JoAnn M. Moulds
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Editors

BeadChip Molecular Immunohematology

Toward Routine Donor and Patient Antigen Profiling by DNA Analysis

Springer

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Preface

We have entered the era of molecular immunohematology, in which DNA is analyzed to predict red blood cell, leukocyte and platelet phenotypes. Molecular immunohematology is changing practice in blood donor centers, hospital blood banks, and reference laboratories. Many blood bankers and transfusion medicine physicians know little about the development of molecular immunohematology or how it is impacting blood banking and transfusion medicine. Many are wondering how it will impact their labs or their jobs even though they realize that this advanced technology may be better for donor and patient care. Indeed, some might be concerned that their serologic expertise may become outdated.

Serology is not becoming outdated and indeed several leading serology laboratories are the very same ones that have been the early adopters of molecular immunohematology. These labs are using molecular techniques to supplement, not replace, serology. Originally, the early laboratories that adopted this technology used labor-intensive molecular biology techniques with laboratory-specific customized protocols and reagents. In the first decade of the twenty-first century, additional blood bank laboratories, some of which do not even have full reference laboratory capabilities, adopted molecular techniques. This movement has been facilitated by the commercial development of a molecular testing platform by BioArray Solutions in which multiple molecular assays can be performed simultaneously in a multiplex system.

The impetus for adopting molecular immunohematology has evolved as the technology has advanced. Initially, reference laboratories used molecular techniques to understand complex cases in which serology alone provided confusing or incomplete results. Subsequently, the technology advanced, which allowed more laboratories to perform molecular testing affordably. Additional laboratories realized the potential utility for molecular testing beyond complex serologic cases. Some laboratories have started using molecular testing to determine the antigens predicted on blood donor units, thereby increasing the supply of antigen-negative units. Other laboratories have incorporated molecular testing into less-complex patient testing. For example, some laboratories determine predicted phenotypes of recently transfused patients. In other cases, laboratories are determining the predicted phenotypes of patients at greatest risk of developing red cell allo-antibodies.

Laboratories are also using these techniques to help determine the specificity of anti-platelet antibodies and determine the antigens predicted to be expressed on platelet units.

As more laboratories are adopting molecular techniques, some facilities have been trying approaches that would have been prohibitively expensive with serologic techniques alone. For instance, molecular approaches have allowed for the relatively inexpensive extended typing of large numbers of red blood cell units. This has allowed facilities to match for more than the ABO and RhD antigens for select patients. As an example of enhanced clinical utility, some centers have prospectively matched for additional antigens for patients with autoimmune hemolytic anemia.

This book is intended for those who want to understand how molecular immunohematology is changing blood bank and transfusion medicine, whether or not their own laboratories have already adopted molecular immunohematology.

Chapter 1 introduces a history of serology, which has been the foundation of blood banking. Serology suffers from limitations in the manufacturing of reagents and the use of these reagents in transfusion medicine laboratories. Chapter 2 provides an overview of gene expression, how genetic variations translate into antigenic differences on the surfaces of cells, and techniques that have been developed to detect genetic variations. Chapter 3 describes the BeadChip™ technology developed by BioArray Solutions, the molecular immunohematology system that has been most widely adopted in blood bank laboratories.

Subsequent chapters describe how molecular immunohematology is being used in various settings for different purposes. Because the laboratory settings and use of molecular techniques vary widely, Chaps. 4–9 describe the use of molecular techniques in different types of laboratories and for a variety of purposes. While these chapters illustrate examples of molecular immunohematology as it is currently being used, additional approaches are likely to be developed in the coming years.

In the near future, we expect the increased use of molecular testing to detect polymorphisms in RH genes. This direction has been particularly challenging because of the large number and types of polymorphisms, which include more than just the single nucleotide polymorphisms responsible for most red blood cell and platelet antigenic polymorphisms. Significant advances have been made to incorporate Rh testing on the BeadChip™ platform. The testing and potential use of that system is described in Chaps. 10 and 11.

Although this book provides information on the development and current uses of molecular techniques, the field of molecular immunohematology is still ripe for further advancement. Each site involved in testing donor units and/or patient samples has its own specific needs. Although many sites will incorporate molecular techniques using approaches that differ somewhat from those described in this publication, the information provided in this book should aid many blood bank laboratories, ranging from those who have yet to consider molecular techniques to those that have already adopted molecular immunohematology testing.

The editors offer their thanks to the authors and collaborators who provided their experience and expertise in the chapters that you are about to read. We also appreciate the support of BioArray Solutions, Immucor, and their technical and support staff, all of whom have provided us with capable assistance in the design and implementation of this project.

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Synopsis

This book describes the impact and spectrum of applications of routine multi plex DNA analysis that have emerged three years after the market introduction of BeadChip molecular immunohematology into the field of transfusion diagnostics, during which period antigen determinations for >200,000 donors and recipients have been completed.

In three sections of this book, leaders in the field, representing donor centers, hospital transfusion services, and reference laboratories in the US and overseas, offer a retrospective analysis of progress made to date, as well as an assessment of the potential for future developments.

The first part (Chaps. 1, 2 and 3) provides the background on the classical serological methods and molecular techniques followed by a description of BeadChip™ technologies and assay format it supports including upcoming automation and further including a summary of a novel paradigm for inventory management in medical centers, enabled by routine and rapid DNA analysis of leukoreduced blood.

The second section (Chaps. 4 and 5) contains reviews of the performance of BeadChip™ mih in the clinical setting, including its ability to reliably predict the expression of antigens on the red cells of donors and prospective recipients; the third section (Chaps. 6, 7, 8, and 9) contains reviews of the performance of BeadChip™ mih in the clinical setting, at international blood banks and medical centers and also provides an outlook of emerging applications, including the routine determination of HPA antigens for complex situations by a national reference laboratory (to avoid complications associated with neonatal allo-disorders); the fourth section (Chaps. 10, 11 and 12) describes the use of new designs in connection with the rapid allele determination for RhCE and RhD variants, the former increasingly important for patient care, the latter primarily aiming at the confirmation of the D-status of potential donors; and the final section provides detailed description of algorithms used for data analysis used by BeadChip™ systems. The volume thus is intended for patient care providers and the suppliers of blood products in blood banks, while providing supplementary reading in the teaching of the molecular biology of blood groups and its application in the field of molecular immunohematology.

Warren, NJ Ghazala Hashmi

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Ghazala Hashmi, PhD, the Technical Editor, coordinated all aspects of the realization of this project, with Yi Zhang, PhD, who supported donor and medical centers in the aggregation and analysis of large data sets underlying especially in Chaps. 4 and 5, and the project coordination support of Tyler Hutchinson, BA, both of BioArray Solutions and editorial support from Ingrid Sprague, BS. Ghazala Hashmi and Michael Seul, PhD, developed the concept for this volume and established the relationship with Springer Verlag where editorial team guided the project to its publication.

List of Abbreviations, Institutions & Terms

Institution	Abbreviation	First mentioned in
BioArray Solutions	BAS	Chap. 1
Cedars-Sinai Medical Center	CSMC	Chap. 3
Central Illinois Blood Center	CICBC	Chap. 4
Centre National de Référence pour les Groupes Sanguins	CNRGS	Chap. 7
Children's Hospital Boston	CHB	Chap. 5
Children's National Medical Center	CNMC	Chap. 5
Gulf Coast Regional Blood Center	GCRBC	Chap. 4
Institut National de la Transfusion Sanguine	INTS	Chap. 6
LifeShare Blood Center	LBC	Chap. 4
LifeSouth Community Blood Center	LSCBC	Chap. 4
Mayo Clinic	MC	Chap. 5
New York Blood Center	NYBC	Chap. 4
Term	**Abbreviation**	**First mentioned in**
Acute hemolytic reaction	AHR	Chap. 9
Allele specific polymerase chain reaction	AS-PCR	Chap. 2
American Rare Donor Program	ARDP	Chap. 4
Amplification, discrimination, identification steps	ADI	Chap. 3
Anti-human globulin	AHG	Chap. 1
Anti-human globulin test	AHGT	Chap. 1
Applied Biosystems	ABI	Chap. 2
Array Imaging System	AIS	Chap. 3
Automated BeadChip Deployment	ABCD	Chap. 3
BioArray Solutions Information System	BASIS™	Chap. 3
Charged-coupled device	CCD	Chap. 3
Clinical Laboratory Improvement Amendments	CLIA	Chap. 4
College of American Pathologists	CAP	Chap. 5
Complementary deoxyribonucleic acid	cDNA	Chap. 10
Conformité Européenne	CE	Chap. 3
Current procedural terminology	CPT	Chap. 4
Data exchange robot	dxBOT™	Chap. 3
Delayed hemolytic reaction	DHR	Chap. 9
Deoxyribonucleic acid	DNA	Chap. 3

(continued)

Term	Abbreviation	First mentioned in
Dideoxy nucleoside triphosphate	ddNTP	Chap. 2
Direct antiglobulin test	DAT	Chap. 1
Elongation-mediated analysis of polymorphisms	eMAP	Chap. 3
Extended human erythrocyte antigen	xHEA	Chap. 3
Extractable nuclear antigens	ENA	Chap. 3
Food and Drug Administration	FDA	Chap. 1
Hemoglobin S	HbS	Chap. 4
Hemolytic disease of fetus or newborn	HDFN	Chap. 11
Hemolytic disease of newborns	HDN	Chap. 5
Human erythrocyte antigen	HEA	Chap. 3
Human immunodeficiency virus	HIV	Chap. 1
Human lymphocyte antigens	HLA	Chap. 3
Human platelet antigen	HPA	Chap. 3
Hybridization-mediated analysis of polymorphisms	hMAP	Chap. 3
Identification of discordances	ID	Chap. 4
IMGT/HLADB IMmunoGeneTics/Human Leucocyte Antigen DataBase	IMGT/HLADB	Chap. 12
Immunoglobulin G	IgG	Chap. 1
Immunohematology Reference Laboratory	IRL	Chap. 4
Indeterminate call	IC	Chap. 4
Indirect antiglobulin test	IAT	Chap. 1
International Society of Blood Transfusion	ISBT	Chap. 2
Laboratory developed tests	LDT	Chap. 2
Low signal	LS	Chap. 9
Maximum likelihood	ML	Chap. 12
Mean	μ	Chap. 12
Messenger ribonucleic acid	mRNA	Chap. 2
Molecular Immunohematology	mih	Chap. 3
Monoclonal antibody-specific immobilization of platelet antigens	MAIPA	Chap. 6
National Heart Lung and Blood Institute	NHLBI	Chap. 10
Normal allele	N	Chap. 3
Operational qualification	OQ	Chap. 4
PCR with sequence-specific primers	PCR-SSP	Chap. 2
Polymerase chain reaction	PCR	Chap. 2
Quality assurance	QA	Chap. 4
Quality control	QC	Chap. 4
Random Encoded Array Detection	READ	Chap. 3
Real-time PCR	RT-PCR	Chap. 2
Red blood cell	RBC	Chap. 1
Research-use only	RUO	Chap. 3
Restriction fragment length polymorphism	RFLP	Chap. 2
RH immune globulin	RhIG	Chap. 11
Rhesus D	RhD	Chap. 2
Sequence-specific primer analysis	SSP	Chap. 2
Sickle cell disease	SCD	Chap. 4
Sickle cell hemoglobin	Hgbs	Chap. 1

(continued)

Term	Abbreviation	First mentioned in
Single nucleotide polymorphism	SNP	Chap. 1
Small interfering RNA	siRNA	Chap. 2
Sodium dodecyl sulfate	SDS	Chap. 2
Stroke prevention	STOP	Chap. 10
Turn-around time	TAT	Chap. 3
Untranslated region	UTR	Chap. 11
Variance	σ^2	Chap. 12
Variant allele	V	Chap. 3
White blood cell	WBC	Chap. 11

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Chapter 1
An Overview of the Classic Serological Methods: Limitations and Benefits of Serology and DNA Testing

John J. Moulds

Abstract Classic serological testing is based on red blood cell (RBC) agglutination using antisera. This chapter provides a historical perspective of different types of serological reagents and their pros and cons of being used to determine red blood cell antigens. Improvement in robotic and optical technology made automation possible to measure agglutination but certain limitations are still set by the reagent, for example, availability of the antisera is limited by getting sufficiently potent antibodies, the limited source of uncommon specificities, false positives from immunoglobulin coated cells, and recent transfusions. DNA testing on the other hand is not limited by immunoglobulin coating of the red blood cells, by the presence of recently transfused cells, or by the limited antisera, but it has its own challenge of altered expression of antigens due to variant alleles.

