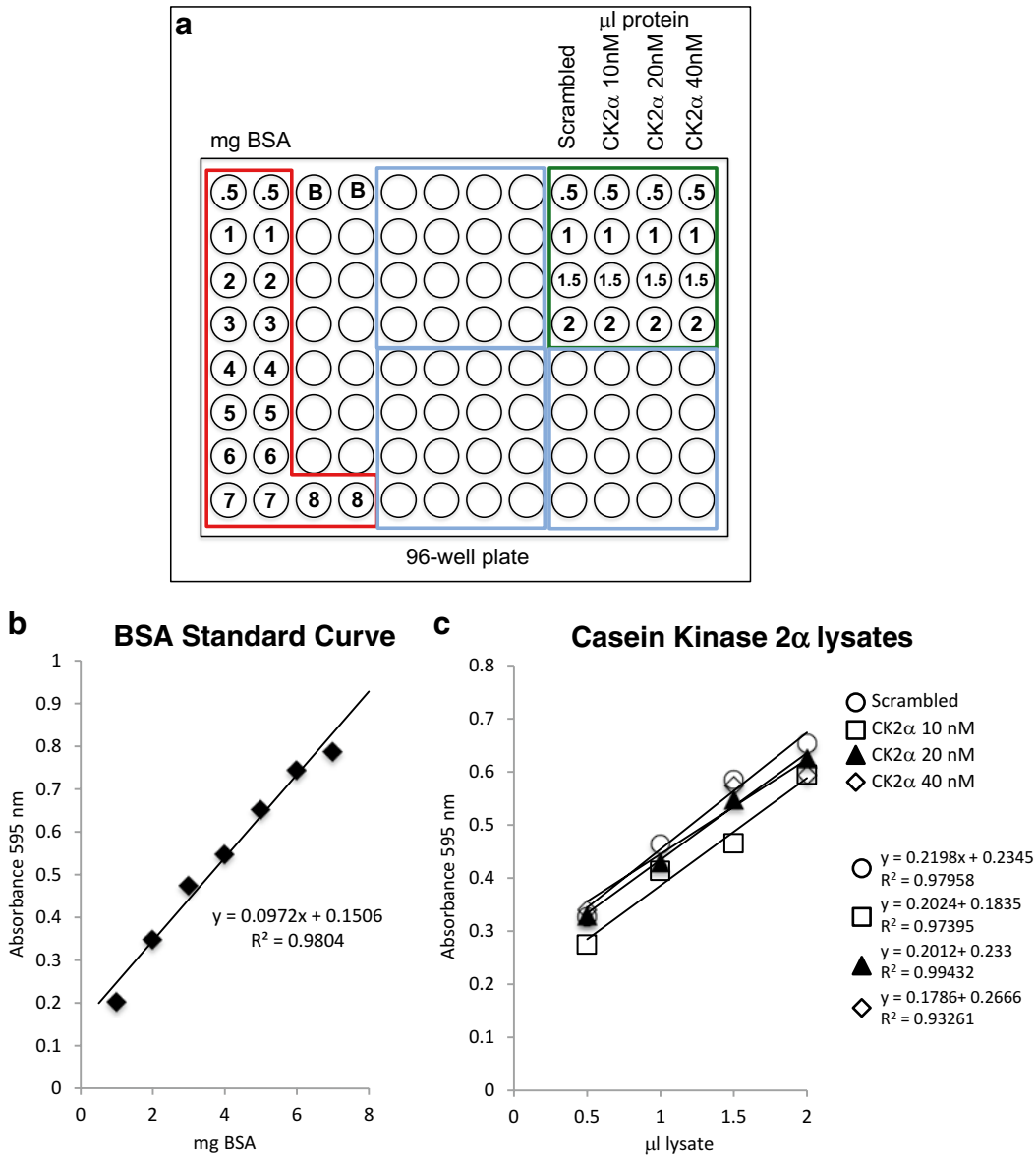

Fig. 3 Cell lysates were harvested at 48 and 72 h post transfection (as per Fig. 1, Day 6) from cells treated with increasing concentrations of siRNA and subjected to SDS-PAGE separation and subsequent Western blotting using an antibody directed to KPNA2 (importin α1). The blot was striped and reprobed with a specific antibody to beta-tubulin as a loading control

6. Aspirate medium from siRNA transfected wells.
7. Add 500 µL of virus master mix to each well and return plate(s) to 37 °C, 5% CO_2 incubator to allow virus to adsorb for 2 h.
8. Rock plate(s) gently every 20 min to ensure even distribution of virus and even infection of cells.
9. After 2 h aspirate viral media from cells and wash two times with pre-warmed 37 °C PBS then add 500 µL of pre-warmed F12-K 2% FBS media and return cells to incubator for 24 h.

3.9 Harvesting Virus from siRNA Transfected and RSV Infected Cells

Both supernatant (released virus) and cell-associated virus should be harvested to determine whether specific gene knockdowns are causing a defect in viral release and thus an accumulation of virus within the infected cell (*see* **Note 14**).

1. For each single sample well pre-label 5 × 1.5 mL screw-capped microcentrifuge tubes with (a) supernatant RT-PCR, (b) supernatant plaque assay, (c) cell-associated RT-PCR, (d) cell-associated plaque assay, (e) supernatant clarification.
2. Into each supernatant plaque assay microcentrifuge tube pipette 400 µL of SPGA buffer and place on ice.
3. 24 h post infection, remove 12-well plate(s) from 37 °C, 5% CO_2 incubator and place in Class II biosafety cabinet.
4. Aspirate off the 500 µL supernatant and place into tubes labeled supernatant clarification.
5. Onto cells remained in 12-well plate, pipette 100 µL of SPGA buffer and 100 µL F12-K 2% FBS medium (*see* **Note 15**).
6. Seal plate(s) with Parafilm and place into a zip-lock bag.
7. Place plates horizontally in −80 °C freezer (~1 h to overnight depending on what is convenient) which will causes the cells to lyse and release any cell-associated virus.
8. Spin supernatant clarification tubes in room temperature microcentrifuge for 1 min at 2000 × *g* to pellet any dead cells/debris.
9. Taking care not to disturb pellet, aliquot 50 µL into tubes labeled supernatant RT-PCR and place on ice.
10. Aliquot remaining 400 µL to microcentrifuge tubes labeled supernatant plaque assay and ensure proper mixing with SPGA buffer and place on ice.
11. Once all supernatant samples have been processed move them to −80 °C freezer.
12. Once frozen, thaw 12-well plate(s) on ice and carefully pipette media within wells up and down five to ten times to ensure that all cells/cellular debris has detached from the plate surface.

13. Remove cell suspension and place into chilled, pre-labeled 1.5 mL microcentrifuge tubes and vortex for 30 s at a moderate speed.
14. Centrifuge 1.5 mL microcentrifuge tubes for 10 min at $3000 \times g$ to pellet cellular debris and remove the virus containing supernatant.
15. Pipette 50 μL virus into screw-capped 1.5 mL microcentrifuge tubes labeled "cell-associated RT-PCR and the remaining ~ 150 μL into "cell-associated plaque assay."
16. Place microcentrifuge tubes into −80 °C freezer until processing for qRT-PCR and plaque assay is required.

3.10 Analyzing the Effect of Host Gene Knockdown on RSV Viral Replication by qRT-PCR Amplification of the RSV N Gene

By utilizing qRT-PCR we can get a quick estimate of how much virus have been produced measuring the amount of RSV-N RNA present in the purified samples.

Perform all the steps on ice to prevent degradation of RNA.

For qRT-PCR amplification of the RSV N gene *see* Chapter 2.

Workspace and all pipettes should be thoroughly cleaned with DNAse/RNAse Away solution (or similar) and filter tips used throughout.

1. Remove 50 μL samples labeled for qRT-PCR from −80 °C freezer and allow to thaw on ice.
2. Once thawed, purify viral RNA using a RNA isolation method of choice. 50–100 μL of purified virus yields sufficient RNA for PCR.
3. Cell associated viral RNA samples may need to be diluted 1:500 to remain within the range of the standard curve ($<10^8$ RSV-N copies/mL). Samples isolated from supernatant do not generally require dilution.
4. Prepare pSFV1-RSV-N (RSV-N plasmid) standard curve (available from Dr. Leon Clay);
   (a) Purify pSFV1-RSV-N (12,235 bp) using your plasmid purification method of choice.
   (b) Standard curve requires pSFV1-RSV-N 10^8, 10^6, 10^4, and 10^2 copies of DNA.
   (c) Using the formula:

$$\text{number of copies} = \left(\text{amount}(x) \times 6.022 \times 10^{23}\right) / \left(\text{length} \times 1 \times 10^9 \times 650\right), \text{solve for}(x);$$

$$1 \times 10^8 = \left(x \times 6.022 \times 10^{23}\right) / \left(12{,}235 \times 1 \times 10^9 \times 650\right) = 1.333\text{ng};$$

Thus 1.333 ng = 1×10^8 copies.

   (d) Dilute 1×10^8 copies stock 1:100 serially to obtain 1×10^6, 1×10^4, and 1×10^2 copies/mL with RNase/DNase free H_2O. To do this label 3 separate 1.5 mL microcentrifuge

tubes (10^6, 10^4 and 10^2) and place 495 μL of DNAse/RNAse free ddH_2O into them. Take 5 μL of the 10^8 RSV-N standard and add it to the 10^6 tube and mix well by pipetting up and down. Then take 5 μL from the 10^6 tube and place into the 10^4 tube as per above, until all standards are made.

(e) Aliquot standards into 40 μL lots and store in −80 °C until required.

5. Prepare a qRT-PCR master mix by combining the following components on ice (example given is for a single reaction using TaqMan Fast Virus 1-step Master Mix, scale up as required).

4× TaqMan Fast Virus 1-Step Master Mix	6.25 μL
RSV N Fwd primer (50 μM)	0.15 μL
RSV N Rev primer (50 μM)	0.15 μL
RSV N FAM probe (10 μM)	0.50 μL
Nuclease free H_2O	7.95 μL
Total volume	15.0 μL

6. Aliquot 15 μL of the master mix into triplicate wells of a 96-well PCR plate for each sample required (45 μL reaction for each sample).
7. Pipette 10 μL of RNA, RSV-N standard or RNAse Free H_2O (negative control) to each associated set of triplicate wells and mix by gentle pipetting.
8. Seal the plate with heat stable optical plate sealer ensuring a tight seal around the edges of the plate.
9. Briefly centrifuge the plate to ensure PCR mixtures are within the base of the plate (30 s at 500 × *g*).
10. Turn on the qRT-PCR machine and associated computer.
11. Log into the qPCR software and complete the plate layout on the template provided.
12. Perform the assay as per the cycle condition shown in Table 4.

Table 4
qRT-PCR cycle condition

Step	Cycles	Temperature (°C)	Time
Reverse transcription	1×	50	5 min
Heat inactivation of RT enzyme	1×	95	20 s
Denaturation	40×	95	3 s
Amplification		60	30 s

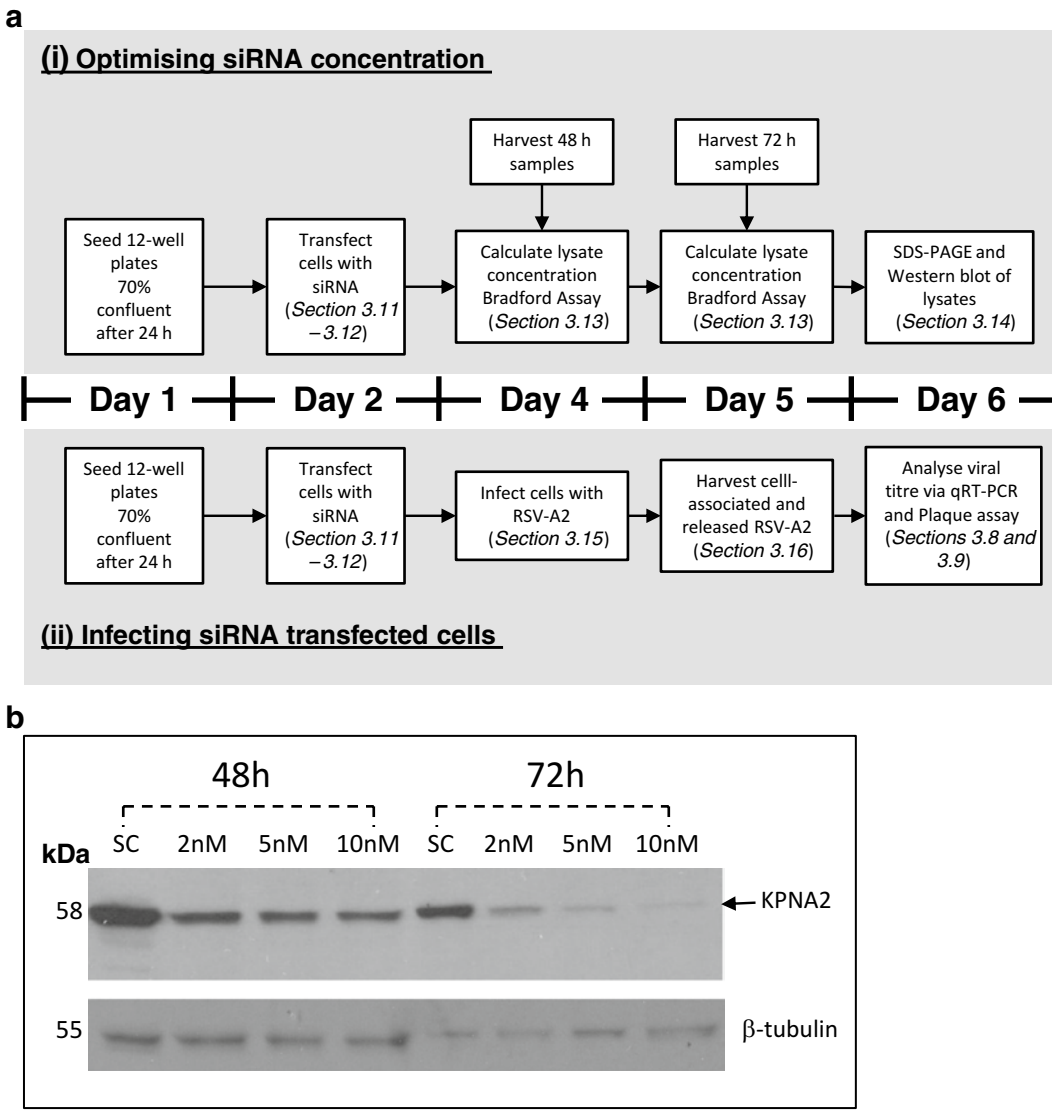

Fig. 4 RSV sample viral titer as determined using qRT-PCR. The RSV-A2 strain was analyzed by qRT-PCR to determine a reading for viral titer. **(a)** Typical qRT-PCR amplification curves plotting normalized FAM fluorescence vs. cycle number. Graph depicts the RSV-N standards (10^8, 10^6, 10^4, 10^2 copies/mL) as indicated and RSV sample of unknown concentration (indicated). **(b)** Standard curve generated from the amplification curves in (**a**). Using CT value obtained for RSV sample in (**a**) concentration can be read off standard curve (*dashed line*)

13. Once the reaction has completed use the analysis function of the qPCR software to create a standard curve and obtain quantitative values for the unknown viral samples. These values (raw data) can be exported to Microsoft Excel for further analysis by the software if required (*see* Fig. 4, **Note 16**).
14. Compare data to that obtained by plaque assay (*see* Fig. 5, **Note 17**).

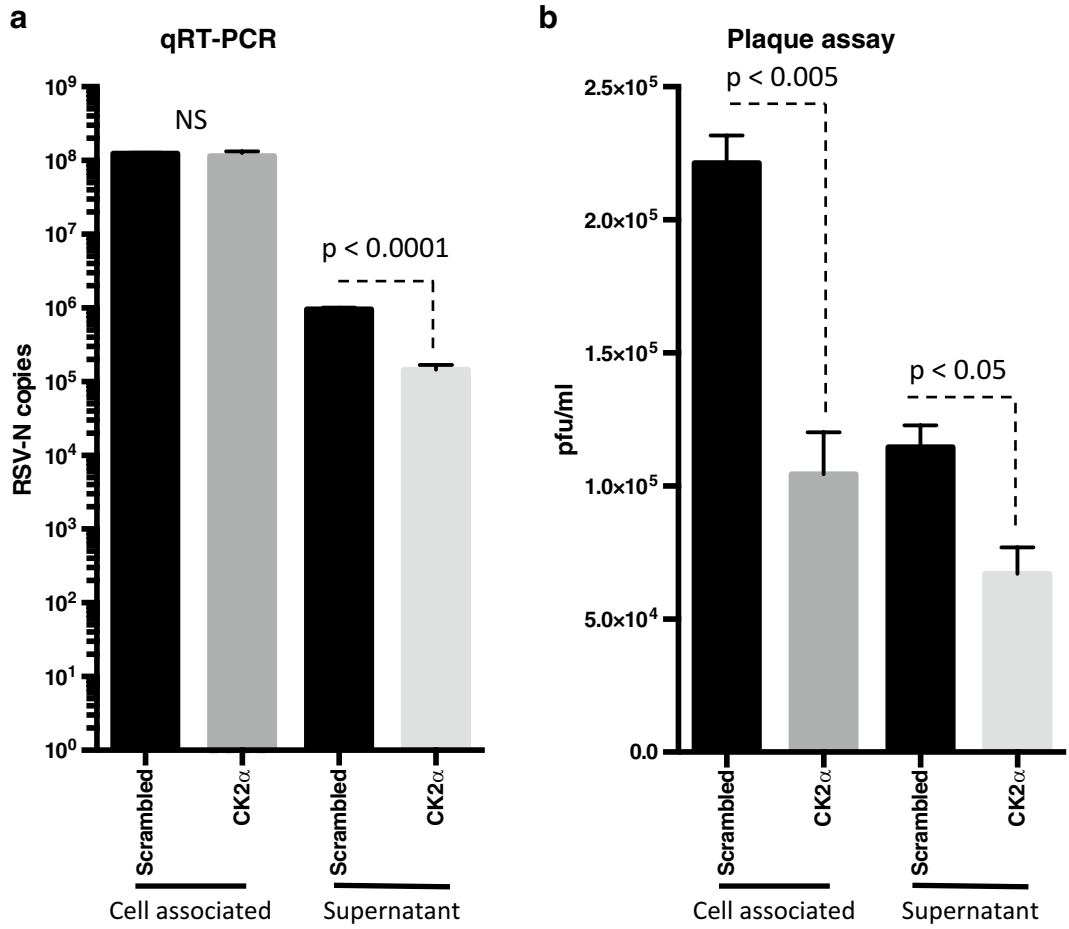

Fig. 5 RSV sample viral titer from A549 cells treated with CK2α siRNA as determined by qRT-PCR and plaque assay. RSV harvested from cell-associated and supernatant fractions was analyzed by qRT-PCR and plaque assay. **(a)** RSV-N copy number was determined using qRT-PCR from both cell-associated and supernatant fractions from cells treated with either negative (scrambled) control or CK2α siRNA (mean ± SD; $n = 3$). **(b)** Plaque assay performed on samples from (**a**) to determine level of infectious virus (pfu/mL) (mean ± SD; $n > 2$)

3.11 Analyzing Infectious Viral Titers by Plaque Assay

1. Remove viral samples prepared in Subheading 3.9 labeled for plaque assay from −80 °C freezer and allow to thaw on ice.
2. Proceed with plaque assay following the protocol from Chapter 3.
3. At completion of assay, calculate PFU/mL and compare to data obtained from qRT-PCR (*see* Subheading 3.10) (an example is shown in Fig. 5, *see* **Note 17** for explanation).

3.12 Using a Selective Inhibitor to Confirm Results Obtained in siRNA Experiments

To confirm the results observed in the siRNA knockdown assay, a selective inhibitor of the protein of interest can be used to confirm the importance of that particular protein in RSV replication. In this protocol we will use 4, 5, 6, 7-tetrabromobenzotriazole (TBB) [18] as an example, since this drug is a selective inhibitor of CK2α.

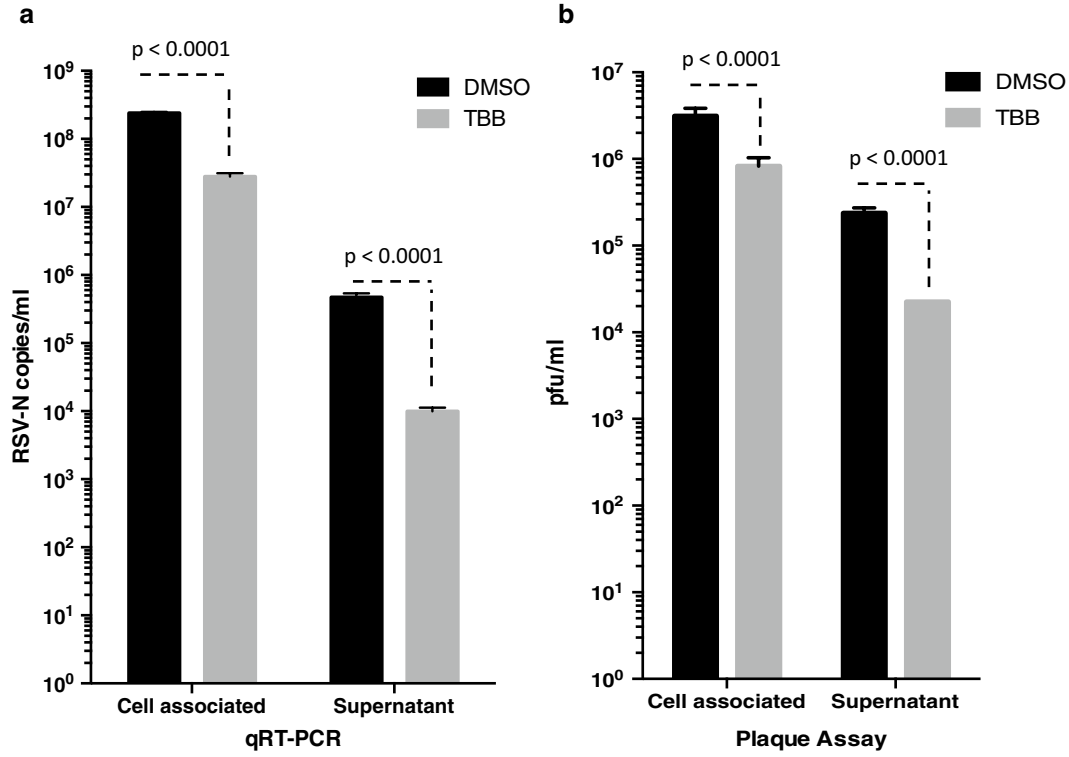

Fig. 6 RSV sample viral titre from TBB-treated A549 cells as determined by qRT-PCR and plaque assay. RSV harvested from cell-associated and supernatant fractions of TBB treated (25 mM) cells was analysed by qRT-PCR and plaque assay. (a) RSV-N copy number was determined using qRT-PCR from both cell-associated and supernatant fractions from cells treated with either DMSO control or TBB (mean +/- SEM; n=6). (b) Plaque assay performed on samples from (a) to determine level of infectious virus (pfu/ml) (mean +/- SD; n=2)

1. Grow enough A549 cells (*see* Subheading 3.1) for the number of samples to be assayed. Each sample requires 1×10^5 cells/well.
2. Place 1 mL F12-K complete medium into each well of a 12-well tissue culture plate and return to 37 °C, 5% CO_2 humidified incubator.
3. Trypsinize A549 cells from pre-prepared flasks (*see* Subheading 3.1) and count using hemocytometer.
4. Seed cells at density of 1×10^5 cells/well and incubate overnight in humidified 37 °C, 5% CO_2 incubator.
5. The next morning confirm that cells are at the required density (~70%).
6. Remove enough RSV viral stock from −80 °C freezer to infect cells at an MOI of 1 (*see* **Note 12**) and allow to thaw on ice.
7. Determine the amount of virus required to infect all of the wells within the experiment and make up a virus master mix (*see* **Note 13**).

8. Aspirate existing media from cells and replace with 500 µL of virus master mix.
9. Incubate plate(s) in 37 °C, 5% CO_2 incubator for 2 h to allow virus to adsorb to cells. Rock plates gently every 20 min to ensure even distribution of virus and even infection of cells (*see* **Note 18**).
10. Dilute enough 25 mM TBB stock solution in F12-K 2% FBS medium to give a final concentration of 25 µM TBB for each well to be treated (500 µL media/TBB required per well) (e.g., 4 wells = 2 mL F12-K media + 2 µL 25 mM TBB).
11. Add 1 µL DMSO to 1 mL of F12-K 2% FBS medium, which acts as a diluent control (DMSO final concentration of 0.1%).
12. After 2 h remove virus containing media and wash cells 2× gently with pre-warmed 37 °C PBS.
13. Add 500 µL of F12-K 2% FBS medium containing 25 µM TBB or 0.1% DMSO to each well and return to 37 °C, 5% CO_2 incubator for 24 h.
14. After 24 h harvest samples are processed as per Subheading 3.9 and analyze via qRT-PCR and plaque assay (*see* Fig. 6, **Note 19**).

4 Notes

1. Design and synthesis of siRNAs can be complicated by either the siRNA failing to target the gene of interest adequately, the siRNA targeting other genes, resulting in off-target effects. Commercially available siRNAs, such as those from Dharmacon, may minimize these problems. For our studies we use Dharmacon's ON-TARGETplus siRNA. Navigate to Dharmacon's (GE Healthcare) website http://dharmacon.gelifesciences.com/sirna/on-targetplus-sirna-reagents-human/ and use the search tool to find your gene(s) of interest and their associated product. We recommend choosing the ON-TARGETplus SMARTpool siRNA which contains a pool of four different siRNA oligos which each target a different region of the mRNA you want to knockdown.
2. Before handing siRNA, make sure you thoroughly clean your work area and all equipment with DNAse/RNAse away spray or equivalent. Always use sterile DNAse/RNAse free filter tips when aliquoting reagents.
3. For most genes, a final siRNA concentration between 2 and 50 nM is sufficient to knock down most genes >75% [16, 17, 19]. Our long-term storage siRNA stocks are 50 µM, which are then diluted to a 5 µM working stock just before use.

4. Avoidance of antibiotic-containing media is advisable for transfection protocols.
5. Due to inherent differences in the properties of various target genes, and their importance for general cell health and viability, it is important to determine the optimal siRNA amount for each gene you want to knock down in each cell line used that gives >75% knockdown. Under certain circumstances, a balance between the level of gene knockdown and cell viability must be met in order to avoid cell death, i.e., a 50% knockdown may represent a compromise between cell viability and gene knockdown. Gene knockdown should be tested at 48 and 72 h post transfection to ensure sustained knockdown.
6. After the addition of RIPA buffer, bend approximately 3–4 mm of the tip of a yellow (200 μL) pipette to form a 90° bend. Use this tip as a cell scraper to mix the RIPA buffer/cells together to ensure complete lysis. This tip can then be used to aspirate the lysate from the plate into a pre-labeled Eppendorf tube.
7. An R^2 value of >0.90 is acceptable.
8. To validate that the siRNA gene knockdown has resulted in a reduction of target protein levels, Western blot analysis is used to directly measure the level of target protein. To ensure that any reduction in protein levels observed is due to gene knockdown and not differences in sample loading, a Bradford assay should be performed to determine the concentration of each separate lysate and 20 μg total protein loaded per sample onto an SDS-PAGE gel. Depending on the size of the protein(s) of interest, choose a suitable concentration of acrylamide (lower % for large proteins and higher % or smaller proteins).
9. Use a disposable transfer pipette or 10 mL pipette to add the solution evenly across the whole gel surface. Avoid bubbles by withholding the last 1 mL of solution in the pipette. Glass plates can be pre-marked
10. The bromophenol blue in the 6× SDS lysis buffer should run off the gel at around the 8 kDa mark on a 12% acrylamide gel. Loss of proteins <8 kDa should not matter if looking for proteins in the 25–130 kDa range. If you wish to preserve these lower MW proteins, do not run the dye off.
11. Surround tank with ice up to approximately ¾ of its depth.
12. Multiplicity of Infection (MOI) = 1.0 implies that there is one viral particle per one cell within the tissue culture flask/disk. An MOI of 1 does not indicate that every cell will be infected with a single virus due to a Poisson distribution for the probability of infection [20].
13. In order to reduce the variability of infection within each well of the experiment, a master mix for the virus stock should be used to ensure homogeneity across all of the wells.

14. It is important to harvest and analyze both the cell-associated virus as well as released virus (supernatant) to determine the effect that specific gene knockdown may have on infection. If both the cell-associated and released viral pools show reduction compared to the negative control, it can be assumed that there is a general decrease/delay in viral replication kinetics. A significant decrease in released virus but not cell-associated virus indicates a potential defect in virus release/budding.
15. SPGA buffer and F12-K (+2% FBS) media can be mixed 1:1 prior to experiment and 200 μL total pipetted onto cells.
16. Figure 4a depicts the typical amplification curves produced by the Eppendorf Mastercycler EP realplex system which automatically determines the baseline fluorescence threshold (red line). The RSV-N standards can clearly be seen, with the 10^8 copies of N gene crossing threshold fluorescence at cycle ~12, with the lowest copy number (10^2 copies) crossing at cycle ~29. The mastercycler software utilizes the cycle numbers which the standards cross the fluorescence threshold to create a standard "curve" (Fig. 4b). This curve is then used to fit any unknown sample(s), in this instance our RSV viral stock, which by reading off the CT value obtained in Fig. 4a, we can determine its concentration (dashed line) of 3.11×10^5 copies/mL, which can then be used to normalize samples between each experiment. It should be noted that qRT-PCR does not give a true indication of the amount of infectious virus within a sample, as immature or incompletely formed virions that contain a viral genome will be measured, as too the newly synthesized viral RNA that has yet to be incorporated into a virion. It is for this reason that we must perform a viral plaque assay, which measures "infectious," and thus viable virions.
17. In the example shown in Fig. 5a the qRT-PCR analysis of RSV-N copy number from CK2α knockdown cells reveals no significant difference in genome copies within the cell associated viral pool, but a significant ($p < 0.0001$) decrease within released virus, indicating that CK2α activity may be important for efficient assembly and subsequent budding/release of RSV to the supernatant. When analyzed by plaque assay, which gives an indication of infectious virus levels, a significant decrease in PFU/mL is observed for both cell-associated ($p < 0.005$) and supernatant ($p < 0.05$) viral fractions. The discrepancy between qRT-PCR and the plaque assay for cell-associated samples implies accumulation of immature virions or non-incorporated viral genomes within the cell in CK2α knockdown samples, implying that CK2α may be required for proper virion assembly and maturation. This example highlights the importance of performing not only qRT-PCR which provides a quick method by which to analyze your viral titers, but

following up with a plaque assay that gives a true indication of the "fitness" and replication competence of your purified virus.

18. By allowing the virus to adsorb to the cells in the absence of drugs it is possible to assume that effects on viral titer are not due to direct inhibition of cell surface adsorption.
19. In the example shown in Fig. 6 when RSV infected cells are treated with the specific CK2α inhibitor TBB, a significant ($p < 0.0001$) decreases in viral titer is detected both via qRT-PCR (Fig. 6a) and plaque assays (Fig. 6b), supporting and validating the results seen when CK2α is knocked down by siRNA.

References

1. Thompson WW et al (2003) Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA 289(2):179–186
2. Elliot AJ, Fleming DM (2008) Influenza and respiratory syncytial virus in the elderly. Expert Rev Vaccines 7(2):249–258
3. Chavez-Bueno S et al (2007) Intravenous palivizumab and ribavirin combination for respiratory syncytial virus disease in high-risk pediatric patients. J Pediatr Infect Dis 26(12):1089–1093
4. Holguera J, Villar E, Munoz-Barroso I (2014) Identification of cellular proteins that interact with Newcastle disease virus and human respiratory syncytial virus by a two-dimensional virus overlay protein binding assay (VOPBA). Virus Res 191C:138–142
5. Dave KA et al (2014) A comprehensive proteomic view of responses of A549 type II alveolar cells to human respiratory syncytial virus infection. Mol Cell Proteomics 13(12): 3250–69
6. van Diepen A et al (2010) Quantitative proteome profiling of respiratory virus-infected lung epithelial cells. J Proteomics 73(9):1680–1693
7. Munday DC, Hiscox JA, Barr JN (2010) Quantitative proteomic analysis of A549 cells infected with human respiratory syncytial virus subgroup B using SILAC coupled to LC-MS/MS. Proteomics 10(23):4320–4334
8. Munday DC et al (2010) Quantitative proteomic analysis of A549 cells infected with human respiratory syncytial virus. Mol Cell Proteomics 9(11):2438–2459
9. Martinez I et al (2007) Distinct gene subsets are induced at different time points after human respiratory syncytial virus infection of A549 cells. J Gen Virol 88(Pt 2):570–581
10. Agrawal N et al (2003) RNA interference: biology, mechanism, and applications. Microbiol Mol Biol Rev 67(4):657–685
11. Konig R et al (2010) Human host factors required for influenza virus replication. Nature 463(7282):813–817
12. Karlas A et al (2010) Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. Nature 463(7282):818–822
13. Panda D et al (2011) RNAi screening reveals requirement for host cell secretory pathway in infection by diverse families of negative-strand RNA viruses. Proc Natl Acad Sci U S A 108(47):19036–19041
14. Yasunaga A et al (2014) Genome-wide RNAi screen identifies broadly-acting host factors that inhibit arbovirus infection. PLoS Pathog 10(2):e1003914
15. Hirsch AJ (2010) The use of RNAi-based screens to identify host proteins involved in viral replication. Future Microbiol 5(2):303–311
16. Kipper S et al (2015) New host factors important for respiratory syncytial virus (RSV) replication revealed by a novel microfluidics screen for interactors of matrix (M) protein. Mol Cell Proteomics 14(3):532–43
17. Bajorek M et al (2014) The Thr205 phosphorylation site within respiratory syncytial virus matrix (M) protein modulates M oligomerization and virus production. J Virol 88(11):6380–6393
18. Sarno S et al (2001) Selectivity of 4,5,6,7-tetra bromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ("casein kinase-2"). FEBS Lett 496(1):44–48
19. Szczepny A et al (2014) Overlapping binding sites for importin beta1 and suppressor of fused (SuFu) on glioma-associated oncogene homologue 1 (Gli1) regulate its nuclear localization. Biochem J 461(3):469–476
20. Knight DM, Howley PM (2001) Fields virology. In: Knipe D.M. Howley P (eds) 4th edn., vol 1. Lippincott Williams & Wilkins, p 3280

Chapter 9

In Vitro Modeling of RSV Infection and Cytopathogenesis in Well-Differentiated Human Primary Airway Epithelial Cells (WD-PAECs)

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Abstract

The choice of model used to study human respiratory syncytial virus (RSV) infection is extremely important. RSV is a human pathogen that is exquisitely adapted to infection of human hosts. Rodent models, such as mice and cotton rats, are semi-permissive to RSV infection and do not faithfully reproduce hallmarks of RSV disease in humans. Furthermore, immortalized airway-derived cell lines, such as HEp-2, BEAS-2B, and A549 cells, are poorly representative of the complexity of the respiratory epithelium. The development of a well-differentiated primary pediatric airway epithelial cell models (WD-PAECs) allows us to simulate several hallmarks of RSV infection of infant airways. They therefore represent important additions to RSV pathogenesis modeling in human-relevant tissues. The following protocols describe how to culture and differentiate both bronchial and nasal primary pediatric airway epithelial cells and how to use these cultures to study RSV cytopathogenesis.

Key words Primary human airway epithelial cells, Well-differentiated human airway epithelial cell culture (WD-PAECs), Respiratory syncytial virus, Cytopathogenesis

1 Introduction

Many of the initial investigations into how RSV infects and causes disease were undertaken in animal models or continuous cell lines. These research models, while providing important discoveries in the area of RSV biology and pathogenesis, do not faithfully reproduce hallmarks of RSV pathogenesis or represent the complexities of the human airway. The development of physiologically and morphologically authentic pseudostratified human airway epithelial cell cultures has added an important new dimension to studying RSV-human host interactions [1, 2].

There is currently an increasing number of research groups worldwide using well-differentiated human airway epithelial cell culture

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(WD-PAEC) models to study RSV infection. This is due primarily to the fact that WD-PAECs replicate many hallmarks of RSV infection of infants including: virus growth kinetics, apical ciliated cell restriction of infection, cell sloughing, increased mucus production, and chemokine responses [3–5]. RSV initially infects the upper respiratory tract but can descend to the lower airways, often causing severe disease, such as bronchiolitis. The development of both well-differentiated primary pediatric bronchial (WD-PBECs) and nasal (WD-PNECs) epithelial cell culture models facilitates investigations into the relative RSV cytopathogenesis in the upper and lower airways [6, 7].

The availability of primary airway epithelial cells from commercial sources has increased rapidly in recent years. This has allowed groups that were previously unable to work with WD-PAECs, due to the requirement for ethical approval and medically trained personnel to acquire the primary cells, to investigate RSV-human host interactions that cannot be successfully studied using traditional models of RSV infection. However, access to pediatric airway epithelial cells from commercial sources remains extremely limited. In contrast, with the appropriate ethical and research governance approvals and clinical infrastructure in place, in our hands these non-injurious protocols have facilitated access to very large numbers of pediatric airway epithelial cells from a range of diverse individuals representative of many different ages (newborn to elderly), phenotypes (e.g., congenital heart disease, bronchopulmonary dysplasia, asthmatics, cystic fibrosis, race) and genotypes (e.g., individuals with single nucleotide polymorphisms associated with severe RSV disease) [8]. Evidently, the protocols described below are also suitable for studying cytopathogenesis of other human or zoonotic respiratory viruses.

2 Materials

2.1 Hardware and Consumables

1. Cell culture incubator at 37 °C, 5% CO_2 and 80% relative humidity.
2. Inverted microscope.
3. Water bath.
4. Liquid nitrogen storage tank.
5. −80 °C Freezer.
6. Tissue culture flasks: 25 cm^2 (T25) and 75 cm^2 (T75).
7. Transwells 6.5 mm diameter (*see* **Note 1**).
8. Neubauer hemocytometer.
9. Isopropanol cell freezing apparatus (to achieve a rate of cooling of approximately −1 °C/min), such as Nalgene Mr. Frosty.
10. 1.5, 15, and 50 mL sterile centrifuge tubes.
11. Cryovials.

2.2 Patient Sample Collection

1. Bronchial cytology brushes 3.0 mm brush diameter 120 cm length.
2. Sterile normal saline interdental brushes 2.7 mm diameter or Cervical cytology brushes.
3. Sterile galley pot and 10 mL.
4. Sterile tweezers.

2.3 Tissue Culture Media and Solutions

1. RSV A and/or B stocks.
2. Collage stock solution: 3 mg/mL bovine collagen solution (*see* **Note 2**).
3. Sterile double distilled water (dd H_2O).
4. Sterile phosphate buffered saline (PBS) cell culture grade.
5. 0.05% trypsin–ethylenediaminetetraacetic acid (EDTA) (*see* **Note 3**).
6. Trypan blue: 0.4% (w/v) trypan blue in PBS.
7. Fetal bovine serum (FBS): Heat-inactivate FBS for 30 min at 56 °C.
8. SF-DMEM: Serum-free Dulbecco's Modified Eagle's Medium (DMEM)—low glucose.
9. 5% FBS/DMEM: SF-DMEM supplemented with 5% (v/v) FBS.
10. 2× P/S-DMEM: SF-DMEM, 200 U/mL penicillin and 200 μg/mL streptomycin.
11. Transport medium (TM): SF-DMEM, 100 U/mL penicillin–streptomycin, 0.1 mg/mL Primocin.
12. Monolayer medium: 500 mL Airway Epithelial Cell Basal Medium (Promocell or equivalent), 1 pack of Airway Epithelial Cell Growth Medium Supplement [final concentration in the medium: 52 μg/mL bovine pituitary extract (BPE), 10 ng/mL human epithelial growth factor (hEGF), 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 0.5 μg/mL epinephrine, 6.7 ng/mL triiodothyronine (T3), 10 μg/mL transferrin, and 0.1 ng/mL retinoic acid (RA)], 100 U/mL penicilin, and 100 μg/mL streptomycin (*see* **Note 4**).
13. 30% bovine serum albumin (BSA): 3 g of BSA dissolved in 10 mL of PBS. Filter sterilize.
14. 150 mg/mL BSA: 1 mL of 30% BSA solution mixed with 1 mL of Airway Epithelial Cell Basal Medium (before supplements are added).
15. 1.5 mg/mL BSA: 10 μL of the 150 mg/mL BSA solution mixed with 990 μL Airway Epithelial Cell Basal Medium (before supplements are added).
16. 3 mg/mL retinoic acid (RA): dissolve 50 mg retinoic acid in 16.67 mL DMSO. Aliquot into 110 μL volumes and store at −20 °C (*see* **Note 5**).

17. 15 μg/mL RA: Dilute 100 μL of the 3 mg/mL RA solution in 20 mL DMSO. Aliquot this into 60 μL volumes and store at −20 °C.
18. 2× air–liquid interface (ALI) medium: 500 mL of Airway Epithelial Cell Basal Medium (Promocell or equivalent), 1 mL of 1.5 mg/mL BSA and 2 packs of Airway Epithelial Cell Growth Medium Supplement (Promocell or equivalent) without triiodothyronine and retinoic acid (final concentration of supplements in the medium: 104 μg/mL BPE, 20 ng/mL hEGF, 10 μg/mL insulin, 1 μg/mL hydrocortisone, 1 μg/mL epinephrine, 10 μg/mL transferrin, 3 μg/mL BSA) (*see* **Note 4**).
19. ALI medium: 25 mL of 2× P/S-DMEM, 25 mL of 2× ALI medium and 50 μL of 15 μg/mL RA (final concentration of supplements in the medium: 52 μg/mL BPE, 10 ng/mL hEGF, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 0.5 μg/mL epinephrine, 10 μg/mL transferrin, 1.5 μg/mL BSA, 15 ng/mL RA, 100 U/mL penicilin, and 100 μg/mL streptomycin). Protect from light. Store at 4 °C (*see* **Notes 4** and **6**).
20. Dimethyl sulfoxide (DMSO): sterile for cell culture.
21. PAECs Freezing medium: 80% monolayer medium, 10% (v/v) FBS, and 10% (v/v) DMSO.

2.4 Cytospin and Cell Staining

1. Cytospin centrifuge.
2. Cytofunnels.
3. Poly-L-lysine coated microscope slides.
4. 4% paraformaldehyde solution (PFA).
5. 0.2% Triton X-100: 0.2% (v/v) Triton X-100 in sterile PBS.
6. 0.5% BSA: 0.5 g of BSA dissolved in 100 mL of PBS.
7. DAPI mounting medium: 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) in PBS.
8. Sterile Pasteur pipettes.
9. Appropriate primary and secondary antibodies for RSV detection.
10. Coverslips and slides.
11. Scalpel.
12. Pasteur pipette.
13. Nail polish.

3 Methods

3.1 Collagen Coating of Tissue Culture Flasks

1. Dilute the collagen stock solution 1:30 with sterile ddH_2O.
2. Coat the tissue culture flask with collagen. For a T10 add 1 mL of collagen solution, for T25 add 2 mL and for a T75 add 3 mL.

3. Ensure collagen solution completely covers the surface.
4. Put flasks in incubator at 37 °C for at least 24 h before use. Flasks can be left in incubator until use.
5. Aspirate any excess collagen solution before use.

3.2 Collagen Coating of Transwells (See Note 2)

1. Dilute the collagen stock solution 1:100 with sterile ddH_2O.
2. Add 100 μL to the apical compartment of each Transwell. Be careful not to damage the Transwell membrane (*see* Fig. 1).
3. Incubate the Transwells at 37 °C for at least 24 h before use. Transwells can be left in the incubator until use. Alternatively, they can be removed, wrapped in cling film and stored at 4 °C for up to 6 months.
4. Aspirate excess collagen solution before use.

3.3 Obtaining Fresh Bronchial Epithelial Cells from Patients (Non-bronchoscopic Method) (See Note 7)

1. Aliquot transport medium (TM) and PBS into 5 mL volumes in separate sterile 15 mL centrifuge tubes.
2. Once the patient is anesthetized and intubated, place 10 mL of normal saline in the sterile galley pot and immerse the bronchial cytology brush in the saline ensuring it is completely wet.
3. Keeping the bronchial cytology brush in its sheath insert it via the ET tube into the lower airways until it meets with resistance. Withdraw the sheathed brush back by 2 cm, advance the brush out of the sheath and obtain the bronchial brushings.
4. Withdraw the brush and place it into the 15 mL sterile centrifuge tube containing the 5 mL of sterile PBS. Wash the brush in the PBS and then place it back into the patient's bronchus for a second brushing if required.
5. Once the bronchial brushings have been completed cut off the excess length of stem of the cytology brush (non-brush end) and add 5 mL of TM to the 5 mL of PBS and bronchial brushings.
6. Seal the tube and mix well by inverting three times.
7. Bring the tube directly to the laboratory for processing (*see* Subheading 3.5).

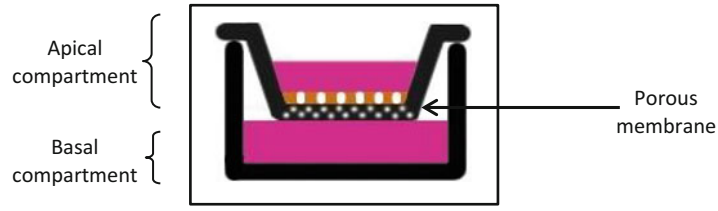

Fig. 1 Diagram of a Transwell

3.4 Obtaining Fresh Nasal Epithelial Cells from Patients (See Note 7)

1. Choose the type of brush depending upon the size of the airways of the patients being sampled. For very young infants interdental brushes are recommended. For older children or adults cervical cytology brushes work well.
2. Insert the brush into the nasal cavity of the conscious patient. The brush should reach the nasal turbinates to obtain a good cell yield. After insertion, rotate the brush to obtain cells.
3. Once the brushing is completed place the brush in the 15 mL sterile centrifuge tube containing the PBS.
4. Cut off the excess length of the stem of the cytology/interdental brush (non-brush end) and add the TM to the PBS and brushings.
5. The tube is then sealed and mixed well by inverting three times.
6. The tube is brought directly to the lab for processing (*see* Subheading 3.5).

3.5 Processing Bronchial or Nasal Brushes from Patients

1. Grip the non-brush end of the cytology brush with tweezers.
2. Using a P1000 micropipette gently expel monolayer medium over the brush bristles into the transport vessel the brushes arrived in. Do this several times over all of the bristles.
3. Discard the brushes.
4. Centrifuge the medium, including the transport medium and the monolayer medium used to dislodge cells, in the 15 mL centrifuge tube at 129 × *g* for 5 min at room temperature.
5. Carefully remove the supernatant and discard.
6. Resuspend the cell pellet in monolayer medium.
7. Put the cell suspension in a collagen-coated flask. It is recommended that a T10 flask is used (*see* **Note 8**).
8. Change medium every 2–3 days. To change medium follow these steps:
   (a) Discard the medium by pouring off or pipetting from the flask.
   (b) Replace with fresh monolayer medium.
   (c) Use 2 mL of medium for a T10 flask and 10–12 mL for a T75 flask.
9. Once the cells are approximately 80% confluent (*see* **Note 9**) passage them into a collagen-coated T75 flask.

3.6 Passage of Human Airway Epithelial Cells (PAECs)

1. Discard the monolayer medium from the flask.
2. Rinse cells gently with 2–3 mL trypsin–EDTA and discard.
3. Add 1 mL trypsin–EDTA (T10 flask) or 3 mL trypsin–EDTA (T75 flask).
4. Incubate the flask at 37 °C for 3–5 min until all cells have detached.

5. Check cell detachment using an inverted microscope.
6. Add 3 mL (T10) or 7 mL (T75) 5% FBS/DMEM to inactivate trypsin–EDTA.
7. Transfer cell suspension to 15 mL sterile centrifuge tube.
8. Spin at 129 × *g* for 5 min at RT.
9. Carefully remove the supernatant.
10. Resuspend the cell pellet in 2 mL of either monolayer medium or ALI medium (depending on whether you are seeding flasks or Transwells, respectively).
11. If you are freezing cells for long term storage (*see* Subheading 3.8), resuspend the cell pellet in monolayer medium.
12. Perform cell count (*see* Subheading 3.7) and proceed to seed the cells:

    (a) To seed tissue culture flasks:

    - Use T75 flasks.
    - Aspirate excess collagen, if any, from tissue culture flasks.
    - Seed cells at a minimum of 5×10^5 cells/T75 flask.
    - Add monolayer medium to ensure final volume of 10–12 mL.
    - Leave flask undisturbed for 2 days to allow the cells to adhere.

    (b) To seed Transwells:

    - Following trypsinization and prior to cell count resuspend the cells in ALI medium.
    - Aspirate excess collagen from the Transwells. Be very careful not to damage the membrane.
    - Sterilize Transwells plates by UV (this step is optional, *see* **Note 1**).
    - For one 6.5 mm diameter Transwells seed 5×10^4 bronchial cells or 1×10^5 nasal cells (*see* **Notes 10** and **11**).
    - Add cells apically in a total volume of 250 µL ALI medium. Be careful not to allow any cells into the basal compartment.
    - Add 750 µL ALI medium to the basal compartment (*see* **Note 12**). *See* Fig. 1 for diagram of Transwell.
    - Leave Transwells undisturbed for 2 days to allow proper adherence of cells.

3.7 Cell Counting of PAECs

1. Fix coverslip across chamber of hemocytometer (*see* Fig. 2).
2. Take 10 µL of cell suspension and add to 10 µL of trypan blue solution. Pipette up and down to mix well.

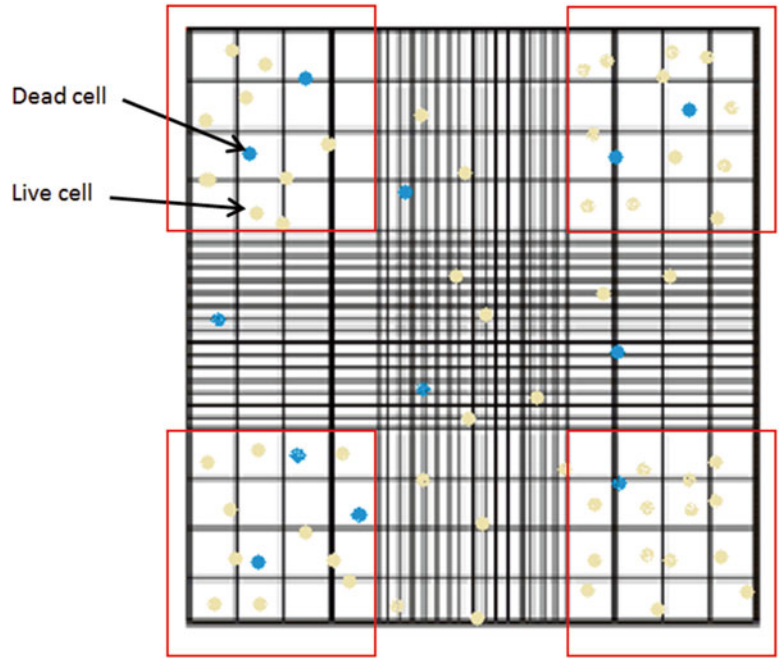

Fig. 2 Diagram of a hemocytometer

3. Take 10 μL of cell suspension–trypan blue mixture and slowly pipette into the hemocytometer chamber so it is completely filled without air bubbles and that the grid is covered.
4. Observe the hemocytometer under the microscope at 10× magnification.
5. Cells stained blue are dead or dying.
6. Record the number of living cells and the number of dead cells separately. Do this for each of the 4 quadrants (shown in red) of the hemocytometer (*see* Fig. 2). Apply the 2-side rule, where cells touching e.g., the top and right outside lines are counted in, while cells touching the bottom and left outside lines are not counted.
7. The numbers of living cells only are used for further calculations. The dead cell count is recorded to ensure the previous processes of trypsinization/resuspension were not overly detrimental to cell survival.
8. Calculate the average of the 4 quadrants. Multiply this by 2, due to the 1:1 dilution of cells with trypan blue. This number is referred to hereafter as "χ."
9. Due to the volume of the hemocytometer chamber the cell count is always: $\chi \times 10^4$/mL.
10. Multiply this by the total volume of cell suspension to obtain the total cell count.

3.8 Freezing of PAECs

This follows on from trypsinization and cell counting of PAECs (*see* Subheadings 3.6 and 3.7).

1. Once the cell count is obtained dilute the cell suspension to 8×10^5 cells/mL using PAECs freezing medium and add 1 mL per labeled cryovial.
2. Put cryovials into isopropanol cell freezing apparatus at room temperature.
3. Store the apparatus at −80 °C for 24 h.
4. Transfer the cryovials to the gaseous phase of the liquid nitrogen storage tank.

3.9 Defrosting PAECs

1. Remove cryovial of cells from liquid nitrogen storage tank and place at room temperature for 1 min.
2. Place the vial in the water bath at 37 °C. Remove when just defrosted, do not leave for longer than required.
3. When defrosted pipette the contents into a 15 mL sterile centrifuge tube. Rinse the cryovial with 1 mL monolayer medium and add this to the 15 mL tube.
4. Spin the cell suspension at $129 \times g$ for 5 min at RT.
5. Discard supernatant without disrupting the cell pellet.
6. Resuspend the cells in 1 mL of monolayer medium.
7. Seed the cells into a collagen-coated T75 flask, following removal of excess collagen.
8. Leave the cells for 2 days to attach to the flask in a cell culture incubator at 37 °C 5% CO_2.
9. Follow normal protocol for changing medium *see* Subheading 3.6.

3.10 Differentiation of PAECs

3.10.1 Submerged Culturing in Transwells (for 6.5 mm Diameter Transwell)

1. Following seeding of PAECs into Transwells incubate for 2 days to allow adherence. After this period the medium must be changed every 2 days.
2. Aspirate the medium from the apical surface being careful not to touch the Transwell membrane, which will cause damage to the cells.
3. Aspirate the basal medium.
4. Add 250 µL fresh ALI medium to the apical compartment.
5. Add 750 µL fresh ALI medium to the basal compartment.
6. Observe cells daily under phase contrast microscope.
7. PAECs should be kept in submerged culture until fully confluent plus 1 day. This should take 4–8 days (*see* **Note 13**).
8. Cells should be kept in the tissue culture incubator at 37 °C, 5% CO_2, 80% RH.

*3.10.2 Air–Liquid Interface (ALI) Culturing (See **Note 11**)*

1. Once the cells are completely confluent an air–liquid interface is initiated in the culture (*see* Fig. 3).
2. Ensure there are no holes in the cell layer before ALI culturing.
3. Aspirate the apical and basal medium from the Transwell.
4. Add 300 μL fresh ALI culture medium basally.
5. No medium is added apically.
6. Replace the basal medium every 2 days (*see* **Note 12**).
7. Observe cells daily under the microscope.
8. Signs of differentiation will begin approximately 7 days post ALI.
9. Mucus production is apparent when liquid is evident on the apical surface of the cells. This should be carefully aspirated from the apical surface to ensure the viability of the air–liquid interface.
10. Ciliated cells will become visible under phase contrast microscopy within a few days of mucus production.
11. Once signs of differentiation begin the apical surface of the cells should be washed once a week to prevent mucus buildup. To wash the apical surface add 200 μL of SF-DMEM to the apical surface and then aspirate off. Ensure complete aspiration without causing damage to cells.
12. PAECs should be left for a minimum of 21 days in ALI before use (*see* Fig. 4). This is a guideline; it may take longer for cultures to be fully differentiated (*see* **Notes 13** and **14**).
13. Ensure good mucus production and extensive cilia coverage throughout culture (*see* **Note 15**, Figs. 5 and 6).
14. Cells should be kept at 37 °C, 5% CO_2, 80% RH.

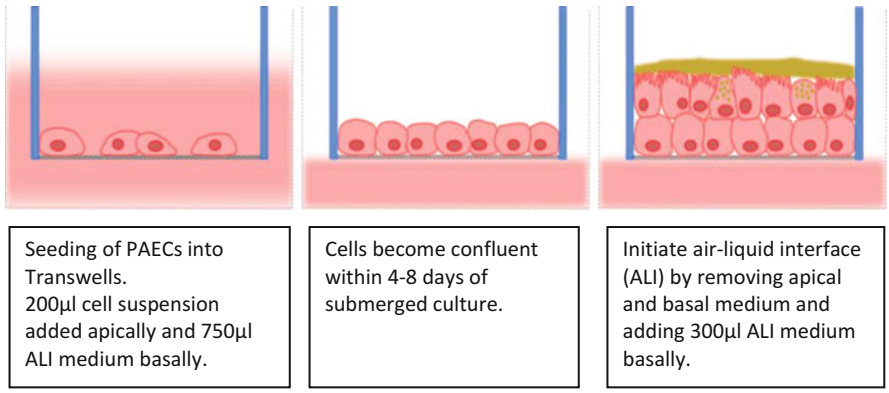

Fig. 3 Diagram of ALI initiation. Seeding of PAECs into Transwells. 200 μL cell suspension added apically and 750 μL ALI medium basally. Cells become confluent within 4–8 days of submerged culture. Initiate air–liquid interface (ALI) by removing apical and basal medium and adding 300 μL ALI medium basally

3.11 RSV Infection of Well-Differentiated PAECs (WD-PAECs)

1. Wash WD-PAECs twice with 200 μL SF-DMEM to remove surface mucus.
2. Calculate volume of virus required to achieve desired MOI. Dilute in SF-DMEM if needed (*see* **Notes 16** and **17**).
3. Add virus apically to cultures. The volume in the apical compartment should not exceed 250 μL (6.5 mm Transwells) (*see* **Note 16**).
4. Incubate at 37 °C for 2 h.
5. Wash apical surface four times with 200 μL SF-DMEM.
6. Wash a 5th time, but retain this sample as the 2 h post-infection (hpi) time point.
7. Place the 2 hpi sample in a pre-labeled cryovial and place on ice.
8. Snap-freeze the 2 hpi sample in liquid nitrogen as soon as possible thereafter and store at −80 °C or below until used.

3.12 Apical Rinsing and Basal Medium Harvesting from RSV-Infected Cultures

1. Apical washes and basal medium should be harvested on a daily basis (or at desired time points) following infection.
2. For apical washes, add 200 μL SF-DMEM to the apical surface of the cultures. Leave to incubate at RT for 5 min. Pipetting up and down gently without incubation is an optional alternative, but this increases the risk of damaging the cultures.
3. For RSV titrations, remove the apical wash entirely, put into a cryovial and place immediately on ice.
4. Snap-freeze in liquid nitrogen as soon as possible thereafter.

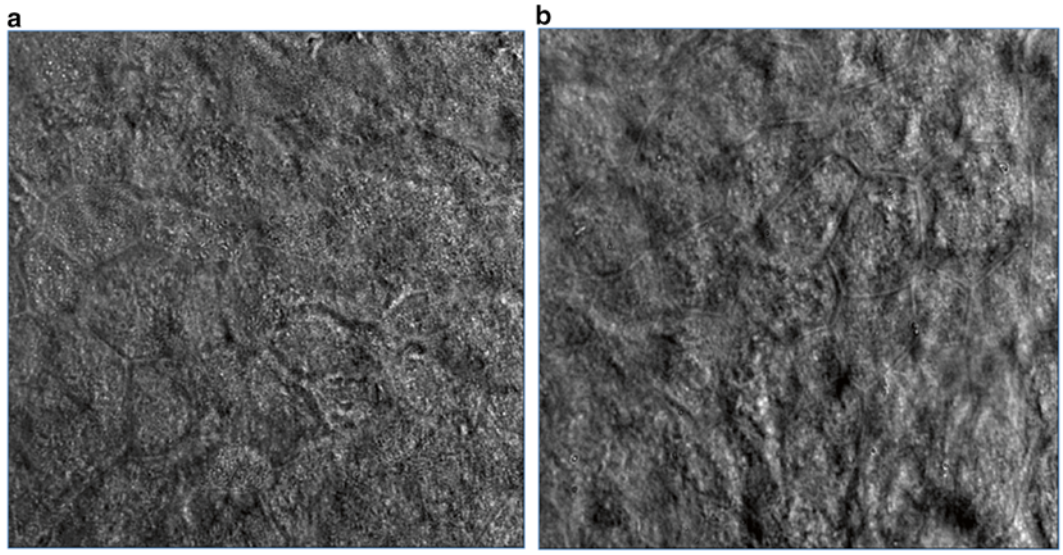

Fig. 4 Confocal phase-contrast microscopy of well-differentiated primary pediatric bronchial epithelial cells (WD-PBECs) (**a**) and well-differentiated primary pediatric nasal epithelial cells (WD-PNECs) (**b**) 21 days post air–liquid interface

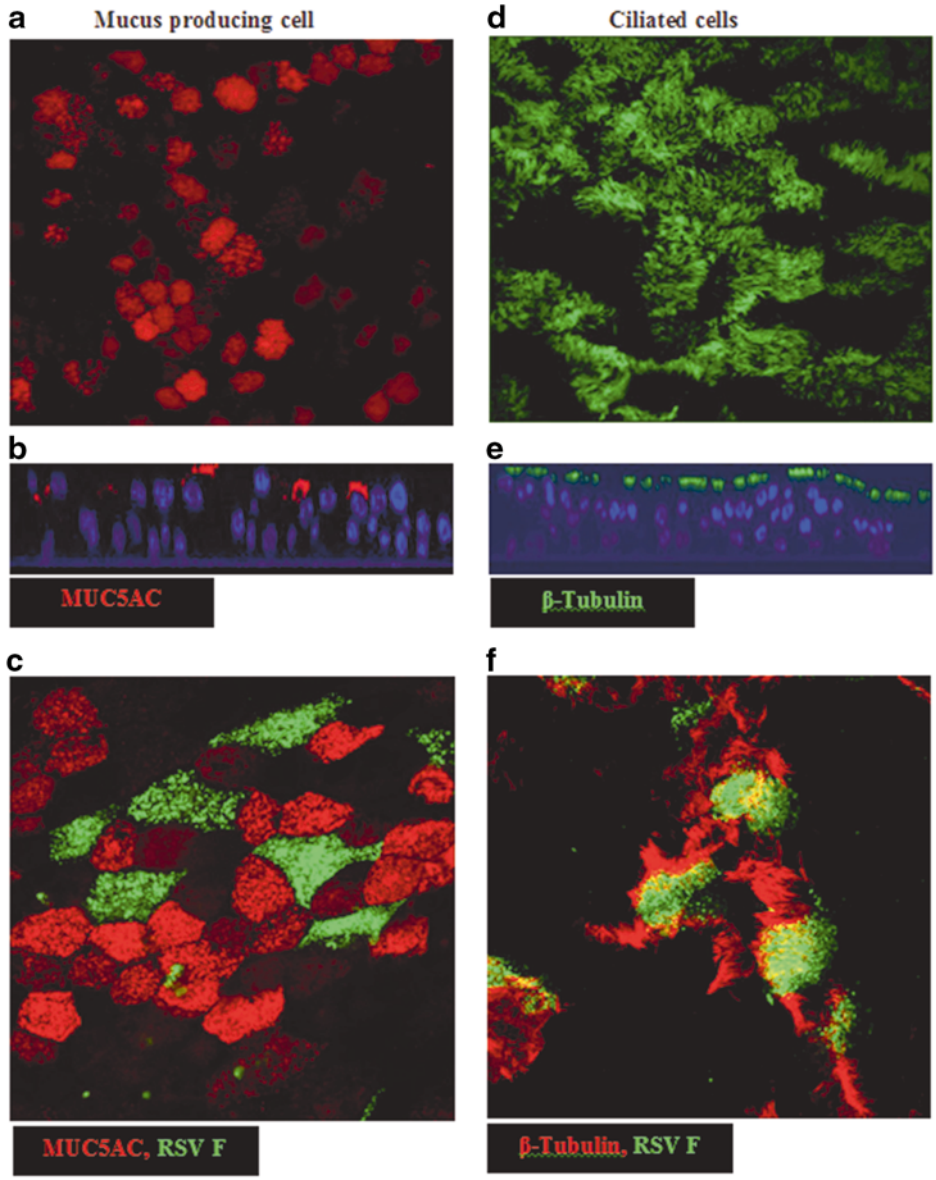

Fig. 5 Well-differentiated primary pediatric bronchial epithelial cells stained for: (**a**) the goblet cell marker Muc5Ac (*red*), (**b**) Muc5Ac (*red*), and DAPI (*blue*), orthogonal section; (**c**) Muc5Ac (*red*) and RSV F protein (*green*); (**d**) ciliated cell marker β-tubulin (*green*); (**e**) β-tubulin (*green*) and DAPI (*blue*), orthogonal section; and (**f**) β-tubulin (*red*) and RSV F protein (*green*)

5. For cytospins of apical washes *see* Subheading 3.13.
6. Transfer basal medium (300 μL) to a cryovial and stored on ice. Snap-freeze in liquid nitrogen.
7. Replace basal medium with 300 μL fresh ALI medium.
8. Store apical washes and basal medium at −80 °C or colder until used.

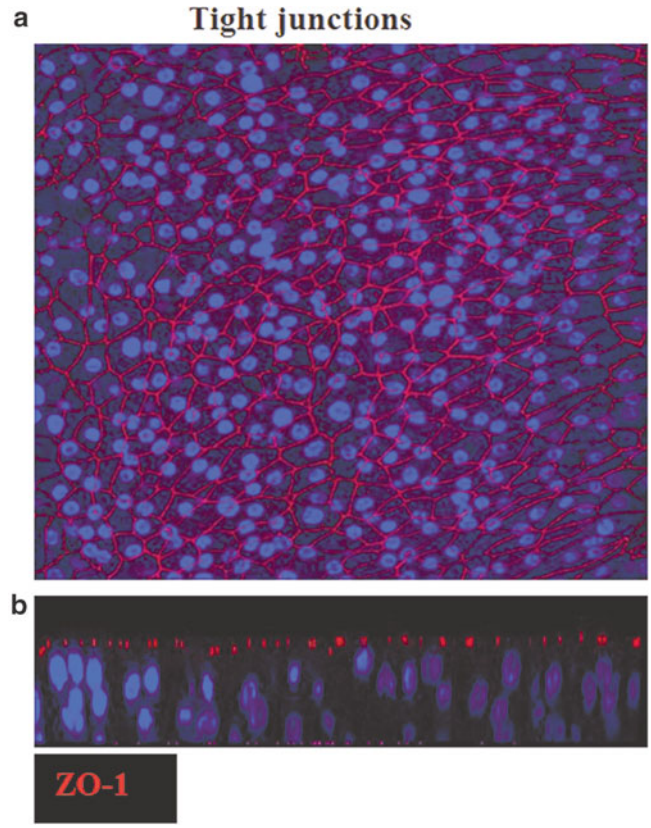

Fig. 6 Well-differentiated primary pediatric bronchial epithelial cells stained for the tight junction protein ZO-1 (*red*) and DAPI (*blue*) (**a**). (**b**) Orthogonal section of WD-PBECs stained for the ZO-1 (*red*) and DAPI (*blue*)

3.13 Cytospin

1. Add 200 μL SF-DMEM to the apical surface of the cultures. Leave to incubate at room temperature for 5 min. Pipetting up and down gently is optional.
2. Insert a coated microscope slide into the cytofunnel.
3. Put fresh apical wash into the sample chamber of the cytofunnel.
4. Spin cytofunnel in a cytospin centrifuge at approximately 335 × *g* for 2 min.
5. Remove slide from cytofunnel and leave to air-dry.
6. Once dry, fix by adding 4% PFA to cover the area of the cytospin on the slide. A PAP pen, or hydrophobic barrier, may be useful to draw around the area of the cytospin to retain the PFA. Leave for 10 min.
7. Remove the PFA and gently wash twice with a few drops of PBS.
8. Store fixed slides at −20 °C.
9. Allow slides to come to room temperature before staining.

10. Add a few drops 0.2% Triton X-100 to the cytospin (the PAP pen should hold this in place. Incubate at RT for 1 h (*see* **Note 18**).
11. Remove 0.2% Triton X-100 and wash once with a few drops PBS.
12. Add a few drops of 0.5% BSA and incubate for 30 min at room temperature. Remove and wash once with PBS.
13. Dilute the primary antibody in 0.5% BSA. Add a few drops to cover the area of the cytospin. Incubate at 37 °C for 1 h (*see* **Note 19**). Wash three times with a few drops of PBS.
14. Dilute the secondary antibody in 0.5% BSA. Add a few drops to cover the area of the cytospin. Protect from light and incubate at 37 °C for 1 h (*see* **Note 19**). Wash three times with a few drops of PBS.
15. Dilute the conjugated antibody in 0.5% BSA. Add a few drops to cover the area of the cytospin. Protect from light and incubate at 37 °C for 1 h (*see* **Note 19**). Wash three times with a few drops of PBS.
16. If staining for multiple proteins, ensure that the sequence of addition of primary and/or secondary antibodies is appropriate to prevent inappropriate cross-reaction of the antibodies with off-target proteins (*see* **Notes 20** and **21**).
17. Add a drop of DAPI mounting medium and place a coverslip on carefully.
18. Seal the edge of the coverslip with nail varnish and store in the dark at 4 °C until used for fluorescent microscopy (*see* Figs. 3, 4, 5, 6, and 7).

3.14 Fixation of WD-PAECs

1. Wash the apical surface of the Transwell with SF-DMEM.
2. Remove the basal medium.
3. Add 200 µL of 4% PFA to the apical surface and 800 µL to the basal compartment.
4. Leave for 20–30 min at room temperature.
5. Aspirate the PFA from both compartments of the Transwell.
6. Add 200 µL 70% ethanol to the apical surface and 800 µL to the basal compartment.
7. Wrap the plate in Parafilm and store at 4 °C. The fixed cultures will be stable for about 1 month. For longer term storage the 70% ethanol will need replaced every few weeks to ensure the Transwells do not dry out.

3.15 Removal of Membrane from Transwell

1. Carefully remove the ethanol from the apical and basal compartments using a Pasteur pipette.
2. Lift the Transwell and turn it upside down.

3. Using a very sharp scalpel, cut around the outside edge of the membrane. Be careful not to cut any of the hard plastic of the actual Transwell (*see* **Notes 22** and **23**).
4. Ensure that the cell side of the membrane is facing up if placing on a slide for staining.

3.16 Staining of WD-PAECs for Immunofluorescence

1. If you are staining the whole Transwell with one or a mixture of compatible antibodies, it is easier to stain the WD-PAECs before cutting the membrane out (*see* **Note 23**).
2. Aspirate the ethanol.
3. Wash the Transwells twice with PBS.
4. If you are not staining intracellularly, skip **steps 5**–7 (*see* **Note 18**).
5. Add enough 0.2 % Triton X-100 to cover the apical surface of the Transwell.
6. Incubate for 2 h at room temperature.
7. Remove Triton X-100 from Transwells.
8. Add enough 0.5 % BSA to cover the surface of the Transwell.
9. Incubate for 30 min at room temperature.

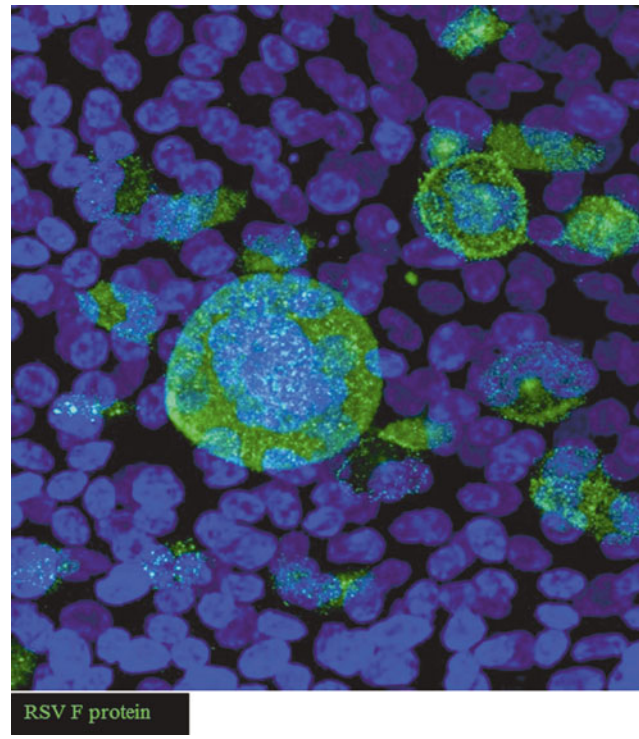

Fig. 7 Well-differentiated primary pediatric nasal epithelial cells stained for RSV F protein (*green*) and DAPI (*blue*). A syncytium can be seen in the center of the image

10. Prepare the primary antibody by diluting in 0.5% BSA to obtain a concentration specified by the manufacturer's instructions or by prior optimization.
11. Add 200 μL of the primary antibody apically. If the primary antibody is conjugated to a fluorophore, protect the Transwells from light as much as is practicable from this point on.
12. Incubate at 37 °C for 1 h or at 4 °C overnight (*see* **Note 19**).
13. Remove primary antibody and add enough PBS to cover the cells. Incubate for 5 min at room temperature.
14. Remove PBS and repeat **step 13** two more times. If a secondary antibody is not required (fluorophore-conjugated primary antibody used), proceed to **step 22**.
15. If a secondary antibody is necessary, dilute it in 0.5% BSA to obtain a concentration specified by the manufacturer's instructions or by prior optimization.
16. Add 200 μL of the secondary antibody apically. Protect the Transwells from light as much as is practicable from this point on.
17. Incubate at 37 °C for 1 h.
18. Remove secondary antibody and wash three times with PBS as in **step 13** above.
19. If staining for multiple proteins, ensure that the sequence of addition of primary and/or secondary antibodies is appropriate to prevent inappropriate cross-reaction of the antibodies with off-target proteins (*see* **Note 19**).
20. Cut out the Transwell as in Subheading 3.15.
21. Ensure membrane is cell-side up on the microscope slide.
22. Add a drop of DAPI mounting medium.
23. Gently cover with a coverslip, avoiding any air bubbles.
24. Seal with nail varnish and store in the dark at 4 °C until used for fluorescent microscopy (*see* Figs. 5, 6, and 7).

4 Notes

1. UV sterilization/cross-linking of the plates is recommended as it helps to assure sterility of the Transwells before use and cross-links the collagen to the Transwell membrane ensuring complete and stable collagen coating. UV sterilization of plates allows the Transwells to be collagen coated in advance and stored at 4 °C. However, this is not essential for successful culture of well-differentiated PAECs. If you do choose to UV-treat the plates, a UV cross-linker set to 184 mW/m^2 is recommended. The lid of the plate must be removed as most plates are not permeable to UV light.

2. Human placental collagen type IV can be used instead of bovine collagen. To prepare this dissolve collagen in sterile filtered glacial acetic acid to a concentration of 2 mg/mL. Incubate at 4 °C for a minimum of 24 h to ensure the collagen has completely dissolved. Keep this stock solution at 4 °C until needed. Dilute the collagen 1:20 with sterile ddH_2O and follow the method for collagen coating described in Subheading 3.1.
3. Trypsin–EDTA is purchased with the following concentrations: 0.5% trypsin and 0.2% EDTA. This is a 10× concentrated solution. To achieve the working concentration of 0.05% trypsin and 0.02% EDTA the stock solution is diluted in sterile PBS. Working concentration trypsin–EDTA can be kept at 4 °C for 3 months. For longer term storage it should be kept at –20 °C. Repeated freeze–thaw cycles should be avoided.
4. The monolayer, 2× ALI, and ALI media often have visible sediment. This is due to the addition of the supplements. This can look like contamination, but it is not. If there is concern about the medium being contaminated, take a small aliquot and incubate at 37 °C to check for bacterial or fungal growth. It is recommended not to do this in the same incubator as any cultured cells to minimize the risk of possible cross-contamination of the cultures. This medium cannot be filtered. Do not heat the medium. Heating may cause degradation of the supplements. Ensure the medium is at room temperature before use.
5. Retinoic acid is extremely light sensitive. Dilution and aliquoting of RA should be carried out in the darkest conditions possible and as rapidly as possible.
6. ALI medium should be made up as needed and not kept for more than 4 weeks.
7. Informed consent must be obtained before sampling from a patient (or parent/guardian) undergoing elective surgery.
8. The T10 flask is a T25 flask placed on its side. The small surface area for culturing is recommended due to the low cell numbers commonly obtained from cytology brushes
9. PAECs should be passaged at between 70 and 85% confluency. Extended culture beyond this level of confluency may compromise successful differentiation of PAECs.
10. The methods above are based on 6.5 mm Transwells. Below are the alterations for using 12 mm Transwells. The method is otherwise the same.
    (a) To collagen coat add 200 μL per Transwell.
    (b) The seeding density of bronchial epithelial cells is 1×10^5/Transwell and 2×10^5/Transwell for nasal epithelial cells. Cell suspension volume for seeding into the apical surface of 12 mm Transwells is 500 μL.

(c) Medium volumes for submerged cultures in 12 mm Transwells: 500 μL apically and 1.5 mL basally.

(d) For ALI culture: 500 μL basally.

(e) To wash cultures during differentiation use 500 μL SF-DMEM.

11. Cells in ALI culture are more sensitive to their environment than standard immortalized cell lines or submerged primary cell cultures. Therefore, it is recommended that you do not use antiseptic water bath/tray treatments in the incubators used for primary cell culture. Such treatments have the potential to damage the cultures and/or change the morphology of the cells. To minimize the risk of contamination of the cultures the incubators should be cleaned every fortnight, and deep cleaned every 8 weeks. If the incubator has a water tray for humidification purposes, the water in the humidity tray should be replaced once a week with sterile distilled water.

12. The medium in ALI cultures should be changed every 2 days. However, it is possible to leave the cultures for 3 days if needed. If 3 days of culture are required before changing the medium, the volume of basal medium should be increased to 350 μL for 6.5 mm and 600 μL for 12 mm Transwells. This should not be done more than once a week.

    Long-reach tips are recommended for changing the medium in the Transwells as well as for apical rinses. They offer more accuracy and reduce the possibility of contamination.

13. For successful differentiation, PAECs should remain in submerged culture until completely confluent, which is commonly attained between for 4–8 days following seeding. This ensures the cells are completely confluent and have formed tight junctions. If ALI is initiated too soon, large holes will appear in the culture and will remain. This renders the culture unusable as there is not an intact layer of cells. Complete differentiation is unlikely as tight junctions are required prior to cilia formation. The range of time required to reach confluency reflects the fact that primary airway epithelial cells from different individuals grow at different rates.

14. Twenty-one days in ALI culture is the minimum time required to obtain cilia coverage and mucus production consistent with appropriate differentiation. As a guideline there should be an average of 60–80% cilia coverage on the apical surface over the whole culture. The center of the cultures is often the last part to reach an adequate level of cilia coverage. Some cultures may take longer to differentiate. For example, cells that are passage more frequently as monolayers will normally take longer to achieve the same cilia coverage. Well-differentiated cultures may be maintained for 3–4 months with no visible signs of deterioration.

15. As indicated above, successful differentiation should result in approximately 60–80% cilia coverage. Cilia are best observed following the aspiration of surface mucus and under phase contrast microscopy at 20× magnification. Cilia will be evident around the periphery of the Transwell initially. When good cilia coverage is observed through the center of the Transwell it is an indication that the cells are ready to be used for experimentation. The transepithelial electrical resistance (TEER) of the Transwells can be measured before use to ensure that tight junctions have formed throughout the culture. Follow the guidelines specific to the TEER meter being used. Ensure the chamber and electrodes are sterile before use. If the meter being used requires a blank reading, use an empty Transwell and ALI medium. Successful cultures should have a reading of 300 Ω/cm^2 or greater.
16. To calculate a multiplicity of infection (MOI) prior to infection of WD-PAECs with RSV, one well should be trypsinized and a cell count undertaken to obtain a representative cell count for all Transwells. The MOI is the ratio of the virus titer to the number of cells counted. It is recommended that a standard total volume is used for apical infection of WD-PAECs. As standard 200 μL of virus inoculum is added apically to 6.5 mm Transwells and 500 μL is added to 12 mm Transwells.
17. WD-PAECs can be infected with different RSV strains. If infecting with a recombinant strain expressing a gene encoding a fluorescent protein, the infection can be monitored in real time with the use of a UV microscope with the correct filter. Although RSV infection does not generally cause substantial damage to WD-PAEC cultures, some strains of RSV may cause slightly more damage to WD-PAEC cultures than others. This can be monitored daily using an inverted light microscope. Damage to WD-PAEC cultures due to RSV infection can include the development of small holes in the culture.
18. Triton X-100 is used to permeabilize cell membranes to make the staining of intracellular proteins possible. However, if you wish to only stain surface proteins then the Triton X-100 step should be removed from the staining protocol. This applies to both the staining of cytospins and WD-PAECs.
19. Depending on the antibody used, incubation of the primary antibody at 4 °C overnight may result in better staining.
20. Where necessary for multi-protein staining in a single culture, the order of addition of primary, secondary and/or conjugated antibodies to the cultures must be carefully planned to avoid off-target cross-reactivities. If all primary antibodies are conjugated to different fluorophores and do not cause target interference, then all may be added together. If, on the other hand, different

protein targets require the use of specific primary and secondary antibody pairs, it is imperative to ensure that the secondary antibodies react only with their cognate primary antibody. Alternatively, where one protein target can be detected directly by a fluorophore-conjugated primary antibody and a second protein target requires a primary/secondary antibody pair, staining the culture sequentially with the primary/secondary antibody pair followed by copious washing before addition of the fluorophore-conjugated antibody will prevent inappropriate cross-reactivity, even if the fluorophore-conjugated antibody is derived from the same animal species as the primary antibody used for the primary/secondary antibody pair. Care is needed in choosing antibodies to ensure there is no cross-reactivity.