Keywords Serological testing • Agglutination • Antisera • Coomb's test • Antibody

1.1 Historical Perspective

For over 100 years, the classic method used to determine human red blood cell group antigens has been serological testing based upon red blood cell agglutination or some form of aggregation of the red blood cells being tested. The testing format initially consisted of slides (or tiles) or test tubes containing the reactants – red blood cells and antisera against potential red blood cell surface antigens. The primary ingredient in all of these test methods was antisera. The first experiments were with human source reagents, predominantly anti-A and anti-B. Experiments with rabbits immunized with human red blood cells produced anti-M, anti-N, and anti-P_1, defining two additional human blood groups. The clinical relevance of

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these red blood cell groups was not understood until the 1940s with the discovery of the Rh and Kell blood group systems. It then was appreciated that immunized humans were an essential source for red cell blood grouping reagents and that multiple red blood cell groups might be of clinical significance. Attempts to produce anti-Rh in rabbits for massive population testing during World War II never made a viable product; hence the sole use of human source of Rh antibodies for the determination of Rh_o(D) and other blood group factors.

Despite a lack of knowledge of immunoglobulin types and subclasses, and only working with empirical knowledge of complete (aka, β_{2M}, γ_{1M}, 19S γ, γM and eventually defined as IgM) and incomplete (aka, γ_2, γ_{ss}, 7S γ, γG and eventually defined as IgG) agglutinins, reagents were formulated for predominantly slide and tube tests. The Rh reagents were in limited supply because of the perceived dependence on direct agglutination. It was common knowledge that recently immunized individuals would produce direct agglutinating (complete) antibodies for only a short time and then it would become a blocking or incomplete antibody. Utilizing the observations reported by Diamond, it was shown that by using serum albumin as a diluent, human serum with high titers of blocking antibodies would directly agglutinate red blood cells if they possessed the antigen to which the antibody was directed [1, 2]. The only way to readily utilize the blocking antibodies was with the incorporation of the antihuman globulin (AHG) or Coomb's test [3]. At one time, many believed this procedure was too complicated to be performed outside of a research laboratory environment.

Throughout the 1950s and 1960s other blood group antibodies were detected and some were found to be detected only by the AHG technique. Only those of the Rh group could reliably be made as direct agglutinants of red blood cells in the presence of high concentration of albumin or some polymers. Therefore, by the early 1970s, there were three forms of reagents that any serologist could have access to (1) direct agglutination reagents (predominantly IgM), (2) high-titer IgG Rh specificities containing high concentration of bovine albumin or other polymers, or (3) those reagent cells that would react only by the indirect AHG test (IAT).

ABO blood grouping reagents were predominantly pools of numerous donors that had been hyperimmunized with A and/or B substance. They contained a mixture of both IgM and IgG antibodies in a solution with 8% or less total protein with a sodium chloride content of greater than 1% to enhance the direct agglutination. The Rh blood grouping reagents were available in two forms. One was called saline reactive reagent which contained predominantly IgM antibodies collected from recently immunized individuals in a solution of less than 8% total protein and intended to work as a direct agglutinating reagent. The other Rh grouping reagent was called slide and modified tube reagent which were predominantly IgG antibodies from hyperimmunized individuals collected over a period of time. The total protein of the reagent was between 15% and 19% with a sodium chloride content of less than 0.8%. It generally contained polymerized bovine albumin and/or other macromolecular structures to enhance the agglutination of IgG sensitized cells.

All of the other blood group specificities for the clinically relevant system antigens (Kell, Duffy, and Kidd) that were commercially available were IgG antibodies in a

6–8% total protein solution, with a sodium chloride content of 0.8% or less and were intended to be tested only by the AHG procedure. Not all human examples of the various specificities could be used as there are minimal potency requirements established by the FDA (Food and Drug Administration) and lot approval prior to commercialization was required [4].

Romans et al. [5] reported the conversion of IgG antibodies, reactive only by the AHG test, into direct agglutinins by modifying the disulfide bonds within the immunoglobulin. This procedure was quickly adapted to utilize the abundant IgG sources of specificities to fill the void of the limited availability of high-titer IgM specificities and was referred to as chemically modified reagents. The additional advantage was that high concentrations of bovine albumin or macromolecular products were not necessary in the formulation, and therefore the reagent was not as prone to give false-positive test results with samples that had positive direct antiglobulin tests (DAT).

By the mid to late 1980s, monoclonal antibodies against human blood group antigens had been described and were considered the obvious way of the future to eliminate the necessity of immunizing individuals with red cells from other sources [6]. This change to monoclonal reagents was driven both by economics, the availability of hyperimmunized plasma sources of various specificities and the increasing fear of an HIV (Human Immunodeficiency Virus) epidemic that made donors and the FDA reluctant to stimulation with red cells from unknown sources or history. Simultaneously, the microtube or column technology described by Lapierre et al. [7] simplified the performance of preparation of testing procedure endpoint readout of the test that was, before that time, considered technically challenging.

In the selection of the specificities for monoclonal reagents, most developers choose saline agglutinating specificities from cell lines secreting IgM in an attempt to eliminate the necessity of the AHGT or Coomb's test and give it a wider application of tests. For the production of monoclonal reagents, murine (mouse) source monoclonal was sufficient for ABO, M and N, P_1, and Lewis specificities. However, attempts to stimulate other specificities in mice were not fruitful. The production of Rh, Kell, and Kidd specificities were not possible until the late 1980s and early 1990s for the development of the human hybridoma or heterohybridoma technology. Unfortunately, the production of a specific stable clone that would secrete the desired IgM specificity is in part a matter of luck or a numbers game. Those that were successful and early to market generally became a standard and few laboratories took the risk to try to develop alternative sources for that same specificity. Many times the specificity of the clone would be very unique only when tested against red cells with rare phenotypes or hybrid structures.

1.2 Automating Blood Grouping

As automation was applied to blood grouping, most of the automation procedures were for ABO and Rh determination and were used in blood donor centers. The reagents were modified FDA-approved slide and modified tube or chemically

modified reagents. Each institution developed its own formulation and performance criteria for acceptability. Screening tests for other than ABO and Rh were usually performed with slide and modified tube or AHG reactive reagents. They were predominantly performed by test tube methods, microtiter plate, or some form of automated procedure. Initially, the tests most frequently consisted of adaptations of the clinical chemistry instruments, e.g., Technicon AutoAnalysers™ [8]. In the beginning such automation was hampered by the ability to determine various degrees of agglutination, so procedural changes were made to read the percent of hemolysis of the unagglutinated red cells. With improvements in robotics and optical reading devices, present instruments are made to directly measure agglutination or adherence of red blood cells onto an antibody coated surface. Whether the system is an automated system, as commonly used in blood centers, or manual, as used in most transfusion services, there has always been certain limitations to serological tests.

1.3 Limitations of Serological Testing

1.3.1 Antisera Availability

For human source or polyclonal reagents, one of the limitations is getting a sufficiently potent and pure antibody that can withstand the processing technologies used in manufacturing and demonstrates sufficient stability for an adequate shelf-life. In addition, the final reagent titer must meet or exceed the FDA requirements [4]. Unfortunately, few individuals respond with a pure antibody specificity, and manufacturers have to use donors other than group AB in order to meet volume requirements. Removal of the unwanted agglutinins, be it either anti-A or anti-B or any other blood group specificity, requires laborious absorption procedures. Not all interfering antibodies can be easily removed. A common response of an immunized individual is an anti-Bg antibody that eventually eliminates the usefulness of that specific donor.

Some specificities, while commonly discussed and the subject of much published literature, may be very uncommon. For example, a human or polyclonal anti-C that is predominantly IgM (for saline reactive Rh reagent) may be not available even in a large immunized donor base. In fact, at one time in the USA, all of the saline reactive anti-C came from one source that was utilized by all manufacturers. The source had a high-titer IgM antibody. She was frequently immunized to encourage IgM immunoglobulin production. When the donor eventually became tired of the frequent injections and refused to be further immunized (coincidentally in the early 1980s), saline reactive anti-C became backordered by all manufacturers. Hence, when monoclonal specificities became available as a continual source with no need for absorption, manufacturers were happy to adjust to this reagent even though certain limitations existed with some monoclonal antibodies.

Monoclonal antibodies may be very unique in the chemical requirements of the buffer to get optimum agglutination, so one uniform diluent for all monoclonal

reagents is not possible. Each reagent must be individually formulated and should not be adulterated by the end-user, lest they deviate from the defined specifications of specificity and potency as claimed from the manufacturer. Some specificities, specifically anti-M or anti-N, can give false-positive reactions with hybrid structures that would not be seen with rabbit or lectin reagents. Furthermore, some specificities that are useful in blood group serology are not available. Based upon the expense of developing a cell line, production of new specificities is unlikely.

However, this is not a limitation of monoclonal source reagents alone. Many human sources of various specificities exist in such small quantities that they are only available in one or two blood group research laboratories. For example, anti-Do^a is extremely useful in both donor screening and patient testing. It is a clinically relevant antibody, so Do(a−) blood should be available for such individuals. However, for a period of 5 years there was less than 15 ml of anti-Do^a in the entire world, which was stored in various blood group reference laboratories and sparingly used. While other examples of anti-Do^a had been found during this period, sufficient quantities had not been collected as most serologists do not consider the availability of rare resources. Anti-Hy and anti-Jo^a are other examples. While these are not uncommon antibodies in multiply transfused African-Americans, these specificities are not available because most serologists deal with patients and do not consider that these individuals may provide suitable supplies of a rare antibody when they are healthy and meet blood donor requirements. The same can be said of almost any blood group specificity.

1.3.2 Red Blood Cell Source

Discrepant red cell grouping results can occur in both patient and donor samples. Both can have immunoglobulin coating their cells that would be reactive with AHG reagents, negating or bringing into question the results of any reagent that used AHG as an endpoint. The degree of sensitization may be minimal, but this may still interfere with the interpretation of results of reagents with limited potency or reactivity. Samples that are heavily coated with IgG may spontaneously agglutinate in some diluents used to formulate the reagents, especially those with high protein content.

The far more common problem in patients is trying to determine their phenotype when they have been recently transfused. The presence of circulating transfused cells may give false-positive tests if sensitive reading techniques are applied. Methods using differential centrifugation or phthalate ester are technically challenging, and frequently result in only small volumes of separated red blood cells. In HgbS (Sickle cell hemoglobin) patients, a method is available to hemolyze HgbA-transfused red blood cells, but this also often does not yield sufficient red blood cells for the performance of a full phenotype. Thus, a genotype offers an alternative answer to this problem.

In some rare individuals or in a certain population of patients, the red blood cells may become polyagglutinable or agglutinated in the presence of any adult normal serum, which was a primary ingredient in the early serological or polyclonal reagents.

The antibodies that define the common forms of polyagglutination (e.g., T, Tx, and Tk) occur in all adult sera. Occasionally, the antibodies to the polyagglutinable characteristic are more abundant than the desired serological specificity. However, testing for the presence of the various forms of antibodies that detect polyagglutination is not a requirement. Generally, the problem is not a major issue in that the naturally occurring antibodies to the various form of polyagglutination are IgM, and degrade upon storage. Therefore, if polyclonal reagents are close to their expiration date or have been heat-treated at 56°C for 3 h prior to testing, false-positive reactions will not occur.

1.4 DNA Testing

DNA testing for red cell blood groups is not influenced by immunoglobulin coating of the red blood cells, the presence of recently transfused red blood cells, or any form of polyagglutination nor by the limitations commonly found with the antisera. DNA can be easily extracted from whole blood, buffy coats, or buccal swabs, and is not influenced by the patient's medications or disease condition with the exception of a transplant.

However, DNA testing is not without its own limitations. While it is simple to test for two or three single nucleotide polymorphisms (SNPs) to determine the potential for a blood group antigen, it is simpler to perform serological testing for ABO determination because of the numerous alleles ($n > 100$) for A and/or B that would have to be tested. Another limitation is that some phenotypes [e.g., Jk(a−b−), Gy(a−), InLu] may appear to have normal genes, but the products are not expressed on the red blood cells. Therefore, DNA typing should only be considered as an adjunct to serological testing. It is not a replacement for but an alternative method to support the serological observations. Even now there are discrepancies between DNA and serology that have not been resolved.

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Chapter 2
Introduction to Molecular Typing

JoAnn M. Moulds and Steven R. Sloan

Abstract The DNA sequences responsible for variations in most blood cell antigens have been determined. Most of these sequence variations are single nucleotide polymorphisms (SNPs) that encode for amino acid substitutions on proteins present on the surface of red blood cells or platelets. However, other variations exist, such as gene deletions and polymorphisms impacting the expression of proteins. To accurately determine blood cell antigens using molecular techniques, sometimes several SNPs need to be analyzed to determine whether a protein is likely to be expressed and which form of the protein will be expressed. Several techniques have been developed to detect these polymorphisms, including sequencing and allele-specific polymerase chain reaction (PCR) techniques. Microarray (DNA assay) techniques based on PCR methods have also been developed that detect multiple SNPs simultaneously. When properly designed and implemented, these techniques have proven to be extremely accurate in predicting the cellular antigens expressed and the microarray techniques also provide a high-throughput process.

Keywords Allele-specific PCR • DNA sequencing • Gene expression • Gene regulation • Microarrays • Molecular techniques • PCR • Sequencing • Transcription • Translation

2.1 Detection of Red Blood Cell Blood Group Polymorphisms

Blood group antigens are inherited polymorphisms found on the red blood cell (RBC), but may also occur on other cells and in secretions. Since the discovery of the ABH blood group at the beginning of the twentieth century, about 300 red cell antigens have been identified and 30 distinct blood group systems have been officially recognized by the International Society of Blood Transfusion (ISBT) [1].

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Until recently, blood group antigens were only detected by hemagglutination methods. However, following the advent of molecular technology, the majority of the blood group genes were identified, sequenced, and the single nucleotide polymorphisms (SNPs) assigned to blood group antigen specificities were defined. These molecular findings were followed closely by the development of laboratory-developed tests (LDT) and, more recently, by commercial assays for the prediction of RBC phenotypes by DNA methods. To fully appreciate the BeadChip™ assay described in this book, one needs to have a better understanding of the molecular basis for blood antigens and the molecular techniques previously available for blood group genotyping, some of which are described below.

2.2 Molecular Determination of Phenotypic Polymorphisms

The molecular mechanisms that determine the specific antigens expressed on the surface of blood cells are the same as those that determine the expression of proteins in all cells throughout the body. The specific proteins expressed by a person's cells are determined by the specific DNA sequences an individual inherits. Alterations in these sequences determine variations in protein expression. Some of these variations determine antigenic variations on the surfaces of cells such as RBCs and platelets. To understand how genetic variations determine antigenic variations, this section will provide an overview on gene expression. Alberts et al. [2] provide a more detailed discussion on gene expression.

2.3 Gene Expression

A gene is said to be expressed when the protein that it encodes is synthesized. This requires that the mRNA for the gene be synthesized (i.e., the gene is transcribed) as well as synthesis of the protein from the mRNA (the transcript is translated).

2.3.1 Transcription

Some genes, such as those involved in basic metabolic functions, are transcribed in almost all cells, but many genes such as the Rh genes are only transcribed in some cells. Transcription factors are important mechanisms in determining whether a gene is transcribed in a particular cell. Transcription factors are proteins that bind to specific DNA sequences and catalyze initiation of transcription of a gene regulated by activity at that binding site. The presence or absence of particular transcription factors in a cell helps determine which genes are transcribed in that cell. Hence, cells that lack particular transcription factors will not express a particular gene. Also, if the DNA binding site for a particular transcription factor is altered, the transcription factor will not bind, the mRNA will not be synthesized, and no protein will be translated.

2.3.2 RNA Processing

RNA that is initially transcribed undergoes further processing before being translated into protein. The RNA initially transcribed from a gene includes both exons, which encode for the corresponding protein, and intervening regions that do not encode for the protein and are called introns. Normally, the introns are removed in a process termed splicing that requires precise sequences to identify the start and end of exons and introns. Most final (mature) mRNAs that are formed are unstable with half-lives of less than a couple of minutes without further modification. However, the stability of mRNAs can vary significantly. This is influenced by proteins and small interfering RNAs (siRNAs) that can bind either in the region of the mRNA that directly codes for a protein or at the ends of the mRNA that are not translated as well as other post-transcriptional RNA processing.

2.3.3 Translation and Additional Processing

The mRNA is transported out of the nucleus to ribosomes where translation occurs. The newly synthesized protein then may undergo modifications and may be transported to specific locations in the cell or on the cellular surface. Each protein has a specific function, but the proteins important in immunohematology generally are proteins expressed or partially expressed on the cell surface, or enzymes that modify cellular molecules that are expressed on the cell surface.

2.4 Allelic Variations

2.4.1 Coding Region SNPs

The most common genetic variation responsible for differences in blood antigens are SNPs in the coding region of a gene. Most commonly, the gene encodes a protein that is expressed on the cell surface and a SNP results in an amino acid substitution. An example of this would be the K/k antigens [3]. Both of these antigens are presented on the Kell protein. If the Kell gene has a T at nucleotide 698 then the protein will have a methionine at position 193 and the K antigen will be present. Alternatively, if the gene has a C at nucleotide 698 then the protein will have a threonine at amino acid position 193 and the k antigen will be present.

SNPs in genes for enzymes can also create blood surface antigen variations. The most important example of this is in the ABO blood group system. These antigens are determined by the activity of a glycosyltransferase. The genes for the A and B versions of the gene differ at seven nucleotides causing changes in four amino acids in the protein [4]. Multiple other different SNPs result in an enzymatic activity creating subtypes of A and B. Blood group O is due to a nonfunctional enzyme that

is most commonly due to a nucleotide deletion causing a frameshift in translation and premature termination of the protein [5, 6]. However, multiple other allelic polymorphisms can also result in a nonfunctional protein and blood group O. Because of the complexity of the ABO system, relatively straightforward serologic approaches to identify the ABO groups are usually successful and most facilities that use molecular techniques do not use molecular techniques for determination of the ABO groups. Molecular testing of the ABO system is usually limited to exceptionally difficult or unique samples.

2.4.2 SNPs in Noncoding Regions

Because gene expression can be regulated by regions of DNA outside of the coding region, polymorphisms in such regions can impact the expression of a gene. While these types of variations could impact splicing or RNA stability, the most important genetic variation known to date that impacts blood surface antigens impacts the binding site for a transcription factor. The Duffy gene is expressed in several types of cells, but its expression in RBCs is dependent on a GATA transcription factor that binds to the DNA sequence GATA that lies near the transcription start site for the Duffy gene. The T in the GATA sequence is altered to a C in many people of African descent and the GATA transcription factor fails to bind the site and no transcription occurs [7, 8]. People with two alleles in which the GATA sequence has been replaced by GACA express no Duffy protein in RBCs and type as Fy(a−b−).

2.4.3 Other Polymorphisms

While SNPs are the most common polymorphisms that determine phenotypic variations between individuals, other genetic polymorphisms are also important. An entire gene can be deleted, as is frequently the case with the *RHD** gene in Rh(D)− individuals. Alternatively, a small region of the gene can be deleted, as sometimes occurs with *RHD**. Other more complex rearrangements can occur with two homologous genes forming hybrid genes, as can occur with the *RHD** and *RHCE** genes (see Chap. 10).

2.5 Molecular Methods Overview

Because most important allelic variations are due to SNPs, high-throughput methods that have been developed are focused on detecting SNPs. Careful consideration has gone into developing and using such approaches. Multiple SNPs may need to be analyzed since one SNP may indicate the presence of a particular allele, while another SNP may prevent the allele from being expressed as can happen with the

Duffy gene. A gene might be absent, as can happen with the *RHD** gene, and controls are needed to ensure that the lack of a signal indicates the lack of a gene rather than a technical failure. Rare complex polymorphisms exist in some genes, such as the Rh genes, making molecular testing design more complex. However, with careful design, implementation, and execution, molecular methods have proven to be robust and reliable for most of the important RBC and platelet antigens.

2.5.1 Methods for the Prediction of RBC Antigens

Currently, there exist a multitude of assays that can be used for SNP detection and these are constantly changing. We will describe only those assays that have been used for the prediction of red cell phenotypes.