21. Cytospins can be stained for mucus producing goblet cells (Muc5Ac) and ciliated cells (β-tubulin) to assess which type of cells are detaching from the culture. A TUNEL assay may also be used to determine if the cytospin contains apoptotic cells. There are a number of appropriate commercial kits available for TUNEL staining. When studying RSV cytopathogenesis, WD-PAEC cultures are routinely stained for mucus producing goblet cells (Muc5Ac), ciliated cells (β-tubulin), tight junctions (zo-1), and RSV. Following staining for RSV syncytia can be seen in WD-PAEC cultures, but they do not occur as frequently in these cultures compared to continuous cell lines *see* Figs. 5, 6, and 7.
22. The best way to ensure the least damage to the culture when cutting out the membrane is to do it in one fluid movement. Insert the scalpel into the underside of the membrane at the edge. Rotate the Transwell while holding the scalpel still. Do not cut the entire way around the edge or the membrane will fall. Leave a small flap attached. Place the Transwell right way up onto a glass slide. The membrane should still be wet enough that it sticks to the slide. If not sufficiently wet, add a small drop of PBS. Tip the Transwell onto its side, in the opposite direction of the flap of membrane that is still attached. The membrane should remain stuck to the slide as you do this. You will see where the membrane is still attached. Take the scalpel and cut through the remaining part attached to the Transwell. The membrane will then be cell-side-up on the slide.
23. Following the removal of the membrane from the Transwell the membrane can halved or quartered. This allows for staining of a broader range of targets to be carried out on the same Transwell. For staining halves or quarters of membranes it is recommended to stain these on a glass slide with PAP pen drawn around it. Alternatively the sections of membrane can be placed in small wells. Be careful to ensure the membrane does not turn over during staining or washing.

References

1. Zhang L, Peeples ME, Boucher RC et al (2002) Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. J Virol 76:5654–5666. doi:10.1128/JVI.76.11.5654-5666.2002
2. Villenave R, Shields MD, Power UF (2013) Respiratory syncytial virus interaction with human airway epithelium. Trends Microbiol 21:238–244. doi:10.1016/j.tim.2013.02.004
3. Gray TE, Guzman K, Davis CW et al (1996) Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. Am J Respir Cell Mol Biol 14:104–112. doi:10.1165/ajrcmb.14.1.8534481
4. Villenave R, Thavagnanam S, Sarlang S et al (2012) In vitro modeling of respiratory syncytial virus infection of pediatric bronchial epithelium, the primary target of infection in vivo. Proc Natl Acad Sci 109:5040–5045. doi:10.1073/pnas.1110203109
5. Pezzulo AA, Starner TD, Scheetz TE et al (2011) The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia. Am J Physiol Lung Cell Mol Physiol 300:L25–L31. doi:10.1152/ajplung.00256.2010
6. Guo-Parke H, Canning P, Douglas I et al (2013) Relative respiratory syncytial virus cytopathogenesis in upper and lower respiratory tract epithelium. Am J Respir Crit Care Med 188:842–851. doi:10.1164/rccm.201304-0750OC
7. Tripp RA (2013) Modeling respiratory syncytial virus cytopathogenesis in the human airway. Am J Respir Crit Care Med 188:766–767. doi:10.1164/rccm.201308-1491ED
8. Doherty GM, Christie SN, Skibinski G et al (2003) Non-bronchoscopic sampling and culture of bronchial epithelial cells in children. Clin Exp Allergy 33:1221–1225. doi:10.1046/j.1365-2222.2003.01752.x

Chapter 10

Reverse Genetics of Respiratory Syncytial Virus

Christopher C. Stobart, Anne L. Hotard, Jia Meng, and Martin L. Moore

Abstract

Respiratory syncytial virus (RSV) is a negative-strand RNA virus that is associated with severe lower respiratory tract infections in young infants and the elderly. RSV remains a leading cause worldwide of infant mortality, and despite the high clinical and economic burden of the virus there are currently no available vaccines. Here, we describe the methods for recovery of recombinant RSV viruses using a bacterial artificial chromosome and methods related to procurement and expansion of stocks of RSV mutants.

Key words Respiratory syncytial virus, RSV, Reverse genetics, Bacterial artificial chromosome, Virus recovery

1 Introduction

Respiratory syncytial virus (RSV) is an enveloped, non-segmented negative-strand RNA virus that is a member of the *Paramyxoviridae* family [1, 2]. RSV was first isolated in 1955 and has since been recognized as a major human pathogen and a leading cause of infant mortality by a virus infection worldwide [3, 4]. The genome of RSV is approximately 15 kb in length and is composed of 10 genes which encode for 11 distinct proteins [2].

The first RSV reverse genetics platform for virus rescue was developed by Peter Collins in 1995 [5]. This platform employs the co-transfection of a plasmid containing the antigenomic cDNA of RSV strain A2 and four helper plasmid constructs encoding the large polymerase (L), phosphoprotein (P), nucleoprotein (N), and matrix 2-1 protein (M2-1) genes of RSV. However, virus rescue systems based on plasmids have demonstrated limited efficiency due to the genetic instability of the cDNA constructs for cloning and recovery [6–8].

Bacterial artificial chromosomes (BACs) are low copy plasmids, which have been successfully used to recover a large number of viruses. BAC constructs offer increased genetic stability and are capable of stable modification through recombination-mediated

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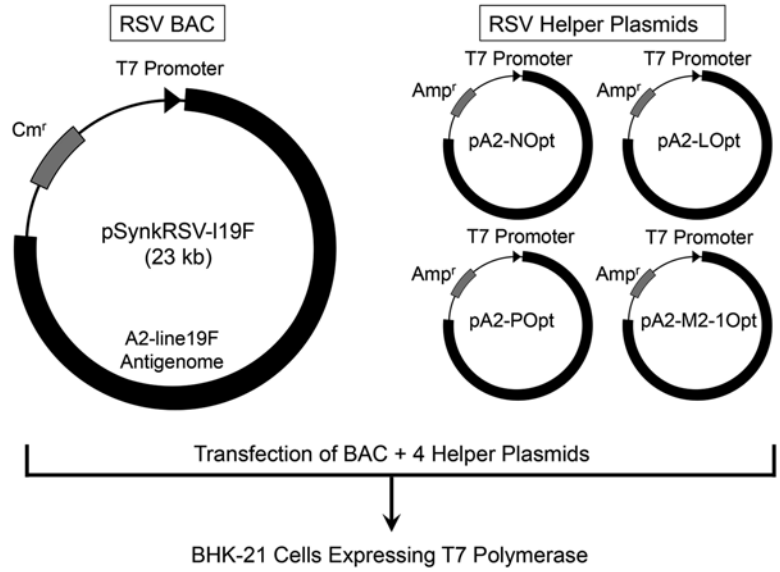

Fig. 1 Overview of the RSV BAC reverse genetics system developed by Hotard et al. [9]. BHK-21 cells expressing T7 polymerase, BSR T7/5 [10] are transfected with BAC construct containing the antigenome of RSV strain A2-line19F under control a T7 promoter (pro) along with four helper plasmids containing human codon bias-optimized sequences for RSV A2 N, L, P, and M2-1 proteins

cloning. We describe here the methods of how the RSV BAC reverse genetics platform is used for recovery of RSV. The method described herein is based on the bacterial artificial chromosome platform developed by Hotard et al. for recovery of RSV [9]. The platform consists of a BAC containing the antigenome of RSV A2-line19F (RSV strain A2 expressing the fusion protein of strain Line 19) under the control of a T7 promoter and four helper plasmids encoding the genes for L, N, P, and M2-1 proteins under the control of a T7 promoter (Fig. 1). Described below is the process of how the BAC constructs, once acquired, are transformed and propagates in bacteria and used for the recovery of RSV infectious clones. Subsequent details are provided which describe how RSV is plaque-purified and how master and working stocks are generated.

2 Materials

Prepare and store all reagents at 4 °C unless otherwise noted.

2.1 Transformation and Expansion of BAC Constructs

1. Competent high efficiency *E. coli* 10-beta cells (stored at −20 °C until use) and SOC Outgrowth Media.
2. Miller's Luria Broth (LB), prepared by suspending 25 g of LB powder in 1 L of water followed by autoclaving.

2.2 RSV Recovery Components

1. BHK-21 cells (ATCC CCL10) expressing the T7 RNA polymerase gene (clone BSR T7/5) were kindly provided by Ursula Buchholz (NIH) [10].
2. 1X phosphate buffered saline (PBS), pH 7.2, without calcium and magnesium.
3. GMEM-3: Glasgow's MEM (GMEM) supplemented with 3% (v/v) fetal bovine serum and 1% antibiotic–antimycotic solution, which contains 10,000 IU/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B.
4. GMEM-10: Glasgow's MEM supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic–antimycotic solution, which contains 10,000 IU/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B.
5. Lipofectamine 2000 Transfection Reagent.
6. Opti-MEM I, Reduced Serum Medium.
7. RSV BAC Antigenome, pSynkRSV-l19F (may be accessed via BEI Resources/ATCC [NR-36460]) (*see* **Note 1**) [9]. This RSV expresses the far-red fluorescent protein monomeric Katushka-2 (mKate2) in the first gene position [6].
8. RSV helper plasmids harboring codon-optimized sequences for L, N, P, and M2-1 proteins: pA2-Lopt [NR-36461], pA2-Nopt [NR-36462], pA2-Popt [NR-36463], and pA2-M2-1opt [NR-36464] (may be accessed via BEI Resources/ATCC) (*see* **Note 2**).

2.3 Plaque Purification and Virus Stock Generation Components

1. HEp-2 cells (ATCC® CCL-23) were used for plaque purification.
2. Minimum essential medium (MEM), serum-free.
3. E-MEM-10: Minimum essential medium (MEM) with Earle's salts (E-MEM) and L-glutamine supplemented with 10% FBS and 1% antibiotic–antimycotic Solution which contains 10,000 IU/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B.
4. E-MEM-20: MEM with Earle's salts (E-MEM) and L-glutamine supplemented with 20% FBS and 1% antibiotic–antimycotic Solution.
5. 2% (w/v) agarose: mix 2 g of agarose with 100 mL of distillated water, sterilize by autoclaving.
6. Overlay medium: Combine equal volumes of E-MEM-20 with 2% (w/v) agarose.

3 Methods

All procedures should be carried out at room temperature unless otherwise noted.

3.1 Transformation and Preparation of RSV BAC Constructs for Transfection

1. To transform the RSV BAC, add 100 ng of the BAC to a thawed tube of 10-beta competent *E. coli* cells on ice. Transform the bacteria following this steps:
   (a) Incubate the mixture of bacteria and BAC construct on ice for 30 min.
   (b) After 30 min, transfer the tube to a 42 °C water bath for a heat shock for 30 s before being returned to ice for 5 min.
   (c) Add 950 μL of room-temperature SOC media and shake the mixture at 250 rpm at 32 °C for 1 h.
   (d) After the 1 h incubation, plate approximately 100 μL of the bacterial suspension on a pre-warmed (32 °C) agar plate containing 12.5 μg/mL of chloramphenicol as a selection agent.
   (e) Incubate the plates at 32 °C overnight (*see* **Note 1**).
2. Following 24 h after transformation, small well-isolated colonies should be visible (*see* **Note 3**). Use one to five colonies to inoculate an equivalent number of cultures of 5 mL of LB broth containing 12.5 μg/mL of chloramphenicol as a selection agent in 14 mL polypropylene round-bottom tubes. Incubate in a shaker at 32 °C at 250 rpm overnight.
3. After 24 h of growth at 32 °C, the 5 ml cultures should be turbid. The small culture(s) may either be used as a starter culture to inoculate directly a larger culture or used for DNA minipreps to analyze constructs:
   (a) Production of a large culture: The 5 mL culture may be added directly to 200 mL of LB broth containing 12.5 μg/mL of chloramphenicol. Similar to the small culture, the large culture is incubated in a shaker at 32 °C at 250 rpm overnight. Following growth, a 0.5 mL aliquot of the bacteria can be mixed with glycerol (1:1 with 50% glycerol or in a final concentration of approximately 20–25% glycerol) and frozen at −80 °C for long-term storage and future growth. Purification of the BAC should be performed using a BAC or large-size DNA purification kit.
   (b) DNA minipreps. Screening by Restriction Digestion: A small aliquot (~5 μL) of the culture suspension may be plated on a pre-warmed (32 °C) agar plate containing 12.5 μg/mL of chloramphenicol as a selection agent for use as a master plate. The bacteria may be pelleted by centrifugation for 10 min at 1200×*g* at room temperature.

Following pelleting, the DNA may be isolated using a miniprep protocol. Special care should be taken to follow miniprep protocols (including all steps), which have been established for recovery of low-copy plasmids, or BAC constructs. BAC constructs should be eluted in nuclease-free water. Restriction digests may be performed to validate the size and orientation of BAC components prior to inoculating a large culture (*see* **Note 4**).

3.2 Recovery of Recombinant RSV A2-Line19F

The protocol described details the recovery of virus using the reverse genetics platform described in Hotard et al. from a BAC [9]. The time frame that is provided may vary considerably based on variations in cell growth, virus attenuation, and replicative dynamics.

3.2.1 Day-1: Preparation of BSR-T7/5 Cells for Transfection

1. In preparation for transfection and virus recovery, remove a confluent 60 cm^2 dish of BSR-T7/5 cells [10], from incubation at 37 °C, and aspirate the supernatant.
2. Wash the cells with a 10 mL of PBS to remove residual media. Remove PBS and add 1 mL of 0.05 % trypsin solution to dissociate the cells.
3. After approximately 3–5 min, the monolayer of cells will be visibly dissociated. Add 26 mL of GMEM-10 to the cells and mix thoroughly to yield a solution of approximately 27 mL of cells.
4. 1 mL of the cell mixture was added to each of six wells in a six well plate. An additional 1 mL of 10 % FBS GMEM was added to each well and the plate placed in a 37 °C in 5 % CO_2 to expand overnight.

3.2.2 Day 0: Transfection for RSV Recovery

In addition to the antigenome of the virus mutant to be recovered, additional controls should be used including a minigenome construct to validate functional helper plasmids, a L gene frameshift mutant antigenomic construct with nonfunctional L as a negative control for rescue, and a wild-type (A2-Line19F) BAC as a positive control for rescue.

1. Evaluate BSR-T7/5 cells for health and confluency. The cells should be at least 95 % confluent at the time of transfection (*see* **Note 5**).
2. Calculate transfection mixture volumes of the antigenome and helper plasmids based on the mass quantities below to yield a total volume of 500 μL per well. Generally, two wells are transfected for each virus recovery. A 3:1 ratio of Lipofectamine 2000 (μL) to DNA (μg) should be used to include the following components:
   (a) RSV Antigenomic BAC (0.8 μg).
   (b) Nucleocapsid (N) Helper Plasmid (0.4 μg).

(c) Phosphoprotein (P) helper plasmid (0.4 μg).

(d) M2-1 helper plasmid (0.4 μg).

(e) Large polymerase (L) helper plasmid (0.2 μg).

(f) Lipofectamine 2000 Reagent (6.6 μL).

(g) Opti-MEM Reagent (up to 500 μL).

3. Following calculation of reaction volumes, add 6.6 μL of Lipofectamine 2000 reagent to the volume of Opti-MEM determined in the previous step. Incubate the resulting mixture for 5 min at room temperature.
4. After 5 min, add RSV antigenomic BAC and helper plasmids to the reaction mixture and incubated for 20 min at room temperature to form complexes. Following this addition, the total volume should be 500 μL for each well to be transformed.
5. After 20 min, remove medium from each well of the six well plate and add the 500 μL transfection complex mixture.
6. Place the plate on a slow rocking platform to incubate at room temperature for 2 h.
7. Following the 2 h incubation, add 500 μL of Opti-MEM to each well (resulting in a total volume of 1 mL) and place the plate in a 37 °C incubator under 5 % CO_2 overnight.

3.2.3 Day 1: Expansion and Recovery of RSV

1. At 16 h post transfection, remove the transfection complex mixtures from each well and wash with PBS to remove residual transfection complex solution (*see* **Note 6**).
2. Add 2 mL of GMEM-3 to each well and incubate at 37 °C under 5 % CO_2 overnight.

3.2.4 Day 2: Transfer of Transfected Cells into Recovery Flask

By Day 2, cells that are producing virus and contain the mKate2 protein (emission wavelength of 633 nm) should be visible under a far-red channel such as Texas Red (*see* Fig. 2) [11]. However, it may remain difficult to identify virus-infected cells by bright field at this time (*see* **Note 7**).

1. Aspirate medium from the plate and wash wells with 1 mL of sterile PBS
2. Dissociate the cells by adding 0.5 mL of 0.05 % trypsin solution to each well to. Gently tap the plate on each side after approximately 3 min to dissociate the cells of the monolayer.
3. Add 3 mL of GMEM-3 to dissociate the cells. Mix thoroughly and transfer to a T25 (25 cm^2) flask. Add 1.5 mL of GMEM-3 to the flask to result in a total volume of approximately 5 mL.
4. Incubated in a 37 °C incubator under 5 % CO_2 overnight.

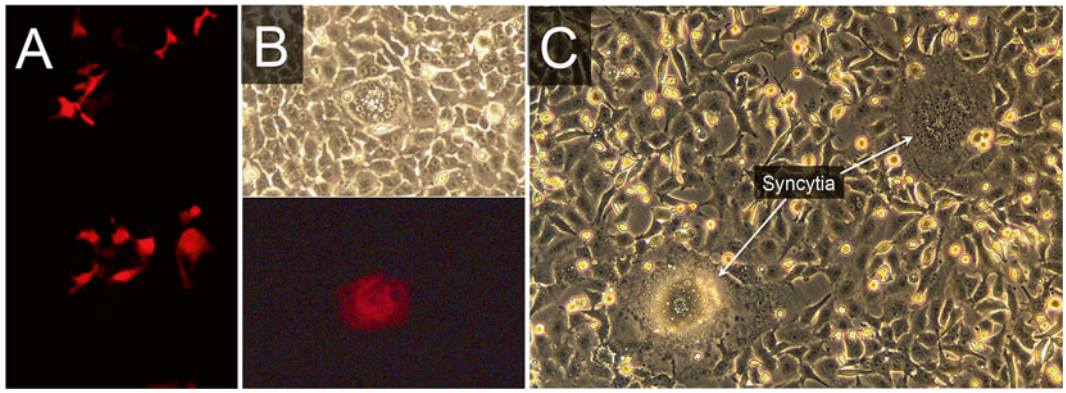

Fig. 2 Images of BSR T7/5 cells transfected with A2-line19F. (**a**) Expression of monomeric Katushka (mKate2) under Texas Red filter channel at 48 h post-transfection. (**b**) An early syncytium in bright-field (*top*) and Texas Red filter channel (*bottom*) at 96 h post-infection. (**c**) CPE evident at 72 h post-transfection. The syncytium on the right remains flattened whereas the syncytium on the left has begun to round up

3.2.5 Day 3–Day 6: Passaging of RSV for Recovery

1. Monitor flasks for cytopathic effects (CPE) over the subsequent days (*see* **Note 8**). When the cells reached 100% confluency, passage them into a subsequent T25 (25 cm^2) flask at a 1:3 split ratio (*see* **Note 9**).
2. Depending on the RSV mutant's growth characteristics, the virus infection will progress from small syncytia, to larger syncytia, to rounded syncytial masses (*see* **Note 8**, Fig. 2).
3. Once the monolayer appears to be involved in greater than 50% syncytial masses, the virus may be harvested. Use of the L frameshift control will result in red cells; however, these cells should not progress to syncytia.

3.2.6 Day 7: Harvest of Virus BSR Stock

1. At the time of harvesting, CPE should be evident throughout the flask with at least half of the monolayer involved in syncytial masses. Five cryovial tubes should be labeled for harvesting of the virus stock.
2. Dissociate the monolayer into the medium by thorough scraping with a cell scraper (*see* **Note 10**). Transfer cell suspension to a 15 mL conical tube and mix by vortexing for approximately 15 s to help dissociate the larger cell/virus masses.
3. After vortexing, aliquot the virus–cell mixture in 1 mL aliquots into the labeled cryovials and placed in storage at −80 °C for plaque purification and/or master stock generation.

3.3 Plaque Purification of RSV

Plaque purification may be performed to enhance genetic homogeneity of a virus stock. Once plaques have been picked, they may be stored for future use at −80 °C.

3.3.1 Day-1: Preparation of HEp-2 Cells for Plaque Purification

In preparation for plaque purification, a 6-well plate of HEp-2 cells should be split for each virus plaque purification to be performed. The HEp-2 cells should be approximately 70% confluent at the time of infection. Described below are the directions for how a 6-well plate may be prepared from a confluent 60 cm^2 dish of HEp-2 cells.

1. In preparation for plaque purification, remove a confluent 60 cm^2 dish of HEp-2 cells from incubation at 37 °C and aspirate supernatant.
2. Wash the cells 10 mL of PBS to remove residual medium.
3. Aspirate PBS and add 1 mL of 0.05% trypsin solution to dissociate the cells.
4. After approximately 3–5 min, the monolayer of cells will be visibly dissociated. Add 25 mL of E-MEM-10 and mix thoroughly to yield a solution of approximately 26 mL of cells.
5. Add 1 mL of the cell mixture to each well of the 6-well plate.
6. Add 1 mL of E-MEM to each well and place the plate in a 37 °C under 5% CO_2.

3.3.2 Day 0: Infection for Plaque Purification of Virus Stock

1. Prepare a 96-well plate for serial dilution of the BSR-derived virus stock. Add 270 μL of cold serum-free MEM to each of the rows (with the exception of the first row) and incubate on ice.
2. Add 300 μL of the BSR-derived stocks in the first row representing the neat dilution.
3. Transfer 30 μL of the neat virus stock to the next row representing a 10^{-1} dilution. Pipette up and down ten times to ensure homogeneity.
4. Repeat process until dilutions were completed through the 10^{-7} dilution.
5. Following completion of the virus dilutions, check the 6-well plates for ~ 70% confluency before the media supernatants is removed by aspiration.
6. Apply 200 μL of each of the virus supernatant for dilutions 10^{-2} to 10^{-7} to wells of the 6-well plate. Slowly rock the plate at room-temperature to ensure that the entire monolayer is infected and remains moist for 1 h to adsorb.
7. After 1 h incubation, add 2 mL of overlay medium to each of the wells (*see* **Note 11**). At the time of addition, the suspension should feel warm to touch (~40–45 °C) without the potential to burn skin.
8. Once the overlays have sat for approximately 5 to 10 min at room temperature, transfer the plates to a 37 °C under 5% CO_2 to incubate.

3.3.3 Day 1–Day ~5: Formation of Visible Plaques

During the next 3–5 days, the visible plaques or fluorescent foci should begin to appear within the monolayer by microscope. Every other day, 1 mL of overlay medium should be added to each well to sustain cell viability. The time required to form visible plaques may vary considerably based on the fitness of the virus.

3.3.4 Day 6: Picking Plaques

Around day 6, visible plaques, which are well isolated, should be present in one of the dilutions in the plate.

1. Holding the plate up to the light, circle potential visible plaques with a fine tipped marker. Examine the circled plaques under a microscope to verify that the circle contains the plaque near center.
2. Using a 1 mL serological pipette, place the opening of the pipette directly over the circled area and firmly press down until it reaches the base of the well (*see* **Note 12**).
3. Use the pipette to scrape the well, dislodge the cells and overlay plug. Once dislodged, aspirate the scrapings into the serological pipette and transfer it into a 1.5 mL microcentrifuge tube containing 1 mL of E-MEM.
4. Vortex the tube before storing at −80 °C or before using. The process of plaque purification may be repeated several times to ensure genetic homogeneity of the resulting culture.

3.4 Generation of Master/Working Stocks

Master stocks represent the first stock of virus generated from either directly from the BSR-derived virus stock or plaque. Typically, a master stock is recovered, split into aliquots, and an aliquot is used for generation of a working stock. RSV stocks may be grown in several different cell lines including VERO or HEp-2 cells. RSV can decline in virus titer over time at −80 °C and requires occasional re-titration of infectivity and regeneration of working stocks.

3.4.1 Day-1: Preparation of Cells for Virus Recovery

The day before infection for master or working stock generation, HEp-2 (or other, e.g., VERO) cells should be passaged to yield a T182 (182 cm^2) flask at approximately 70% confluency next day.

3.4.2 Day 0: Infection for Virus Recovery

The plaque to be used for master or working stock generation should be thawed at 37 °C temperature. Ideally, the flask will be ready for infection near the time of thawing completion.

1. Aspirate the medium from T182 (182 cm^2) and add 2 mL of serum-free MEM to the flask.
2. Add approximately 1 mL of the plaque suspension to the flask yielding a mixture of 3 mL of virus suspension for infection.
3. Incubate at room temperature while rocking (as described above) to ensure adequate infection of the entirety of the cell monolayer.

4. After 1 h, add 47 mL of prewarmed E-MEM-10 and place the flask in a 37 °C incubator under 5 % CO_2 (*see* **Note 13**).
5. The virus will typically take approximately 4–6 days to reach the extent of infection optimal for harvesting the master stock.

3.4.3 Day ~5: Harvest of Virus Stock

Around 5 days post-infection, the monolayer should be at least 50 % involved in syncytia or exhibit the majority of cells infected, as evidenced by mKate2 fluorescence. Common signs of late stage infection include extensive cytoplasmic effects include adherent multinucleated syncytia and large rounded syncytia which have begun the process of detaching from the flask surface. Some strains induce less syncytia than others but spread in HEp-2 monolayers nonetheless, and therefore the mKate2 signal is informative for time of harvest. The flask is ready for harvesting when most of the monolayer exhibits signs of infection and the monolayer may have begun to detach from the flask.

1. Once the flask has reached the point of harvesting, scrape the cells into the supernatant and split the volume between two 50 mL conical tubes.
2. Vortex the tubes of cells for approximately 15 s and incubate on ice for 5 min.
3. Following ice incubation, sonicate the cells on ice at 30 % amplitude for pulses of 1 s with a pause of 1 s between each. The number of pulses is equivalent to the approximate number of mL of cells in the tube.
4. Following sonication, centrifuge the tubes for 10 min at 2000 × *g* at 4 °C to pellet residual cell debris.
5. Pool the supernatant from the tubes into a new 50 mL conical on ice.
6. Aliquot 1 mL of virus per cryovial.
7. Freeze the aliquots by submersion into liquid nitrogen or a bath of dry ice and isopropanol.
8. The virus aliquots should be stored at −80 °C and re-titrated over long storage due to slow degradation of virus.

4 Notes

1. The RSV BAC contains the strain RSV A2 expressing the fusion (F) protein of Line 19. Previous studies have demonstrated that the F protein of Line 19 is associated with higher lung viral load, lung IL-13 levels, increased mucin expression and higher airway dysfunction in mice compared to strains A2 and Long F. The BAC contains selection resistance for chloramphenicol. Generally, the BAC is grown in bacteria at 32 °C

rather than 37 °C for increased genetic stability while cloning and culturing.

2. The BAC and helper plasmids may be accessed through BEI Resources/ATCC as described. The helper plasmids are codon-optimized for expression and are under T7 promoter control. The details for design of these plasmids are described in Hotard et al. [9].
3. The colonies observed following transformation of the BAC might vary in size.
4. There are many different restriction enzymes, which may be used to develop a restriction fingerprint of the BAC. EcoRV digestion of the pSynkRSV-l19F BAC results in seven fragments (7.5, 5.6, 3.3, 3.2, 2.7, 0.2, and 0.2 kb).
5. The confluency of the monolayer must be high at the time of transfection with Lipofectamine. Transfections can be performed with lower levels (<95%) of cell confluency; however, lower transfection efficiencies and thus viral recoveries may occur.
6. The next day the cells will not likely show any apparent signs of virus infection. However, if the virus possesses the monomeric Katushka (mKate) protein, dim red cells should be visible after 24 h infection indicating successful transfection (*see* Fig. 2).
7. At 48 h post-transfection, cells under bright field may start to form rounded syncytia exhibiting 2–8 nuclei. Many times it is difficult to know for sure, however, the mKate2 under red channel should exhibit a strong signature if these masses are true syncytia.
8. Between 3 and 6 days post-transfection, active syncytia should begin to grow and the virus infection should become apparent. The monolayer should go through phases beginning with small syncytia, then larger syncytia, then syncytia that begin to round up, and lastly detachment from the monolayer (Fig. 2).
9. Since RSV is predominately cell associated, very little concern should be made for retaining or recycling media when passaging.
10. It is important that the entire monolayer is scraped into the overlying supernatant to optimize a strong BSR-derived stock for generating master stocks.
11. The temperature of the plaque overlay must be carefully monitored prior to use. If the temperature is too hot (the media is too hot to touch), the cells will be scorched and the monolayer will be lost. If the temperature is too cold (very little or no detectable warmth against the skin), the suspension will harden before there is time for addition. Care should be taken when adding to add the overlay to the side of the wells rather than

directly into the well and not too fast as such actions may detach the monolayer.

12. To ensure disruption of the cells associated with a plaque and that the plaque has been detached, the surface must be adequately disrupted before aspirating the plaque to ensure uptake of the virus and plaque.
13. Some mutants are more sensitive to warmer temperatures and may propagate better at 32 °C, such as A2-line19F-K357T/Y371N [12].

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References

1. Meng J, Stobart CC, Hotard AL, Moore ML (2014) An overview of respiratory syncytial virus. PLoS Pathog 10(4):e1004016. doi:10.1371/journal.ppat.1004016
2. Collins PL, Melero JA (2011) Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years. Virus Res 162(1–2):80–99. doi:10.1016/j.virusres.2011.09.020
3. Blount RE Jr, Morris JA, Savage RE (1956) Recovery of cytopathogenic agent from chimpanzees with coryza. Proc Soc Exp Biol Med 92(3):544–549
4. Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, O'Brien KL, Roca A, Wright PF, Bruce N, Chandran A, Theodoratou E, Sutanto A, Sedyaningsih ER, Ngama M, Munywoki PK, Kartasasmita C, Simoes EA, Rudan I, Weber MW, Campbell H (2010) Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. Lancet 375(9725):1545–1555. doi:10.1016/S0140-6736(10)60206-1
5. Collins PL, Hill MG, Camargo E, Grosfeld H, Chanock RM, Murphy BR (1995) Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5′ proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. Proc Natl Acad Sci U S A 92(25):11563–11567
6. Biacchesi S, Murphy BR, Collins PL, Buchholz UJ (2007) Frequent frameshift and point mutations in the SH gene of human metapneumovirus passaged in vitro. J Virol 81(11):6057–6067. doi:10.1128/JVI.00128-07
7. Stobart CC, Moore ML (2014) RNA virus reverse genetics and vaccine design. Viruses 6(7):2531–2550. doi:10.3390/v6072531
8. Moore ML, Chi MH, Luongo C, Lukacs NW, Polosukhin VV, Huckabee MM, Newcomb DC, Buchholz UJ, Crowe JE Jr, Goleniewska K, Williams JV, Collins PL, Peebles RS Jr (2009) A chimeric A2 strain of respiratory syncytial virus (RSV) with the fusion protein of RSV strain line 19 exhibits enhanced viral load, mucus, and airway dysfunction. J Virol 83(9):4185–4194. doi:10.1128/JVI.01853-08
9. Hotard AL, Shaikh FY, Lee S, Yan D, Teng MN, Plemper RK, Crowe JE Jr, Moore ML (2012) A stabilized respiratory syncytial virus reverse genetics system amenable to recombination-mediated mutagenesis. Virology 434(1):129–136. doi:10.1016/j.virol.2012.09.022
10. Buchholz UJ, Finke S, Conzelmann KK (1999) Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. J Virol 73(1):251–259
11. Shcherbo D, Murphy CS, Ermakova GV, Solovieva EA, Chepurnykh TV, Shcheglov AS,

Verkhusha VV, Pletnev VZ, Hazelwood KL, Roche PM, Lukyanov S, Zaraisky AG, Davidson MW, Chudakov DM (2009) Far-red fluorescent tags for protein imaging in living tissues. Biochem J 418(3):567–574. doi:10.1042/BJ20081949

12. Hotard AL, Lee S, Currier MG, Crowe JE Jr, Sakamoto K, Newcomb DC, Peebles RS Jr, Plemper RK, Moore ML (2015) Identification of residues in the human respiratory syncytial virus fusion protein that modulate fusion activity and pathogenesis. J Virol 89(1):512–522. doi:10.1128/JVI.02472-14

Chapter 11

Use of Minigenome Systems to Study RSV Transcription

Michael N. Teng and Kim C. Tran

Abstract

Minigenome assays have been essential tools in the understanding of viral transcription and RNA replication for respiratory syncytial virus (RSV). Here, we describe the RSV minigenome assay for determining transcription by the viral polymerase in the absence of infection. We detail two different methods of detecting viral RNA synthesis: a firefly luciferase assay for rapid and sensitive measurement of RSV polymerase activity; and a real-time quantitative PCR method for determination of specific effects on the transcription of individual viral genes and the polar transcription gradient of RSV.

Key words RSV transcription, Minigenome, Polymerase, Luciferase assay, Real-time quantitative PCR assay

1 Introduction

The advent of reverse genetics systems over 20 years ago revolutionized the study of viral transcription for non-segmented negative sense RNA viruses. Initial studies utilized viral genome analogs cloned from defective interfering (DI) particles. Expression of these DI genomes in virus-infected cells resulted in the production of new DI particles [1]. Alternatively, synthetic viral genome analogs (minigenomes) were constructed where a reporter gene (chloramphenicol acetyltransferase, CAT) whose transcription was directed by viral transcription start and stop sequences was cloned between the 3′ and 5′ ends of the viral RNA (vRNA) (e.g., [2, 3]). Thus, these minigenomes contained the signals for viral transcription and RNA replication without encoding any viral proteins. The minigenomes were transcribed in vitro, transfected into virus-infected cells, and viral transcription measured by CAT activity. Further refinement of these minigenome systems used co-transfection of cells of a plasmid expressing the minigenome under the control of a T7 RNA polymerase promoter with plasmids encoding the various viral polymerase components, also under the control of a T7 RNA polymerase promoter. The T7 RNA

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polymerase was then provided in *trans* by simultaneous infection with recombinant vaccinia virus (VV-T7) (e.g., [4]). This transfection/infection system allowed for the transient expression of high levels of viral proteins and minigenome, which then could be replicated and transcribed. Importantly, T7 RNA polymerase expression in the cytoplasm allowed for correct subcellular targeting of the transcribed minigenome, since NNS RNA viruses save bornaviruses replicate in the cytoplasm. Cytoplasmic transcription also obviated the potential problem of cryptic splicing of viral protein mRNAs and minigenomes.

The recovery of recombinant NNS RNA virus soon followed [5–7]. However, there were a number of problems resulting from the use of VV-T7, including contamination of newly produced recombinant virus with the vaccinia virus and homologous recombination between transfected plasmids. Therefore, a cell line stably transfected to express T7 RNA polymerase was developed [8]. This cell line, BSR-T7/5, could then be transfected with the T7 plasmids to produce recombinant virus.

Use of the minigenome system for respiratory syncytial virus (RSV) was essential for identifying the M2-1 protein as an essential component of the viral polymerase [9]. In addition, the minigenomes have been further developed to include multiple transcription units and different reporter genes (e.g., green fluorescent protein, firefly luciferase) [10–12]. Currently, we use two different methods to detect viral transcription in minigenome assays. First, when examining the effect of *trans*-acting factors, we evaluate transcription by simple luciferase assay using a minigenome that encodes firefly luciferase as the only gene [12]. This assay shows global effects on RSV polymerase activity but does not distinguish between effects on viral transcription and RNA replication. For more detailed analysis of viral transcription and RNA replication, including the effect of *cis*-acting sequences, we use a real-time quantitative PCR (QPCR) assay to detect viral RNA products from a modified minigenome assay which replaces the luciferase minigenome with the full-length antigenome cDNA used to recover recombinant RSV. The QPCR assay allows us to examine the transcription of individual RSV genes as well as global effects on RSV transcription and the polar transcription gradient. This assay relies on the use of expression plasmids encoding codon-optimized versions of the RSV polymerase genes so that transcripts synthesized by the viral polymerase can be differentiated from those derived from T7 RNA polymerase transcription. In addition, the QPCR assay can be adapted for use in cells or tissues infected by RSV (A subgroup).

2 Materials

2.1 Transfection

1. BSR-T7/5 cells (courtesy of Klaus Conzelmann).
2. Dulbecco's minimal essential medium (DMEM). Store at 4 °C.
3. Complete Medium (CM): DMEM containing 10% (v/v) fetal bovine serum and (FBS) 1× MEM nonessential amino acids. Store at 4 °C.
4. Transfection reagent (GeneJuice, Novagen or equivalent). Store at 4 °C.
5. Plasmids: pcDNA3.1-N, pcDNA3.1-P, pcDNA3.1-M2-1, pcDNA3.1-L (courtesy of Martin Moore, Emory University); C2L and D53 (courtesy of Peter Collins, NIAID); phRL-TK (Promega). Store at −20 °C.
6. 24-well plates.

2.2 Luciferase Assay

1. Dual luciferase reporter assay kit (Promega or equivalent). Store at −80 °C.
2. Phosphate buffered saline (PBS): 10× (80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4{\cdot}7H_2O$, 2 g KH_2PO_4 in 1 l ultrapure water). Dilute to 1× in ultrapure water before use.
3. Black flat-bottom 96-well plate.
4. Plate reading luminometer.
5. Distilled water.
6. 70% ethanol in distilled water.

2.3 Real-Time Quantitative PCR (QPCR) Assay

1. RNAzol.
2. Phosphate buffered saline (PBS): 10× (80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4{\cdot}7H_2O$, 2 g KH_2PO_4 in 1 l ultrapure water). Dilute to 1× in ultrapure water before use.
3. Isopropanol.
4. Nuclease-free water.
5. 75% ethanol in nuclease-free water.
6. cDNA synthesis kit. Store at −20 °C.
7. 2× SYBR Green qPCR kit. Store at −20 °C.
8. Primers (*see* Table 1).
9. 96-well PCR plate, 0.2 ml well.
10. Optical Film PCR plate cover.
11. Standard PCR machine.
12. Real-time PCR machine.

Table 1
List of QPCR primers

RSV gene	Primer sequence (5′ to 3′)	Antigenome position	Fragment size
NS1	F: CACAACAATGCCAGTGCTACAA R: TTAGACCATTAGGTTGAGAGCAATGT	329–343 411–386	82
NS2	F: TGAATGCATAGTGAGAAAACTTGATG R: GGAAAGTGCCATATTTTGTGTTG	762–787 901–879	139
N	F: AAGGGATTTTTGCAGGATTGTTT R: CTCCCCACCGTAGCATTACTTG	1857–1880 1923–1908	66
P	F: TGACCAATGACAGATTAGAAGCT R: TCATTGTCACTATCATTCCCTTCC	2905–2927 3049–3026	144
M	F: ATGTGCTAATGTGTCCTTGGATGA R: TGATTTCACAGGGTGTGGTTACA	3530–3543 3597–3575	67
G	F: AGCCCACAGAAGAGCCAACCA R: TTCCTGTGGTGTTGGAGGTGAGT	5373–5393 5450–5428	77
F	F: TAAGCAGCTCCGTTATCACATCTC R: ATTGGATGCTGTACATTTAGTTTTGC	6865–6888 6938–6913	73
M2	F: CATGAGCAAACTCCTCACTGAACT R: TCTTGGGTGAATTTAGCTCTTCATT	7899–7922 7978–7954	79
L	F: CACTCTACAAAACAAAAAGACACAATCA R: AGGATGCTGCATTGAACACATT	9026–9053 9097–9076	71
Le-NS1	F: TGCGTACAACAAACTTGCAT R: GCTGCCCATCTCTAACCAAG	12–31 107–88	95
SH-G	F: TTAACATCCCACCATGCAAA R: GCATTTGCCCCAATGTTATT	4575–4594 4683–4664	108
L-tr	F: CACAGGTAGTCTGTTATACAACTTTCA R: ACAGTGTTAGTGTATAGCTATGGGAAT	14959–14985 15045–15019	86
18S rRNA	F: GTAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG		

3 Methods

Perform all incubations at room temperature unless otherwise noted.

3.1 Transfection (See Note 1)

1. Plate BSR-T7/5 cells in 24-well clusters (~2×10^4 cells/well) in complete medium (CM). Seed enough wells for triplicate samples.
2. Incubate overnight at 37 °C/5% CO_2.

3. Mix support plasmids in a sterile 1.5 ml microfuge tube (150 ng pcDNA3.1-N, 100 ng pcDNA3.1-P, 50 ng pcDNA3.1-M2-1, 100 ng pcDNA3.1-L per sample) with: either (a) firefly luciferase expressing minigenome (150 ng C2L) and *Renilla* luciferase transfection control (20 ng phRL-TK); or (b) full-length antigenome cDNA (300 ng D53) (*see* **Note 2**).
4. Make a master mix of transfection reagent (3 µl GeneJuice/µg DNA) in serum-free DMEM (250 µl/tube). Incubate for 5 min.
5. Add 250 µl of transfection reagent master mix to each tube, mix by gentle agitation, and incubate for 30 min (*see* **Note 3**).
6. Remove media from 24-well plate of BSR-T7/5 cells.
7. Add 83 µl of DNA/GeneJuice mix per well and incubate at 37 °C/5% CO_2 for 24 h (*see* **Note 4**).
8. Harvest cells for luciferase (*see* Subheading 3.2) or QPCR (*see* Subheadings 3.3–3.5) assay (*see* **Note 5**).

3.2 Luciferase Assay (Dual Luciferase Reporter Assay System Method)

1. Dilute the 5× Passive Lysis Buffer (PLB) in distilled water to make a 1× solution. Make sure PLB is at room temperature before use.
2. Remove media from cells and wash with 1× PBS by rocking plate gently to remove detached cells and residual medium. Completely remove PBS before adding PLB.
3. Add 100 µl PLB to each well (24-well plate), swirl to cover cells. Rock plate for 30 min (*see* **Note 6**). Transfer lysate to 0.5 ml microfuge tube and keep on ice.
4. Spin samples at max speed (13,000 × *g*) for 1 min to clarify (*see* **Note 7**).
5. Prepare Luciferase Assay Reagent II (LAR II) by solubilizing the lyophilized Luciferase Assay Substrate in 10 ml of the Luciferase Assay Buffer II (*see* **Note 8**).
6. Preparation of Stop & Glo Reagent. (Prepare immediately prior to use). Make 1× Stop & Glo Reagent by mixing 1 volume of 50× Stop & Glo Substrate to 50 volumes of Stop & Glo Buffer in glass vial (100 µl reagent per assay) (*see* **Note 9**).
7. Program plate reader luminometer to perform a 2-s premeasurement delay, followed by a 10 s measurement period for each reporter assay. Program the injector system to dispense **50 µl** of *diluted* LARII reagent per well and **50 µl** of *diluted* Stop & Glo reagent per well.
8. Purge all storage liquid (distilled water or 70% ethanol wash solution) from injector. Prime empty injector system with LAR II (Injector #1) or Stop & Glo Reagent (Injector #2) and collect priming reagent in appropriate vials.

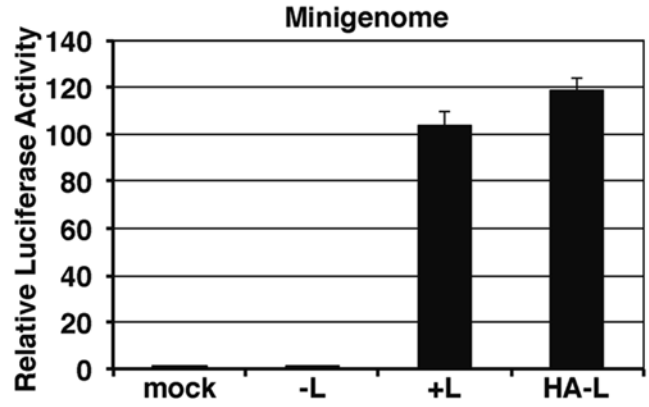

Fig. 1 Luciferase detection for minigenome assay. BSR-T7 cells were transfected with expression plasmids encoding N, P, M2-1, and luciferase (transfection control) plus a firefly luciferase-expressing RSV minigenome plasmid (C2L). Cells were co-transfected with empty vector (–L) or expression plasmids encoding wild-type L (+L) or HA-tagged L (+HA-L). Mock transfected cells were used as a negative control. 24 h post-transfection, lysates were harvested and subjected to Dual Luciferase Assay. Luciferase activities were normalized to those from mock transfected cells. Shown are the averages ± SEM of triplicate samples

9. Dispense **30 µl** (for most luc assays from p24, I usually use 30 µl) of cell lysate samples into appropriate well of black 96-well microplate (*see* **Note 10**). Place the microplate in plate reader (*see* **Note 11**).
10. Run Dual Luciferase Assay program. Make sure injection volume is set to **50 µl** as the SynergyMx will inject 100 µl otherwise.
11. Download the data from the Excel file. Calculate firefly luciferase numbers relative to *Renilla* luciferase. An example of the level of minigenome transcription detectable is shown in Fig. 1, normalizing to mock transfected cells.

3.3 General Injector Wash Protocol (See Note 12)

1. Purge Stop & Glo Reagent from injector lines by repeated priming/washing with distilled water (about 3 pump void volumes).
2. Prime injector system with at least 5 ml 70% ethanol to completely replace the void volume and rinse the injector plumbing. It is preferable to soak in this wash solution for 30 min prior to rinsing with distilled water.
3. Rinse with enough distilled water (at least 3 pump void volumes) to thoroughly remove all traces of ethanol.

3.4 RNA Isolation (See Note 13)

1. Aspirate medium from wells of transfected cells.
2. Wash cells with 1 ml ice-cold PBS and aspirate.

3. Add 250 µl of RNAzol and incubate for 5 min at room temperature.
4. Harvest the RNAzol mixture by carefully swirling the viscous liquid around the well and add to a 1.5 ml microfuge tube.
5. Add 100 µl nuclease-free water to each tube, mix by inversion 6–10 times, and incubate for 15 min.
6. Centrifuge tubes for 10 min at 12,000 × *g* (*see* **Note 14**).
7. Transfer supernatant to fresh microfuge tube, add 1 vol. of isopropanol and mix by inversion. Incubate for 15 min.
8. Centrifuge at 12,000 × *g*, for 10 min.
9. Aspirate supernatant, add 250 µl 75 % ethanol, and centrifuge at 4000 × *g*, for 2 min.
10. Repeat **step 9**.
11. Resuspend pellet in 10 µl of nuclease-free H_2O.

3.5 First Strand cDNA Synthesis (iScript Method) (See Note 15)

This section describes the protocol for the iScript cDNA synthesis kit from Bio-Rad. Follow the manufacturer's recommendation if you are using a different kit.

1. Reaction setup (*see* **Note 16**)

RNA template (500 ng)	*x*µl
5× reaction mix	4 µl
Reverse transcriptase	1 µl
RNase/DNase-free H_2O	*x*µl
Total volume	20 µl (0.2 ml microfuge tube)

2. Reaction protocol (performed in a standard thermocycler):

   5 min., 25 °C

   60 min, 42 °C

   5 min, 85 °C

   Hold at 4 °C

3.6 Real-Time Quantitative PCR Protocol (See Note 17)

1. Reaction setup (*see* **Notes 18–20**).

cDNA	2 µl
Forward primer (10 µM)	1.25 µl
Reverse primer (10 µM)	1.25 µl
RNase/DNase-free H_2O	9 µl
2× SYBR Green mix	12.5 µl

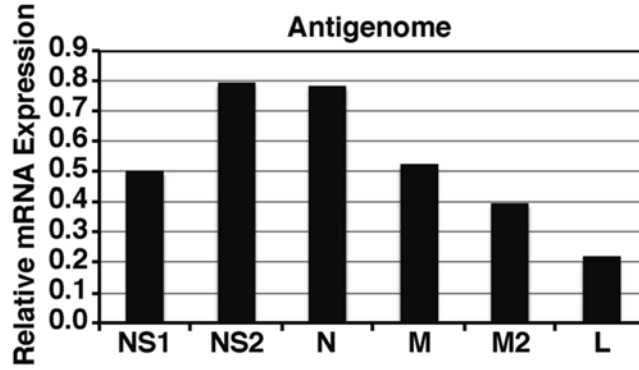

Fig. 2 QPCR assay for antigenome assay. BSR-T7 cells were transfected with expression plasmids encoding N, P, M2-1, and L plus a plasmid containing the full-length antigenome cDNA of RSV (D53). 24 h post-transfection, total RNA was harvested and first strand cDNA was produced using random hexamer priming. QPCR was performed with primers for the indicated genes and SH-G (as a measure of genome/antigenome) and 18S rRNA. Transcript levels were calculated by the ΔΔC(t) method. Shown are the averages of duplicate samples

Run protocol (*see* **Note 21**)
95 °C, 5 min.
40 cycles: 95 °C for 5 s and 60 °C for 15 s

2. Download data and calculate fold increase using ΔΔC(t) method. An example of this analysis is shown in Fig. 2 (*see* **Note 22**).

4 Notes

1. The transfection protocol has been optimized for 24-well plates. However, the protocol can readily be scaled up or down for larger or smaller plates.
2. For negative control, replace pcDNA3.1-L with an equal amount of empty pcDNA3.1.
3. Making a master mix of DNA/transfection reagent for each triplicate then dispensing 1/3 of the volume per well gives us the least amount of sample-to-sample variability.
4. Best results occur with transfection for 6–8 h followed by addition of fresh media (800 μl CM/well).
5. Alternatively, an additional (fourth) well can be transfected to harvest to check for protein expression by immunoblotting.
6. We generally lyse for a minimum 30 min and up to 3 h.
7. Samples can be stored at −80 °C at this point for later analysis.

8. LARII can be stored in 1 ml aliquots at –80 °C (good for up to 1 year). Make sure LAR II is at room temperature before use. Mix LAR II by inversion or gentle vortexing prior to use.
9. LARII and Stop & Glo can be diluted to a working stock before use. We generally use a 1:5 dilution.
10. It is essential to use opaque microplates to prevent cross-talk between samples.
11. The microplate can be washed with water then EtOH and reused 4–8 times.
12. It is essential to carefully clean the injection apparatus after each use.
13. Other RNA isolation methods can be used; however, we use RNAzol because of the lack of DNA contamination of the RNA sample. For other methods, treatment with DNase is recommended prior to cDNA synthesis to avoid spurious results due to contamination of the RNA by the transfected plasmids.
14. It is important to centrifuge specifically at 12,000 × *g*. Higher centrifugation speeds will compress the pellet containing the DNA/protein contaminants.
15. We have had good success using a variety of first strand cDNA synthesis kits though the iScript kit works the best in our hands.
16. We add the RNA to the tube and make up the volume to 5 μl with nuclease-free H_2O. Then, we make a master mix of the 5× reaction mix (4 μl/reaction), reverse transcriptase (1 μl/reaction), and nuclease-free H_2O (10 μl/reaction) and dispense 15 μl of master mix per tube.
17. We usually perform duplicates of each sample to minimize the effects of well-to-well variation.
18. We make a master mix of the forward and reverse primers then dispense 2.5 μl per well. In addition, we make a master mix of the cDNA, H_2O, and 2× SYBR Green reagent then dispense 22.5 μl per well.
19. We generally use human 18S rRNA primers as a housekeeping control. Although BSR-T7/5 cells are derived from the BHK-21 cell line, we see good C(*t*) values with these primers. We have not seen an improvement in inter-assay variation using multiple housekeeping controls.
20. The SH-G primer pair gives the most consistent C(*t*) values between assays for genome/antigenome. The L-tr primer pair gives C(*t*) values that are somewhat higher than the other two pairs.
21. Alternatively, a melt curve protocol (increasing temperature by 0.5 °C/15 s steps) can be added to the end of the run to ensure the relative specificity of the PCR.

22. The fold increase for the viral RNAs is not as high as that seen in infection, likely due to the comparison with excess production of T7-transcribed antigenome that is not properly encapsidated.

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References

1. Pattnaik AK et al (1992) Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell 69(6):1011–1020
2. Park KH et al (1991) Rescue of a foreign gene by Sendai virus. Proc Natl Acad Sci U S A 88(13):5537–5541
3. Collins PL, Mink MA, Stec DS (1991) Rescue of synthetic analogs of respiratory syncytial virus genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. Proc Natl Acad Sci U S A 88(21): 9663–9667
4. Calain P et al (1992) Molecular cloning of natural paramyxovirus copy-back defective interfering RNAs and their expression from DNA. Virology 191(1):62–71
5. Schnell MJ, Mebatsion T, Conzelmann KK (1994) Infectious rabies viruses from cloned cDNA. EMBO J 13(18):4195–4203
6. Whelan SP et al (1995) Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. Proc Natl Acad Sci U S A 92(18):8388–8392
7. Collins PL et al (1995) Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. Proc Natl Acad Sci U S A 92(25):11563–11567
8. Buchholz UJ, Finke S, Conzelmann KK (1999) Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. J Virol 73(1):251–259
9. Collins PL et al (1996) Transcription elongation factor of respiratory syncytial virus, a non-segmented negative-strand RNA virus. Proc Natl Acad Sci U S A 93(1):81–85
10. Kuo L, Fearns R, Collins PL (1996) The structurally diverse intergenic regions of respiratory syncytial virus do not modulate sequential transcription by a dicistronic minigenome. J Virol 70(9):6143–6150
11. Hardy RW, Harmon SB, Wertz GW (1999) Diverse gene junctions of respiratory syncytial virus modulate the efficiency of transcription termination and respond differently to M2-mediated antitermination. J Virol 73(1):170–176
12. Teng MN, Collins PL (1998) Identification of the respiratory syncytial virus proteins required for formation and passage of helper-dependent infectious particles. J Virol 72(7):5707–5716

Chapter 12

Screening for Host Factors Directly Interacting with RSV Protein: Microfluidics

Sarit Kipper, Dorit Avrahami, Monika Bajorek, and Doron Gerber

Abstract

We present a high-throughput microfluidics platform to identify novel host cell binding partners of respiratory syncytial virus (RSV) matrix (M) protein. The device consists of thousands of reaction chambers controlled by micro-mechanical valves. The microfluidic device is mated to a microarray-printed custom-made gene library. These genes are then transcribed and translated on-chip, resulting in a protein array ready for binding to RSV M protein.

Even small viral proteome, such as that of RSV, presents a challenge due to the fact that viral proteins are usually multifunctional and thus their interaction with the host is complex. Protein microarrays technology allows the interrogation of protein–protein interactions, which could possibly overcome obstacles by using conventional high throughput methods. Using microfluidics platform we have identified new host interactors of M involved in various cellular pathways. A number of microfluidics based assays have already provided novel insights into the virus–host interactome, and the results have important implications for future antiviral strategies aimed at targets of viral protein interactions with the host.

Key words RSV–host interaction, Integrated microfluidics, Protein arrays, Virus–host interactions

1 Introduction

A key question in proteomics is how to measure a large number of specific interactions in any given proteome. Even small proteome, such as that of respiratory syncytial virus (RSV) having just 11 ORFs, presents a challenge due to the fact that viral proteins are usually multifunctional and thus their interaction with the host is complex [1]. Several high-throughput methods for screening protein–protein interactions have been commonly used, such as Yeast 2_Hybrid (Y2H) [2, 3] and affinity purification and mass spectrometry [4, 5]. However, many obstacles still remain such as high false positives rates in Y2H and low sensitivity in affinity purification. Overall, most of the interactions found in each of the two methods are not overlapping. Another disadvantage of the traditional protein–protein interaction screening is

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their low success when it comes to measuring interaction of membrane proteins [1].

Protein microarrays technology allows the interrogation of protein–protein interactions, which could possibly overcome the obstacles mentioned above [6]. Such technologies are limited by the need to purify proteins, inadequate functionality and by the inherent inability to measure interactions at equilibrium. Recently, a solution emerged by integrating microfluidic technologies with protein arrays [1, 7–12]. The microfluidic device contains miniature tubing and thousands of embedded valves, used to manipulate liquids on the array. These specialized protein arrays start from DNA arrays and produce fresh proteins on chip. The arduous protein expression and purification is thus eliminated. Another advantage of in vitro protein expression is the ability to match the extract to the protein. For example, bacterial proteins can be expressed with bacterial extracts or microsomal membranes can be added to mammalian membrane proteins [11]. The use of mechanically induced trapping of molecular interactions (MITOMI) to capture interactions at equilibrium allows the detection of weak interactions as well as interactions with fast off-rates, increasing sensitivity [12].

The network of RSV-host protein interactions is still mostly unknown, with limited targets identified. We used an in vitro protein expression and interaction analysis platform based on a highly parallel and sensitive microfluidics affinity assay [13] to identify new host factors interacting with RSV matrix (M). This is the first time microfluidics has been used to screen for host factors interacting with a protein from a negative strand RNA virus. The M protein plays multiple key roles in virus life cycle and thus its interaction with the host is complex. Early in infection M localizes in the nucleus via the action of the nuclear transport protein importin β1 [14], probably serving a dual role of inhibiting host cell transcription [15] as well as preventing inhibition of viral transcription in the cytoplasm [16]. Nuclear targets of M have thus far not been reported. Later in infection, M traffics to the cytoplasm through the action of the nuclear export protein CRM-1 [17] to associate with inclusion bodies (IBs), presumably the site of RSV transcription and replication. It was recently suggested that IBs also serve to sequester cellular proteins involved in the host innate immune response [18]. M localization into IBs is believed to represent a potential switch between viral transcription and assembly [19], with M playing a key structural role in infectious viral particles production [20]. The microfluidics screen identified a range of host factors for the first time, including proteins involved in host transcription and translation regulation, innate immunity response, plasma membrane remodeling, cytoskeleton regulation, and cellular trafficking. A small microfluidics evaluation screen was performed with a number of positive hits verified further by co-precipitation.

2 Materials

2.1 Microfluidics

1. Flexible plastic tubing with internal diameter of 0.02″ (Tygon®).
2. Stainless steel pin (New England Small Tubes Corporation).
3. MicroGrid 610 (Bio Robotics).
4. SMT-S75 silicone pins (Parallel Synthesis, USA).
5. Rabbit reticulocyte Lysate (RRL) quick coupled transcription and translation reaction (Promega) or equivalent RRL transcription/translation System.
6. Microfluidics devices fabricated by the Bar Ilan Microfluidic foundry according to the procedure published [10].
7. Synthetic genes carrying a His tag in C-terminus and a c-Myc tag in N-terminus.
8. Synthetic RSV M gene (or gene of choice) carrying a V5 tag in C-terminal.
9. 384 wells plates.
10. PEG-Trehalose: 1.25% (w/v) Polyethylene glycol (PEG) and 125 mg/ml D-trehalose dihydrate.
11. MicroGrid 610 Microarrayer or equivalent.
12. Biotinylated-Bovine Serum Albumin (BSA), 1 μg/μl.
13. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 mM.
14. Strepavidin: 0.5 μg/μL Neutravidin.
15. Penta-His-Biotin, 0.16 μg/μL.

2.2 Antibodies

1. Anti-Penta-His biotinylated.
2. anti-c-Myc IgG-Cy3-labeled, 0.01 mg/ml.
3. anti-V5 IgG Alexa Fluor 647-labeled.
4. FluoroTect™ GreenLys in vitro Translation Labeling System (Promega).

2.3 Co-precipitation

1. 293 T cells.
2. DMEM medium supplemented with 10% FBS and 1% L-glutamine.
3. Fetal Bovine Serum (FBS).
4. DNA transfection reagent of choice.
5. Lysis buffer: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 supplemented with protease inhibitor cocktail.
6. Wash buffer: 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Triton X-100.
7. StrepTactin Sepharose (slurry).

3 Methods

3.1 Microfluidics

Coding regions for 500 proteins from the human proteome are assembled using PCR and selected clones from the public genome-scale lentiviral sequence-confirmed expression library of human ORFs (open reading frames) in a Gateway vector system [21] as templates. PCR products are generated by using specific primers encoding c-Myc and Penta-His tags at the N and C terminal respectively for all of the ORFs (Fig. 1) (*see* **Notes 1** and **5**).

3.1.1 DNA Arraying and Device Alignment

1. Produce synthetic genes by assembly PCR. The synthetic genes are compose of T7 promoter, ribosome binding site (RBS), ORF with two epitope tags (*see* **Note 4**) (c-Myc and His tag one at each end), and T7 terminator. The genes can vary in length from 100 bp up to, at least, 5000 bp.
2. Prepare the synthetic genes for arraying by dispensing 2 μl of PEG-Trehalose per reaction into 384 wells plate. This solution will reduce irreversible binding of the DNA to the glass as well as for visualization during alignment [22].
3. Transfer the synthetic genes to the 384 wells plate. Usually DNA concentrations can range from 10 ng/μl to 100 ng/μl final concentration. Add dH_2O to a final volume of 20 μl (depends on microarray device used and pin type).
4. Spot a series of synthetic genes onto epoxy coated glass substrates using a microarrayer. We use MicroGrid 610 with

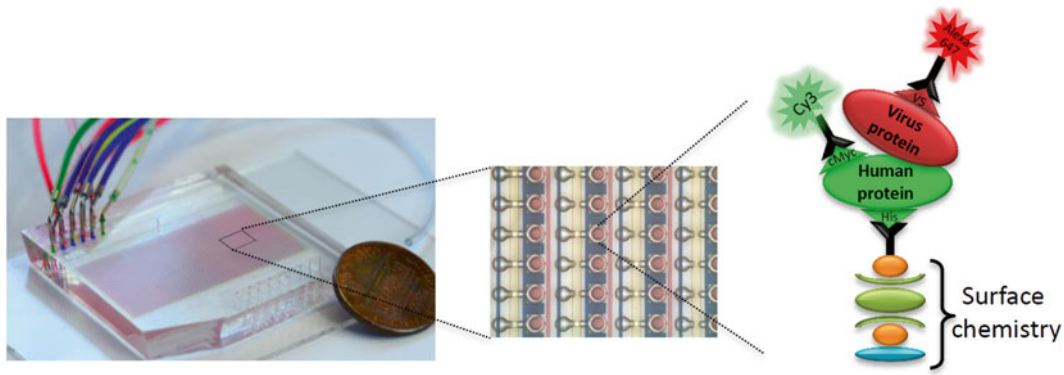

Fig. 1 An illustration of a protein array on-chip, surface chemistry and protein interaction. Protein array on-chip is shown on the *left*. Individual DNA and protein chambers are shown in the middle. Zoom-in on protein chamber is shown on the *right*. DNAs encoding human ORFs with N-terminal c-Myc and C-terminal Penta-His tags were expressed in vitro using TNT (transcription and translation mix). Biotinylated BSA was bound to the surface within the microfluidic device to enable streptavidin to form a sandwich between the surface-bound BSA and biotinylated anti-Penta-His antibody, to enable expressed human proteins to be immobilized onto the surface via the Penta-His tag. Viral protein M was produced separately with a V5 tag at the C terminus, and then flowed into the device and incubated in the reaction chamber to allow binding to human proteins. Interaction was detected by labeling human proteins with Cy3-labeled anti c-Myc antibody and by labeling M with fluorescent Alexa Fluor 647

SMT-S75 silicone pins. Contact printing of the DNA, with these specific pins, results in spots with diameter of about 100 μm on the glass surface. Each pin load is enough for approximately 100 spots. Make sure the column and row pitches correspond to the specific device used. The device we use contains 16 columns and 40 rows with a pitch of 680 μm by 320 μm, respectively (*see* **Note 11**).

5. Manually align the microfluidic device to the gene array under a stereoscope. The DNA spot should be in the middle of the DNA chambers. Start the alignment by locating the first row of spots below the first row of DNA chambers. Then, bring into line the rest of the rows finishing with fine tuning of the whole device. Finally, bond the device to the glass slide by overnight incubation on a hot plate at 80 °C (*see* **Note 8**).
6. Connect the device to a controller and activate pressures to fill valves with water. Test each valve as it fills for functionality. Once control valves are primed, the device is ready for the experiment (*see* **Note 10**).

3.1.2 Surface Chemistry

In order to facilitate the self-assembly of a protein array on the surface and prevent nonspecific adsorption within the microfluidic device, the surface is chemically modified.

1. To flow a component through the device (*see* **Note 2**), connect a new tube with the required solution to one of the flow channels in the device. Connect the free side of the tube to the manual manifold and open the air pressure flow (5 PSI).
2. Load 40 μl of biotinylated-BSA in a new tube and flow approximately half of it for 20 min through the device, the BSA will bind to the epoxy surface.
3. Use HEPES (*see* **Note 12**) for washing unreacted substrate between each of the different surface chemistry steps.
4. Flow 25 μl streptavidin for 20 min on top of the biotinylated-BSA.
5. Wash with HEPES for 5 min.
6. Close the "button" valve and flow the rest of the biotinylated-BSA (as described above), passivating the surface surrounding the button.
7. Wash with HEPES for 5 min.
8. Release the "button" valve and flow 30 μl of Penta-His-biotin for 20 min. The antibody will bind to the exposed streptavidin: specifically to the area under the "button" creating an anti His-tag array.

3.1.3 Protein Expression and Labeling

Express the proteins on the device using RRL quick coupled transcription and translation reaction. Protein expression from the spotted synthetic genes on the device creates an array of proteins ready to use.

1. Open the "neck" valves and flow 12.5 μl RRL quick coupled transcription and translation reaction through the device into the DNA chamber. Next, close the "sandwich" valves and separate each gene from its environment.
2. Incubate the device on a hot plate for 2.5 h at 32 °C. Expressed proteins will diffuse from the DNA chamber to the reaction chamber spontaneously (neck valve is open) and bind to the anti-His antibody under the "button" valve immobilizing the protein through its C-terminus tag.
3. Label the proteins with 0.01 mg/ml Cy3-labeled anti-c-Myc antibody. The antibody will bind to its corresponding epitope located at the protein N-terminus.
4. Determine protein expression levels with a microarray scanner using a 532 nm laser and 575 nm emission filter.
5. Express the RSV M protein in an Eppendorf microfuge tube (*see* **Note 3**). Produce the M clone using the same PCR approach as above, but with C-terminal primer encoding the V5 tag (*see* **Notes 4** and **6**). Express the protein by in vitro transcription/translation from the linear PCR fragment in a final volume of 25 μL. Incubate as above, before flushing into the device.

3.2 Small Evaluation Microfluidics Screen

1. Add 1 μg of PCR-generated human ORF linear expression templates directly to RRL off-chip in an Eppendorf microfuge tube in a final volume of 25 μL in the presence of a FluoroTect™ GreenLys in vitro Translation Labeling System (*see* **Note 9**). Proteins (Bait) will be immobilized to the surface through their Penta-His tag.
2. Separately express the RSV M protein (Prey) in an Eppendorf microfuge tube in a final volume of 25 μL by RRL quick coupled transcription/translation system.
3. Flush Bait proteins into the device for 20 min each. Then flush the RSV M protein on top of the whole device. Close the sandwich valve and incubate Prey with Bait proteins for 30 min.
4. Open sandwich valve and close button valve. Wash the device with wash buffer for 5 min after each step.
5. Flush the fluorescently labeled antibodies (diluted 1:100) (*see* **Note 13**). Close sandwich valve and open button for 30 min. Then open sandwich, close button valves and wash excess antibodies for 5 min.
6. Scan the device in a microarray scanner. Bait fluorescent excitation is done at 488 nm and emission at 530 nm. Prey fluorescent excitation is done at 635 nm and emission at 670 nm. The signals Bait should be normalized to the number of Lysines in each protein [10].

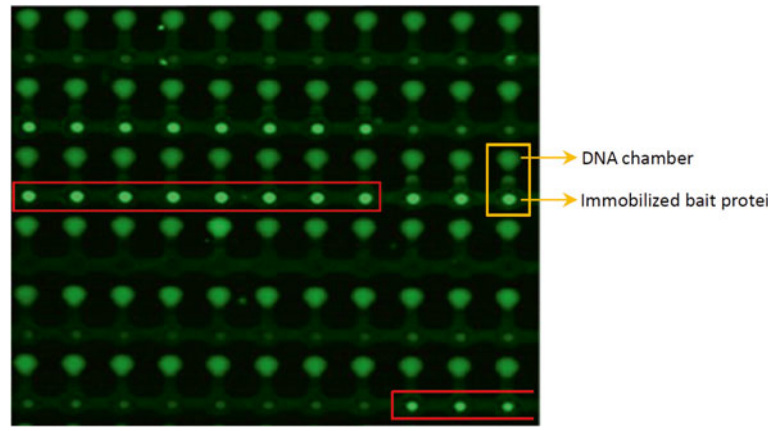

Fig. 2 A microarray laser scanning image of microfluidic device expressing a human protein array ready for RSV M protein interaction screen. The proteins were expressed from spotted gene array using cell free transcription and translation system. Proteins were immobilized to the device surface by their C-terminus tag forming a protein array, and labeled by a fluorescent antibody specific to their N-terminus tag (*lower green dots*). *Red frame* encompasses repeats of the same Bait protein ($n=8$), *yellow box* represents a single cell with DNA chamber and immobilized Bait protein

3.3 Protein Network Interaction Generator (PING)

1. Extract the Bait and RSV M (Prey) median signals for each position in the array (Fig. 2).
2. Calculate the fluorescence ratio of RSV M signal over the Bait signal (*see* **Note 7**). Average the repetitions and calculate the standard deviation.
3. Ratios that are more than 3 standard deviations above negative controls are considered as interactions. In our case, negative controls can be proteins that are known controls or positions with Bait protein, but not Prey.

3.4 Validation of Interactions by Co-precipitation

1. Seed 293 T cells in DMEM medium supplemented with 10% FBS and 1% L-glutamine at 1.5×10^6 cells per 60 mm dish.
2. 24 h after seeding transfect cells with 4 μg of DNA encoding the One-STREP-FLAG (OSF) tagged M protein and Myc-tagged host protein using the transfection reagent of choice.
3. Harvested cells 36 h post-transfection and lyse in 300 μL Lysis buffer for 10 min.
4. Clarify the lysates by centrifugation at $11{,}600 \times g$, for 6 min at 4 °C.
5. Incubate the lysate containing StrepTactin-tagged M and Myc-tagged host proteins affinity with StrepTactin Sepharose for 2 h (40 μl slurry).
6. Wash the beads 3 times in wash buffer and detect bound proteins by Western blot (*see* Chapter 13, Subheadings 3.4 and 3.6).

4 Notes

1. Make sure that the stop codon is removed at the end of the ORF before adding an epitope tag.
2. Spin down all reagents before loading on the device to prevent clogging.
3. For proteins that are synthetized in an Eppendorf tube and flushed on the microfluidics chip, we recommend using freshly synthesized proteins. However, freezing at –80 °C also works.
4. Both Bait and Prey proteins can be tagged with different epitope tags, depending on the availability of biotinylated and fluorescent antibodies.
5. Since Bait proteins are labeled from both directions, we observe only full-length proteins. Another benefit is that we can switch directions and immobilize the protein from their N-terminus instead of their C-terminus. Bait and Prey can also be switched (RSV M on the surface and human proteins in solution).
6. In our screen, we discovered that RSV M N-terminus is hidden and the tag cannot be labeled.
7. In case of protein oligomerization, signals reflect avidity.
8. During DNA arraying and device alignment; once the array is aligned care should be taken to alleviate any stress in the PDMS, by lifting it locally. If there is tension left in the PDMS it may not bond well to the microarray.
9. Before using FluoroTect™ GreenLys for labeling, make sure that your protein's sequence contain above five lysine residues.
10. Make sure air bubbles do not go into the device, it can affect the device modified surface chemistry.
11. Microarray print is good for over a month if kept in dry conditions.
12. Wash buffer can be stored at room temperature.
13. Diluted antibodies for microfluidics need to be fresh.

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References

1. Gerber D, Maerkl SJ, Quake SR (2009) An in vitro microfluidic approach to generating protein-interaction networks. Nat Methods 6(1):71–74. doi:10.1038/nmeth.1289
2. Fields S, Song O (1989) A novel genetic system to detect protein-protein interactions. Nature 340(6230):245–246. doi:10.1038/340245a0
3. Parrish JR, Gulyas KD, Finley RL Jr (2006) Yeast two-hybrid contributions to interactome mapping. Curr Opin Biotechnol 17(4):387–393. doi:10.1016/j.copbio.2006.06.006
4. Deshaies RJ, Seol JH, McDonald WH, Cope G, Lyapina S, Shevchenko A, Verma R, Yates JR III (2002) Charting the protein complexome in yeast by mass spectrometry. Mol Cell Proteomics 1(1):3–10
5. Ho Y, Gruhler A, Heilbut A, Bader GD, Moore L, Adams SL, Millar A, Taylor P, Bennett K, Boutilier K, Yang L, Wolting C, Donaldson I, Schandorff S, Shewnarane J, Vo M, Taggart J, Goudreault M, Muskat B, Alfarano C, Dewar D, Lin Z, Michalickova K, Willems AR, Sassi H, Nielsen PA, Rasmussen KJ, Andersen JR, Johansen LE, Hansen LH, Jespersen H, Podtelejnikov A, Nielsen E, Crawford J, Poulsen V, Sorensen BD, Matthiesen J, Hendrickson RC, Gleeson F, Pawson T, Moran MF, Durocher D, Mann M, Hogue CW, Figeys D, Tyers M (2002) Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature 415(6868):180–183. doi:10.1038/415180a
6. Chen R, Snyder M (2010) Yeast proteomics and protein microarrays. J Proteome 73(11):2147–2157. doi:10.1016/j.jprot.2010.08.003
7. Meier M, Sit R, Pan W, Quake SR (2012) High-performance binary protein interaction screening in a microfluidic format. Anal Chem 84(21):9572–9578. doi:10.1021/ac302436y
8. Bates SR, Quake SR (2009) Highly parallel measurements of interaction kinetic constants with a microfabricated optomechanical device. Appl PhysLett95(7):73705.doi:10.1063/1.3211382
9. Ben-Ari Y, Glick Y, Kipper S, Schwartz N, Avrahami D, Barbiro-Michaely E, Gerber D (2013) Microfluidic large scale integration of viral-host interaction analysis. Lab Chip 13(12):2202–2209. doi:10.1039/c3lc00034f
10. Glick Y, Avrahami D, Michaely E, Gerber D (2012) High-throughput protein expression generator using a microfluidic platform. J Vis Exp 66, e3849. doi:10.3791/3849
11. Einav S, Gerber D, Bryson PD, Sklan EH, Elazar M, Maerkl SJ, Glenn JS, Quake SR (2008) Discovery of a hepatitis C target and its pharmacological inhibitors by microfluidic affinity analysis. Nat Biotechnol 26(9):1019–1027. doi:10.1038/nbt.1490
12. Maerkl SJ, Quake SR (2007) A systems approach to measuring the binding energy landscapes of transcription factors. Science 315(5809):233–237. doi:10.1126/science.1131007
13. Kipper S, Hamad S, Caly L, Avrahami D, Bacharach E, Jans DA, Gerber D, Bajorek M (2015) New host factors important for Respiratory Syncytial Virus replication revealed by a novel microfluidics screen for interactors of matrix protein. Mol Cell Proteomics. doi:10.1074/mcp.M114.044107
14. Ghildyal R, Ho A, Wagstaff KM, Dias MM, Barton CL, Jans P, Bardin P, Jans DA (2005) Nuclear import of the respiratory syncytial virus matrix protein is mediated by importin beta1 independent of importin alpha. Biochemistry 44(38):12887–12895. doi:10.1021/bi050701e
15. Ghildyal R, Baulch-Brown C, Mills J, Meanger J (2003) The matrix protein of Human respiratory syncytial virus localises to the nucleus of infected cells and inhibits transcription. Arch Virol 148(7):1419–1429. doi:10.1007/s00705-003-0112-y
16. Ghildyal R, Mills J, Murray M, Vardaxis N, Meanger J (2002) Respiratory syncytial virus matrix protein associates with nucleocapsids in infected cells. J Gen Virol 83(Pt 4):753–757
17. Ghildyal R, Ho A, Dias M, Soegiyono L, Bardin PG, Tran KC, Teng MN, Jans DA (2009) The respiratory syncytial virus matrix protein possesses a Crm1-mediated nuclear export mechanism. J Virol 83(11):5353–5362. doi:10.1128/JVI.02374-08
18. Lifland AW, Jung J, Alonas E, Zurla C, Crowe JE Jr, Santangelo PJ (2012) Human respiratory syncytial virus nucleoprotein and inclusion bodies antagonize the innate immune response mediated by MDA5 and MAVS. J Virol 86(15):8245–8258. doi:10.1128/JVI.00215-12
19. Li D, Jans DA, Bardin PG, Meanger J, Mills J, Ghildyal R (2008) Association of respiratory syncytial virus M protein with viral nucleocapsids is mediated by the M2-1 protein. J Virol 82(17):8863–8870. doi:10.1128/JVI.00343-08
20. Bajorek M, Caly L, Tran KC, Maertens GN, Tripp RA, Bacharach E, Teng MN, Ghildyal R, Jans DA (2014) The Thr205 phosphorylation site within respiratory syncytial virus matrix (M) protein modulates M oligomerization and virus production. J Virol. doi:10.1128/JVI.03856-13

21. Yang X, Boehm JS, Salehi-Ashtiani K, Hao T, Shen Y, Lubonja R, Thomas SR, Alkan O, Bhimdi T, Green TM, Johannessen CM, Silver SJ, Nguyen C, Murray RR, Hieronymus H, Balcha D, Fan C, Lin C, Ghamsari L, Vidal M, Hahn WC, Hill DE, Root DE (2011) A public genome-scale lentiviral expression library of human ORFs. Nat Methods 8(8):659–661. doi:10.1038/nmeth.1638
22. Kusnezow W, Hoheisel JD (2003) Solid supports for microarray immunoassays. J Mol Recognit 16(4):165–176. doi:10.1002/jmr.625

Chapter 13

A Proteomic-Based Workflow Using Purified Respiratory Syncytial Virus Particles to Identify Cellular Factors as Drug Targets

Tra Nguyen Huong, Boon Huan Tan, and Richard J. Sugrue

Abstract

The identification of cellular factors that play a role in respiratory syncytial virus (RSV) replication is an alternative strategy in the identification of druggable cellular protein that are essential for RSV replication. In this regard experimental strategies that are able to screen relevant proteins from the vast array of proteins in the cellular milieu will facilitate the identification of potential drug targets. In this chapter we describe a procedure where RSV particles are purified from cells that are permissive for RSV infection, and the protein composition of the purified virus particles characterized using a proteomics-based strategy. This procedure revealed that actin, several actin-binding proteins, and the chaperones HSP70 and HSP90 also co-purified with the virus particles. The relevance of the HSP90 protein to virus replication was then further validated using imaging, gene silencing and by using an established small molecule HSP90 inhibitor.

Key words Respiratory syncytial virus, Paramyxovirus, LC MS/MS, Proteomics, HSP90, Virus morphogenesis, Cell factors, Cytoskeleton, Virus filaments

1 Introduction

Respiratory virus (RSV) morphogenesis occurs on the surface of infected cells, and the virus matures with a filamentous morphology. These structures are referred to as virus filaments, and they can be easily visualized on RSV-infected cells using light and electron microscopy [1]. Furthermore, inhibition of virus filament formation correlates with impaired virus transmission [2], suggesting that these structures may play a direct role in cell-to-cell transmission. Therefore, drugs that target the process of virus filament formation represent a potential antivirus strategy to prevent and treat RSV infection.

Previous studies have indicated that a variety of different cellular factors play a role in the formation of these virus filaments. The involvement of lipid raft microdomains in the formation of

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virus particles has been demonstrated [3–6], suggesting that destabilizing lipid raft domains in permissive cells can inhibit RSV infectivity. The cortical cytoskeleton network also plays an important role in this RSV morphogenesis [2, 7, 8], and rhoA kinase is a raft-associated signaling molecule that mediates actin remodeling [9] and which is required for virus filament formation [10]. Phosphoinositide 3-kinase (PI3K) is a cellular enzyme involved in regulating rhoA activity, and PI3K is also required for virus filament formation [6, 8]. Although the cellular processes that lead to assembly of the mature virus filaments are still not fully understood, these previous observations highlight the importance of cellular factors in mediating the process of RSV morphogenesis.

Druggable cellular factors that participate in virus particle assembly can offer an alternative antiviral strategy to the current paradigm of directing anti-virus strategies against virus proteins with defined activities. In this respect, the identification of cellular proteins that interact with the virus particles will facilitate the identification of protein and cellular pathways that play a role in RSV maturation. Proteomic-based technologies can lead to the high throughput identification of cellular factors involved in virus-replication (reviewed in ref. 11), and the application of these technologies to RSV replication should aid in the development of novel drug targets for the treatment and prevention of RSV infection.