2.5.2 Assays Based on Gel Electrophoresis

2.5.2.1 PCR-RFLP

The earliest laboratory defined test (LDT) utilized polymerase chain reaction (PCR) to amplify specific regions of the target blood group gene. The primers were designed to amplify a region of the blood group gene known to contain the SNP causing different antigens to be expressed. Based on the sequence change, a specific restriction enzyme could be chosen to cut the PCR product into DNA fragments. This process was known as a restriction enzyme digest. The digested PCR products could then be separated using agarose or sodium dodecyl sulfate gel electrophoresis. The resulting restriction fragment length polymorphism (RFLP) could be visualized under ultraviolet light using ethidium bromide dye. The resulting band pattern was dependent on whether a restriction site was lost or gained when the blood group SNP was present. For example, when a *JO* allele is present, there is a loss of an *Xcm* I restriction site resulting in a single band of 220 kDa as compared to the two bands of 167 and 53 that are observed with a Jo(a+) person. The major problem with this type of assay was its subjectivity.

2.5.2.2 Allele-Specific PCR

Another somewhat faster adaptation of the basic PCR method is allele-specific PCR (AS-PCR) also known as sequence-specific priming PCR (PCR-SSP). This test omits the restriction digest step. In this assay, a primer that only detects the allele of interest is used in the PCR and a band is observed on the gel only when the gene of interest is present. A multiplex PCR-SSP has been used to screen donors for the following high-incidence antigens: Kp^b, Co^a, Yt^a, and Lu^b [9].

In addition to LDT, there are commercially available kits that can be used to test for *RHD* variants and other blood group genes such as *KEL*01/*02*, *FY*A/FY*B*, and *JK*A/JK*B*. In general, these methods are labor-intensive and can only test a few samples at a time. Thus, they are not suitable for large-scale genotyping, but lend themselves well to a reference laboratory or hospital laboratory.

2.5.2.3 Other PCR-Based Methods

Some other assays that can be considered as medium-throughput use real-time PCR (RT-PCR). This procedure follows the general principle of PCR. However, its key feature is that the amplified DNA is detected as the reaction progresses in real time. In general, there are two common methods for the detection of products using real-time PCR. The first method uses nonspecific fluorescent dyes that intercalate with any double-stranded DNA (i.e., SYBR® Green). The second method uses sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target (i.e., TaqMan®). The former has been used in combination with various instruments, e.g., Lightcycler, ABI 7500, to predict the following antigens*:* RH 1–5 (D, C/c, E/e), Jk^a/Jk^b, and K1/2 [10].

In addition, real-time PCR can be used in conjunction with melting curve analysis of the PCR product. Until recently, melting and analysis of the entire PCR product was not generally successful in finding single base variants. However, with higher resolution instruments and advanced dyes, amplicon melting analysis of a single SNP is now possible. Detection of RBC blood group SNPs by melting curve analysis has been used to genotype for *KEL*01/*02*, *JK*A/*B*, *FY*A/*B*, *FY*, *FY*X*, *MNS1-4*, *DO*A/*B*, *CO*A/*B*, *LU*A/*B*, *YT*A/*B*, and *DI*A/*B* [11–14].

2.5.3 Sequencing-Based Assays

2.5.3.1 Sanger DNA Sequencing

Two methods developed for DNA sequencing were the Maxam–Gilbert method or chemical sequencing technique and the Sanger chain termination method. Both use gel electrophoresis for detection of the DNA. However, they differ significantly from the earlier described assays. The dye-terminator method, an adaption of the Sanger method, has become the method of choice. Dye-terminator sequencing utilizes labeling of the chain terminator dideoxynucleoside triphosphates (ddNTPs), which permits sequencing in a single reaction rather than four reactions as in the labeled-primer method. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labeled with separate fluorescent dyes, each with different wavelengths of fluorescence. Dye-terminator sequencing is now the mainstay in automated sequencing [15]. Although DNA sequencing is the gold standard for the initial determination of blood group polymorphisms, its application for large-scale screening is limited.

2.5.3.2 Minisequencing

A platform that may be more applicable to RBC genotyping is minisequencing or the SNaPshot assay (Applied Biosystems). Cyclic minisequencing reactions with fluorescently labeled dideoxynucleotides are performed in solution using multiplex PCR product as template and detection primers that are designed to anneal immediately adjacent and upstream of the SNP site. Following the hybridization and extension steps, the fluorescent signals from the array are measured and the genotypes are deduced by cluster analysis.

A minisequencing method to genotype the ABH blood group system has been described and found to be a reproducible strategy to type for the most common ABH alleles [16]. It is more rapid than PCR-RFLP and newly discovered mutations could be readily investigated by the addition of new extension primers into the minisequencing multiplex reaction. The SNapShot method has been used for typing for the following antigens: Fy^a/Fy^b, Do^a/Do^b, Jo^a, Hy, LW^a/LW^b, Co^a/Co^b, Sc1/Sc2, Di^a/Di^b, Jk^a/Jk^b, Lu^a/Lu^b, MNSs, and K/k [17].

2.5.3.3 Pyrosequencing

Pyrosequencing is a method of DNA sequencing based on the sequencing by synthesis principle. It differs from the common Sanger sequencing by relying on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides. The method allows sequencing of a single strand of DNA by synthesizing the complementary strand along it, one base pair at a time, with detection of the base that was actually added at each step. Pyrosequencing has been successfully used for some RBC genotyping, but presently is limited to only a few blood group systems including Kell, Duffy, and Kidd [18].

2.5.3.4 DNA Array Methods

The ability to have true high-throughput testing became a reality with the development of microarrays. Although the earliest assays were expression arrays, the technology has been applied successfully and adapted for SNP typing of genomic DNA. Some microarray methods use glass slides dotted with DNA probes. Others are bead-based and still others are fluidic arrays. Commercialization has resulted in the successful introduction of this technology into the area of immunohematology although the actual methods may differ from company to company. All of the genotyping assays face issues raised earlier in this chapter, e.g., the detection of silencing mutations that will directly affect their ability to make correct RBC antigen predictions. However, they offer us a new insight into the complexity of each blood group system.

The following chapters will describe the early introduction of BeadChip™ arrays into blood centers and transfusion medicine departments. It is clear, from the authors' descriptions, that DNA technology will be a part of the field of immunohematology for a long time to come.

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Chapter 3
The BeadChip System: A Flexible Array Format for Complex Nucleic Acid and Protein Analysis

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Abstract This chapter provides an overview of key elements of the bead array technology and related manufacturing steps as well as key components in the deployment of the BeadChip™ system. The BeadChip™ molecular immunohematology has a spectrum of applications which can utilize multianalyte (multiplex) nucleic acid and protein analysis by Random Encoded Array Detection.

Keywords DNA Array • BeadChip • Phenotype • Molecular diagnostic techniques

3.1 Introduction

The association of the majority of human erythrocyte antigens (HEA) and human platelet antigens (HPA) with single-nucleotide polymorphisms (SNPs) [1, 2] and their utility in identifying RhCE and RhD variants with hybrids, insertions, and deletions provides the basis for determining blood group antigen expression by identifying alleles of the encoding genes. The BeadChip™ system provides a platform for performing multianalyte (multiplexed) protein and nucleic acid analysis. Over the years, BioArray Solutions has established a wide range of applications (Fig. 3.1) from SNP/allele determination to therapeutic drug monitoring. A group of assays most relevant to transfusion medicine has enabled BeadChip™ molecular immunohematology (mih) (Table 3.1). This technology reduces the need for increasingly rare serologic reagents and also permits reliable determination of a

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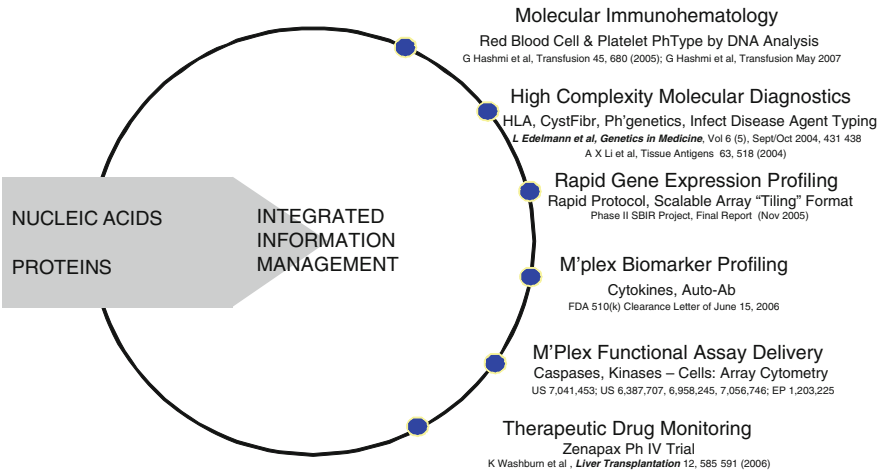

Fig. 3.1 A universal platform: the spectrum of BeadChip™ applications

Table 3.1 Current BeadChip™ mih applications

BeadChip™ kit	Target(s)	Clinical application
HEA	Minor erythrocyte antigens	Special patients, donors
RhCE	Principal CE variants	Patient and special donors
RhD	Principal D variants	Donors, patients
HPA	Platelet antigen markers	Platelet donors, patients
HLA-A	Leukocyte antigen markers	Platelet donors, patients
HLA-B	Leukocyte antigen markers	Platelet donors, patients
Reference panels	HEA, HPA	Validation

phenotype in situations that are difficult to resolve by serologic methods, especially when the available antibody reagents are only weakly reactive (Fig. 3.2) (see Chap. 1). BeadChip™ mih represents an example of multiplex analysis of polymorphisms. As documented in this volume, the BeadChip™ system, since its introduction for DNA analysis of HEA in 2006 followed by kits for platelet antigen and HLA analysis, facilitated the adoption of BeadChip™ mih for routine analysis of donors as well as prospective recipients of transfusion. The various components comprising the BeadChip™ system are described in this chapter. Chapter 12 gives further detail regarding the design of algorithms for Bayesian allele assignment for complex allele determinations of HLA and RH gene variants. The book chapters in this volume that report the results of BeadChip™ systems for HEA, HPA, HLA, RHCE, and RHD variant analysis illustrate the reliability of this method in the clinical setting. Table 3.1 enumerates the six BeadChip™ mih kits currently available as research use only (RUO) products. Two of those kits (HEA and HPA) are also available as CE (*Conformité Européenne*), marked for use in Europe.

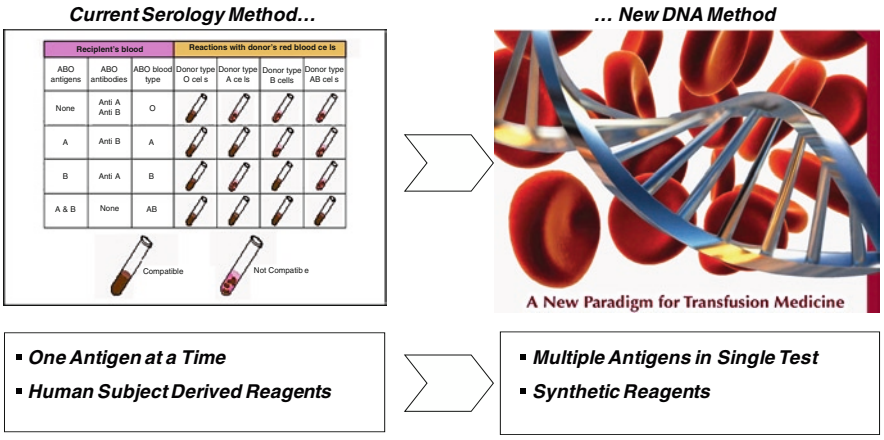

Fig. 3.2 Pretransfusion diagnostics, present and future, are shown from serological hemagglutination to DNA analysis of blood group antigens

3.2 BeadChip™ Technology

3.2.1 BeadChip™ Production

The BeadChip™ system provides a platform for performing multianalyte (multiplexed) protein and nucleic acid analysis. Central to the system is an array format [3]. A solid-phase (semiconductor) substrate supports a bead array, with each bead array being composed of thousands of small color-encoded microparticles or beads that are fluorescently encoded. Each bead displays a specific detection agent (probe), which can be read out in a Random Encoded Array Detection (READ™) format. The READ™ mechanism employs an array imaging system (AIS) and associated image analysis software which takes assay snapshot images, processes the images, and extracts assay information in real time. Since each bead in the array has been identified with a specific probe displayed on its surface during BeadChip™ manufacturing, the AIS imaging analysis software is able to generate an assay response, in terms of intensity statistics, for all the specific probes presented by the array from one snapshot image.