In this chapter we describe a proteomics-based workflow whereby the RSV particles are purified and the protein composition of the purified particles examined. Identifying cell proteins that co-purify with the virus particles is a first step to screen for cellular factors that may be involved in virus filament formation. Following the identification of candidate cell proteins by mass spectrometry an important next step is to confirm the presence of these proteins in the virus preparation. However, it is also anticipated that during the virus purification procedure low levels of irrelevant contaminating cell proteins may also be present in the virus preparation. The final step in the validation process is to determine the association of the cell proteins with virus particles in virus-infected cells, and to determine if the cellular proteins identified also play a role in virus infection. The validation step is an essential part of the proteomic workflow, and examples of these strategies to validate a role for HSP90 in RSV morphogenesis will be described.

2 Materials

2.1 RSV Production and Purification

1. 175 cm^2 tissue culture flask.
2. HEp-2 cells (ATCC® CCL-23).
3. RSV virus stock.

4. Cell maintenance medium: Dulbecco's modified Eagle's medium (DMEM), 2 mM GlutaMAX, 2% (v/v) heat-inactivated fetal bovine serum (FBS) and 1× penicillin–streptomycin.
5. Cell culture medium: DMEM, 2 mM GlutaMAX, 10% (v/v) FBS and 1× penicillin–streptomycin.
6. Sterile acid-washed 3 mm diameter glass beads.
7. Polyethylene glycol (PEG)-6000.
8. Hanks' buffered saline solution (HBSS).
9. Ultracentrifuge.
10. 20% (w/v) sucrose: 20 g of ultrapure sucrose, HBSS to 100 mL.
11. 35% (w/v) sucrose: 35 g of ultrapure sucrose, HBSS to 100 mL.
12. 45% (w/v) sucrose: 45 g of ultrapure sucrose, HBSS to 100 mL.
13. 60% (w/v) sucrose: 60 g of ultrapure sucrose, HBSS to 100 mL.
14. Phosphate-buffered saline (PBS) 1×.
15. Formvar-coated grid.
16. 2% PTA: 2 g of phosphotungstic acid in water. Adjust pH to 7.5.
17. Whatman 3MM filter paper.
18. JEOL 1200 EX electron microscope.

2.2 RSV Protein Analysis by SDS-PAGE

1. Sonicator.
2. 1× boiling mixture: 1% (w/v) sodium dodecyl sulfate (SDS), 15% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, 60 mM sodium phosphate, pH 6.8.
3. SDS-polyacrylamide gel electrophoresis (PAGE) reagents: 15% polyacrylamide gel, vertical electrophoresis system, power supply, running buffer, protein standard.
4. Coomassie brilliant blue solution: 0.25% (w/v) Coomassie Brilliant Blue G-250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid.
5. Destaining solution: 45% (v/v) methanol and 10% (v/v) glacial acetic acid.
6. Fixing solution: 50% (v/v) methanol and 7% (v/v) glacial acetic acid.
7. Wash solution: 10% (v/v) methanol and 7% (v/v) glacial acetic acid.
8. SYPRO® Ruby Red gel stain solution.
9. Ultrapure water.
10. Typhoon Trio Imager.

2.3 One Dimensional LC-MS/MS (1D LC MS/MS)

1. 25 mM ammonium bicarbonate (ABC) containing 2% (w/v) SDS.
2. 40% acrylamide–bisacrylamide (29:1) gel matrix.

3. Ammonium persulfate (10%): Dissolve 1 g ammonium persulfate in 10 mL of H_2O and store at 4 °C. Ammonium persulfate decays slowly in solution, so replace the stock solution every 2–3 weeks.
4. *N*,*N*,*N*′,*N*′-tetramethylethane-1,2-diamine (TEMED).
5. 25% (v/v) ammonium bicarbonate (ABC) and 50% (v/v) acetonitrile (ACN).
6. 25 mM ABC and 10 mM dithiothreitol (DTT).
7. 25 mM ABC and 55 mM iodoacetamide.
8. 25 mM ABC and trypsin (protein–trypsin = 10:1, g/g).
9. 5% (v/v) formic acid (FA) in water.
10. 5% (v/v) formic acid in acetonitrile.
11. Buffer A: 0.1% (v/v) formic acid and 2% (v/v) acetonitrile.
12. Buffer B: 0.1% (v/v) formic acid and 90% (v/v) acetonitrile.
13. A nanoflow liquid chromatography system (UltiMate Plus, Dionex).
14. Nano-LC column (C18 PepMap100, 3 μm, 0.075 × 150 mm, Dionex).
15. LTQ Orbitrap (Thermo, Bremen, Germany).

2.4 Western Blot

1. 15% SDS-PAGE.
2. Blotting apparatus.
3. PVDF membrane.
4. Blocking solution: 5% (w/v) Skim milk, 1× PBS, and 0.05% (v/v) Tween 20.
5. PBS-T: 1× PBS and 0.05% (v/v) Tween 20.

   Primary Antibodies: Anti-RSV serum, anti-F (MAb19) (G. Taylor, IAH), anti-G (Mab30), anti-N (MRC), anti-M2-1 (MRC), anti-F (polyclonal) (Jose Melero, Madrid), anti-β-actin, anti-filamin-1, anti-HSC70, anti-HSP70, anti-actin, anti-HSP90A, anti-cofilin-1 (Cytoskeleton).
6. Secondary antibodies: anti-mouse or rabbit IgG conjugated to antibodies conjugated to HRP.
7. HRP Chemiluminescent Substrate (ECL)

2.5 Immunostaining

1. 12-mm glass coverslips.
2. 3% paraformaldehyde (PFA): mix 3 g of paraformaldehyde with 50 mL of water. Add 1 mL of 1 M NaOH and stir gently on a heating block at ~60 °C until the paraformaldehyde is dissolved. Add 10 mL of 10× PBS and allow the mixture to cool to room temperature. Adjust the pH to 7.4 with 1 M HCl, then adjust the final volume to 100 mL with H_2O. Filter the

solution through a 0.45-μm membrane filter to remove any particulate matter.

3. 0.1% (w/v) saponin: 0.1 g of saponin in 100 mL 1× PBS.
4. 0.1% (w/v) BSA: 0.1 g bovine serum albumin in 100 mL 1× PBS.
5. Primary Antibodies: Anti-RSV serum, anti-F (MAb19) (G. Taylor, IAH), anti-G (Mab30), anti-N (MRC), anti-M2-1 (MRC), anti-F (polyclonal) (Jose Melero, Madrid), anti-β-actin, anti-filamin-1, anti-HSC70, anti-HSP70, anti-actin, anti-HSP90A, anti-cofilin-1 (Cytoskeleton).
6. Secondary antibodies: anti-mouse IgG conjugated to FITC and anti-rabbit IgG conjugated to Cy3.
7. Fluorescence mounting medium (Dako).
8. Nail varnish.
9. Tanespimycin (17-*N*-allylamino-17-demethoxygeldanamycin, 17-AAG 2 μM 17AAG diluted in MEM containing 2% FBS, and 1% penicillin–streptomycin.

2.6 Transfection

1. Transfection reagent: Lipofectamine® 2000 or equivalent.
2. Opti-MEM I Reduced-Serum Medium.
3. siRNAs: 100 nM siGFP and 100 nM siHSP90. SiGlow (Dharmacon) prepare in RNAase-free water.

3 Methods

This chapter focuses on the procedures used in specimen preparation, the protein analysis and some subsequent strategies for validation.

3.1 RSV Production

1. HEp-2 cells are cultured in cell culture medium at 37 °C, 5% CO_2.
2. Plate cells in 175 cm^2 tissue culture flask when the cells reach confluence 80–90%, the cells are ready for infection.
3. Remove cell culture medium. Dilute the RSV inoculum in 10 mL cell maintenance medium and add to the cells in each flask. The cell monolayer is infected using a multiplicity of infection (MOI) of 0.1.
4. Incubate the flask at 33 °C, 5%CO_2 for 2 h, rocking every 15 min to redistribute.
5. After 2 h, top up the tissue culture flask with 10 mL of cell maintenance medium. Incubate at 33 °C, 5%CO_2.
6. At 48 h post-infection, harvest both cell maintenance medium and cells using acid-washed glass beads (3 mm diameter) at 4 °C (approximately 2 mL beads/flask). Gently shake the flask

until the remaining cells are removed from monolayer. The virus–cell suspension can be used immediately in the steps below or stored at −80 °C until further use (*see* **Note 1**).

3.2 RSV Purification

1. Since most of the virus is associated with infected cells, further release of virus can be achieved by gentle sonication. Place virus suspension on ice afterward.
2. Clarify the harvested virus suspension (maximum of 30 mL virus suspension per 50 mL Falcon tube) by centrifugation at 5000 × *g* for 20 min at 4 °C. Harvest the clarified supernatant.
3. Centrifuge the clarified supernatant again at 5000 × *g* for 20 min at 4 °C. Harvest the clarified supernatant.
4. Adjust the clarified supernatant to contain 10% (w/v) PEG-6000. Slowly add PEG-6000 while the virus suspension is gently stirred using a magnetic stirrer. Once added, continue the incubation at 4 °C for 1 h with continual stirring.
5. Centrifuge the PEG-treated lysate at 5000 × *g* for 20 min at 4 °C. Pellet should be visible.
6. Resuspend the pellet in 20% (w/v) sucrose prepared in Hanks' buffered saline solution (HBSS) (*see* **Note 2**). This is then layered onto a 30% (w/v) sucrose cushion prepared in HBSS.
7. Centrifuge at 51,000 × *g* for 1 h at 4 °C and collect the resulting pellet.
8. Resuspend pellet in 20% (w/v) sucrose in HBSS (*see* **Note 2**).
9. Prepare a discontinuous gradient consisting of 35, 45, 60% (w/v) sucrose in HBSS, and then layer the resuspended virus suspension onto the discontinuous gradient. Mark each interface on the wall of the centrifuge tube using a permanent marker. Centrifuge at 165,000 × *g* for 1 h at 4 °C (*see* **Notes 3** and **4**).
10. Harvest the opalescent band at the 35 and 45% (w/v) sucrose interface, dilute with HBSS (1 part virus to 2 parts HBSS), and layer the resuspended virus preparation onto a continuous 30–60% (w/v) sucrose gradient prepared using HBSS (*see* **Note 5**). This may require more than one tube. Centrifuge at 165,000 × *g* for 3 h at 4 °C. Harvest the virus containing band (Fig. 1a).
11. Dilute the virus suspension with HBSS (typically 1:10 ratio), centrifuge at 284,000 × *g* for 90 min at 4 °C.
12. Collect virus pellet and resuspend in 100 μL of HBSS.

3.3 Morphological Analysis of Purified RSV Preparation Using Transmission Electron Microscopy (See Note 6)

1. Dilute virus suspension 1:10 using PBS.
2. Place a 20 μL droplet of the virus suspension on a Formvar-coated grid for 10 min.
3. Blot off virus suspension using 3 mm blotting paper.
4. Add 20 μL droplet of PTA (phosphotungstic acid, pH 7.5) for 10 min.

5. Blot off PTA from grid using 3MM paper and allow to air dry.
6. View stained specimen in a JEOL 1200 EX electron microscope at 80 kV (Fig. 1b).

3.4 Protein Analysis to Assess Level of Purity (See Note 7)

1. Heat an aliquot of the virus preparation in 1× boiling mixture and sonicate sample using a sonicating probe (2 s high power) and immediately heat sample at 100 °C for 2 min.
2. Use 15 % SDS-PAGE to separate the proteins in the virus preparation. Use a protein standard as Precision Plus protein standards (Bio-Rad) or similar.
3. To visualize protein bands in the polyacrylamide gel, stain using either Coomassie Brilliant Blue (G-250) or SYPRO Ruby Red (Fig. 1c).

3.4.1 Coomassie Brilliant Blue

1. Stain the gel in Coomassie Brilliant Blue solution for 16 h at room temperature using gentle agitation at 100 rpm.
2. Destain the stain polyacrylamide gel using destaining solution using gentle agitation 100 rpm (*see* **Note 8**).
3. Scan the destained polyacrylamide gel and quantify the individual protein bands using a densitometer.

3.4.2 SYPRO Ruby Red

1. Fix protein bands in gel with fixing solution for 30 min.
2. SYPRO Ruby Red staining solution's volume of at least 10 times the volume of the gel is added to totally cover the gel,

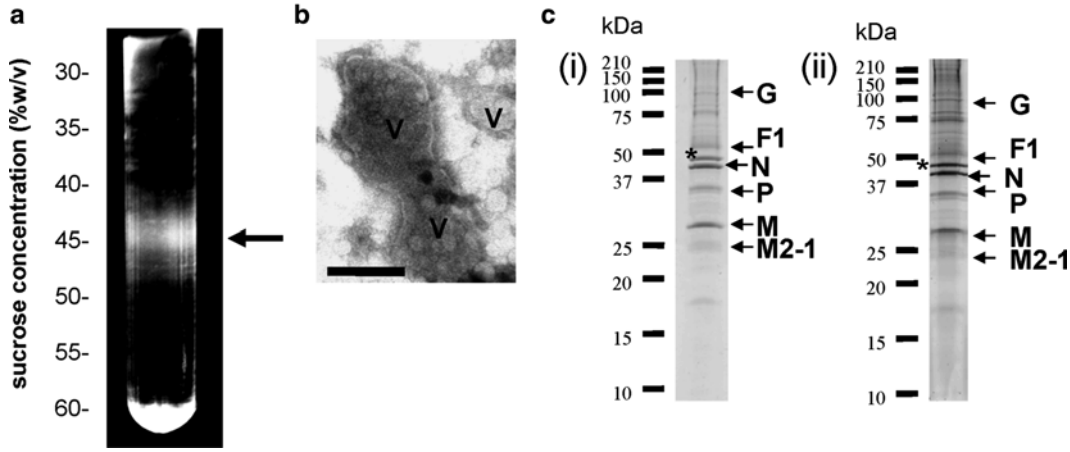

Fig. 1 (**a**) Purification of RSV using a 30–60 % discontinuous gradient. After centrifugation, the virus band (*highlighted by the black arrow*) (at approximately 45 % sucrose) was detected by using a focused light beam and harvested. (**b**) Transmission electron microscopy was used to examine the harvested virus band to confirm the presence of virus particles (V) (*bar*, 100 nm). (**c**) Proteins from purified virus preparation were separated by SDS-PAGE, and the protein visualized by staining the polyacrylamide gel with (*i*) SYPRO Ruby Red or (*ii*) Coomassie. Bands corresponding to viral proteins with known sizes are shown. *Asterisk* is an abundant protein band in the purified virus preparation that was independently identified as actin by using MALDI TOF/TOF

keep in the dark, shake with moderate speed for at least 3 h (*see* **Note 9**).

3. Remove the stain and wash the gel twice with wash solution for 30 min each.
4. Rinse the gel with ultrapure water for 5 min.
5. Scan using Typhoon Trio Scanner at an emission of 630 nm.
6. Quantification of protein bands detected by SYPRO Ruby Red can be performed using Image-Quant™ TL.

3.5 One Dimensional LC-MS/MS (1D LC MS/MS)

3.5.1 Preparation of Peptide Digest from Virus Preparation (See **Note 10**)

1. Dissolve 5 μg of purified virus pellet in 25 mM ammonium bicarbonate (ABC) containing 2% (w/v) SDS; incorporated into a 40% acrylamide–bisacrylamide (29:1) gel matrix. The gel mix is polymerized using ammonium persulfate (10%) and TEMED.
2. After polymerization cut the gel into small pieces (approx 1 mm^3); wash 3 times with 25% ABC containing 50% acetonitrile (ACN), and then vacuum-dried.
3. Treat the dried gel in ABC containing 10 mM DTT at 60 °C for 30 min.
4. Treat the dried gel in ABC containing 55 mM iodoacetamide at 25 °C in the dark for 30 min.
5. Wash the gel pieces with 25 mM ABC, dehydrate with 100% CAN and vacuum-dry.
6. Perform proteolytic digestion by ABC containing trypsin (protein/trypsin = 10:1, g/g) at 37 °C for 16 h.
7. Extract peptides from gel by sequential extraction using 25 mM ABC, 5% formic acid (FA) in water, 5% FA in ACN, and 100% ACN.
8. Vacuum dry the pooled peptide extracts.

3.5.2 1D LC MS/MS Analysis (See **Note 11**)

1. Reconstitute the peptide mixture in 30 μL of buffer A.
2. Inject aliquots (maximum volume of 1 μL) for LC separation using an autosampler. Peptide separation is achieved by using a nanoflow liquid chromatography system (UltiMate Plus, Dionex) with a nano-LC column (C18 PepMap100, 3 μm, 0.075 × 150 mm, Dionex).
3. Use solvent gradient with buffer B increasing from 0 to 45% in 45 min to elute peptides, followed by a 10-min wash with 80% buffer B and a 25-min equilibration with buffer A. Flow rate at 300 nl/min.
4. Couple the column to a Linear Trap Quadrupole (LTQ) Orbitrap (Thermo Fisher Scientific) having a T-union and a nanospray tip (20 μm ID tubing, 10 μm ID tip) and a spray voltage of 1.8 kV through the T-union.

5. Operate the mass spectrometer in data-dependent mode to switch between MS and MS/MS.
6. Acquire the full scan MS spectra from m/z 350–1800 Da in the Orbitrap with resolution of 60,000 at m/z 400.
7. Select five most intense precursor ions for MS/MS scan with collision-induced dissociation (CID) in the linear ion trap LTQ.

3.5.3 Database Search (See Table 1)

1. Process the acquired raw spectra with RawDistiller (v.1.0). The peak list generated in the MGF format is used for database search with Mascot search engine (v.2.2 Matrix Science, London, UK) and NCBI non-redundant database (released July 23, 2008).
2. The search settings as follow: mass values, monoisotopic; precursor mass tolerance, ±20 ppm; fragment mass tolerance ±0.5 Da; enzyme, trypsin; maximum missed cleavage allowed, 1; fixed modification, carbamidomethyl Cys; variable modification, oxidation of Met, phosphorylation of Ser, Thr, and Tyr.
3. Perform the searches with the addition of a decoy database to allow estimation of peptide false discovery rate.
4. Use Scaffold (version Scaffold_2.02.01, Proteome Software Inc., Portland, OR) to validate MS/MS based peptide and protein identifications.

3.6 Confirmation That the Proteins Detected in the 1D LC MS/MS Analysis Are Present in the Virus Preparation (See Note 12)

3.6.1 Distribution of the Cellular Proteins in the Continuous Sucrose Gradient to Confirm Their Co-migration with Virus Particles

1. Perform centrifugation of the virus preparation (as indicated in Subheading 3.2).
2. Fractionate the gradient into 11 equal 1 mL fractions. The gradients can be carefully fractionated by hand starting at the top and progressively working down to the last fraction.
3. Harvest the pelleted material by adding 1 mL to the bottom of the tube and using a sonicating probe to resuspend the material (e.g., 2 × 5 s at high power).
4. Vortex each fraction briefly for approximately 30 s to mix the contents of the gradient.
5. Add 200 μL of each fraction to 50 μL of 5× Boiling Mixture and heat the sample at 100 °C for 2 min.
6. Separate the proteins by electrophoresis in a 15% SDS-PAGE.
7. The total proteins in the sucrose gradient can be determined by staining of polyacrylamide gel using SYPRO Ruby Red (Fig. 2a).
8. After SDS-PAGE, transfer the proteins from polyacrylamide gel onto PVDF membrane.
9. After transference, wash the membrane with PBS and placed in blocking solution at 4 °C for 18 h.

Table 1
Purified virus sample was processed using in-gel digestion to become peptide mixture, which was analyzed using LC MS/MS

Protein name	Accession number	Protein probability	Peptides identified (unique peptides)	Sequence coverage	Group probability
Virus proteins					
Nucleoprotein (protein N) (nucleocapsid protein)	sp|P03418|NCAP_HRSVA	1/1	218(11)/165 (12)	51.4/51.2	1/1
Matrix protein (M)	sp|P03419|MATRX_HRSVA	1/1	239 (12)/192 (12)	69.5/69.5	1/1
Fusion glycoprotein	sp|P03420|FUS_HRSVA	1/1	144(14)/135 (13)	39.9/44.4	1/1
Phosphoprotein (protein P)	sp|P03421|PHOSP_HRSVA	1/1	97 (6)/48 (6)	51.9/44	1/1
Matrix M2 (envelope-associated 22-kDa protein)	sp|P04545|M21_HRSVA	1/1	90(9)/94 (7)	60.8/59.8	1/1
Polymerase L	sp|P28887|L_HRSVA	1/1	31(8)/37 (10)	7.2/8.8	1/1
Major surface glycoprotein G	sp|P03423|GLYC_HRSVA	1/0.9904	5(1)/1 (1)	5.4/5.4	1/0.9904
Host cell proteins					
Chaperones					
Heat shock protein HSP90α	sp|P07900|HS90A_HUMAN/group	0.9937/NA	4 (2)/NA	4.4/NA	0.9999/1
Heat shock protein HSP90β	sp|P08238|HS90B_HUMAN	0.9937/1	4 (2)/14 (3)	4.4/14.9	0.9999/1
Heat shock cognate 71-kDa protein	Group/sp|P11142|HSP7C_HUMAN	NA/1	NA/11 (5)	NA/21.1	1/1
Heat shock 70-kDa protein 6	Group/sp|P17066|HSP76_HUMAN	NA/0.9584	NA/1 (1)	NA/5.1	1/1

(continued)

Table 1
(continued)

Protein name	Accession number	Protein probability	Peptides identified (unique peptides)	Sequence coverage	Group probability
Cytoskeleton					
ß-Actin-like protein 2	Group/ sp|Q562R1|ACTBL_ HUMAN	NA/1	NA/22 (3)	NA/14.1	1/1
Cofilin-1	sp|P23528|COF1_ HUMAN	1/1	23(3)/7 (2)	31.3/27.7	1/1
Keatin, type II cytoskeletal 1	sp|P04264|K2C1_ HUMAN	1/1	9 (2)/13 (6)	6.1/16.3	1/1
Moesin	sp|P26038|MOES_ HUMAN	0.9979/1	5 (1)/39 (5)	6.4/23.3	0.9984/1
Filamin A	sp|P211333|FLNA_ HUMAN	0.9989/1	2 (2)/22 (7)	1/4.2	0.9989/1
Tubulin, ß	Group/group	NA/NA	NA/NA	NA/NA	1/1
Tubulin, α-1B	sp|P68363|TBA1B_ HUMAN/group	1/NA	6 (2)/NA	11.8/NA	1/1
Putative tubulin-like protein α-4B	Group/ sp|Q9H853|TBA4B_ HUMAN	NA/0.9999	NA/6 (2)	NA/10.8	1/1

Virus and Host proteins identified are shown. Homologous proteins are not listed when there is a specific protein identified in the same group. Individual proteins with the chaperone and cytoskeleton protein groups are shown. Numbers in parentheses are the number of unique peptides identified. Statistics collected from two individual virus preparations (sample 1 and 2) are separated by "/". When peptides identified can only infer a group of proteins, these entries are labeled with "group" in the "Accession number" column, and other statistics referring to a single protein are labeled with "NA". In the table we present a subset of the protein identified. A more extensive list of proteins identified is available [12]

10. Incubate membrane with the specific primary antibody for 1 h. Select antibodies that recognize the virus and cellular proteins identified by the MS/MS analysis.
11. Wash membrane with PBS-T for 5 min. Repeat this step 3 times.
12. Incubate membrane with the appropriate secondary antibody (anti-mouse or anti-rabbit IgG) conjugated to HRP for 1 h.
13. Wash membrane with PBS-T for 5 min. Repeat this step 3 times.
14. Visualize the protein bands using ECL protein detection system or another chemiluminescent HRP substrate of choice (Fig. 2b).

3.6.2 Detection of Virus and Cellular Proteins in the Purified Virus Preparation (As Indicated in Subheading 3.2)

1. Samples are made up in 1× Boiling Mixture and heated at 100 °C for 2 min.
2. Run SDS-PAGE and perform immunoblotting as described above.
3. Visualize the virus and cellular protein bands using ECL protein detection system (Figs. 3a, b).

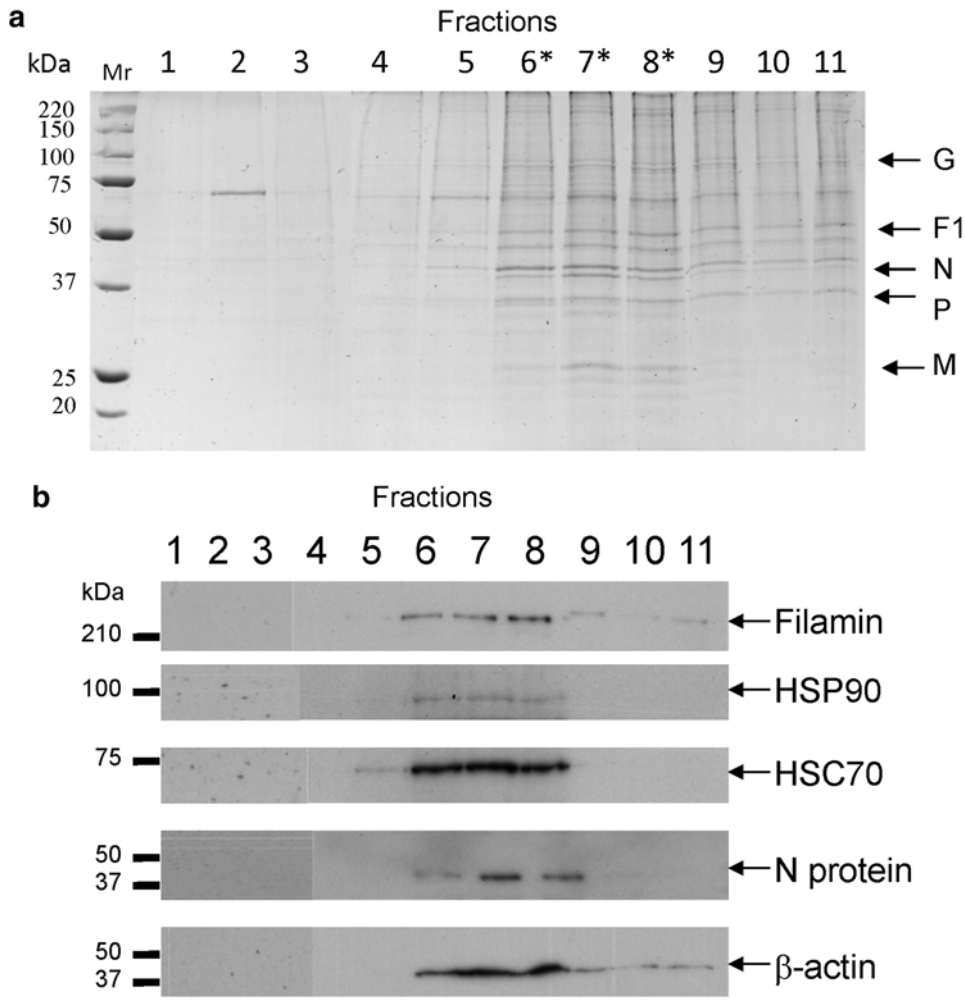

Fig. 2 Co-purification of identified cell proteins with RSV particles. 30–60 % sucrose gradient was fractionated into 11 fractions. Proteins in each fraction were separated by SDS-PAGE. (**a**) SYPRO Ruby Red staining of polyacrylamide gel shows the total proteins within each fraction. *Asterisk* indicates the peak fraction. Mr is the protein marker lane (**b**) Western blot results of all fractions using antibodies against Filamin-1, HSP90, HSC70, N protein and β-actin

3.7 Demonstration of Relevance of Cellular Proteins Identified in the Proteomic Analysis to Virus Replication in RSV-Infected Cells (See Note 13)

3.7.1 Immunolocalization of HSP90 in Virus Filaments by Imaging Co-stained Virus-Infected Cells (See **Note 14**)

1. Culture HEp-2 cells on 12-mm glass coverslips at 37 °C.
2. Infect cells with RSV in cell maintenance medium using a MOI of 3 and incubate at 33 °C.
3. At 24 h post infection (hpi), rinse the cells using PBS and fix them on the glass coverslips using 3% PFA for 30 min at 4 °C. In case of anti-β-actin staining, fix cells with methanol for 5 min at 4 °C prior to antibody staining.
4. Soak coverslips in freshly made 1× PBS at 25 °C
5. Continue to wash with 1× PBS for 5 min. Repeat washing step for 3 times.
6. Permeabilize the cells using 0.1% saponin in PBS for 20 min.
7. Wash with 1× PBS for 5 min. Repeat washing step for 3 times. Block with 0.1% BSA in PBS for 10 min and wash with 1× PBS for 5 min. Repeat washing step for 3 times.
8. Remove excess PBS by gently blotting using tissue paper.
9. Place a 30 μL droplet containing the diluted primary antibodies (anti-F and anti-HSP90) on plastic dish and invert coverslip (containing cells) onto droplet (cell-face down). Place lid on dish and incubate for 1 h at room temperature. Place moist tissue paper into dish to avoid drying of sample.
10. Remove coverslips from primary antibodies; wash with freshly made 1× PBS for 5 min. Repeat washing for 3 times.
11. Remove excess of PBS by gently blotting using tissue.
12. Place a 30 μL droplet containing the diluted secondary antibodies (anti-mouse IgG conjugated to FITC and anti-rabbit IgG conjugated to Cy3) on plastic dish and invert coverslip (containing cells) onto droplet (cell-face down). Place lid on dish and incubate for 1 h at room temperature. Place moist tissue paper into dish to avoid drying of sample.
13. Remove the coverslip from the secondary antibody mix; wash with freshly made 1× PBS for 5 min. Repeat washing for 3 times.
14. The coverslip is blotted dry and placed on microscope slides (cell-side down) using mounting media. Seal the coverslip by applying nail varnish to boundary of coverslip and once dry the stained cells are examined using confocal microscope to determine the presence of co-stained virus filaments (Fig. 4) (*see* **Note 15**).

3.7.2 Effect of HSP90 Gene Silencing on RSV Morphogenesis (See **Note 16**)

1. Grow HEp-2 cells on coverslips in cell culture medium (without antibiotics) until confluence of 50–70% at 37 °C in 5%CO_2.
2. Mix Lipofectamine with serum-free Opti-MEM media and incubate at room temperature for 10 min.

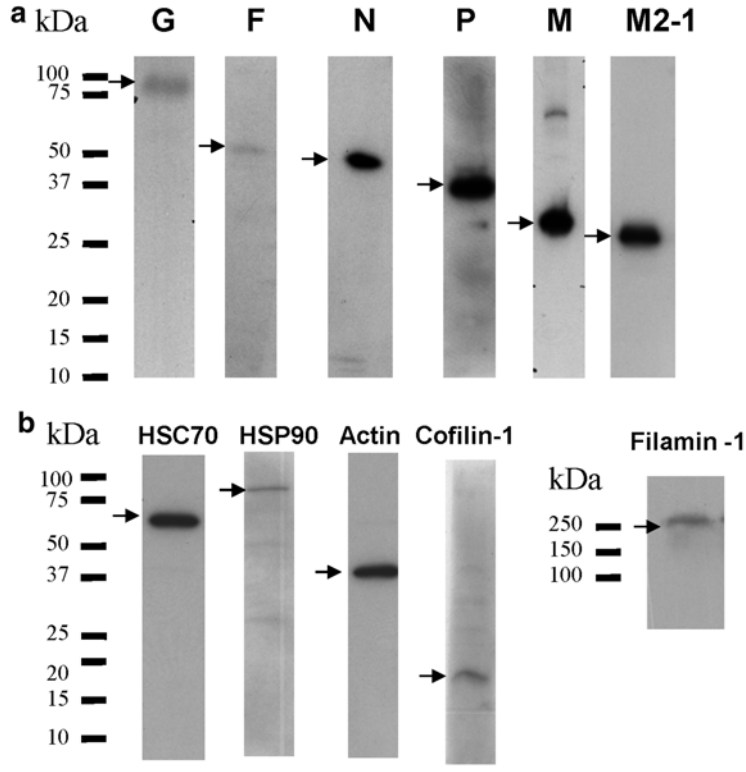

Fig. 3 Identification of virus and cell proteins in purified RSV preparation by Western blot using (**a**) virus specific antibodies and (**b**) antibodies against several cellular proteins that were detected using mass spectrometry. Proteins of the expected size are indicated by *black arrows*

3. Mix 100 nM siRNA (siGFP or siHSP90) with serum-free Opti-MEM media and incubate at room temperature for 5 min (*see* **Note 17**).
4. Add mixture at **step 2** into mixture at **step 3** and incubate at room temperature for 20–25 min.
5. Add siRNA–Lipofectamine complexes to the HEp-2 cells (dropwise) and incubate for 6 h at 37 °C in 5 %CO_2.
6. Change media to cell maintenance medium. Incubate cells at 33 °C in 5 %CO_2.
7. At 36 h post-transfection, infect cells with RSV at MOI of 1. Incubate for 2 h at 33 °C in 5 % CO_2.
8. Remove the virus inoculum and replace medium for cell maintenance medium. Incubate at 33 °C in 5 %CO_2.
9. At 24 h post-infection, rinse the cells using PBS and fix them on the glass coverslips using 3 % PFA for 30 min at 4 °C. Permeabilize and stain the cells with appropriate antibodies following the same protocol from Subheading 3.7.1

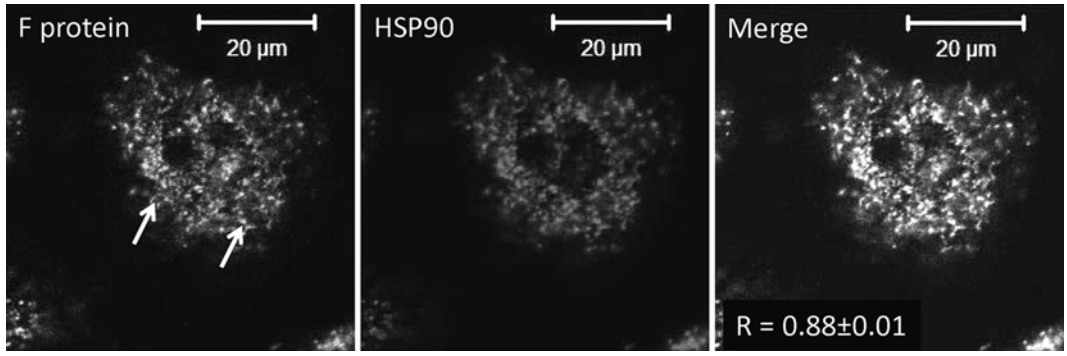

Fig. 4 Association of HSP90 with virus filaments on RSV-infected cells. Virus-infected cells were fixed and stained using anti-HSP90 and anti-F at 24 hpi and imaged using confocal microscopy. The presence of co-stained virus filaments (VF) is indicated. The co-localization in the merged image was quantified using the Pearson's coefficients (R and R^2). The cells were viewed in a confocal microscope at an optical plane that allows imaging of the virus filaments

10. Examine stained cells using confocal microscopy to determine the presence of virus filaments.

*3.7.3 The Effect of the HSP90 Inhibitor 17-Allyaminogeldanamycin. (17AAG) on RSV Filament Formation (See **Note 18**)*

1. Grow HEp-2 cells on coverslips until confluence of 70 %. Infect the cell monolayer with RSV at MOI of 3.
2. After first 2 h of virus absorption remove the virus inoculum, change media with cell maintenance medium. Incubate at 33 °C in 5 % CO_2.
3. At 8 h post-infection the cells are either non-treated or treated with 2 µM 17AAG (prepared in cell maintenance medium) (*see* **Note 19**).
4. At 24 h post-infection, fix, permeabilize, and stain the cells with anti-RSV and anti-mouse IgG conjugated to FITC (follow protocol from Subheading 3.7.1).
5. Examine the stained cells using confocal microscopy to compare the staining pattern on non-treated and 17AAG-treated cells to determine if virus filament formation is impaired (Fig. 5).

3.7.4 The Effect of the HSP90 Inhibitor 17-Allyaminogeldanamycin (17AAG) on RSV Transmission in a HEp-2 Cell Monolayer

1. Grow HEp-2 cells on coverslips until semi-confluent (80–85 %). Infect the cell monolayer with RSV at MOI of 0.1.
2. After first 2 h of virus absorption remove the virus inoculum, and add fresh cell maintenance medium. Incubate at 33 °C in 5 % CO_2.
3. At 18 h post-infection add 2 µM 17AAG dilute in cell maintenance medium (*see* **Note 19**).
4. At 30 h post-infection, fix, permeabilize, and label cells with anti RSV and anti-mouse IgG conjugated to FITC (follow protocol from Subheading 3.7.1).

5. Examine the anti-RSV stained cell monolayers using low power fluorescence microscope microscopy. Staining pattern non-treated cells compared with that on treated cells (Fig. 6).
6. The number of cells in the infected cell clusters is counted (*see* **Note 20**).

4 Notes

1. This is an important first step in the analysis. Most of the virus remains cell-associated. It is important to release as much virus from the cell to improve the yield of released virus. This will have significant consequences in the proteomic analysis which requires adequate protein yields for protein detection. We estimate that 2×10^9 HEp-2 cells yields approximately 100 μg protein in the final virus preparation.
2. Care should be taken to ensure that the virus pellet is completely resuspended prior to proceeding to the next stage. This can affect the final protein yield and adversely affect the proteomic analysis.
3. HBSS buffer contains high levels of magnesium which can improve recovery rate of RSV during purification process and enhanced stability upon storage at −80 °C.
4. Virus band to be harvested is located at a position in the tube that corresponds to the interface between the interface between the 35 and 45 % (w/v) sucrose concentrations.

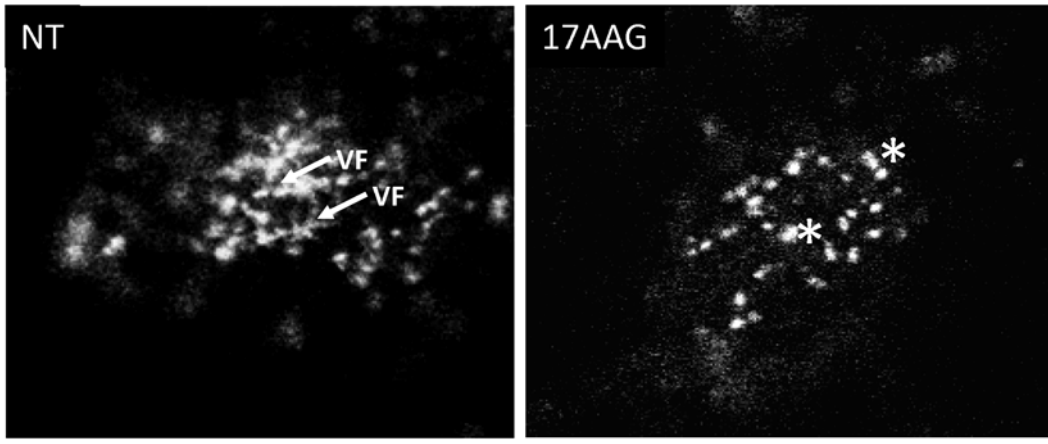

Fig. 5 Inhibition of HSP90 impairs virus filament formation. Cells were infected with RSV and at 8 hpi the cells were either left non-treated (NT) or alternatively treated with 2 μM 17AAG. . At 18 hpi, the cells were fixed, labeled with anti-F and then examined by confocal microscopy. The virus filaments (VF) and punctuate staining pattern in the presence of 17AAG (*asterisk*) are highlighted

5. Prior to harvesting, the opalescent bands are visualized by passing transmitted light through the centrifuge tube in the dark using a COLD LIGHT L-150A lamp. The virus particles are typically seen as a broad opalescent band that is located between 40 and 50% (w/v) sucrose.
6. The use of transmission electron microscopy to view negative stained preparations is recommended. This confirms the presence of virus particles in the virus preparation. Several different staining protocols can be used. In this section we use PTA.
7. The purity of the recovered purified virus is assessed. The proteins in the virus sample are separated by 15% SDS-PAGE. Following SDS PAGE the polyacrylamide gel is stained using Coomassie Blue or SYPRO Ruby Red stain. This allows visualization of the total proteins in the sample, as well as some

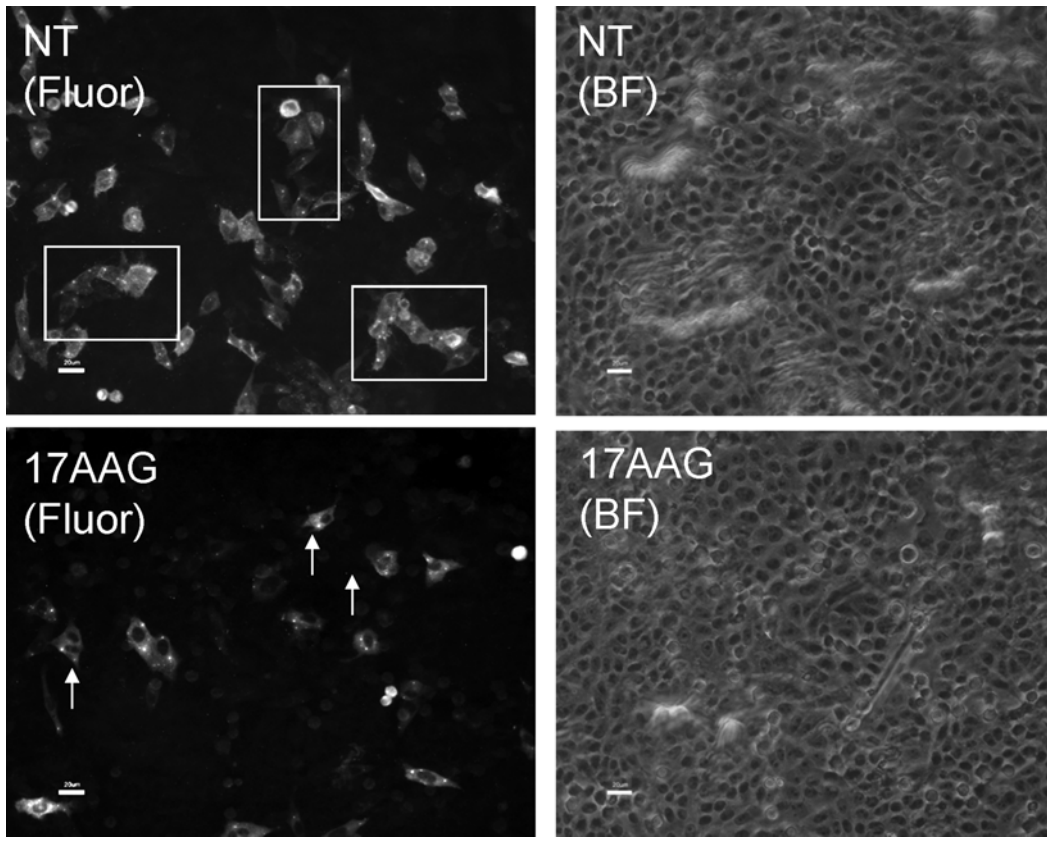

Fig. 6 Cells infected by RSV using a MOI of 0.1 and the HSP90 inhibitor 2 μM 17AAG was added at 18 h post-infection. At 30 h post-infection, cells were fixed and stained using anti-RSV. The same cell area was examined using a ×20 objective lens using fluorescent (Fluor) and bright field (BF) microscopy. Scale bar, 20 μm. Compare the presence of cluster of cells within *white boxes* (non-treated cell) with individual cells at *white arrows* (treated cells) in fluorescence microscopy images. Estimate the average number of cells per cluster; 8.75 ± 2.7 cells per cluster in the non-treated cell monolayer and 1.3 ± 0.60 cells per cluster in 17AAG-treated cell monolayer

other information, e.g., numbers of protein bands present, size of the protein bands, and their relative abundance. It also gives a good indication of how well the purification procedure has worked and the levels of protein recovered; both important considerations prior to performing the proteomic analysis.

8. To reduce time taken for destaining of the Coomassie stained polyacrylamide gel, place small amount of absorbent tissue in destain solution to facilitate destaining of polyacrylamide.
9. In SYPRO Ruby Red staining of polyacrylamide gel, the volume of the staining solution used is important to get equal staining of all proteins in the polyacrylamide gel.
10. Protein identification is performed by using LC-MS/MS. However, the specimen must be first digested and an in-gel trypsin digestion technique will be described. We have found that this technique gives reproducible results between the different batches of virus preparation.
11. In this 1D LS MS/MS section we describe one chromatography procedure to separate the peptides prior to ms analysis. The separation procedure used will depend on several factors, which include for example the chromatography column and the machine being used. This procedure may therefore need to be optimized to increase the peptide separation and hence the resolution of protein identification.
12. The proteomic analysis is a predictive tool in which proteins are identified on the basis of the mass of detected peptides that are compared with a virtual library of peptides. However, the presence of the proteins that have been identified in the data analysis will need to be confirmed before further validation is attempted. This is performed by immunoblotting using antibodies against proteins of interest. This is an important step in the characterization of the virus preparation.
13. It is envisaged that while some co-purifying proteins will have a functional association with RSV, other co-purifying cellular proteins will have no structural or functional relevance to RSV replication (e.g., their presence may reflect the presence of contaminating exosomes). Therefore, once the list of proteins has been identified and their presence in the virus preparation confirmed, their relevance to virus infection should be assessed using cells that are permissive for RSV infection. This is an essential final stage in the workflow. In general we use HEp-2, A549 and T239 cells to perform this analysis. Several strategies can be adopted and three procedures will be described (1) localization of the respective cellular protein with virus antigen, (2) gene silencing using siRNA, and (3) inhibiting the activity of the cellular protein using small molecule inhibitors.

14. Immunofluorescence microscopy using specific antibodies applied in virus-infected cells allows us to examine interested proteins and to determine their association with virus filaments. It is necessary to determine the optimal dilution of each antibody to be used to ensure specificity of labeling in both single-stained and co-stained cells.
15. The co-localization between virus antigen in the virus filaments and different cellular proteins identified in the proteomic analysis may indicate a role in virus replication. The degree of association in the co-stained cells may be assessed by using the Zeiss imaging tools. This allows a quantitative estimate of co-localization between pairs of antibody staining by determining parameters of co-localization such as the Pearson's correlation coefficient. In addition the pixel distribution and staining distribution in individual cells can be determined.
16. If there is an issue with low siRNA transfection efficiency in HEp-2 cells the effect of protein knock down on virus filament formation can be demonstrated using imaging of several individual co-stained cells.
17. siGFP is used as a siRNA negative control.
18. This step will be demonstrated using the protein HSP90 as an example. There are several established specific small molecules that inhibit the HSP90 activity. However, the general procedure used for other proteins will depend to a great extent on the nature of the cellular proteins that is being validated and the availability of suitable inhibitors. HSP90 protein was detected within the virus filaments, suggesting a role in virus assembly. Thus, RSV-infected cells were either non-treated or treated with the HSP90 small molecule inhibitor 17-allyaminogeldanamycin (17AAG). The effect of drug treatment on virus filament formation and on RSV transmission in a cell monolayer was examined.
19. The time at which the drug is added will depend on the MOI used and the effect being studied. In the case of normal MOI (i.e., MOI = 3) we examined impaired virus filament formation. The drug is added approximately 5 h post-infection. This is to ensure that the initial phase of infection takes place (but prior to virus filament formation) and the cells examined at between 18 and 20 hpi. When using low MOI (MOI = 0.1–0.01) to examine virus transmission in the cell monolayer the drug is added later. In general we can add the drug at 8–16 hpi depending on the end point of the experiment and the nature of the inhibitor (usually 30–40 hpi). It is important to independently check that the drug used has no general cytotoxic effects (e.g., by measuring LDH release) during the time frame of the experiment.

20. In non-treated cells the presence of relatively large numbers of stained cells in each cluster indicates efficient transmission, while reduced cell number in the clusters indicate impaired transmission.

Acknowledgments

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References

1. Jeffree CE, Rixon HW, Brown G, Aitken J, Sugrue RJ (2003) Distribution of the attachment (G) glycoprotein and GM1 within the envelope of mature respiratory syncytial virus filaments revealed using field emission scanning electron microscopy. Virology 306(2):254–267
2. Ravi LI, Liang L, Wong PS, Brown G, Tan BH, Sugrue RJ (2013) Increased hydroxymethylglutaryl coenzyme A reductase activity during respiratory syncytial virus infection mediates actin dependent inter-cellular virus transmission. Antiviral Res 100:259–268
3. Brown G, Rixon HW, Sugrue RJ (2002) Respiratory syncytial virus assembly occurs in GM1-rich regions of the host-cell membrane and alters the cellular distribution of tyrosine phosphorylated caveolin-1. J Gen Virol 83: 1841–1850
4. McCurdy LH, Graham BS (2003) Role of plasma membrane lipid microdomains in respiratory syncytial virus filament formation. J Virol 77:1747–1756
5. Brown G, Jeffree CE, McDonald T, Rixon HW, Aitken JD, Sugrue RJ (2004) Analysis of the interaction between respiratory syncytial virus and lipid-rafts in Hep2 cells during infection. Virology 327:175–185
6. Yeo DS, Chan R, Brown G, Ying L, Sutejo R, Aitken J, Tan BH, Wenk MR, Sugrue RJ (2009) Evidence that selective changes in the lipid composition of raft-membranes occur during respiratory syncytial virus infection. Virology 386:168–182
7. Kallewaard NL, Bowen AL, Crowe JE Jr (2005) Cooperativity of actin and microtube elements during replication of respiratory syncytial virus. Virology 331:73–81
8. Jeffree CE, Brown G, Aitken J, Su-Yin DY, Tan BH, Sugrue RJ (2007) Ultrastructural analysis of the interaction between F-actin and respiratory syncytial virus during assembly. Virology 369:309–323
9. Brown JH, Del Re DP, Sussman MA (2006) The Rac and Rho hall of fame: a decade of hypertrophic signaling hits. Circ Res 98:730–742
10. Gower TL, Pastey MK, Peeples ME, Collins PL, Mc Curdy LH, Hart TK, Guth A, Johnson TR, Graham BS (2005) RhoA signaling is required for respiratory syncytial virus induced-syncytium formation and filamentous virion morphology. J Virol 79:5326–5336
11. Zheng J, Sugrue RJ, Tang K (2011) Mass spectrometry based proteomic studies on viruses and hosts--a review. Anal Chim Acta 702(2):149–159
12. Radhakrishnan A, Yeo D, Brown G, Myaing MZ, Ravi LI, Fleck R, Tan BH, Aitken J, Sanmun D, Tang K, Yarwood A, Brink J, Sugrue RJ (2010) Protein analysis of purified respiratory syncytial virus particles reveals an important role for heat shock protein 90 in virus particle assembly. Mol Cell Proteomics 9:1829–1848

Chapter 14

MicroRNA Profiling from RSV-Infected Biofluids, Whole Blood, and Tissue Samples

Lydia Anderson, Patricia A. Jorquera, and Ralph A. Tripp

Abstract

Several studies have shown that respiratory syncytial virus (RSV) can modulate the host innate immune response by dysregulation of host microRNAs (miRNAs) related to the antiviral response, a feature that also affects the memory immune response to RSV (Thornburg et al. MBio 3(6), 2012). miRNAs are small, endogenous, noncoding RNAs that function in posttranscriptional gene regulation. Here, we explain a compilation of methods for the purification, quantification, and characterization of miRNA expression profiles in biofluids, whole blood samples, and tissue samples obtained from in vivo studies. In addition, this chapter describes methods for the isolation of exosomal miRNA populations. Understanding alterations in miRNA expression profiles and identifying miRNA targets genes, and their contribution to the pathogenesis of RSV, may help elucidate novel mechanism of host-virus interaction (Rossi et al., Pediatr Pulmonol, 2015).

Key words Respiratory syncytial virus, MicroRNAs, Exosomes, RT-qPCR, RNA isolation

1 Introduction

MicroRNAs (miRNAs) are small, endogenous RNAs, approximately 20–25 nucleotides long that can govern posttranscriptional gene expression [1]. miRNA genes constitute one of the most abundant gene families, which are conserved across all eukaryotes and have been substantially expanded during evolution with only 12 miRNAs having been lost from deuterostomes to mammals [2–4]. The miRNA gene family makes up a global regulatory network controlling homeostasis, cell proliferation, cell differentiation, cell migration, disease progression, and inflammatory responses [5, 6]. Altered microRNA expression and their diagnostic potential have been evaluated with various disorders [7], autoimmune diseases [8–13], and infectious diseases [14–20]. For example, distinct microRNA expression patterns have been identified in the nasal epithelium of infants with acute RSV disease [21]. RSV-positive infants showed a distinct profile of microRNA downregulation and

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upregulation as compared to healthy controls, where miR-125a and miR-429 were regulated in mild but not severe RSV disease [21]. Understanding the changes in miRNA expression profiles and identifying miRNA targets genes and their contribution to disease pathogenesis may help elucidate novel mechanism of host–virus interaction [22, 23].

MicroRNA genes are located on the sense/antisense strands in genic/intergenic and introns of genes as independent transcription units called mirtrons [24, 25]. Parent genes are typically transcribed by RNA polymerase II [26, 27], and in some cases by RNA polymerase III [28] producing long primary transcripts, pri-miRNAs, of varying length in the nucleus [5]. Pri-miRNAs are processed by the microprocessor complex consisting of Drosha and DGCR8 into ~60 nucleotide long hairpin structured pre-miRNAs, which are then exported out of the nucleus by exportin 5 (XPO5) [29]. Exported pre-miRNAs are processed further by a Dicer and Argonaute complex to produce mature miRNA duplexes, 20–25 nucleotides long, that consist of a "guide" strand and a passenger strand, which is generally degraded [5]. The guide strand nucleotides 2–8 represent the "seed site" which recognizes a 6–8 nucleotide complementary sequence in the target gene(s) causing either a translational block or transcript decay in a RNA protein complex termed as RNA induced silencing complex (RISC) [5, 29]. Some miRNAs can also bind with perfect complementarity with the target transcript [5]; therefore, the degree of base pairing between the miRNA guide strand and mRNA and the composition of the RISC complex determine the downstream pathways [5]. Due to the short sequence complementarity between a miRNA and its target, a single miRNA can regulate the expression of multiple genes [29, 30]. Accumulating evidence has shown that miRNAs, as well as those derived from exosomal vesicles, can stably exist in body fluids, including saliva, urine, breast milk, and blood [6, 31–35].

Previous studies have also shown that miRNA profiles detectable in biofluids and blood samples are concentrated in exosomes, and can potentially undergo modulation during viral infection [31, 36, 37]. Exosomes are 40–100 nm microvesicles that are released from many cell types into the extracellular space after fusion with the plasma membrane [6]. In addition to the proteins, various nucleic acids have recently been identified in exosomes, including mRNAs, miRNAs, and other noncoding RNAs [6]. These vesicles are widely distributed in various biofluids, and exosomal miRNAs have been shown to have an important role in disease progression.

In this chapter, we define a compilation of protocols that can be employed for the purification, quantification, and characterization of miRNA expression profiles in biofluids, whole blood samples, and tissue samples obtained from in vivo studies. In addition, we discuss methods for the isolation of exosomal miRNA populations from biofluids and blood samples. Therefore, this chapter can be

used as a guideline for studying the total miRNA and exosomal miRNA populations in in vivo samples from studies focusing on RSV pathogenesis and disease progression.

2 Materials

2.1 Exosome Isolation from Biofluids

1. Exosome precipitation: ExoQuick Exosome Precipitation Solution (System Biosciences, Inc.) or equivalent.
2. Biofluid samples: Collected from clinical samples or research experiments using appropriate methods. Store at −80 °C.

2.2 Plasma Separation and Storage from Total Blood

1. Blood collection: Primary blood collection tube containing EDTA.
2. Refrigerated microcentrifuge.

2.3 Serum Separation and Storage from Whole Blood

1. Blood collection: Primary blood collection tube without clot activator and without anticoagulants such as EDTA or citrate.
2. Refrigerated microcentrifuge.

2.4 RNA Isolation

1. Purified exosome samples:
   (a) RNAzol® RT (Molecular Research Center, Inc.) or equivalent
   (b) RNA Precipitation: Polyacryl Carrier (Molecular Research Center, Inc.) or equivalent
   (c) Isopropanol
   (d) 75 % ethanol
2. Whole blood samples:
   (a) Blood miRNA isolation kit: we recommend miRNeasy Serum/Plasma Kit from QIAGEN. This kit includes RNAeasy® MiniElute spin columns, QIAzol® Lysis reagent, Buffer RWT, Buffer RPE, and Ce_miR39_1 miScript® primer assay.
   (b) Chloroform (without added isoamyl alcohol)
   (c) Ethanol (70 %, 80 %, and 100 %)
   (d) miRNeasy Serum/Plasma Spike-In Control or *C. elegans* miR-39 miRNA mimic
3. Tissue sample RNA isolation:
   (a) RNAzol® RT (Molecular Research Center, Inc.) or equivalent.
   (b) gentleMACS™ Dissociator and gentleMACS™ M Tubes or equivalent

(c) RNA Precipitation: Polyacryl Carrier (Molecular Research Center, Inc.)

(d) Isopropanol

(e) 75 % Ethanol

4. Eppendorf Vacufuge Concentrator or equivalent
5. Nuclease-free water

2.5 RNA Quantification

1. Total RNA: Extracted from samples of interested using appropriate purification method outlined in Subheading 3.4.
2. Sample quantification: Epoch microplate spectrophotometer (BioTek) or equivalent spectrophotometer.
3. Take3 Micro-Volume Plate (BioTek) or equivalent
4. Nuclease-free water

2.6 RT-qPCR

1. Quantified total RNA from samples of interested as outlined in Subheading 3.5.
2. miRNA first-Strand cDNA Synthesis Kit (Agilent Technologies) or equivalent miRNA amplification kit
3. Brilliant III Ultra-Fast SYBR Green QPCR Master Mix or equivalent qPCR kit containing SYBR green.
4. qPCR forward primer: a unique forward primer that allows for specific amplification of the miRNA of interest (*see* **Note 1**).
5. Nuclease-free water

3 Methods

3.1 Exosome Isolation from Biofluid Samples

1. Collect biofluid and centrifuge at 3000 × *g* for 15 min to remove cells and cellular debris (if harvesting samples prior to this step see storage details in **Note 2**).
2. Transfer supernatant to a sterile microcentrifuge tube and add the appropriate volume of ExoQuick Exosome Precipitation Solution to the biofluid sample (*see* Table 1). Mix well by inverting of flicking the tube.
3. Store at 4 °C for ≥12 h (*see* **Note 3**) for ascites fluid, urine, and spinal fluid samples or 30 min for serum samples (*see* **Note 4**).
4. Centrifuge the ExoQuick–biofluid mixture at 1500 × *g* for 30 min at 4 °C. After centrifugation, the exosome may appear as a beige or white pellet at the bottom of the tube.
5. Aspirate the supernatant.
6. Spin down residual ExoQuick solution by centrifugation at 1500 × *g* for 5 min at 4 °C. Remove all traces of fluid by aspiration (*see* **Note 5**).
7. Proceed with RNA isolation, as described in Subheading 3.2.3.

Table 1
Example of the appropriate volume of exosome precipitation solution to add per biofluid sample

Biofluid	ExoQuick type	Sample volume	ExoQuick volume	Incubation time
Serum	ExoQuick	250 µl	63 µl	30 min
Ascites fluid	ExoQuick	250 µl	63 µl	≥12 h
Urine	ExoQuick-TC	5 ml	1 ml	≥12 h
Spinal fluid	ExoQuick-TC	5 ml	1 ml	≥12 h

Example of the appropriate volume of ExoQuick and ExoQuick-TC solution to add for each biofluid sample, more specifically serum, ascites fluid, urine, and spinal fluid. The optimal sample volume for serum and ascites fluid is 250 µl and for urine and spinal fluid is 5 ml, respectively

3.2 Plasma Separation from Whole Blood Samples

1. Collect whole blood in primary blood collection tube containing EDTA as anticoagulant. Store tubes at room temperature and process within 1 h (*see* **Note 6**).
2. Centrifuge blood samples in primary blood collection tubes at 1900 × *g* for 10 min at 4 °C.
3. Transfer the upper (yellow) plasma phase to a new tube without disturbing the intermediate buffy coat layer.
4. Centrifuge plasma samples at 16,000 × *g* for 10 min at 4 °C.
5. Carefully transfer cleared supernatant to a new tube without disturbing the pellet.
6. Store at 4 °C until further processing, if plasma will be used for RNA isolation on the same day. For longer storage, keep plasma frozen in aliquots at −80 °C.
7. Before using frozen plasma for RNA isolation, thaw at room temperature (*see* **Note 7**).

3.3 Serum Separation from Whole Blood Samples

1. Collect whole blood in primary blood collection tube without clot activator and without anticoagulants such as EDTA or citrate. Store tubes at room temperature for 30 min to 1 h to allow for complete clotting.
2. Continue with **step 2** of the protocol as described in Subheading 3.2.

3.4 RNA Isolation

3.4.1 Purified Exosome Samples

1. Resuspend the exosome pellet obtained in Subheading 3.1 using 1 ml of RNAzol® RT per 0.4 ml of biofluid starting sample volume (for example *see* Table 2).
2. Add 0.4 ml of sterile water per 1 ml of RNAzol® RT used for homogenization.
3. Shake the resulting sample mixture vigorously for 15 s and incubate it for 15 min at room temperature.
4. Centrifuge samples at 12,000 × *g* for 15 min at 4 °C.

5. Transfer 1 ml of the supernatant (or approximately 75% of total supernatant volume) to a new microcentrifuge tube, leaving a layer of the supernatant above the DNA/protein pellet.
6. Add to the tube containing the supernatant an equal volume of isopropanol.
7. Add 1 μl of Polyacryl Carrier to the isopropanol–supernatant mixture. Incubate mixture for 15 min at room temperature.
8. Centrifuge at 12,000 × *g* for 10 min at 4 °C.
9. Aspirate supernatant, wash RNA pellet twice by adding 0.6 ml of 75% ethanol (v/v) (*see* **Note 8**). Centrifuge the pellets at 8000 × *g* for 3 min at 4 °C. Aspirate the alcohol solution.
10. Dry the RNA pellet at 45 °C for 6 min using the Eppendorf Vacufuge Concentrator or equivalent.
11. Dissolve the RNA pellet in 20 μl of nuclease-free water per 1 ml of starting sample volume.
12. Proceed to RNA quantification or store the reactions at −20 °C. For long-term storage, store the reactions at −80 °C.

3.4.2 Total Blood Samples

1. Prepare serum or plasma or thaw frozen samples.
2. Use the miRNeasy Serum/Plasma Kit (QIAGEN), and add 5 volumes of QIAzol Lysis Reagent (*see* **Note 9**). Mix by vortexing (*see* **Note 10**).
3. Incubate the tube containing the lysate at room temperate for 5 min.
4. Add chloroform of an equal volume to the starting sample to the tube containing the lysate. Shake vigorously for 15 s (*see* **Note 11**). Incubate the tube at room temperature for 3 min.
5. Centrifuge at 12,000 × *g* for 15 min at 4 °C.

Table 2
Example of the appropriate volume of RNAzol® RT solution to add to exosome pellet per biofluid sample

Biofluid exosome pellet	Sample volume	RNazol® RT volume
Serum	250 μl	625 μl
Ascites fluid	250 μl	625 μl
Urine	5 ml	12.5 ml
Spinal fluid	5 ml	12.5 ml

Example of the appropriate volume of RNAzol® RT solution to add to exosome pellet per biofluid sample, specifically serum, ascites fluid, urine, and spinal fluid. For liquid samples, homogenize liquid samples using 1 ml of RNAzol®RT per up to 0.4 ml of a liquid sample. For processing a small volume sample, mix the sample with 1 ml of RNAzol®RT and supplement the mixture with water to approach the sample + water volume of 0.4 ml

6. Transfer the upper aqueous phase to a new tube. Add 1.5 volumes of 100% ethanol and mix thoroughly by pipetting up and down several times.
7. Pipette up to 700 µl of the sample into an RNeasy MinElute spin column in a 2 ml collection tube.
8. Close the lid gently and centrifuge at ≥8000 × *g* for 15 s at room temperature. Discard the flow-through.
9. Repeat **step 8** using the remainder of the sample. Discard the flow-through after each centrifugation.
10. Add 700 µl Buffer RWT to the RNeasy MinElute spin column. Close the lid gently and centrifuge at ≥8000 × *g* for 15 s at room temperature. Discard the flow-through.
11. Add 500 µl Buffer RPE onto the RNeasy MinElute spin column. Close the lid gently and centrifuge at ≥8000 × *g* for 15 s at room temperature. Discard the flow-through.
12. Add 500 µl of 80% ethanol onto the RNeasy MinElute spin column. Close the lid gently and centrifuge at ≥8000 × *g* for 15 s at room temperature. Discard the collection tube with the flow-through.
13. Place the RNeasy MinElute spin column into a new 2 ml tube. Open the lid of the spin column, and use the Eppendorf Vacufuge Concentrator at 45 °C for 6 min to dry the membrane. Discard the collection tube with the flow-through.
14. Place the RNeasy MinElute spin column in a new 1.5 ml tube. Add 10 µl nuclease-free water directly to the center of the spin column membrane. Close the lid gently, and allow the water to saturate the spin column membrane for 3–5 min at room temperature.
15. Centrifuge for 1 min at full speed to elute the RNA.
16. Repeat **steps 13** and **14** using 5 µl of nuclease-free water (*see* **Note 12**).
17. Proceed to RNA quantification or store the reactions at –20 °C. For long-term storage, store the reactions at –80 °C.

3.4.3 Tissue Samples

1. Collect tissue sample in gentleMACS™ M Tubes and add 1 ml of RNAzol® RT per 100 mg tissue (*see* **Note 13**).
2. Homogenize tissue samples in the gentleMACS™ Dissociator using the appropriate pre-set gentleMACS programs.
3. Centrifuge the homogenized tissue sample at 700 × *g* for 10 min at room temperature.
4. Transfer supernatant to a sterile microcentrifuge tube, and add 0.4 ml of sterile water per 1 ml of RNAzol® RT used for homogenization.
5. Continue with **step 3** of protocol described in Subheading 3.2.1.

3.5 RNA Quantification

1. Quantify RNA using appropriate methods, if using the Epoch Microplate Spectrophotometer, then blank the plate by adding 2 µl of nuclease-free water (*see* **Note 14**) per microspot on Take3 Micro-Volume Plate.
2. Remove water from microspot(s) using Kimwipes or equivalent.
3. Add 2 µl of RNA sample per microspot on Take3 Micro-Volume Plate or equivalent.
4. Use the Gen5 Data Analysis software interface (BioTek) program to obtain information regarding the quality and quantity of total RNA per sample or equivalent software per method (*see* **Note 15**).
5. Proceed to RT-qPCR or store the reactions at −20 °C. For long-term storage, store the reactions at −80 °C.

3.6 RT q-PCR

3.6.1 Polyadenylation Reaction

1. Extract total RNA using the appropriate protocol as described in Subheading 3.4.
2. Standardize the concentration of total RNA to be used per reaction across all samples (*see* **Note 16**).
3. Calculate the necessary amount of each polyadenylation reaction component for the total number of reactions, plus the noPAP control reaction (*see* **Note 17**). If you are using the miRNA First-Strand cDNA Synthesis Kit (Agilent Technologies), add the following components *in order* for ×1 reaction:

(a)	Nuclease-free water	= *N*× amount to 19 µl final volume
(b)	5× poly A polymerase buffer	= *N*× 4.0 µl
(c)	rATP (10 mM)	= *N*× 1.0 µl
(d)	Total RNA (30 ng to 1 µg)	= *x*µl (variable)
		Total reaction volume: 19 µl

4. Add 1.0 µl of *E. coli* poly A polymerase to each reaction and mix gently (*see* **Note 18**). This will result in a final reaction volume of 20 µl per 1 reaction. Briefly centrifuge the reactions to collect the contents at the bottoms of the tubes and remove any bubbles.
5. Incubate the reactions at 37 °C for 30 min followed by 95 °C for 5 min to terminate adenylation, and then hold at 4 °C. Immediately transfer the tubes to ice.
6. Proceed to cDNA synthesis or store the reactions at −20 °C. For long-term storage, store the reactions at −80 °C.

3.6.2 cDNA Synthesis

1. Use or thaw on ice polyadenylated RNA obtained using the appropriate protocol as described in Subheading 3.6.1.
2. Determine the number of polyadenylation reactions (N) to prepare for the assay, using the following guidelines:
   (a) For the number of samples (n), including the noPAP control reaction (*see* **Note 17**) then $N = n + 1$.
3. Calculate the amount of cDNA synthesis mastermix to be prepared for the assay. If you are using the miRNA First-Strand cDNA Synthesis Kit (Agilent Technologies), add the following components *in order* for N reactions:

(a) Nuclease-free water	$= N \times 11.2$ µl
(b) 10× AffinityScript RT Buffer	$= N \times 2.0$ µl
(c) dNTP mix (100 mM)	$= N \times 0.8$ µl
(d) RT adaptor primer (10 µM)	$= N \times 1.0$ µl
(e) AffinityScript RT/RNase Block	$= N \times 1.0$ µl
Total volume	$= N \times 16$ µl

4. Gently mix the mastermix without creating bubbles (do not vortex), and then distribute the mixture to individual PCR reaction tubes.
5. Add 4.0 µl of polyadenylation reaction from Subheading 3.6.1 to the individual PCR reaction tubes to bring the final reaction volume to 20 µl.
6. Briefly centrifuge the reactions to collect the contents at the bottoms of the tubes and remove any bubbles.
7. Incubate the reactions using the following thermocycling conditions:
   (a) 55 °C for 5 min
   (b) 25 °C for 15 min
   (c) 42 °C for 30 min
   (d) 95 °C for 5 min
   (e) Hold at 4 °C
8. Dilute the cDNA 1:5 using nuclease-free water (e.g., for 20 µl cDNA synthesis reaction volume add 80 µl of nuclease-free water).
9. Place the completed first-strand cDNA synthesis reactions on ice for immediate use in qPCR. For long-term storage, keep the reactions at −20 °C.

3.6.3 qPCR

1. Use or thaw on ice cDNA obtained using the appropriate protocol as described in Subheading 3.6.2.

2. Determine the number of qPCR reactions (N) to prepare for the assay, using the following guidelines:
   (a) For the number of samples (n), including the noPAP control reaction and no-template control (NTC) reaction then $N = n + 2$.
   (b) If using endogenous control genes for normalization of miRNA expression levels, then for each endogenous control (x control) gene include an additional reaction: $N = (x \text{ control} \times n) + 2$ (*see* **Note 19**).
3. If a passive reference dye is needed, dilute the provided reference dye using nuclease-free water to specifications of your qPCR instrument. If using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies), then follow these guidelines (*see* **Note 20**):
   (a) Dilute the dye 1:50 (for a final concentration of 300 nM in the reactions) for the ABI StepOnePlus instrument.
   (b) Dilute the dye 1:500 (for a final concentration of 30 nM in the reactions) for the Stratagene Mx3000P or Mx3005P instrument or the ABI 7500 Fast instrument.
   (c) Bio-Rad CFX96, the Roche LightCycler 480, and the QIAGEN Rotor-Gene Q instruments do not require the use of the reference dye.
4. Calculate the amount of qPCR mastermix to be prepared for the assay. If you are using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix, add the following components *in order* for N reactions (*see* **Note 21**):

(a) Nuclease-free water	$= N \times 3.32$ µl
(b) 2× SYBR Green QPCR master mix	$= N \times 6.0$ µl
(c) Forward primer	$= N \times 0.12$ µl
(d) Reverse primer (*see* **Note 22**)	$= N \times 0.38$ µl
(e) Diluted reference dye	$= N \times 0.18$ µl
Total volume	$= N \times 10$ µl

5. Gently mix the mastermix without creating bubbles (do not vortex), and then distribute the mixture to individual PCR reaction tubes.
6. Add 2 µl of the diluted cDNA obtained in Subheading 3.6.2 to each reaction to bring the final reaction volume to 12 µl.

7. Gently mix the reactions without creating bubbles (do not vortex), and then centrifuge the reactions at briefly (*see* **Note 23**).
8. Place the reactions in the qPCR instrument, and amplify using the appropriate thermocycler conditions (*see* **Note 24**). If using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix and the Stratagene Mx3000P/Mx3005P instrument, use the following thermocycler conditions as a guideline:

   (a) 95 °C for 3 min ×1 cycle

   (b) 95 °C for 15 s
   (c) 55 °C for 30 s } ×40 cycles
   (d) 60 °C for 30 s

   (e) 95 °C for 1 min
   (f) 55 °C for 30 s } ×1 cycle
   (g) 95 °C for 30 s

3.7 Data Analysis

1. Generate an excel spreadsheet with the C_T values from the qPCR cycle.
2. Normalize C_T values using the average C_T value of the endogenous controls, to obtain the ΔC_T values.
3. Normalize the ΔC_T values using the average C_T value of uninfected/mock samples, to obtain the $\Delta\Delta C_T$ values.
4. Use the following formula: 2^−($\Delta\Delta C_T$) to calculate the miRNA expression in fold change/mock.

4 Notes

1. To obtain the most recent update of mature miRNA sequences for the design of unique forward primers for miRNA profiling we recommend miRBase: the microRNA database or the Sanger database.
2. In order to maintain optimal viability of the exosomes in biofluid samples store the samples at −80 °C prior to exosome isolation.
3. Biofluids that require ≥12 h of incubation time can be left overnight.
4. The tubes do not need to be agitated during this period.
5. When aspirating the residual ExoQuick solution from the sample, take great care not to disturb the precipitated exosomes in the pellet.

6. Do not use heparin-containing blood collection tubes as this anticoagulant can interfere with downstream assays, such as RT-qPCR.
7. If using thawed plasma samples for RNA isolation step, centrifuge thawed plasma samples at 16,000 × *g* for 5 min at 4 °C to remove cryoprecipitates. Then transfer supernatant to new tube.
8. The RNA isolation can be interrupted and samples can stored in 75% ethanol overnight at room temperature, for at least 1 week at 4 °C, or at least 1 year at −20 °C.
9. Recommend using 50–200 μl per RNA sample. Processing of more than 200 μl sample is not recommended as the amounts of contaminants introduced by larger sample volumes can interfere with the purification process and downstream assays.
10. After addition of QIAzol Lysis Reagent, lysates can be stored at −80 °C for several months.
11. Thorough mixing is important for subsequent phase separation.
12. This elution method provides a higher concentration of RNA. Do not elute with less than 10 μl for the first elution step, as the spin column membrane will not be sufficiently hydrated.
13. When processing tissues with high DNA content such as spleen use 50 mg of tissue/1 ml reagent. And to further prevent RNA degradation in tissues with high level of RNase, perform homogenization in cold RNAzol® RT.
14. If RNA samples were eluted in a solution other than nuclease-free water, use this to blank apparatus for RNA quantification.
15. RNA samples with $OD_{260/280}$ ratios of 1.8–2.0 are optimally pure for downstream assays such as RT-qPCR.
16. In order to obtain accurate information on the miRNA expression levels within the samples of interest it is important to use a standard concentration of RNA across all samples for RT-qPCR. The range of RNA concentrations that can be used is 30 ng to 1 μg.
17. To screen for contamination, include a noPAP control cDNA template. The noPAP control cDNA is prepared from a polyadenylation reaction in which the poly A polymerase is omitted from the reaction.
18. Enzymes (such as poly A polymerase) should be mixed gently without generating bubbles. Do not vortex. Also pipette the enzymes carefully and slowly to avoid pipetting errors due to the viscosity of the 50% glycerol in the buffer.

19. Carefully select a set of several endogenous control genes based on the species, tissues, or biofluids used in your study. Alternatively, or in addition to, use specific miRNAs that demonstrate the least variability across experimental conditions under investigation.
20. Prepare fresh dilutions of the reference dye prior to setting up the reactions, and keep all solutions containing the reference dye protected from light at all times.
21. SYBR Green dye is light sensitive, solutions containing the master mix should be protected from light as much as possible. Once the tube containing the 2× SYBR Green QPCR master mix is thawed, store it on ice while setting up the reactions. Following initial thawing of the master mix, store the unused portion at 4 °C for up to 3 months or return to −20 °C for long term storage.
22. For the reverse primer, use the universal reverse primer provided in the miRNA first-strand cDNA synthesis kit (QIAGEN). The universal reverse primer anneals to the cDNA sequence tag added to the 5′ end of all cDNA species by the RT adaptor primer during first-strand cDNA synthesis.
23. Make sure all reactions are free of any bubbles, as bubbles interfere with fluorescence detection during qPCR cycling.
24. The optimal PCR program will vary based on several factors such as the instrument you are using, the qPCR kit recommendations, and the T_m of the primers.

References

1. Oglesby IK, McElvaney NG, Greene CM (2010) MicroRNAs in inflammatory lung disease–master regulators or target practice? Respir Res 11:148
2. Ha M, Kim VN (2014) Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol 15(8):509–524
3. Berezikov E (2011) Evolution of microRNA diversity and regulation in animals. Nat Rev Genet 12(12):846–860
4. Grimson A et al (2008) Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. Nature 455(7217):1193–1197
5. Barichievy S, Bakre A (2014) Host-encoded mirnas involved in host-pathogen interactions (Chapter 6). In: Tripp RA, Karpilow J (eds) Frontiers in RNAi, vol 1. Bentham Science, Sharjah, UAE, pp 107–143
6. Zhang J et al (2015) Exosome and exosomal microRNA: trafficking, sorting, and function. Genomics Proteomics Bioinformatics 13(1): 17–24
7. Hebert SS, De Strooper B (2009) Alterations of the microRNA network cause neurodegenerative disease. Trends Neurosci 32(4):199–206
8. Amarilyo G, La Cava A (2012) miRNA in systemic lupus erythematosus. Clin Immunol 144(1):26–31
9. Bostjancic E, Glavac D (2008) Importance of microRNAs in skin morphogenesis and diseases. Acta Dermatovenerol Alp Pannonica Adriat 17(3):95–102
10. Chan EK, Ceribelli A, Satoh M (2013) MicroRNA-146a in autoimmunity and innate immune responses. Ann Rheum Dis 72(Suppl 2):ii90–ii95
11. Moser JJ, Fritzler MJ (2013) Relationship of other cytoplasmic ribonucleoprotein bodies (cRNPB) to GW/P bodies. Adv Exp Med Biol 768:213–242
12. Persengiev SP (2012) miRNAs at the crossroad between hematopoietic malignancies and autoimmune pathogenesis. Discov Med 13(70): 211–221

13. Wittmann J, Jack HM (2011) microRNAs in rheumatoid arthritis: midget RNAs with a giant impact. Ann Rheum Dis 70(Suppl 1):i92–i96
14. Al-Quraishy S et al (2012) Organ-specific testosterone-insensitive response of miRNA expression of C57BL/6 mice to Plasmodium chabaudi malaria. Parasitol Res 111(3):1093–1101
15. Delic D et al (2011) Hepatic miRNA expression reprogrammed by Plasmodium chabaudi malaria. Parasitol Res 108(5):1111–1121
16. Ge Y et al (2013) Serum microRNA expression profile as a biomarker for the diagnosis of pertussis. Mol Biol Rep 40(2):1325–1332
17. Ma L et al (2014) miRNA-1236 inhibits HIV-1 infection of monocytes by repressing translation of cellular factor VprBP. PLoS One 9(6), e99535
18. Podolska A et al (2012) Profiling microRNAs in lung tissue from pigs infected with Actinobacillus pleuropneumoniae. BMC Genomics 13:459
19. Singh PK, Singh AV, Chauhan DS (2013) Current understanding on micro RNAs and its regulation in response to Mycobacterial infections. J Biomed Sci 20:14
20. Wang C et al (2011) Comparative miRNA expression profiles in individuals with latent and active tuberculosis. PLoS One 6(10):e25832
21. Inchley CS et al (2015) Nasal mucosal microRNA expression in children with respiratory syncytial virus infection. BMC Infect Dis 15:150
22. Thornburg NJ, Hayward SL, Crowe JE Jr (2012) Respiratory syncytial virus regulates human microRNAs by using mechanisms involving beta interferon and NF-kappaB. MBio 3(6)
23. Rossi GA, Silvestri M, Colin AA (2015) Respiratory syncytial virus infection of airway cells: Role of microRNAs. Pediatr Pulmonol. 50(7):727–32. doi:10.1002/ppul.23193
24. Sibley CR et al (2012) The biogenesis and characterization of mammalian microRNAs of mirtron origin. Nucleic Acids Res 40(1):438–448
25. Berezikov E et al (2007) Mammalian mirtron genes. Mol Cell 28(2):328–336
26. Cai X, Hagedorn CH, Cullen BR (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 10(12):1957–1966
27. Lee Y et al (2004) MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23(20): 4051–4060
28. Borchert GM, Lanier W, Davidson BL (2006) RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol 13(12):1097–1101
29. Fabian MR, Sonenberg N, Filipowicz W (2010) Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 79:351–379
30. Li JH et al (2014) starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic Acids Res 42(Database issue):D92–D97
31. Gallo A et al (2012) The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PLoS One 7(3):e30679
32. Gutzeit C et al (2014) Exosomes derived from Burkitt's lymphoma cell lines induce proliferation, differentiation, and class-switch recombination in B cells. J Immunol 192(12):5852–5862
33. Lv LL et al (2013) Isolation and quantification of microRNAs from urinary exosomes/microvesicles for biomarker discovery. Int J Biol Sci 9(10):1021–1031
34. Michael A et al (2010) Exosomes from human saliva as a source of microRNA biomarkers. Oral Dis 16(1):34–38
35. Zhou Q et al (2012) Immune-related microRNAs are abundant in breast milk exosomes. Int J Biol Sci 8(1):118–123
36. Jia HL et al (2014) MicroRNA expression profile in exosome discriminates extremely severe infections from mild infections for hand, foot and mouth disease. BMC Infect Dis 14:506
37. Shimbo K et al (2014) Exosome-formed synthetic microRNA-143 is transferred to osteosarcoma cells and inhibits their migration. Biochem Biophys Res Commun 445(2):381–387

Chapter 15

Mouse and Cotton Rat Models of Human Respiratory Syncytial Virus

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Abstract

Human respiratory syncytial virus (hRSV) is a common respiratory virus that is usually no cause for alarm. Symptoms of hRSV usually resemble those of the common cold and can go undiagnosed. However, infants as well as the elderly are at risk for developing severe cases, which can lead to high morbidity and mortality rates especially if there are underlying health issues. Despite many years of effort, no vaccine or specific treatments exist and RSV is still the leading cause of infant hospitalizations worldwide. Here, we describe methods to infect two widely used small animal models: laboratory mice and cotton rats.