BeadChip™ production starts with bead synthesis. Methods of production and staining have been developed to synthesize a batch of core-shell beads of 3 μm diameter. This method has enabled the rapid production of three-color cluster maps producing over 110 spectrally distinguishable bead types by CCD (charge-coupled device) cameras within AIS and deployed in the field. The surface of the colored beads is chemically modified followed by covalent attachment of application-specific capture agents, such as oligonucleotides, antibodies, antigens, or peptides. Typically, a bead of 3 μm diameter accommodates approximately 10^6 oligonucleotide probes of 20 nucleotides in length for DNA analysis applications.

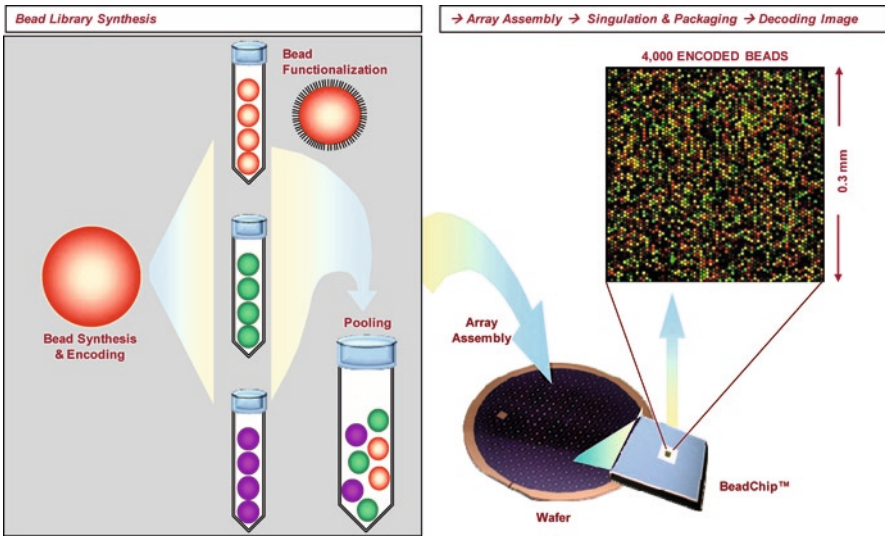

Fig. 3.3 BeadChip™ production. Three principal components of the BeadChip™ manufacturing process: production of an application-specific collection (library) of encoded functionalized beads, wafer-scale assembly of planar arrays composed of a mixture (pool) of encoded beads and segregation of wafer sections into individual BeadChips™, and acquisition of decoding images recording unique array configurations

The array assembly process, as illustrated in Fig. 3.3, comprises the steps of producing a pool of beads from the selected library and its application to (sections of) a silicon wafer with each chip further patterned to display a designated central area – in the current commercial designs of 300 μm side length. The array assembly processes permit the concurrent assembly of several dozen bead arrays with high feature density, typically ~4,000 particles of ~100 different types (colors). Wafer sections with assembled arrays are "cut" by way of a standard process and individual chips are assembled onto pretreated glass carriers in a 1×8 (slide) or 12×8 (plate)-well configuration. Decoding images are generated and the data are extracted from them by image processing. These are then placed into a production database for archiving and distribution of cluster maps to customer installations where they are used in connection with merging assay and decoding information. Figure 3.4 illustrates an automated array imaging, analysis and decoding, which was enabled by AIS.

3.2.2 Thresholding

Final acceptance testing of BeadChip™ kits in a given production lot calls for the completion of the assay in question using a set of independently characterized clinical (reference) samples. To that end, lot-specific thresholds are first determined, so

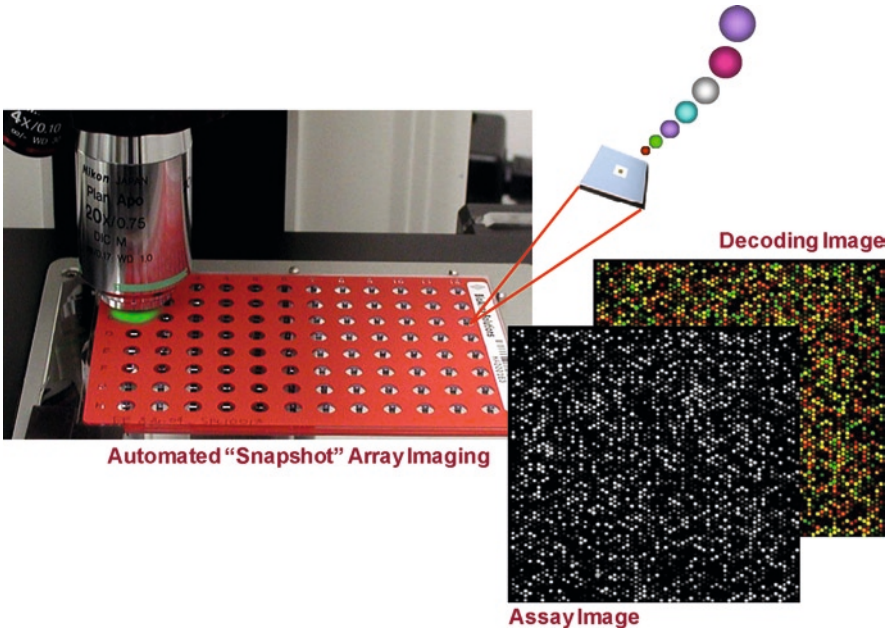

Fig. 3.4 Assay and decoding images – array composition: ~4,000 colored beads assembled in an area of ~300×300 μm², embedded in a 1×1-mm² silicon chip – for each chip two images are produced by automated snapshot imaging (see also: AIS, below), namely, a decoding image to record the unique (compositionally random) configuration of each "as-assembled" array and an "assay image" to record the patterns of assay signal intensities reflecting assay outcome. The superposition (merge) of these two images – automatically performed as part of the analysis by BAS software – associates signal intensities with bead identity

as to permit the grouping of normalized assay intensities into, depending on the application, either two or three categories.

For the hybridization-mediated multiplexed analysis of polymorphisms (hMAP®) format of multiplex allele determination of HLA analysis, thresholds are applied to normalized probe intensity profiles (Fig. 3.5). For the elongation-mediated multiplexed analysis of polymorphism (eMAP®) format of multiplex allele determination for HEA and HPA analysis, thresholds are applied to a discrimination ratio, Δ, derived from the assay signal intensities for members of each pair of elongation probes (Fig. 3.6): Δ varies between 1 (indicating homozygous normal, N) and −1 (indicating homozygous variant, V), a value of 0 indicating heterozygous NV. In this way, thresholding converts assay signal intensity patterns into reaction patterns in the form of (1, 0) or (1, 0, −1) strings. As with any measurement, experimental error gives rise to a "*gray zone*" containing Δ values whose assignment to a category is ambiguous (Fig. 3.6). The early version of this methodology, described in greater detail by Hashmi et al. [4], has been considerably refined to permit the systematic removal of statistical

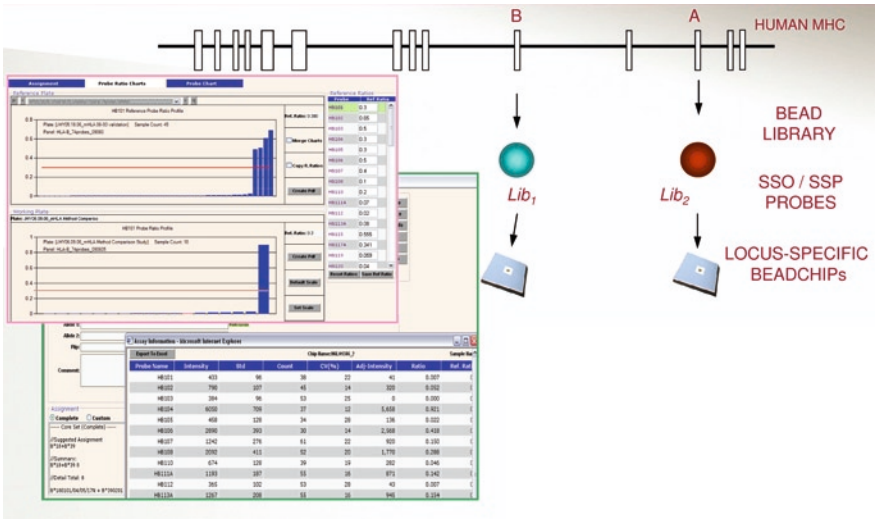

Fig. 3.5 BeadChip™ HLA analysis

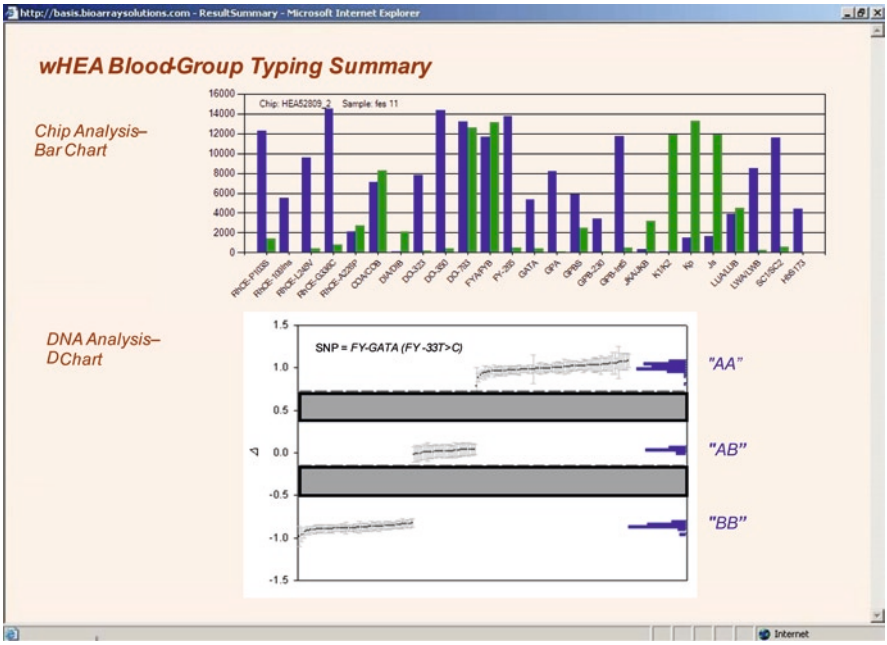

Fig. 3.6 An example of DELTA plot with thresholds and *gray zones*

outliers, based on the analysis of experimental error associated with Δ. Figure 3.7 illustrates overlapping probability density functions resulted from nonideal distribution of Δs from different typing groups.