Key words Human respiratory syncytial virus, Methodology, Propagation, Titration, In vivo infections

1 Introduction

Human respiratory syncytial virus (hRSV) is the leading global cause of serious lower respiratory tract infection in children, infants, elderly, and immunocompromised adults [1, 2]. Disease symptoms resulting from hRSV infection are age dependent [3], and can range from mild cold-like symptoms to bronchiolitis, recurrent wheezing, and asthma in severe cases. Furthermore, hRSV is the leading cause of hospitalization in children less than 24 months and can cause severe morbidity and mortalities in premature infants and children with other chronic illnesses [4, 5]. It has been estimated that globally each year, hRSV is responsible for over 34 million episodes of lower respiratory tract infections leading to 3.4 million hospitalizations and 66,000–200,000 deaths [6, 7]. Despite being a huge social and economic burden, only supportive care is usually administered to hRSV patients due to the lack of vaccines and specific treatments. In vivo models are indispensable tools towards vaccine development and generation of novel therapeutics. There are several animal models of hRSV that are commonly used; however, none can replicate the

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complete disease spectrum as seen in humans. The choice of an animal model highly depends on the specific research questions under consideration. For instance, small animals such as mice are widely used in therapeutic research and immunology [8, 9]. One of the major benefits in using mice as an hRSV model is their relative permissiveness to the virus and their ability to recapitulate similar time-course disease outcomes in human infections [10, 11]. Since the late 1970s, the cotton rat (*Sigmodon hispidus*) has also been widely used in hRSV research [12]. This small animal model is also highly permissive to hRSV infection and is frequently used to assess the efficacy of novel vaccines, antivirals, and neutralizing antibodies such as palivizumab [13]. In fact, it has been reported that cotton rats are 100 times more susceptible to hRSV infections than mice and they develop both upper and lower respiratory illnesses following intranasal infection [12, 14]. Here, we present methods for hRSV propagation, viral titration by immunostaining, and plaque methods [15] in addition to in vivo infection of both mice and cotton rat models.

2 Materials

2.1 Animals

1. Neonate (≤7 days old) or adult (6–8 weeks old) female BALB/c mice. C57BL/6 mice can also be used, but they are less susceptible to RSV infection than BALB/c mice.
2. 5–8-Week-old female cotton rats (*Sigmodon hispidus*).
3. RSV A or B stocks.

2.2 Anesthetics

1. Avertin (2, 2, 2-tribromoethanol) stock solution: Combine 25 g avertin with 15.5 mL tert-amyl alcohol (2-methyl-2-butanol) and mix at room temperature for ~12 h (Heating to approximately 40 °C helps dissolve the avertin). The final concentration of the solution is 1.6 g/mL. Store at RT in a dark bottle.
2. Avertin (20 mg/mL): Mix 0.5 mL of avertin stock solution and 39.5 mL of 0.9% saline. Filter the solution through a 0.2 μm filter into a sterile dark bottle. Store at 4 °C and replace at least once a month, ideally fortnightly. Dosage is 180–250 mg/kg given intraperitoneally (i.p.) in mice.
3. Isoflurane: Isoflurane should be delivered to adult mice as a mixture of 4–5 % isofluorane and 95 % oxygen for induction and lowered to 1–3 % isofluorane for maintenance. For neonates, 2–4 % isofluorane should be given.
4. Ketamine-xylazine (KX): Combine 2 mL of ketamine 50 mg/mL, 0.8 mL of xylazine 20 mg/mL, and 7.2 mL of sterile water. Store at 4 °C for a maximum of 2 weeks. Dosage is 40 mg/kg of ketamine and 6 mg/kg of xylazine for an adult mouse and 100 mg/kg ketamine and 16 mg/kg xylazine for an adult cotton rat.

2.3 Infection of Mice and Cotton Rats with RSV

1. Mouse restraint.
2. Induction box for inducing inhaling anesthesia with animals.
3. PBS.
4. Micropipette.
5. 200 μL Tips.
6. Scalpels.
7. Blood collection tubes.
8. 23G and 25G needles.
9. 1 mL Syringes.
10. Ice.
11. Digital scale.

3 Methods

Before performing any procedures in mice and cotton rats, protocols and the experimental conditions must be approved by the animal care and use committee of the university/organisation where the experiments will be conducted.

3.1 RSV Infection of Adult and Neonate Mice (Mus musculus)

1. Group 5–6 adult female mice per cage (depending on the specific type) using standard mouse cages on a ventilated rack under controlled conditions. If working with neonates keep them with their mothers under the same environmental conditions as the adult mice. Handling of neonates needs to be quiet, gentle, and swift to limit stress.
2. Keep the mice in a 12-h diurnal cycle to maintain the animals' natural circadian rhythms.
3. Provide food and water *ad libitum*.
4. Prepare the virus inoculum by diluting the RSV stock virus in PBS to reach the desired challenge dose (*see* **Note 1**). Keep the virus on ice.
5. Anesthetize adult mice by injecting 200–250 μL of avertin i.p., and 50–100 μL of KX intramuscularly (i.m.) or by isoflurane inhalation. For neonates isoflurane is the preferred anesthetic (*see* **Note 2**).
6. Test mice for rear foot reflexes before proceeding to the infection, and continue observation of respiratory pattern, mucous membrane color, and responsiveness to manipulations throughout the procedure.
7. Weigh the mice using a digital scale and record as the starting weight.
8. Firmly scruff the neck of the animal and hold them upright with their neck fully extended. Using a micropipette, carefully

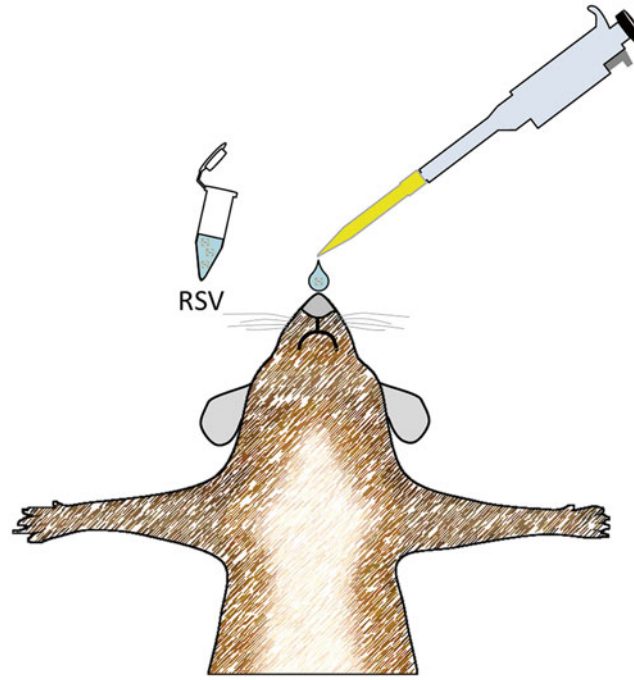

Fig. 1 Intranasal delivery of RSV to a mouse or cotton rat

administer the inoculum drop by drop into the nostrils alternating sides: use 50–100 μL for adults and 10–20 μL for neonates (*see* **Note 3**, Fig. 1). Mock-infected mice should receive an equivalent volume of PBS instead of virus.

9. Place anesthetized mice in a clean cage and observe to ensure that they recover from the anesthetic. Remember to keep the mice warm during recovering. This can be done by placing the cage on a heat mat on a low setting.
10. Monitor mice daily for signs of disease including weight loss (*see* **Note 4**).
11. Lung tissue collection for viral titration, cytokine evaluation, and/or histopathology may be done following euthanasia of animals by cervical dislocation or any other accepted method of euthanasia (*see* **Note 5**).
12. Signs of infection and disease can be detected in different tissues:
    (a) The first sign of disease is ruffled fur seen on day 5 p.i. Peak illness is seen around day 7–8 p.i. Older mice have more severe signs of disease compared to their younger counterparts. Severity of RSV appears to be age dependent. Signs of illness include lethargy, ruffled fur, and substantial weight loss (*see* **Note 6**). However, animals generally recover by day 10 p.i.

(b) Peak viremia is observed on day 4 post-infection (p.i.) and is usually cleared by day 8 p.i. Viremia may be monitored by collecting blood via the tail vein (*see* **Note 7**).

(c) Peribronchovascular inflammation is observed as early as day 3 p.i. but peaks at about day 6 post-infection (p.i.) (*see* **Note 5**).

(d) RSV replication in the lungs peaks at day 5 post-infection. Lungs can be harvested and viral titer quantified by plaque assay (see **Chapter 3**).

(e) Infected neonates can develop long-term "asthma" characterized by increased airway hypersensitivity, mucus hyperproduction, Th2 cytokine and cellular responses, and airway remodeling. A small percentage may succumb immediately following infection.

3.2 RSV Infection in Cotton Rats (Sigmodon hispidus) (see Note 9)

1. House animals in groups of 2–5 cotton rats per cage using static filter-top polycarbonate rat cages or microisolation rat boxes on a ventilated rack.
2. Keep the cotton rats in a 12-h diurnal cycle to maintain the animals' natural circadian rhythms.
3. Provide food and water *ad libitum*.
4. Prepare the virus inoculum by diluting the RSV stock virus in PBS to reach the desired inoculation dose (*see* **Note 10**). Keep the virus on ice.
5. Anesthetize the cotton rats by isoflurane inhalation followed by IP injection of KX (100 mg/kg ketamine and 16 mg/kg xylazine per animal) (*see* **Note 11**).
6. Using a micropipette, carefully administer the inoculum drop by drop into the nostrils alternating sides: use 50–100 μL of inoculum per cotton rat (*see* Fig. 1). Mock-infected rats should receive an equivalent volume of PBS instead of virus (*see* **Note 12**).
7. Monitor animals daily for signs of disease (*see* **Notes 13** and **14**).
8. Peak of lung inflammation can be seen on days 5–6 post-infection. Lung tissue collection for viral titration, cytokine evaluation, and/or histopathology may be done following euthanasia of animals by cervical dislocation or any other accepted method of euthanasia (*see* **Note 5**).
9. Animals develop viremia with peak titers seen on day 4 p.i. and viral clearance is achieved by day 7 p.i. Blood can be collected via the retro-orbital plexus:

   (a) As a general rule, do not collect more than 10 % of total circulating blood volume in healthy, normal, adult animals on a single occasion. 7.5 % can be removed every 7 days, and 1 % every 24 h. When using the retro-orbital vein, a minimum of 10 days should be allowed for tissue repair

before repeat sampling from the same orbit. Otherwise the healing process may interfere with blood flow.

(b) Anesthetize the cotton rats using the method described in **step 5** (*see* **Note 11**).

(c) Once a deep plane of sleep is reached, insert a Pasteur pipette into the retro-orbital plexus applying light pressure.

(d) To increase yield apply gentle pressure to the jugular vein with the other hand. Blood flow should stop by applying gentle pressure over the closed eyelid for approximately 30 s.

(e) Carefully monitor for adverse effect. Training is required to perform this task on animals.

4 Notes

1. Adult mice should receive between 6×10^4 and 1×10^7 PFU of RSV, while neonates should be administered a dose between 4×10^4 and 2×10^5 PFU/g of body weight.
2. Adult mice can be anesthetized by intraperitoneal injection of avertin (180–250 mg/kg) or intramuscular injection of ketamine (40 mg/kg) and xylazine (6 mg/kg) [16]. When using isoflurane, it should be administered as a vapor as a mixture of 4–5 % isoflurane and 95 % oxygen for induction and lowered to 1–3 % isoflurane for maintenance. For neonates, 2–4 % isoflurane should be given [17].
3. We recommend to use 10 μL and 50 μL of virus for neonates and adult mice, respectively. Large volumes of virus can induce choking and death by asphyxia. If a mouse is struggling to breathe, gently massage its chest until it recovers.
4. Body weight loss can be used to measure disease severity. By weighing mice daily, % of body weight loss can be calculated by comparing with the starting weight prior to RSV infection (consider 100% body weight). The peak of % of body weight loss after RSV challenge is normally ~5–10%; Humane end points are usually around 10–15 % total weight loss. Please abide by the protocols and the experimental conditions approved by the animal care and use committee of the university/organization where the experiments will be conducted. Values higher than 15–20 % are unusual and may indicate another underlying condition.
5. Mice can be euthanized by an injection of 2X the dose of avertin followed by cervical dislocation. Cotton rats can be euthanized by an injection of 2X the dose of KX followed by cervical dislocation. If bronchioalveolar lavage is going to be

collected, exsanguination is recommended instead of cervical dislocation. Other methods of euthanasia include cardiac puncture or CO_2 inhalation. Perfusions may also be performed on animals using 4 % paraformaldehyde diluted in PBS 1X if only histology samples are to be collected. Please abide by the recommendations of the local animal ethics committee and national guidelines.

6. It has been shown that 4-week-old mice do not present signs of disease while 8-week-old mice get ruffled fur and slight weight loss. At 15 and 32 weeks animals are ruffled and lethargic and undergo significant weight loss up to 20 % without succumbing to the virus infection. The more severe signs of illness were seen in the eldest animals [16].
7. Tail vein collection is a quick and simple way to collect blood to monitor viremia. Heating of the animal with a heat lamp for a few minutes can help dilate the blood vessel. However, in our experience this is not often necessary. The animal should be placed comfortably into a restraint. Then, blood may be collected by generating a small nick into the lateral tail vein using a scalpel or by inserting a 23G needle into the vein. 50–200 μL of blood can be collected into a heparinized or EDTA-treated Minicollect® tube using this method. Blood flow should be stopped by applying light pressure to the area for approximately 30 s before the animal is returned to its cage.
8. Two inbred strains of cotton rats are also currently available from Iffa Credo (Lyon, France) and Harlan Inc. (Indianapolis, USA). The outbred colony developed at the National Institutes of Health (Bethesda, USA) was established from the capture of wild hispid cotton rats [18]. Outbred animals can also be purchased from Sage labs (Boyertown, USA).
9. Adult cotton rats should receive between 1×10^4 and 1×10^6 PFU of RSV per animal.
10. The behavior of the cotton rats is not conducive to easy handling; thus anesthesia should be planned for all procedures. Cotton rats should be first transferred to anesthesia box and exposed to 5 % isoflurane in oxygen. This process minimizes the stress of handling and prevents dropping or escape. Once anesthetized, cotton rats should be injected with KX to facilitate the prolonged sedation required for RSV infection. KX injection provides muscle relaxation, loss of consciousness, and analgesia. Other acceptable anesthetics include ketamine (25 mg/kg) and acepromazine (2.5 mg/kg)/diazepam (10 mg/kg) or ketamine (22 mg/kg), xylazine (10 mg/kg), and acepromazine (5 mg/kg) [18].
11. Animals should be held upright taking care of holding their snouts shut with the thumb to ensure virus delivery to the

lungs. Using a micropipette, carefully administer the virus drop by drop into the nostrils without provoking sneezing. No more than 100 μL may be used in 6-week-old or younger animals and up to 200 μL of inoculum can be used for older (adult) cotton rats.

12. All cotton rats are susceptible to RSV infection regardless of age. However, it has been shown that viral clearance is reduced in elderly animals ranging from 6 to 16 months [19].
13. Disease severity is monitored by weight loss or for juvenile animals as the inability to gain weight. Humane end points are usually around 10–15 % total weight loss. Please abide by the protocols and the experimental conditions approved by the animal care and use committee of the university/organization where the experiments will be conducted.

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References

1. McNamara PS, Smyth RL (2002) The pathogenesis of respiratory syncytial virus disease in childhood. Br Med Bull 61(1):13–28
2. Walsh EE. Respiratory syncytial virus infection in adults. Seminars in respiratory and critical care medicine 2011; 32(4):423–32
3. Munywoki PK, Koech DC, Agoti CN, et al. Influence of age, severity of infection, and co-infection on the duration of respiratory syncytial virus (RSV) shedding. Epidemiology and infection 2015;143(4):804–12
4. Turner TL, Kopp BT, Paul G, Landgrave LC, Hayes D Jr, Thompson R (2014) Respiratory syncytial virus: current and emerging treatment options. Clinicoecon Outcomes Res 6:217–225. doi:10.2147/CEOR.S60710
5. Hall CB, Weinberg GA, Blumkin AK, Edwards KM, Staat MA, Schultz AF, Poehling KA, Szilagyi PG, Griffin MR, Williams JV, Zhu Y, Grijalva CG, Prill MM, Iwane MK (2013) Respiratory syncytial virus-associated hospitalizations among children less than 24 months of age. Pediatrics 132(2):e341–e348. doi:10.1542/peds.2013-0303
6. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD (1999) Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet 354(9178):541–545. doi:10.1016/S0140-6736(98)10321-5
7. Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, O'Brien KL, Roca A, Wright PF, Bruce N, Chandran A, Theodoratou E, Sutanto A, Sedyaningsih ER, Ngama M, Munywoki PK, Kartasasmita C, Simoes EA, Rudan I, Weber MW, Campbell H (2010) Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. Lancet 375(9725):1545–1555. doi:10.1016/S0140-6736(10)60206-1
8. Klimczak L, Burkholder A, Wiltshire T, Fargo D, Kleeberger S, Verhein K, McCaw Z (2014) Gene expression profiling predicts response to respiratory syncytial virus (rsv) in inbred strains of mice. Am J Respir Crit Care Med 189:A3820
9. Bolger G, Lapeyre N, Dansereau N, Lagacé L, Berry G, Klosowski K, Mewhort T, Liuzzi M (2005) Primary infection of mice with high titer inoculum respiratory syncytial virus: characterization and response to antiviral therapy. Can J Physiol Pharmacol 83(2):198–213
10. Kong X, Hellermann GR, Patton G, Kumar M, Behera A, Randall TS, Zhang J, Lockey RF, Mohapatra SS (2005) An immunocompromised BALB/c mouse model for respiratory syncytial virus infection. Virol J 2(3)
11. Domachowske JB, Bonville CA, Rosenberg HF (2004) Animal models for studying respiratory syncytial virus infection and its long

term effects on lung function. Pediatr Infect Dis J 23(11):S228–S234

12. Prince GA, Jenson AB, Horswood RL, Camargo E, Chanock RM (1978) The pathogenesis of respiratory syncytial virus infection in cotton rats. Am J Pathol 93(3):771–791
13. Prince GA, Hemming VG, Horswood RL, Baron PA, Chanock RM (1987) Effectiveness of topically administered neutralizing antibodies in experimental immunotherapy of respiratory syncytial virus infection in cotton rats. J Virol 61(6):1851–1854
14. Boukhvalova MS, Prince GA, Blanco JC (2009) The cotton rat model of respiratory viral infections. Biologicals 37(3):152–159. doi:10.1016/j.biologicals.2009.02.017
15. McKimm-Breschkin JL (2004) A simplified plaque assay for respiratory syncytial virus—direct visualization of plaques without immunostaining. J Virol Methods 120(1):113–117
16. Graham BS, Perkins MD, Wright PF, Karzon DT (1988) Primary respiratory syncytial virus infection in mice. J Med Virol 26(2): 153–162
17. Gargiulo S, Greco A, Gramanzini M, Esposito S, Affuso A, Brunetti A, Vesce G (2012) Mice anesthesia, analgesia, and care, part I: anesthetic considerations in preclinical research. ILAR J 53(1):E55–E69. doi:10.1093/ilar.53.1.55
18. Niewiesk S, Prince G (2002) Diversifying animal models: the use of hispid cotton rats (Sigmodon hispidus) in infectious diseases. Lab Anim 36(4):357–372. doi:10.1258/002367702320389026
19. Curtis SJ, Ottolini MG, Porter DD, Prince GA (2002) Age-dependent replication of respiratory syncytial virus in the cotton rat. Exp Biol Med (Maywood) 227(9):799–802

Chapter 16

In Vivo Assessment of Airway Function in the Mouse Model

Azzeddine Dakhama and Erwin W. Gelfand

Abstract

This chapter describes two procedures commonly used to examine airway function in mice. Airway function can be assessed in vivo using noninvasive or invasive methods. Noninvasive methods can be used to monitor respiratory function in mice without the involvement of restraint, anesthesia, or surgery. The methods allow for multiple animals to be monitored simultaneously and can be used in longitudinal studies requiring repeated measurements on the same animals. Invasive methods are used to assess airway function under anesthesia, in mechanically ventilated mice. Although used as terminal procedure, the invasive methods are most appropriate for direct assessment of lower airway dysfunction.

Key words Airway function, Respiratory function, Airway hyperresponsiveness, Noninvasive method, Invasive method, Mouse model

1 Introduction

Airway function is an important attribute of respiratory health. The primary function of the airways is to conduct air to the periphery of the lung where vital gas exchanges take place. The airways are also constantly exposed to the environment, which makes them vulnerable to the adverse effects of noxious factors including respiratory pathogens. Pathogens such as viruses that enter the airways and establish successful infection not only cause inflammation but can also alter the function of the airways leading to serious illness [1]. Because the function of the airways is closely coupled to its structure, an altered airway function may reflect alterations in airway structure due to inflammation, tissue damage, or abnormal tissue remodeling. Thus, airway function is an important feature to monitor when evaluating the impacts of infection on respiratory health and the efficacy of therapeutic interventions.

Mice are the most common species used in lung research because they are the lowest phylogenetic mammalian species that offers many advantages including a well-defined genome, a

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well-characterized immune system, availability of a wide variety of specific immunologic and pharmacologic reagents, and the availability of transgenic models [2]. Additionally, despite the small size of their lung, measuring airway function in mice is no longer a challenge owing to the development of sensitive tools and methods [3]. In mice, airway function can be assessed in vivo using two approaches: (1) noninvasive whole-body plethysmography (WBP), measuring respiratory function in unrestrained, spontaneously breathing animals [4–6], or (2) invasive methods, measuring lung resistance and compliance in anesthetized, mechanically ventilated animals [7–10].

The noninvasive approach consists of placing the animal inside a plethysmograph and measuring the changes in airflow that occur inside the chamber, as a result of the animal's respiration. The system correlates the flows inside the chamber to the animal's respiratory flow and calculates various parameters of respiratory function using Boyle's law [11, 12], including respiratory rates, lung volumes, and flows, all measured without the stress of restraint or depressive effects of anesthesia. Multiple animals can be monitored at the same time and the same animals can be evaluated repeatedly over time [13], making the approach suitable for longitudinal studies, particularly those relating to respiratory breathing control. Integrated measurements of box temperature and humidity allow real-time corrections to tidal volume calculations from the box, producing more accurate flow-derived measurements. However, because mice are obligate nose breathers and changes in resistance in the upper airways (nasal resistance) can influence lung function, direct measurements (e.g., using invasive methods) are necessary for proper assessment of lower airway function [14].

The invasive approach allows for direct measurement of lung function (resistance and compliance) in animals that are ventilated via the trachea, thus bypassing the upper airways. Classically, dynamic lung resistance and compliance are measured under mechanical ventilation and the values are derived by fitting the measurements of pressure, flow, and volume to an equation of motion [7, 15]. With the introduction of the forced oscillation technique (FOT), new opportunities are added that allow for parametric distinction between alterations occurring in central airways and in peripheral tissue [16]. The technique consists of applying an oscillatory waveform to the airways opening (trachea) and measuring the pressure, flow, and volume signals. Although it can be perceived as time consuming and not suitable for longitudinal studies, the invasive approach is considered to be the gold standard for assessment of lower airway function in the mouse model [17].

2 Materials

2.1 Equipment

2.1.1 Noninvasive, Unrestrained Whole-Body Plethysmography

The basic system consists of a WBP (*see* **Note 1**), a volumetric pressure transducer (one for each plethysmograph), a bias flow regulator (*see* **Note 2**), a preamplifier unit, and a computer with the appropriate data acquisition and analysis software. An aerosol delivery system can be added for animal inhalation challenge. An example of noninvasive, unrestrained WBP system is shown in Fig. 1.

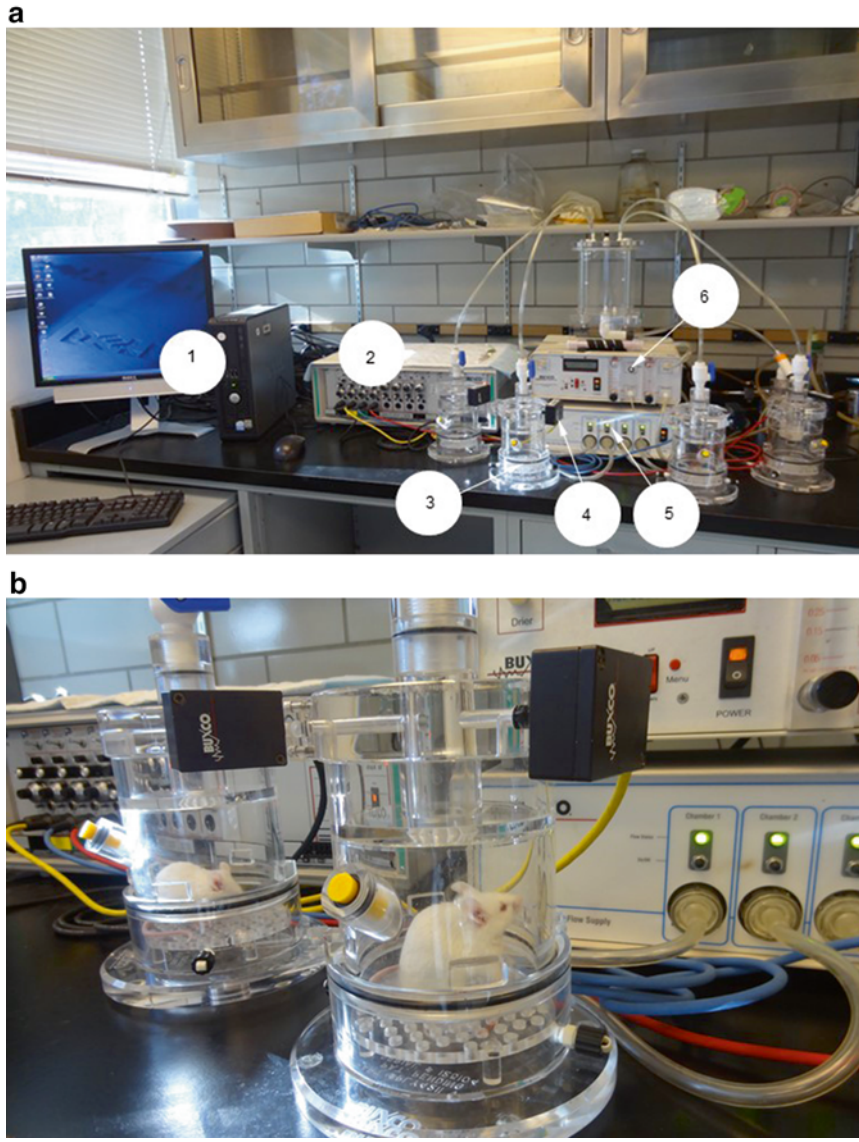

Fig. 1 Noninvasive, unrestrained plethysmography system. (**a**) A complete system consists of a computer with data acquisition and analysis software (1), a preamplifier unit (2), whole-body plethysmographs (3), transducers (4), a bias flow regulator (5), an aerosol delivery system including an aerosol distribution chamber, a nebulizer and its controller (6). (**b**) Detailed view of plethysmographs with attached transducer and unrestrained mice inside the chambers

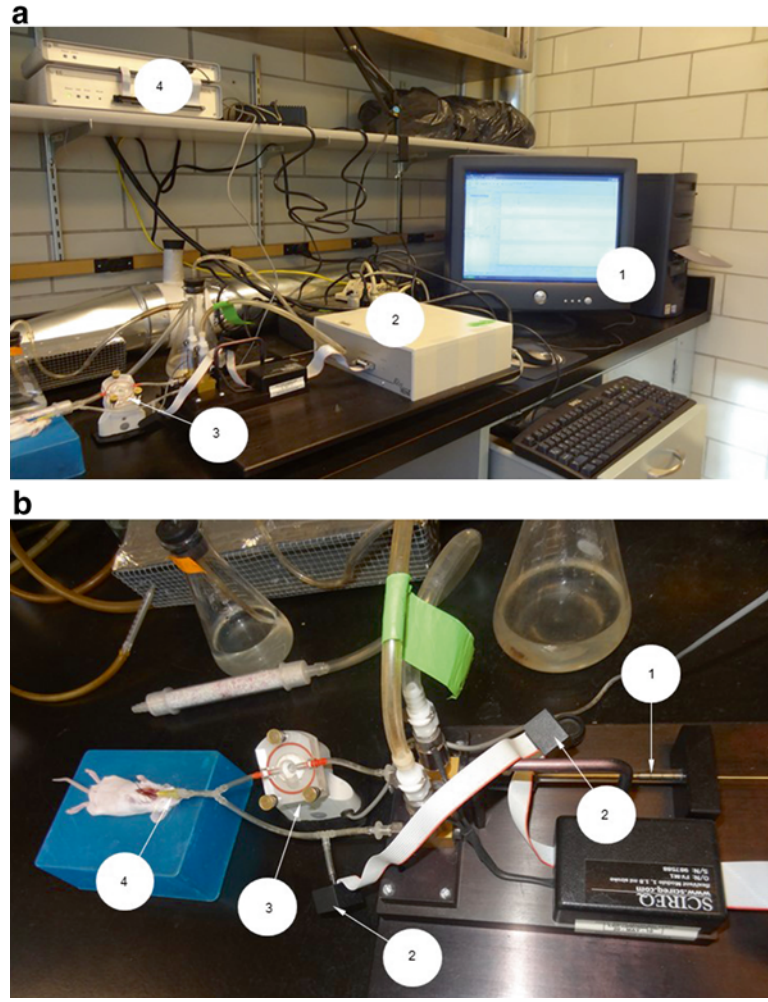

Fig. 2 Invasive airway physiology system. (**a**) The basic system consists of a computer with data acquisition and analysis software (1), a mechanical ventilator (2), a nebulizer (3), and its controller unit (4). (**b**) Detailed view showing the ventilator piston (1), the transducers (2), an inline nebulizer (3), and a mouse connected via the cannula (4)

2.1.2 Invasive Airway Physiology System

The invasive system consists of a mechanical ventilator, a set of transducers (one to monitor the cylinder pressure and another one to monitor airway pressure opening), an aerosol delivery system for airway challenge, and a computer with data acquisition and analysis software. An example of invasive system is shown in Fig. 2.

2.1.3 Small Equipment

1. Weighing scale.
2. 1 ml Syringe and 21 g × 1–1/2 in needle.
3. Dissection tools: Scissors, forceps, etc.
4. 20- or 18-Gauge blunt-end needle for cannulation.

5. 4-0 Surgical silk suture.
6. Personal safety equipment: Lab coat, gloves, face mask, etc.

2.2 Reagents and Solutions

1. 70% Ethanol.
2. Anesthetic agents (*see* **Note 3**): Sodium pentobarbital (80–100 mg/kg body weight), ketamine (50–100 mg/kg body weight)/xylazine (10 mg/kg body weight).
3. Saline: 0.9% Sodium chloride (*see* **Note 4**).
4. Methacholine (acetyl β-methylcholine chloride) (*see* **Note 5**): Prepare a stock solution of 100 mg/ml in saline. Make serial dilutions with saline (50, 25, 12.5, 6.25, 3.125, and 1.56 mg/ml).

3 Methods

3.1 Noninvasive, Unrestrained Whole-Body Plethysmography

For detailed procedure on running the equipment, the operator is referred to the instruction manual of the instrument. The following step-by-step procedure is simplified to facilitate understanding and how to use the system to assess respiratory function at baseline and after methacholine challenge using the Buxco system as an example.

1. Set up the hardware and make sure that all components are connected properly (*see* **Note 6**).
2. Turn on the power for the preamplifier, the bias flow regulator, the aerosol controller, and the computer. Let the preamplifier unit and transducers warm up for 30 min.
3. Start up the system's software.
4. Close the aerosol ports and calibrate the flow inside each plethysmograph (*see* **Note 7**).
5. Create a file name for the experiment and save it.
6. Place the animals inside the plethysmographs and close (*see* **Note 8**).
7. Allow the animals to acclimatize to the plethysmographs for 15 min.
8. Measure baseline respiratory function (*see* **Note 9**).
9. Perform the methacholine (MCh) dose-response measurements.
   (a) Load saline in the nebulizer cup, open the aerosol port on the plethysmographs, and start the nebulizer (*see* **Note 10**): nebulization is set for 1.5 min followed by a 2-min drying cycle.
   (b) Close the aerosol ports and start data recording. Data are recorded continuously over a 3-min period, starting by the end of each nebulization.

(c) Load the first dose of MCh (e.g., 3.25 mg/ml) and start the nebulizer again.

(d) Record data.

(e) Repeat the challenge with subsequent doses and record data after each dose.

10. End the experiment after completion of the measurements.
11. Remove animals from the plethysmographs and return them to their cages.
12. Detach the transducers and disconnect the tubing from the plethysmographs.
13. Rinse the plethysmographs with distilled water to remove all traces of MCh.
14. Dry the plethysmographs (*see* **Note 11**).
15. Rinse the nebulizer cup, the aerosol distribution chamber, and attached tubing with distilled water, and dry the unit.
16. Store all components in dust-free area or reset for a new series of measurements.

3.2 Invasive Airway Physiology Measurements

The following procedure is simplified to provide understanding of how to measure mouse lung function at baseline and after MCh challenge using the *flexiVent* (first generation) as an example of invasive system. For detailed operating procedure, the reader is referred to the instruction manual of the instrument. The system consists of a computer-controlled piston that serves a multiple purpose: (1) ventilate the lung, (2) inflate and deflate the lung in a stepwise fashion to generate pressure-volume curves for analysis of static compliance (reflecting elastic recoil or stiffness) of the lung, and (3) perform the forced oscillation technique (FOT). When performed at a single frequency, the FOT allows for measurements of dynamic resistance (R), elastance (E), and compliance (C) of total lung. When performed under a wide range of frequencies, both below and above the subject's breathing frequency, the FOT can allow for partitioned analysis of respiratory mechanics using the constant-phase model (Hantos 1992) to obtain parametric distinction between central airway resistance (parameter R_N: Newtonian resistance) and peripheral tissue resistance (parameter G: tissue damping) and elastance (parameter H).

1. Make sure that all components are connected properly. Set up the PEEP (positive end-expiratory pressure) to 3 cmH_2O (*see* **Note 12**).
2. Turn on the computer and start the software.
3. Start a new experiment from an existing template. A template includes a selection of parameters to be measured, e.g., pressure-volume, SnapShot (for single-frequency FOT), and Prime-8 (for broadband FOT).

4. Create a file for the new experiment and save it.
5. When prompted, enter the subject's identification (species/strain, group, body weight, gender), and confirm the subject's weight (*see* **Note 13**).
6. The system performs an auto-calibration and the transducers warm up for about 2 min.
7. Perform the channel calibration as instructed (*see* **Note 14**).
8. Perform the tube calibration as instructed (*see* **Note 15**).
9. Prepare the animal.
   (a) Anesthetize the animal (*see* **Note 16**).
   (b) Cannulate the trachea. Place the animal on its back and spray the neck with 70% ethanol. Make a small incision in the skin in the frontal area of throat to expose the trachea and make a partial incision in the trachea just below the larynx. Insert the cannula in the trachea (about 2–3 cartilage rings deeper) and securely tie in place with a suture thread (*see* **Note 17**).
10. Connect the animal to the ventilator (*see* **Note 18**).
11. Perform a deep inflation maneuver (*see* **Note 19**).
12. Measure baseline airway function. Three perturbations can be used: (1) P-V loop, to generate pressure-volume curves for static lung compliance (*see* **Note 20**); (2) SnapShot, to measure lung resistance (R), elastance (E), and dynamic compliance (C); (3) Prime-8, to measure Newtonian resistance (R_N), tissue damping (G), and tissue elastance (H).
13. Start the MCh dose-response measurements.
   (a) Load saline first, in the nebulizer, and start the nebulization (*see* **Note 21**).
   (b) Measure lung function using SnapShot and Prime-8 perturbations (*see* **Note 22**).
   (c) Load the first dose of MCh, start the nebulizer, and measure lung function parameters as described in **step 13b**.
   (d) Challenge with the next dose of MCh and repeat the measurements after each dose (*see* **Note 23**).
14. After completion of all measurements, stop the ventilation.
15. Disconnect the animal and euthanize (*see* **Note 24**).
16. Rinse the nebulizer, tubing, and cannula to remove all traces of MCh.
17. Enter a new subject and confirm its weight.
18. Perform the measurements by repeating all **steps 9–13**.
19. At the end the experiment, save the results and close the session, and turn off the system.

3.3 Output Parameters and Interpretation

3.3.1 Noninvasive, Unrestrained Whole-Body Plethysmography

Several parameters are derived from the measurements, including respiratory rates (or frequency measured in breaths/min), tidal volume, peak inspiratory flow, peak expiratory flow, and expiratory time, all of which can be altered with altered breathing pattern. A dimensionless parameter termed enhanced pause (Penh) is also derived from the measurement and is often misinterpreted as an index of airway resistance, but there is a common consensus that this parameter should not be used or interpreted as a true measurement of lower airway resistance [14].

3.3.2 Invasive Airway Physiology Measurements

Several parameters of lower airway function can be obtained by this approach. These include static lung compliance, dynamic lung resistance and compliance, and parameters of the constant-phase model when using the FOT. Static compliance (Cst) reflects lung tissue stiffness and is a useful indicator of alveolar enlargement (increased Cst) and tissue fibrosis (decreased Cst). Dynamic lung resistance (R_L) and compliance (C or C_{dyn}) are good parameters reflecting airway narrowing and are useful in assessment of bronchoconstriction response to airway challenge. These two parameters are generally coupled (i.e., increased R_L is associated with decreased Cdyn), but in some circumstances they may appear uncoupled due to differential responses related to central versus peripheral airways [17]. Increased R_L indicates increased airway resistance to airflow as a result of airway constriction. This parameter is commonly used in analysis of airway hyperresponsiveness (AHR), a characteristic alteration of airway function in reactive airway diseases such as asthma. AHR is determined by analyzing changes in RL in response to airway challenge with a pharmacologic agonist (typically MCh). AHR is characterized by increased airway sensitivity (leftward shift in the MCh dose-response curve) and increased airway reactivity (upward shift in the MCh dose-response curve) to increasing doses of MCh challenge (Fig. 3). Airway sensitivity to MCh can be expressed more concretely as PC_{100} or PC_{200}, representing the provocative concentration (or dose) of MCh causing 100% or 200% increase in R_L over baseline value (R_L measured after challenge with normal saline), respectively. With the FOT, additional parameters derived from the constant-phase model can be used to gain more insights into airway mechanics. An increase in Newtonian resistance (R_N) reflects increased resistance in the central airways, and an increase in tissue damping (G) and tissue elastance (H) reflects increased resistance in the peripheral airway tissue compartment. As with R_L and C_{dyn}, the changes in RN and G (or H) values may also be uncoupled under circumstances that remain to be explored.