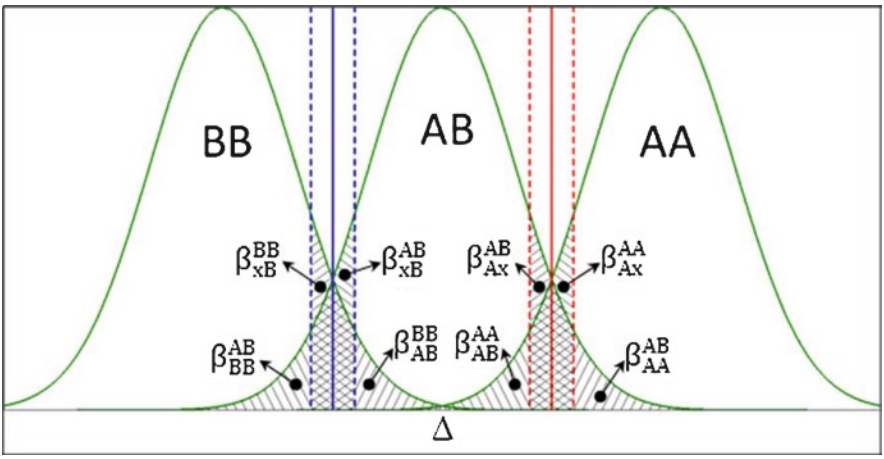

Fig. 3.7 The definition of transition probability/overlap

3.2.3 Deployment

The AIS, deployed at the user site, takes a snapshot of the entire array providing a walk-away operation with algorithms that facilitate the recording of assay signal intensities in ~5 min for a 8-chip carrier or around 30 min for a 96-chip carrier. Concurrent image processing, the first steps of which are performed locally to extract assay image data, are then uploaded to a central server within BioArray Solutions Information System (BASIS™) for further analysis and application data management. Concurrent processing ensures that, by the time the assay image acquisition is complete, the generation of merged array image data is available for interpretation.

3.2.4 BioArray Solutions Information System (BASIS™)

The BASIS™ system is designed as a web-based, database-driven, and service-oriented information system to process and manage all BeadChip™-related data and information. BASIS™ provides a centralized database management system to manage all application (and on a separate system of similar design, production) data. The system architecture is illustrated in Fig. 3.8. Authorized users access individual accounts holding assay analysis encrypted by chip ID, by way of a password-protected log-in to a portal that provides tabs for individual applications (such as HEA, HPA, and others). An example is shown in Fig. 3.9.

The retrieval of data and reports may be performed interactively (following association of chip ID with user-controlled sample ID) or automatically, namely by scheduled “downloads” using a program for automated data exchange (dxBOT™)

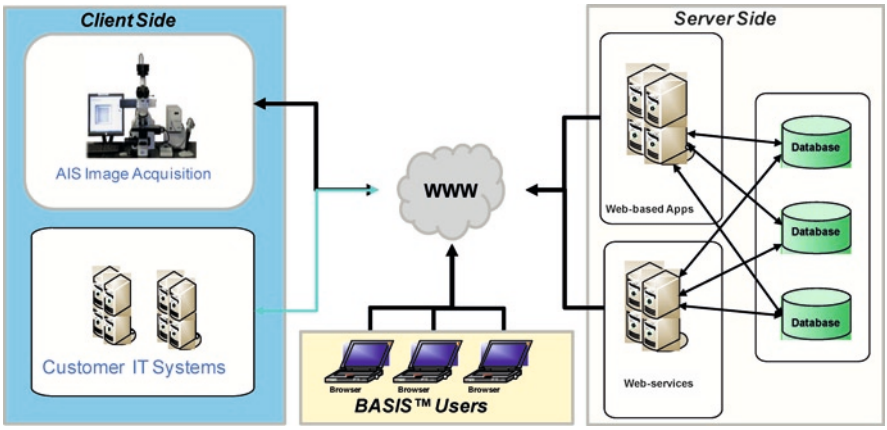

Fig. 3.8 BASIS™ system architecture

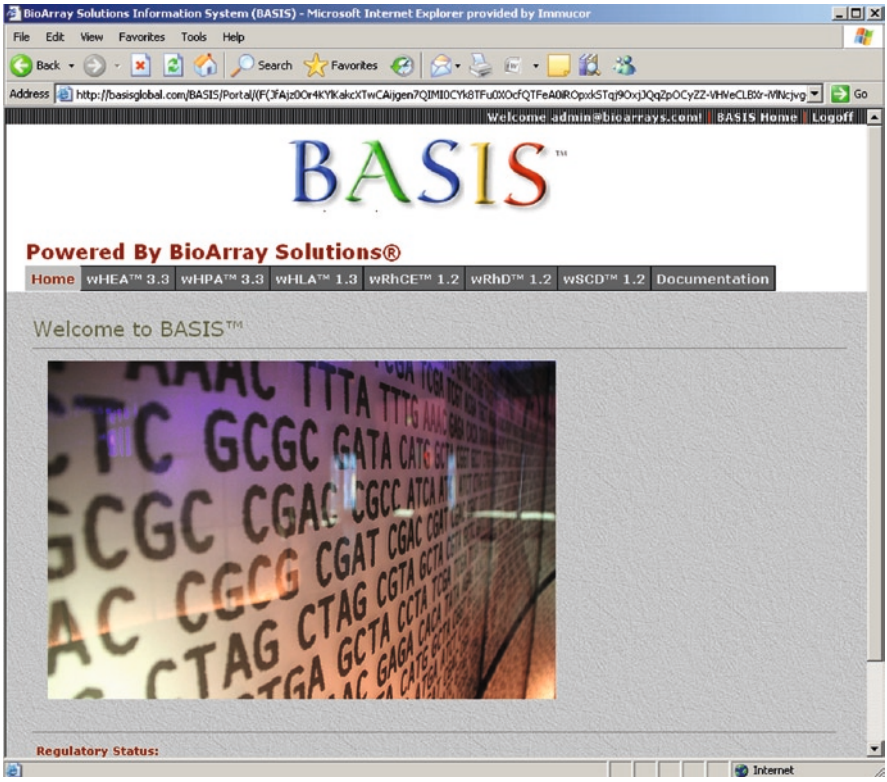

Fig. 3.9 The BASIS™ portal showing tabs for individual applications

in a staging database outside of the (regulated) Blood Establishment Computer Software (Fig. 3.10). As the assay data flows from AIS to BASIS™ database for further management, as illustrated in Fig. 3.11 via a stack of software, it is transformed

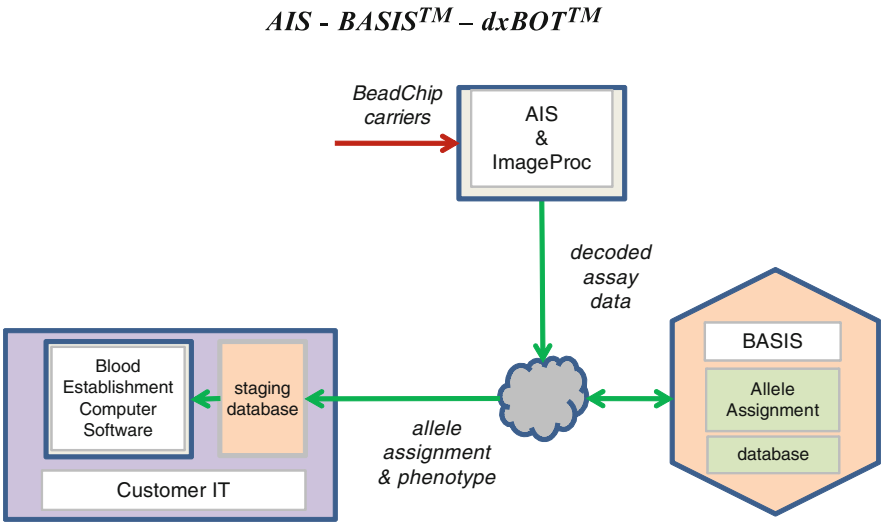

Fig. 3.10 Data flow in BASIS™

Fig. 3.11 The software tiers within the BeadChip™ system

from the BeadChip™ image, by way of real-time image processing, to a set of fluorescence signals linked to individual probe clusters, which is further transformed by application software hosted on BASIS™ to a reaction pattern and to allele assignments and/or predicted phenotypes. The results are handled and managed by BASIS™ database, interface, and transmitted by dxBOT™.

3.3 BeadChip™ Molecular Analysis of Proteins and Nucleic Acids

3.3.1 Protein Analysis

Protein analysis is also supported by the BeadChip™ system, which converts traditional "sandwich" immunoassays into BeadChip™ format. For example, a simple

design captures circulating antibodies to cognate antigens displayed on encoded beads, followed by labeling with a common anticlass (most frequently antihuman IgG) (Fig. 3.12). This format enabled the development of a BeadChip™ kit for the profiling of autoantibodies in a patient's serum. Figure 3.13 shows an example of the response of autoantibody (ENA) panel to different doses of anti-SCL079 antibody. This application, in a configuration comprising autoantigens associated with type I diabetes, lupus psoriasis, and others, received 510(k) FDA clearance in 2005 [5].

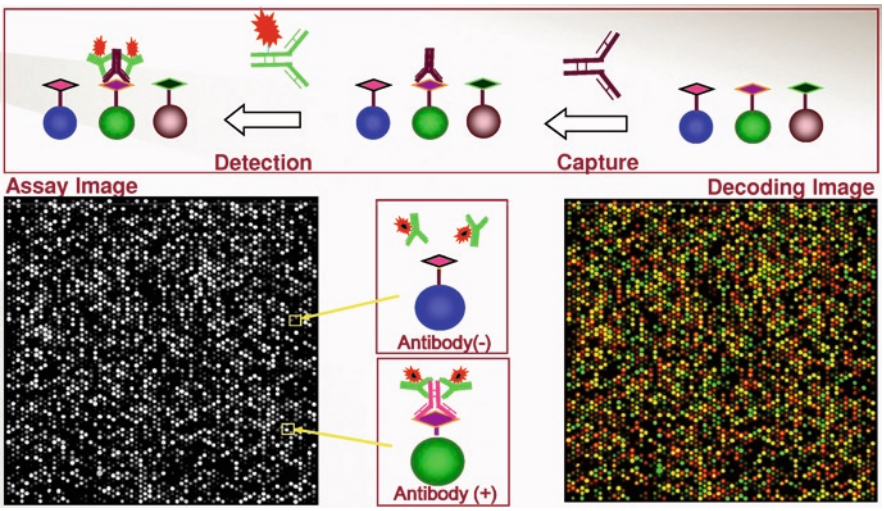

Fig. 3.12 Autoantibody profiling assay design

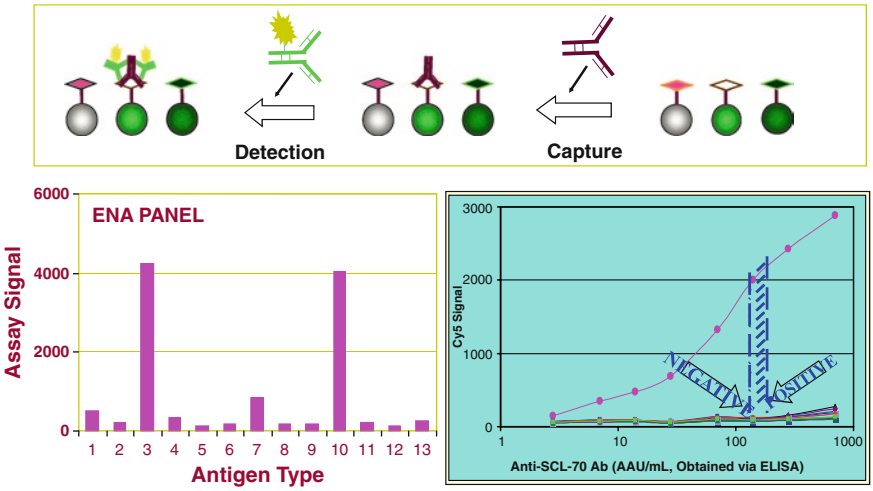

Fig. 3.13 Autoantibody profiling and an example of dose response

3.3.2 *Nucleic Acid Analysis*

3.3.2.1 Multiplex Analysis of Polymorphisms: Allele Discrimination

The identification of alleles, in the form of SNPs or small insertions or deletions, represents a principal interest in the field of molecular diagnostics and has offered opportunity for the development of BeadChip™ applications in contexts as diverse as: blood group typing, determination of carrier status for molecular disorders (notably cystic fibrosis) [6], and related lysozyme storage disorders [7].

Two formats have been developed for multiplex analysis of polymorphisms, hMAP® and eMAP®.

hMAP® has proved particularly robust in situations requiring the interrogation of a select number of designated polymorphisms within the context of highly variable sequences, as illustrated by the analysis of the highly polymorphic multilocus HLA gene complex. Signal intensity patterns produced in the hMAP designs call for analysis of intensities produced by an entire set of probes for the identification of a pair of alleles by reference to hit tables.

eMAP® has been the preferred method for the analysis of large sets of isolated SNPs, insertions or deletions, requiring a substantial degree of multiplexing in single-tube PCR to generate the requisite number (typically ~20) of amplicons (Fig. 3.14). Signal intensity patterns produced in the eMAP® designs are analyzed one at a time – as in the case of a one-to-one correspondence between SNPs and antigen type (Chap. 6). Signal intensity patterns may also be analyzed a few at a time in the event of genetic linkage, as in the case of *FY*B* or *GYPB* silencing (Fig. 3.15) [4, 8] and handled (within BASIS™) by a special section of code,

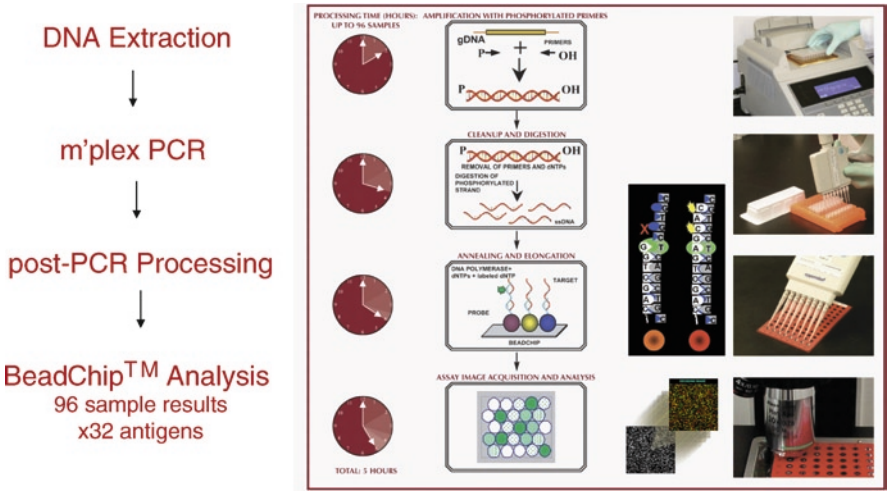

Fig. 3.14 Elongation-mediated multiplexed analysis of polymorphism protocol steps for DNA analysis are shown

Blood Group	Antigen		Blood Group	Antigen
Rh	Cc		Lutheran	Lu^a/Lu^b
	V, VS		Diego	Di^a/Di^b
	Ee		Colton	Co^a/Co^b
Kell	K/k		Dombrock	Do^a/Do^b
	Js^a/Js^b			Jo(a+)/Jo(a-)
	Kp^a/Kp^b			Hy+/Hy-
Kidd	JK^a/JK^b			
Duffy	Fy^a/Fy^b		Landsteiner-Wiener	LW^a/LW^b
Duffy-GATA	Silencing FY		Scianna	Sc1/Sc2
	Fy^x [$Fy(b+^W)$]			
MNS	GYPA (M/N)			
	GYPB (S/s)			
	U - , U ^{var}		Hemoglobin S	HbS

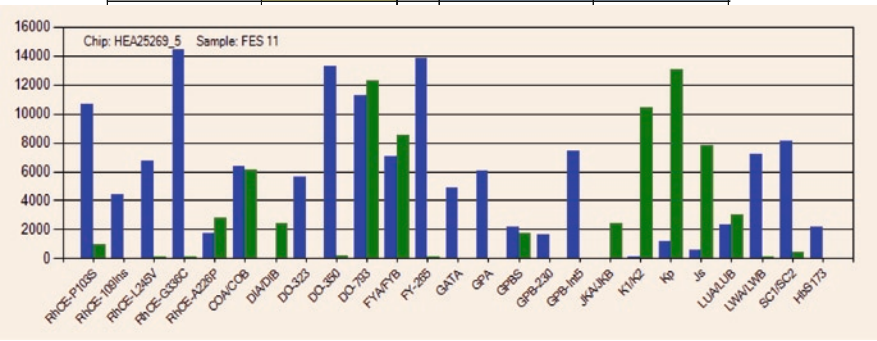

Fig. 3.15 HEA panel design

preferably by referencing look-up tables. In contrast, the analysis of variants of *RHCE* and *RHD* calls for a two-stage assignment of alleles or haplotypes, comprising readings for a multiplicity of probe pairs. The requisite analysis, preferably within a Bayesian framework (below), combines the challenges accommodating multiple entries for the identification of alleles.

3.3.2.2 Bayesian Analysis of Reaction Patterns and Allele Assignment

The signal intensity pattern reflecting the interaction of a set of probes with specific target subsequences contains the desired allele or haplotype information, and this information is extracted by comparing reaction patterns (see Section 2.4) to (pairwise superposition of) the entries in a hit table of binary strings characterizing known alleles in terms of the probe set in the array design. Bayesian analysis, given allele frequencies and the (experimentally determined) error rates for probes in the array design, provides a framework for the quantitative assessment of uncertainty, determined by algorithms that produce an assignment confidence scores. When applied in the context of HEA analysis, allele and haplotype assignments are translated by reference to look-up tables, into predicted phenotypes, taking into account genetic linkage information [4].

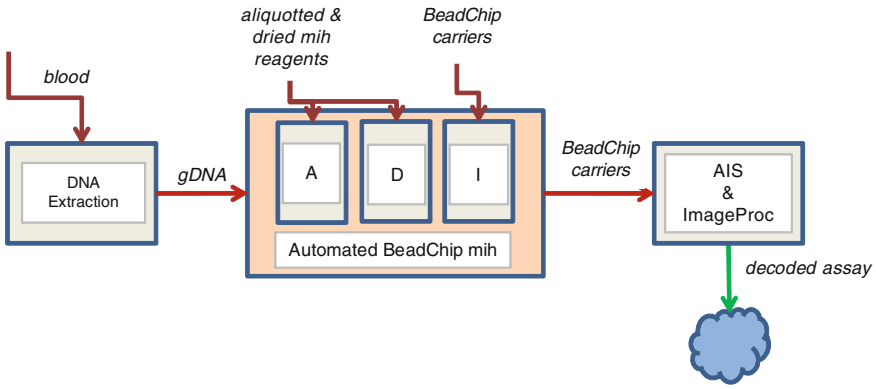

Fig. 3.16 Amplification, discrimination, incubation (ADI) module is provided

3.4 Automation (Automazione)

To render the complex molecular analysis more widely available, the development of a system for Automated BeadChip™ Deployment has been initiated. In accordance with a modular design (Fig. 3.16), the central processing module performs amplification ("A"), discrimination ("D"), and incubation ("I") steps, leaving DNA extraction and snapshot image acquisition as stand-alone operations and thus permitting the use of preexisting systems.

The layout of the ADI module conforms to the three-step eMAP-S® (elongation-mediated multiplex analysis of polymorphisms in solution) protocol comprising precisely these steps: PCR-mediated amplification, followed by allele discrimination, and incubation following placement of the product of the discrimination reaction onto BeadChip™ carriers. This permits the capture of labeled elongation products to a set of universal capture probes. Signal intensity patterns are then read after transfer of the carrier to the stage of the AIS for image acquisition. To facilitate protocol automation, hMAP® and eMAP® assays are being ported to eMAP-S® format.