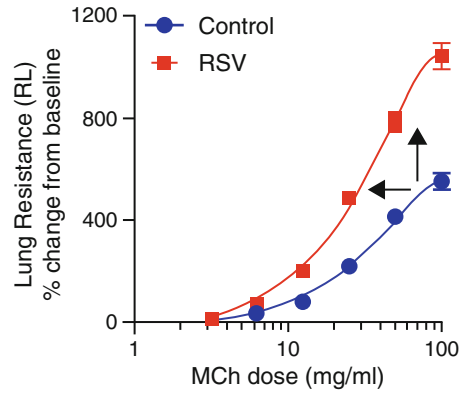

Fig. 3 Airway responsiveness to methacholine. Lung resistance (R_L) was measured in anesthetized, mechanically ventilated control (uninfected) and RSV-infected mice following airway challenge with increasing doses of inhaled methacholine (MCh). Data are expressed as percent increase in R_L from baseline (saline) value. The leftward (*horizontal arrow*) and upward (*vertical arrow*), respectively, indicate increased airway sensitivity and reactivity to MCh, characteristic of airway hyperresponsiveness. *RSV* respiratory syncytial virus

4 Notes

1. The plethysmograph consists of a double chamber, with a large chamber housing the animal, and a smaller one used as reference. The chambers are made of transparent plexiglass allowing for the animals to be observed from all sides during the entire experiment. Multiple plethysmographs can be used to monitor several animals simultaneously.
2. During animal respiration, CO_2 is expired inside the plethysmograph. The bias flow continuously draws fresh air into the plethysmograph to remove CO_2 buildup and/or to draw aerosol into the chamber.
3. Anesthetic agents are controlled substances. They should be handled responsibly and stored safely in an approved drug cabinet that can be locked and physically attached to the wall or kept in a location with access limited to the user only. Usage should be documented, and expired or unused amounts should be disposed off according to the applicable regulations.
4. Use non-pyrogenic, endotoxin-free solutions (pharmaceutical or tissue culture grade).
5. Methacholine is a potent bronchoconstrictor that should be handled carefully. Methacholine powder is hygroscopic and is normally stored desiccated at −20 °C. Stock solutions containing ≥50 mg/ml of MCh diluted with saline can be stored in aliquots at −20 °C for up to 6 months without loss of potency.

However, for animal challenge, all serial dilutions should be made fresh and administered at room temperature.

6. Make sure that the plethysmographs are clean and all ports are unobstructed before connecting the unit.
7. Calibration is the most critical step of the procedure. It ensures communication between the software and transducers, and assigns units to the signals detected by the transducers.
8. Handle all animals gently to avoid stress. Make sure not to pinch the tail or toes when closing the chambers; this will cause pain and influence the results. Record the strain, age, weight, and sex. For tracking, animals must be labeled appropriately (tail marks, ear punch, or tattoos). Tracking is critical in longitudinal studies with repeated measurements.
9. A protocol can be created to assist with the measurement of all respiratory parameters.
10. Make sure that the nebulizer is running and aerosol is delivered to the plethysmographs.
11. Compressed air may be used to remove water trapped inside the plethysmograph ports. Careful: Do not blow air on the transducers at any time, as this will alter the calibration.
12. A PEEP of 3 cmH_2O is sufficient to maintain good gas exchange during mechanical ventilation and to prevent alveolar de-recruitment (collapse) at the end of expiration. Higher PEEP values can cause excessive alveolar distension (stretch) and reduced airway constriction in response to MCh challenge.
13. The body weight entered will be used to set up the tidal volume (V_T) for ventilation (normally 10 ml/kg).
14. This calibration assigns units to the signals recorded by the transducers. A manometer is required to set up a 2-point pressure calibration, e.g., between 0 and 20 cmH_2O.
15. The goal of this calibration is to exclude the resistance due to the cannula itself. Ideally, the internal diameter of the cannula should be as close as possible to the caliber of the trachea.
16. The purpose of anesthesia is to sedate the animal and to abolish spontaneous breathing during mechanical ventilation, which interferes with the measurements. This can be achieved with deep anesthesia using sodium pentobarbital (80–100 mg/kg) or xylazine (10 mg/kg) and ketamine (50–100 mg/kg). In some cases, a muscle paralytic agent (pancuronium, 1 mg/kg, i.p.) may be added to completely abolish spontaneous breathing.

17. It is important that a seal is maintained to prevent any leak of air (loss of pressure) during ventilation and measurements.
18. Make sure that the animal is aligned with the ventilator to avoid any distortion that may obstruct the cannula. The default ventilation is performed at a frequency of 150 breaths per minute (i.e., 2.5 Hz or breaths/s) and a tidal volume of 10 ml/kg.
19. This procedure is important to recruit the alveolar compartment (open up all airways) and to standardize the volume history of the lung. This can be achieved by increasing the pressure to 25–30 cmH_2O and holding for 2–3 s. The short-time hold may serve to check if there is air leak, as indicated by a drop in pressure, originating at or below the position of the cannula.
20. Static compliance should be measured at baseline, but not after MCh challenge because the P-V curves can be distorted after airway constriction.
21. Perform the challenge with different ventilation settings (60 breaths/min and V_T of 20 ml/kg) and deliver ten breaths of aerosol challenge. The higher V_T ventilation is to allow for aerosol to be delivered to all the airways.
22. Return to the default ventilation and perform the SnapShot and Prime-8 perturbations to measure lung function parameters after each challenge. The timing of the peak response can vary depending on the dose of MCh challenge, the strain of mice used, and the model studied (allergen, infection, irritant, etc.). Accordingly, the measurements should be repeated serially every 20 s over a 3-min period to capture the maximum response values. A script can be written with the software to automate these measurements.
23. Make sure that airway resistance has returned close to baseline values before administering the next dose of MCh. If the airways are still constricted, the aerosol cannot be delivered completely.
24. Use a secondary method to complete euthanasia under terminal anesthesia (e.g., overdose anesthesia, cervical dislocation, exsanguination, removal of vital organs such as the lung).

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References

1. Dakhama A, Lee YM, Gelfand EW (2005) Virus-induced airway dysfunction: pathogenesis and biomechanisms. Pediatr Infect Dis J 24(11 Suppl):S159–S169
2. Taube C, Dakhama A, Gelfand EW (2004) Insights into the pathogenesis of asthma utilizing murine models. Int Arch Allergy Immunol 135:173–186
3. Irvin CG, Bates JH (2003) Measuring the lung function in the mouse: the challenge of size. Respir Res 4:4
4. Hamelmann E, Schwarze J, Takeda K et al (1997) Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am J Respir Crit Care Med 156:766–775
5. Tripp RA, Dakhama A, Jones LP et al (2003) The G glycoprotein of respiratory syncytial virus depresses respiratory rates through the CX3C motif and substance P. J Virol 77:6580–6584
6. Miyahara S, Miyahara N, Lucas JJ et al (2008) Contribution of allergen-specific and nonspecific nasal responses to early-phase and late-phase nasal responses. J Allergy Clin Immunol 121:718–724
7. Martin TR, Gerard NP, Galli SJ et al (1988) Pulmonary responses to bronchoconstrictor agonists in the mouse. J Appl Physiol 64:2318–2323
8. Levitt RC, Mitzner W (1988) Expression of airway hyperreactivity to acetylcholine as a simple autosomal recessive trait in mice. FASEB J 2:2605–2608
9. Takeda K, Hamelmann E, Joetham A, Shultz LD, Larsen GL, Irvin CG, Gelfand EW (1997) Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. J Exp Med 186(3):449–454
10. Lee YM, Miyahara N, Takeda K et al (2008) IFN-gamma production during initial infection determines the outcome of reinfection with respiratory syncytial virus. Am J Respir Crit Care Med 177:208–218
11. Sinnett EE, Jackson AC, Leith DE et al (2003) Fast integrated flow plethysmograph for small mammals. J Appl Physiol Respir Environ Exerc Physiol 50:1104–1110
12. O'Neil JJ, Raub JA (1984) Pulmonary function testing in small laboratory mammals. Environ Health Perspect 56:11–22
13. Delaunois A, Dedoncker P, Hanon E et al (2009) Repeated assessment of cardiovascular and respiratory functions using combined telemetry and whole-body plethysmography in the rat. J Pharmacol Toxicol Methods 60:117–129
14. Bates J, Irvin CG, Brusasco V et al (2004) The use and misuse of Penh in animal models of lung disease. Am J Respir Cell Mol Biol 31:373–374
15. Irvin CG, Tu YP, Sheller JR et al (1997) 5-Lipoxygenase products are necessary for ovalbumin-induced airway responsiveness in mice. Am J Physiol 272:L1053–L1058
16. Hantos Z, Daróczy B, Suki B et al (1992) Input impedance and peripheral inhomogeneity of dog lungs. J Appl Physiol 72:168–178
17. Bates JH, Irvin CG (2003) Measuring lung function in mice: the phenotyping uncertainty principle. J Appl Physiol 94:1297–1306

Chapter 17

Evaluation of the Adaptive Immune Response to Respiratory Syncytial Virus

Cory J. Knudson, Kayla A. Weiss, Megan E. Stoley, and Steven M. Varga

Abstract

Evaluation of the adaptive immune response is critical to the advancement of our basic knowledge and understanding of respiratory syncytial virus (RSV). The cellular composition in the lung following RSV infection is often evaluated using flow cytometry. However, a limitation of this approach has been the inability to readily distinguish cells that are within the lung parenchyma from cells that remain in the pulmonary blood vessels. Herein, we detail a procedure to evaluate the adaptive immune response via flow cytometric analysis that incorporates an in vivo intravascular staining technique. This technique allows for discrimination of immune cells in the lung tissue from cells that remain in the pulmonary vasculature following perfusion. Therefore at any given time point following an RSV infection, the leukocytic populations in the lung parenchyma can be quantified and phenotypically assessed with high resolution. While we focus on the T lymphocyte response in the lung, this technique can be readily adapted to examine various leukocytic cell types in the lung following RSV infection.

Key words Cell localization, Intravascular staining, Lung, Respiratory syncytial virus, T lymphocytes

1 Introduction

The adaptive immune response plays a crucial role in mediating protection against reinfection from viruses. While long-lived immunity against RSV is not achieved [1, 2], previous work has indicated that high serum titers of neutralizing antibodies correlate with increased protection [3–7]. While far less is known about CD8 T cells, the cytotoxic T lymphocytic response is critical for mediating viral clearance during primary RSV infection [8, 9]. Therefore, proper analysis of the adaptive immune response to RSV infection is necessary to further our understanding of this important human pathogen. Flow cytometry is a common, yet powerful tool used to quantify and characterize the immune response on a single-cell basis in the murine model of RSV infection. Due to the vast network of blood vessels in the lung, a large fraction of the immune response in the lung parenchyma may be

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localized to the pulmonary vasculature depending on the timing of analysis and tissue tropism of the pathogen [10, 11]. Therefore, in vivo intravascular staining has been utilized in combination with flow cytometric analysis to further distinguish the localization of immune cells within the lung parenchyma [10–13]. Herein, we detail the general procedure to process and assess the adaptive immune response following RSV infection focusing on the T cell response. Since RSV replication is restricted to the respiratory tract [14, 15], the majority of the responding T cells are localized to the lung parenchyma, lung airways, draining lymph node, and blood. The procedure begins with the intravenous administration of a fluorochrome-conjugated antibody to target immune cells circulating in the bloodstream. Intravascular staining allows for the discrimination of immune cells that localize within peripheral tissues, e.g., the lung, that remain unlabeled by antibody. This is in contrast to cells located in the pulmonary vasculature as they will be exposed to the antibody in the bloodstream and become labeled after a short period of time. Organs and tissues are harvested, processed into a single-cell suspension, and prepared for flow cytometric analysis. Cells are stained with fluorochrome-conjugated antibodies to target extracellular markers for phenotypic analysis. Subsets of antigen-specific T cells can also be stimulated with individual peptides to known epitopes and subsequently stained intracellularly for cytokine production. Alternatively, all antigen-experienced T cells can be activated via nonspecific stimulation with substrates such as phorbol 12-myristate 13-acetate (PMA) and ionomycin and stained intracellularly for cytokine production. Overall, we describe a general method to process and analyze the principal organs and tissues associated with the adaptive immune response following RSV infection. This approach ensures a high yield of leukocytes from the peripheral tissues and a broad platform for assessment of many immune cell types.

2 Materials

2.1 Intravenous Labeling

1. 27-Gauge x ½ in. needle.
2. 1 ml Syringes.
3. Fluorescently labeled antibody (*see* Table 1).
4. Sterile PBS.
5. Heat lamp.
6. Apparatus for mouse tail vein injection.
7. Timer.

2.2 Cell Isolation, Stimulation, and Staining

1. Heparinized glass collection tubes.
2. Razor blade.
3. 1 ml Syringe.

Table 1
Antibody dilutions for i.v. labeling following RSV infection

Antibody	Concentration (μg/mouse)	Fluorochromes tested
CD4 (GK1.5)	1	PE, APC
CD8α (53-6.7)	1	PE, APC
CD45 (30-F11)	1–3	FITC, PE, APC
CD90.1 (HIS51) CD90.2 (53-2.1)	1	PE, APC

4. Dissecting tools: Scissors, forceps (*see* **Note 1**).
5. 5 ml Round-bottom polystyrene tubes.
6. 15 ml Conical polypropylene tubes.
7. Cannulation needle: 20-Gauge × 1 in. needle and plastic tubing with slightly larger diameter (*see* **Note 2**).
8. HBSS with $CaCl_2$ and $MgCl_2$.
9. DNase I.
10. Collagenase.
11. Reagent to block Fc receptors such as FcBlock (BD Biosciences) or anti-mouse CD16/CD32 (eBioscience).
12. 2% RPMI: RPMI 1640, 10% fetal calf serum, 5 nM 2-mercaptoethanol (ME), 2 mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin sulfate, 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids.
13. 10% RPMI: RPMI 1640, 2% fetal calf serum, 5 nM 2-ME, 2 mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin sulfate, 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids.
14. Adjustable-volume pipetters (0.5–10, 2–20, 20–200, and 100–1000 μl).
15. Tips for pipetters.
16. Pipette controller or pipet-aid.
17. Serological pipettes (5 and 10 ml).
18. Stainless steel cell dissociation sieves (Bellco Glass, Inc.) or tissue grinder kits.
19. Petri dishes—60 × 15 mm.
20. Microscope slides with frosted ends.
21. Lyse/Fix Solution Buffer (eBioscience or BD Biosciences).
22. 0.4% Trypan blue solution.

23. NH_4Cl: 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA in sterile H_2O, pH 7.4.
24. Hemocytometer.
25. 96-Well round-bottom plates.
26. FACS buffer: PBS, 2% fetal calf serum, 0.02% (w/v) sodium azide.
27. Tube rotator for 15 ml conical tubes (Miltenyi Biotec, *see* **Note 3**).

3 Methods

3.1 Intravascular Labeling of Immune Cells (See Note 4)

1. Dilute the fluorescently labeled antibody to the appropriate concentration (*see* Table 1) in sterile PBS to administer 200 µl/mouse (*see* **Note 5**).
2. Place mice under heat lamp until tail veins dilate.
3. Restrain mouse in tail vein injection apparatus and administer 200 µl of diluted antibody intravenously via the tail vein.
4. Release mouse, let rest for 3 min, and then immediately collect peripheral blood via an approved method for collection. Place blood in 1 ml of 2% RPMI and place on ice (*see* **Note 6**) for further processing later (*see* Subheading 3.5).
5. Immediately perform an approved form of euthanasia on the mouse (*see* **Note 7**).
6. Within 12 min collect all tissue samples desired for analysis.

3.2 Cell Collection and Preparation: Bronchoalveolar Lavage (BAL)

1. Expose trachea by removing the tissue and membranes.
2. Using a razor blade make a small horizontal incision near the middle of the trachea and hold the trachea in place with forceps above the incision while inserting the cannulation needle.
3. Insert 1 ml of 2% RPMI media slowly into the lungs by tracheal cannulation. Slowly remove the 1 ml of media in the lungs by creating negative pressure with the syringe and needle still inserted into the trachea. Dispense cells and media into a tube (*see* **Note 8**).
4. Repeat **step 3** twice more.
5. Centrifuge at 400×*g* for 5 min at 4 °C. Decant supernatant and resuspend the cell pellet (*see* **Note 9**).
6. Add 1 ml of 2% RPMI to the resuspended pellet and mix thoroughly.
7. Dilute and count cells using 0.4% trypan blue on a hemocytometer.
8. Centrifuge tube with BAL cells at 400×*g* at 4 °C for 5 min. Decant supernatant and resuspend the cell pellet.

9. Add the appropriate volume (*see* **Note 10**) of either 2% or 10% RPMI depending on if you are performing ex vivo staining or in vitro stimulation, respectively.
10. Plate cells 100 µl/well in a 96-well round-bottom plate.

3.3 Cell Preparation: Draining Lymph Node (Mediastinal)

1. Expose the lung by removing the rib cage.
2. Harvest the mediastinal lymph node using fine dissection forceps (*see* **Note 11**).
3. Place into 15 ml conical tube with 1 ml 2% RPMI (*see* **Note 12**).
4. Pour media and draining lymph node into small petri dish and dissociate lymph node using frosted ends of microscope slides.
5. Use 4 ml of 2% RPMI to rinse slides and petri dish and pipet single-cell suspension back into 15 ml conical.
6. Rinse petri dish with 4 ml of 2% RPMI and pipet into tube.
7. Centrifuge at 400 × *g* for 5 min at 4 °C. Decant supernatant and resuspend in 2 ml 2% RPMI.
8. Dilute a small amount of each sample and count cells using 0.4% trypan blue on a hemocytometer.
9. Resuspend cells in 10% RPMI for vitro stimulation or in 2% RPMI for extracellular staining at 2.0×10^7 cells/ml (*see* **Note 13**).
10. Plate cells 100 µl/well in a 96-well round-bottom plate.

3.4 Cell Preparation: Lungs

1. Perfuse the lung tissue with 10 ml of PBS via the right ventricle.
2. Remove lung ensuring that the peribronchial lymph nodes are removed from the connective tissue between the lung lobes.
3. Cut into smaller pieces and place in a tube with 4 ml of HBSS with $CaCl_2$ and $MgCl_2$.
4. Supplement media with 60 U/ml DNase I and 125 U/ml collagenase (*see* **Note 14**).
5. Digest lungs for 30 min at 37 °C using either tube rotator or gently shaking every 5 min.
6. Press lungs through 190 µm wire mesh screen into a petri dish. Use 4 ml of 2% RPMI to wash out screen.
7. Pipet cells in 15 ml conical. Rinse petri dish with 4 ml of 2% RPMI and pipet into 15 ml conical.
8. Centrifuge at 4 °C to reach approximately 200 × *g* and immediately stop centrifuge. Pour supernatant into a new 15 ml conical tube. This step will remove large cellular debris; cells will remain in the supernatant.
9. Centrifuge at 4 °C at 400 × *g* for 5 min. Decant supernatant and resuspend cell pellet.

10. Pipet 5 ml of NH_4Cl in tube with cells and mix thoroughly to lyse red blood cells.
11. Incubate on ice for 5 min.
12. Centrifuge at 4 °C at 400 × *g* for 5 min. Decant supernatant and resuspend cell pellet.
13. Wash cells 2× with 5 ml of 2% RPMI.
14. Centrifuge at 4 °C at 400 × *g* for 5 min. Decant supernatant and resuspend cell pellet.
15. Add 5 ml of 2% RPMI to the resuspended cell pellet and mix thoroughly.
16. Dilute a small portion of each sample in 0.4% trypan blue and count cells on a hemocytometer (*see* **Note 15**).
17. Centrifuge at 4 °C at 400 × *g* for 5 min. Decant supernatant and resuspend cell pellet.
18. Resuspend cells in 10% RPMI for vitro stimulation or in 2% RPMI for extracellular staining at 2.0×10^7 cells/ml.
19. Aliquot 100 µl/well in a 96-well round-bottom plate.

3.5 Processing Cells: Peripheral Blood Leukocytes (PBL)

1. Centrifuge the tube with the peripheral blood in 2% RPMI at 400 × *g* at 4 °C for 5 min (all centrifuge steps in this protocol section will be under these conditions unless otherwise noted).
2. Remove the majority of supernatant by pipetting (*see* **Note 16**).
3. Resuspend cell pellet and pipet 2 ml of NH_4Cl in tube with cells and mix thoroughly to lyse red blood cells.
4. Centrifuge at 4 °C at 400 × *g* for 5 min. Decant supernatant and resuspend cell pellet.
5. Pipet 2 ml of NH_4Cl in tube with cells and mix thoroughly to lyse any remaining blood cells.
6. Immediately centrifuge down cells. Decant supernatant and resuspend cell pellet.
7. Wash cells 2× with 2 ml of 2% RPMI.
8. Centrifuge at 4 °C at 400 × *g* for 5 min.
9. Decant supernatant and resuspend cell pellet in 1 ml of 2% RPMI.
10. Dilute a small portion of each sample in 0.4% trypan blue.
11. Count cells using hemocytometer.
12. Centrifuge, decant supernatant, and resuspend cells in 10% RPMI for vitro stimulation or in 2% RPMI for extracellular staining at 2.0×10^7 cells/ml.
13. Aliquot 100 µl/well of cells in 96-well round-bottom plate.

3.6 In Vitro Stimulation (If Not Performing a Stimulation, Advance to Subheading 3.7)

1. For either peptide or PMA and ionomycin stimulation prepare a 2× concentration in 10% RPMI to add 100 µl/well with:
   2 µM of peptide and 20 µg/ml of brefeldin A for peptide, or
   100 ng/ml PMA, 1 µg/ml ionomycin, and 20 µg/ml of brefeldin A for PMA and ionomycin stimulation.
2. Add 100 µl/well of the appropriate 2× stimulation dilution to the 100 µl of cells in the 96-well round-bottom plate.
3. Incubate at 37 °C for either 5 h for peptide stimulation or 4 h for PMA and ionomycin stimulation, respectively.

3.7 Cell Staining

1. Centrifuge plate at 4 °C at 400×*g* for 5 min (same speed and duration throughout protocol). Decant liquid by inverting plate and flicking once. Resuspend cells by vortexing plate at a medium speed.
2. For MHC class I tetramer staining, prepare tetramer stain with Fc blocking reagent in FACS buffer at the appropriate concentration to add 50 µl/well (*see* **Note 17**). Otherwise skip **steps 2–4** and continue at **step 5**.
3. Incubate cells with tetramer at 4 °C for 30 min (*see* **Note 18**).
4. Prepare extracellular antibody stain at 2× concentration to add 50 µl/well (final volume of 100 µl).
5. Prepare extracellular antibody stain with Fc blocking reagent in FACS buffer at the appropriate concentration to add 100 µl/well (*see* **Note 19**).
6. Incubate cells with antibody at 4 °C for 30 min.
7. Add 100 µl/well of FACS buffer.
8. Centrifuge at 4 °C at 400×*g* for 5 min. Decant supernatant and resuspend cells.
9. Wash cells 2× with 200 µl/well FACS buffer.
10. Centrifuge at 4 °C at 400×*g* for 5 min. Decant supernatant and resuspend cells.
11. Lyse red blood cells and fix cells by adding 100 µl/well of lyse/fix solution. Incubate for 5–10 min at room temp in the dark.
12. Add 100 µl/well FACS buffer and centrifuge plate.
13. Wash 2× in 200 µl/well of FACS buffer.
14. Centrifuge plate at 4 °C at 400×*g* for 5 min. Decant supernatant and resuspend cells.
15. If only staining extracellular proteins, resuspend cells in FACS buffer for collection on the flow cytometer and protocol ends here. For intracellular protein staining, continue with protocol.
16. Wash 2× in 200 µl of FACS buffer with 0.5% saponin (*see* **Note 20**).

17. Centrifuge at 4 °C at 400×*g* for 5 min. Decant supernatant and resuspend cells.
18. Prepare intracellular antibody stain in FACS buffer with 0.5% saponin in the appropriate dilution to add 100 µl/well. Incubate cells with antibody for 30 min at 4 °C in the dark.
19. Add 100 µl/well of FACS buffer with 0.5% saponin.
20. Centrifuge at 4 °C at 400×*g* for 5 min. Decant supernatant and resuspend cells.
21. Wash with 200 µl/well of FACS buffer with 0.5% saponin.
22. Centrifuge at 4 °C at 400×*g* for 5 min. Decant supernatant and resuspend cells.
23. Wash with 200 µl/well of FACS buffer.
24. Centrifuge at 4 °C at 400×*g* for 5 min. Decant supernatant and resuspend cells.
25. Resuspend cells in 100–200 µl/well FACS buffer, transfer samples to tubes, and collect samples on the flow cytometer. To confirm intravascular staining protocol was performed correctly, virtually all target cells in the PBL should be labeled whereas BAL cells should remain unlabeled (Fig. 1).

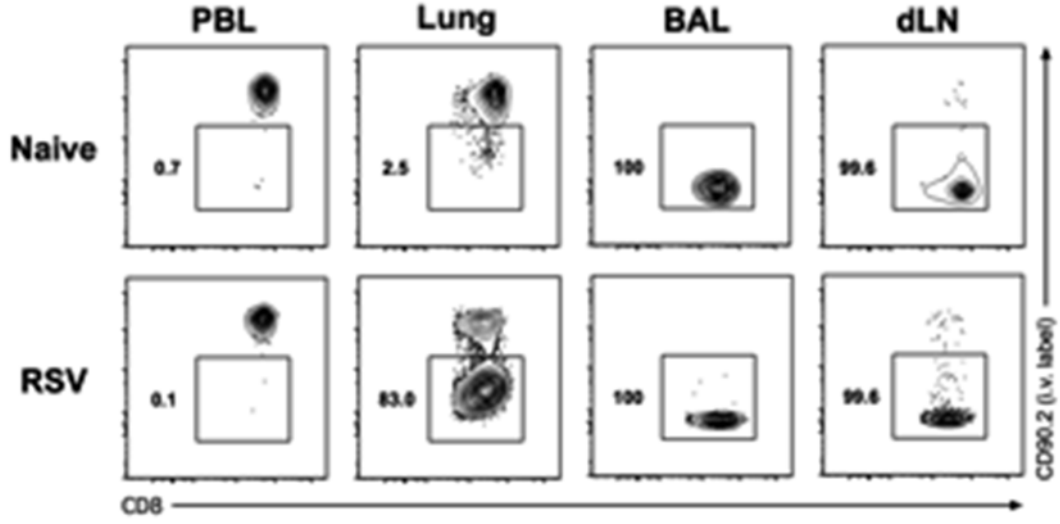

Fig. 1 Representative flow plots illustrating intravascular staining across tissues. Gated populations indicate the frequency of unlabeled (localized to the lung tissue) CD8 T cells in the PBL, lung, BAL, and draining lymph node (dLN). Comparison between naive and RSV-infected BALB/c mice day 8 p.i. All plots are gated on CD8 T cells ($CD90.2^{+}CD8^{+}$). Note that nearly all cells in the PBL are labeled following i.v. injection of the fluorescent antibody. Cells in the BAL and the draining lymph node should remain unlabeled. The frequency of unlabeled immune cells in the lung will vary depending on factors including day of infection, tissue tropism of infection, and cell type of analysis

4 Notes

1. We use a combination of straight and curved fine-point dissection forceps. The straight forceps are for general dissection, while the curved fine-point dissection forceps are used to remove extraneous tissue and acquire the draining lymph node.
2. We cut and file down the end of a 20-gauge needle until it is smooth. Then we take approximately a 1 cm piece of the plastic tubing and place on the end of the needle. We make 45° cut on the end of the tubing to allow for easy insertion of the cannulation needle into the cut trachea. Alternatively, one can use approximately an 18-gauge needle that has been filed and smoothed with a slight angle at the end. However, this may require tying a piece of string around the trachea after insertion of the cannulation needle as media may leak out.
3. Alternatively, samples can be gently mixed over a 30-min time period by gentle shaking every 5 min.
4. When using the intravascular labeling technique, it is important to keep all tissues and samples in the dark or covered. Exposure to bright light can cause photo-bleaching, resulting in a loss of fluorescence.
5. While a number of antibodies can be utilized for intravascular staining, we would recommend the use of CD45 antibody when performing in vitro stimulation on cells (Fig. 2). CD4 and CD8α expression downregulates with T cell activation. The fluorescent intensity of CD90 also seems to significantly decrease over time during T cell stimulation. However, regardless of the antibody used for intravascular staining, immune cells can typically be stained with the same antibody clone conjugated to a different fluorochrome. We have previously used CD90.2-PE for intravascular staining and subsequently stained the samples with CD90.2 in another fluorochrome.
6. After collection all tissues/cells should be kept on ice or 4 °C throughout the experiment, unless otherwise noted.
7. Method of euthanasia may significantly alter results. We have found that euthanasia via CO_2 inhalation significantly alters cell trafficking in the lung of mice. Furthermore, euthanasia should be performed rapidly following administration of the labeling antibody to prevent the antibody from entering tissues.
8. Do not expect all media to be collected back. A typical BAL pull will only obtain approximately 0.7–0.9 ml. Also if there is a pool of blood around the trachea, you will want to wash the area with 70% ethanol or PBS and dry prior to cutting the trachea. Any blood pulled into the BAL sample

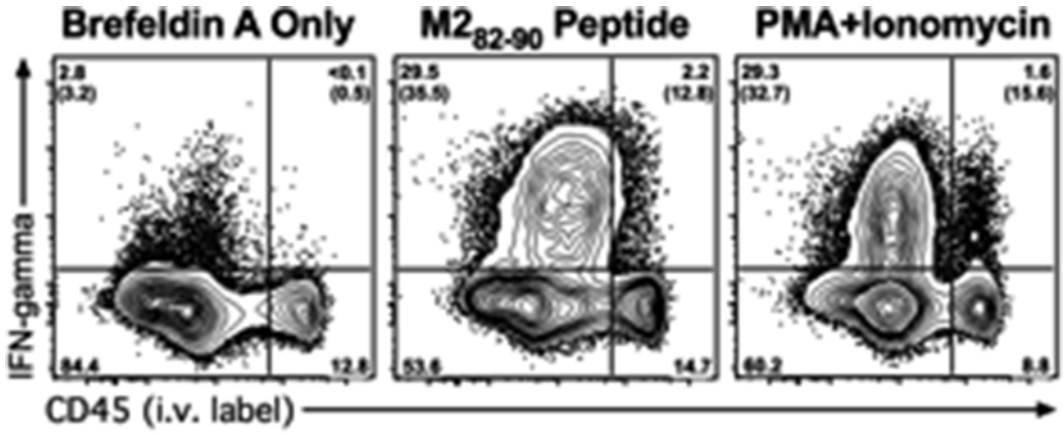

Fig. 2 Concatenated flow plots demonstrating intravascular staining technique paired with intracellular cytokine staining. Lung cells from day 7 p.i. RSV BALB/c mice were stimulated with either $M2_{82–90}$ peptide or PMA and ionomycin for 5 h and 4 h, respectively, in the presence of brefeldin A. Cells were stimulated with media containing brefeldin A alone as a control for 5 h. Flow plots are concatenated plots from four mice and gated on $CD90.2^+CD8^+$ T cells. Numbers in parenthesis indicate the frequency of CD8 T cells within either the lung tissue ($CD45^-$) or the pulmonary vasculature ($CD45^+$) that are producing IFN-γ

will add the intravascular labeling antibody and BAL cells should be unlabeled as they are located within the airways.

9. To resuspend cell pellets, we vortex tubes or rapidly rub the bottom of the tube against the side of a test tube rack with pegs. Sometimes the pellet can be hard to resuspend; just ensure that there is no pellet at the bottom of the conical tube following resuspension regardless of the method.
10. A naïve lung has very few lymphocytes in the BAL, so we advise to use the sample for only one stain or pool samples as necessary. At the peak of RSV infection the BAL contains approximately one million cells.
11. Ensure that you are harvesting lymph nodes and not the thymus or adipose tissue. You can *gently* roll the lymph node on a paper towel and a lymph node will remain intact while thymus will disintegrate. When placing the lymph node into media, it should also sink in the media whereas adipose tissue will float. Sometimes some adipose or connective tissue will pull away with the lymph node, try to remove as much as possible without damaging the lymph node.
12. The lymph node can also be enzymatically digested similar to lungs to maximize cell numbers (also see note 14). For enzymatic digest, place the draining lymph node in 1 ml of HBSS. Supplement with 60 U/ml of DNase and 125 U/ml of collagenase and incubate at 37 °C for 20 min using tube rotator or gently shaking every 5 min.

13. Due to the lower number of cells in the draining lymph node, it may not be possible to resuspend the cells at a final concentration of 2.0×10^7 cells/ml. We typically resuspend the sample to the appropriate volume of media dependent upon the number of stains to be performed (0.3 ml for three stains for example). As the draining lymph node will contain primarily lymphocytes, a small number of cells can be used for staining. We try to ensure that there are at least 1×10^5 cells for each stain.
14. Enzymatic digestion facilitates the release of immune cells that are tightly held in the lung tissue. This may alter surface expression of some molecules, thus preventing their detection via antibody staining. For example, CXCR3 expression is diminished following the enzymatic digestion step. We suggest that each antibody be tested with or without enzymatic digestion if fluorescent intensity of staining is not optimal. While enzymatic digestion is not necessary, it may alter release of some cell populations and/or impact numbers of cells obtained from the lung.
15. The dilution of lung cells is dependent on the context of the experiment as well. Naïve lungs will have about 10–15 million total lung cells, whereas at the peak of cellular infiltration at day 8 following RSV infection of BALB/c there are approximately 30–40 million total lung cells.
16. Do not decant the supernatant after the first centrifugation of PBL as the cell pellet is very loose and cells will be lost! Not all the liquid has to be removed, just the majority so the NH_4Cl is not diluted.
17. Tetramer staining is an excellent method to track a subset of antigen-specific T cells in the lung over time (Fig. 3). The immunodominant CD8 T cell epitope in BALB/c mice is $M2_{82\text{-}90}$, with several additional subdominant epitopes (see [16] for a complete list). The immunodominant epitope in C57BL/6 mice is $M_{187\text{-}195}$. All tetramers should be evaluated prior to use to determine optimal staining concentration.
18. Staining conditions for tetramers can vary. We have found that the majority of tetramers can be stained at 4 °C for 30 min. However, some tetramers may require incubation at higher temperatures for optimal staining. These conditions should be determined prior to experimentation.
19. All antibodies should be titrated to determine optimal staining concentrations prior to use in experiments. Additional phenotypic antibody markers can be utilized to further characterize antigen-specific CD8 T cells (Fig. 4).
20. Alternatively, intracellular staining can be performed the next day. Resuspend wells in 200 μl FACS buffer and refrigerate plate overnight at 4 °C. The next day, centrifuge the plate, decant supernatant, and continue with protocol.

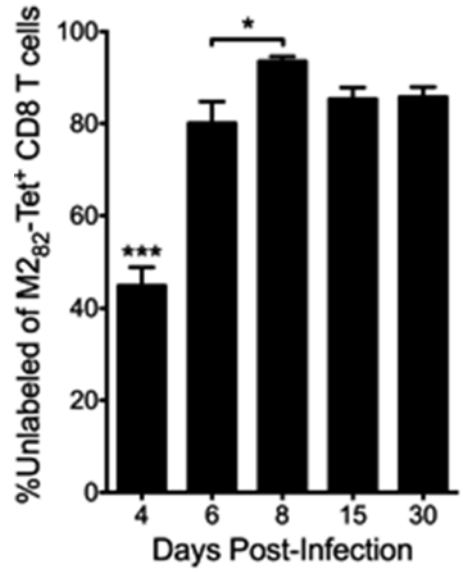

Fig. 3 Frequency of antigen-specific CD8 T cells localized within the lung tissue following RSV infection. The frequency of unlabeled $M2_{82}$-tetramer-specific CD8 T cells was assessed in the lung of RSV-infected BALB/c mice over time. The frequency of unlabeled leukocytes will vary with time following RSV infection

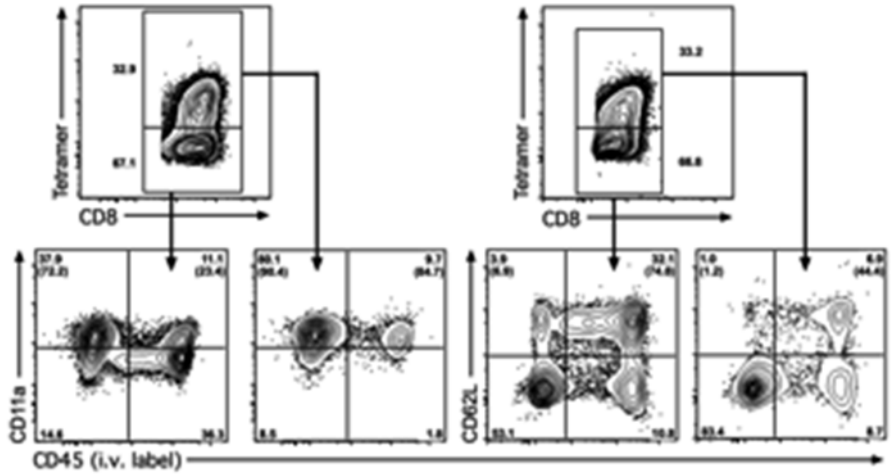

Fig. 4 Characterization of the antigen-specific CD8 T cell response and localization within the lung parenchyma. Concatenated lung samples of four BALB/c mice at day 10 p.i. following RSV infection. *Top panels* show tetramer staining for $M2_{82}$-specific and tetramer$^-$ CD8 T cells. *Bottom panels* illustrate the expression of CD11a or CD62L on either $M2_{82}$-tetramer-specific or tetramer$^-$ CD8 T cells and their localization within the lung tissue (CD45$^-$) or the pulmonary vasculature (CD45$^+$). Numbers in the parenthesis indicate the frequency of CD8 T cells within the lung tissue (CD45$^-$) or in the pulmonary vasculature (CD45$^+$) that express either CD11a (*top panels*) or CD62L (*bottom panels*)

References

1. Hall CB, Walsh EE, Long CE, Schnabel KC (1991) Immunity to and frequency of reinfection with respiratory syncytial virus. J Infect Dis 163(4):693–698
2. Glezen WP, Taber LH, Frank AL, Kasel JA (1986) Risk of primary infection and reinfection with respiratory syncytial virus. Am J Dis Child 140(6):543–546
3. Glezen WP, Paredes A, Allison JE, Taber LH, Frank AL (1981) Risk of respiratory syncytial virus infection for infants from low-income families in relationship to age, sex, ethnic group, and maternal antibody level. J Pediatr 98(5):708–715
4. Groothuis JR, Simoes EA, Levin MJ, Hall CB, Long CE, Rodriguez WJ, Arrobio J, Meissner HC, Fulton DR, Welliver RC et al (1993) Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. The Respiratory Syncytial Virus Immune Globulin Study Group. N Engl J Med 329(21):1524–1530. doi:10.1056/NEJM199311183292102
5. McIntosh K, Masters HB, Orr I, Chao RK, Barkin RM (1978) The immunologic response to infection with respiratory syncytial virus in infants. J Infect Dis 138(1):24–32
6. Graham BS, Bunton LA, Rowland J, Wright PF, Karzon DT (1991) Respiratory syncytial virus infection in anti-mu-treated mice. J Virol 65(9):4936–4942
7. Graham BS, Bunton LA, Wright PF, Karzon DT (1991) Reinfection of mice with respiratory syncytial virus. J Med Virol 34(1):7–13
8. Graham BS, Bunton LA, Wright PF, Karzon DT (1991) Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 88(3):1026–1033. doi:10.1172/JCI115362
9. Bangham CR, Openshaw PJ, Ball LA, King AM, Wertz GW, Askonas BA (1986) Human and murine cytotoxic T cells specific to respiratory syncytial virus recognize the viral nucleoprotein (N), but not the major glycoprotein (G), expressed by vaccinia virus recombinants. J Immunol 137(12):3973–3977
10. Anderson KG, Sung H, Skon CN, Lefrancois L, Deisinger A, Vezys V, Masopust D (2012) Cutting edge: intravascular staining redefines lung CD8 T cell responses. J Immunol 189(6):2702–2706. doi:10.4049/jimmunol.1201682
11. Knudson CJ, Weiss KA, Hartwig SM, Varga SM (2014) The pulmonary localization of virus-specific T lymphocytes is governed by the tissue tropism of infection. J Virol 88(16):9010–9016. doi:10.1128/JVI.00329-14
12. Teijaro JR, Turner D, Pham Q, Wherry EJ, Lefrancois L, Farber DL (2011) Cutting edge: Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. J Immunol 187(11):5510–5514. doi:10.4049/jimmunol.1102243
13. Galkina E, Thatte J, Dabak V, Williams MB, Ley K, Braciale TJ (2005) Preferential migration of effector CD8$^+$ T cells into the interstitium of the normal lung. J Clin Invest 115(12):3473–3483. doi:10.1172/JCI24482
14. Zhang L, Peeples ME, Boucher RC, Collins PL, Pickles RJ (2002) Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. J Virol 76(11):5654–5666
15. Tyrrell DA, Mika-Johnson M, Phillips G, Douglas WH, Chapple PJ (1979) Infection of cultured human type II pneumonocytes with certain respiratory viruses. Infect Immun 26(2):621–629
16. McDermott DS, Knudson CJ, Varga SM (2014) Determining the breadth of the respiratory syncytial virus-specific T cell response. J Virol 88(6):3135–3143. doi:10.1128/JVI.02139-13

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