3.5 Future Directions

3.5.1 A Novel Operational Paradigm for Transfusion Service

A recently published multi-institutional, prospective observational study (Fig. 3.17) demonstrated the feasibility of increasing the number of antigen-matched red blood cell (RBC) units available for transfusion by establishing and maintaining an inventory of RBC units of known extended human erythrocyte antigen (xHEA) phenotype [9]. To that end, leukoreduced donor units were selected from those already on hand

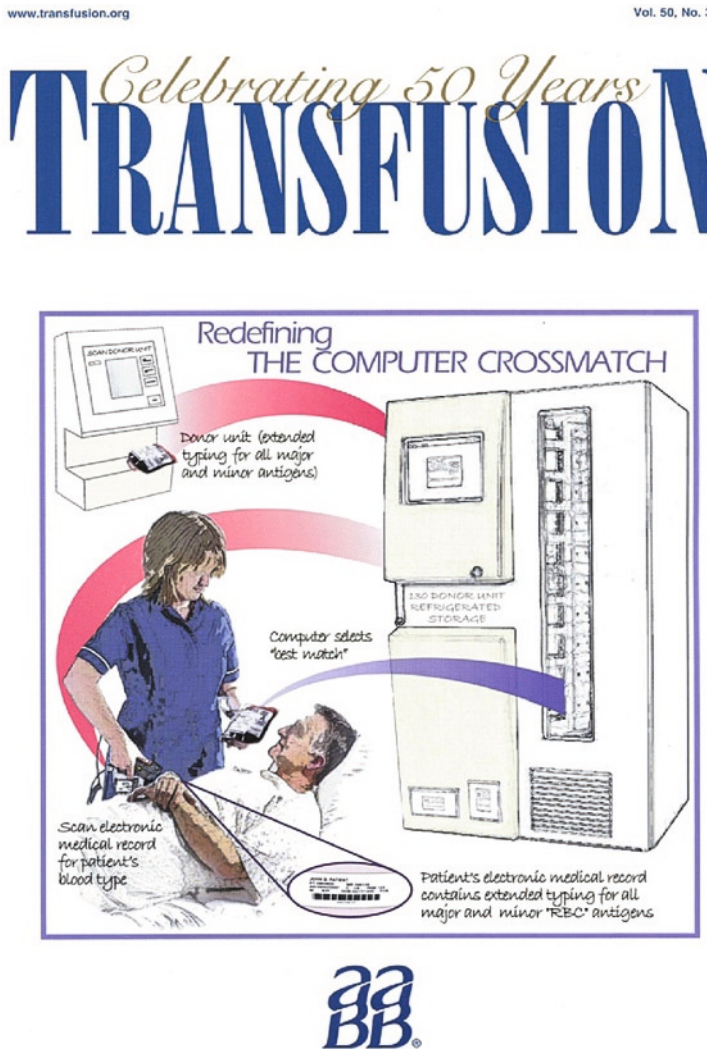

Fig. 3.17 Electronic cross-matching of extended phenotype is shown on the cover of "Transfusion" [9]

at the transfusion service for determination of the xHEA type which, as with that of the patient, was determined by BeadChip™ DNA analysis. The prototype of a novel inventory management system was designed and implemented to simulate (essential aspects of) blood order processing in the transfusion service, and to facilitate the assignment of xHEA-typed units from the simulated special inventory to the alloimmunized as well as nonalloimmunized transfusion recipients included in the study. The fraction of requests fulfilled, or fill fraction, was determined at

four levels of increasing antigen matching stringency and found to be substantial even for highly immunized patients.

Subsequent to the completion of this study, Abumuhor et al. showed that by adopting this practice at their transfusion service at Cedars-Sinai Medical Center, they were able to significantly reduce the turnaround time (TAT) for procurement of antigen-negative units, from an average of 6 to 2 hours. As a result, they were able to decrease the overall turnaround time and cost of procuring blood [10].

Acknowledgment We acknowledge the contributions of all members of BioArray Solutions' technical organization for the development and implementation of the technology.

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Chapter 4
Implementation of HEA at Blood Centers: Prescreening, Rare Donors, Inventory Management

JoAnn M. Moulds, for the BeadChip User Group at Donor Centers*

Abstract The BeadChip™ system, one of the commercial platforms currently available for predicting human erythrocyte antigen (HEA) phenotypes by DNA analysis, has shown utility in blood bank operations and hospital transfusion services since its introduction in 2006. This chapter compiles information on the implementation and performance of extended HEA typing using the BeadChip™ platform in US-based blood centers and hospital transfusion services. LifeShare Blood Centers, Gulf Coast Regional Blood Center, LifeSouth Community Blood Center, and the New York Blood Center represent some of the nation's largest independent blood centers and together collect over 1.5 million blood units annually. The combined experience of these centers provides an overview of the impact of HEA BeadChip™ implementation on the provision of compatible blood for patients, on complex patient work ups, and also on blood bank operation by discussing the process flow, concordance, and economic impact.

Keywords Blood groups • DNA array • DNA testing • Minor blood groups

4.1 Introduction

The 2007 National Blood Collection and Utilization Survey Report [1] indicated that 14,461,000 units of allogeneic blood (whole blood/red blood cells) were transfused in 2006, double the number transfused in 1997 [2]. Alloimmunization to one of the

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308 blood group antigens now recognized is possible whenever donor red cells express antigens which transfusion recipients lack [3]. In clinical practice, the prevalence of alloimmunization ranges from 1% to 8% in unselected patient populations to 76% in patients with sickle cell disease (SCD) in the UK where the blood donor population is predominantly white [4]. Clinical studies and animal models suggest that alloimmunization to blood group antigens depends on genetically controlled immune responses [5, 6] as well as environmental factors [7]. Hemovigilance data from the FDA and the UK indicate that despite improvements in antibody detection methods, delayed hemolytic transfusion reactions continue to cause significant morbidity and mortality [8, 9]. These reactions often occur because antibodies are weak or undetectable. Although blood bank information systems perform historical checks to prevent the release of blood positive for antigens to which a patient is immunized, such information may be available only locally. Prospective antigen matching of units selected for transfusion is recommended to prevent delayed hemolytic reactions and limit further alloimmunization [10]. Extension of this practice to more patient groups may be necessary to further reduce delayed hemolytic transfusion reactions [11].

Using serological techniques to screen blood is expensive and time-consuming [12]. Antisera from human sources are often in short supply and may be very expensive. Moreover, licensed serological reagents are not available for all clinically important antigens. Manual polymerase chain reaction (PCR) techniques are useful for the initial identification of an allele but are unsuitable for donor screening. High-throughput DNA analysis is ideally suited to generate phenotyped donor inventories to provide compatible units for alloimmunized patients [13–18]. The BeadChip™ system, one of the several platforms currently available, has shown concordance with other DNA methods and with available serological types. The availability of BeadChip™ kits for HLA and HPA will also permit blood centers to screen donors for these antigens without investing in additional technology.

The initial evaluation of the BeadChip™ system on donors involved more than 2,000 samples and included Caucasian, African-American, Hispanic, and Asian cohorts [16, 19]. In these studies, the phenotype prevalencies and allele frequencies matched those obtained by serologic testing, and 19,474 new antigen-negative types and 21 rare donors were identified [Co(a−b+) $n=1$, Jo(a−) $n=6$, S−s− $n=12$, and K+k− $n=2$]. Subsequent studies confirmed the accuracy and reproducibility of BeadChip™ technology for red blood cell characterization [19–21]. This chapter provides information on the implementation and performance of BeadChip™ human erythrocyte antigen (HEA) in ten US-based blood centers and six hospital transfusion services that also collect blood donations. The combined experience of four large blood centers, LifeShare Blood Centers (LBC), Gulf Coast Regional Blood Center (GCRBC), LifeSouth Community Blood Center (LSCBC), and New York Blood Center (NYBC), provides an overview of the impact of HEA BeadChip™ implementation on the identification of blood for alloimmunized patients. The introduction of BeadChip™ technology has heralded a paradigm shift in routine operations for donor screening and inventory management of extended matched blood allowing rapid identification and removal of special units from

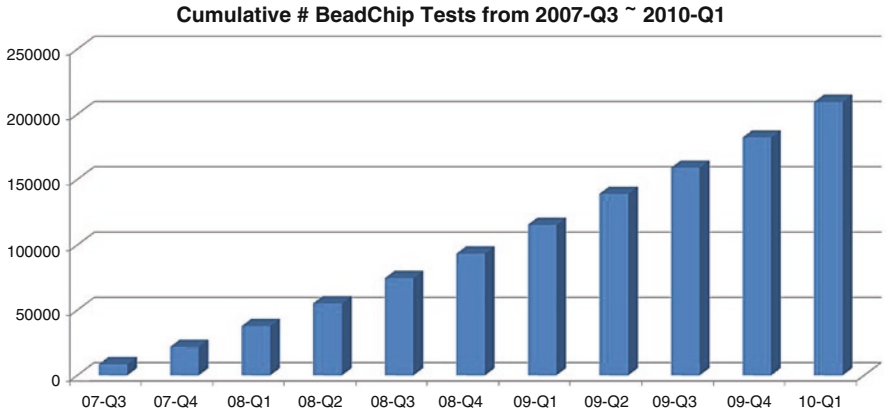

Fig. 4.1 Cumulative BeadChip™ usage during 2007-Q3 to 2010-Q1 is shown

routine inventories and permitting provision of fresh liquid units instead of frozen deglycerolized units in many instances.

The cumulative number of BeadChip™ tests performed each quarter from third quarter 2007 through the first quarter 2010 is presented in Fig. 4.1. The cumulative number of 200,000 tests included a small number of tests using HLA and HPA BeadChips™. The user base for this data was 60% blood centers, 20% hospital transfusion services, and 20% reference laboratories (includes both donor centers and hospital transfusion services). Figure 4.2 captures BeadChip™ usage in the ten largest blood centers, including GCRBC, LSCBC, NYBC, and LBC, which represent some of the nation's largest independent blood centers, together collecting over 1.5 million blood units a year. The majority of the blood centers were motivated by the cost and limited availability of antisera, and decided to adopt the technology to address the growing demand for rare blood units. Central Illinois Community Blood Center (CICBC) is a relatively small start-up blood center, which adopted the BeadChip™ platform for routine use of RBC and platelet management, using HEA, HPA, and HLA BeadChip™ assays to avoid the time and cost associated with sending samples out for testing.

Over the years, BeadChip™ usage has increased to a relatively steady level while providing an alternative to serological methods for donor and patient testing. The current semiautomated mode of running the assays, even after data acquisition and interpretation have been automated, limits system capacity (see Chap. 3). To address this, an automated platform is currently under development. Other areas of improvement include downstream data management for further enhancing the value of extended characterized blood [22].

Decisions regarding the integration of BeadChip™ into blood center operations have depended upon the type of products and services requested by clients. LBC in Louisiana, East Texas, and Southern Arkansas serve over 130 patients, including 49 dialysis centers, extended care facilities, and surgical centers, to provide compatibility testing as well as blood products. Initially, the BAS system was acquired as a

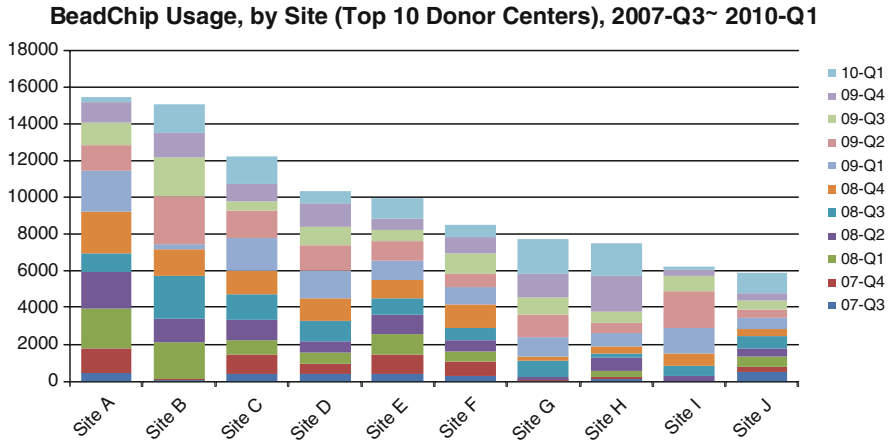

Fig. 4.2 BeadChip™ usage at top ten donor centers during 2007-Q3 to 2010-Q1 is depicted

research tool. After an internal review of the Immunohematology Reference Laboratory (IRL), it was determined that patients with SCD accounted for almost 50% of complex cases and the selection of phenotype-matched units identified by DNA-based typing donor was implemented. The use of DNA-based techniques to identify compatible units was then extended to other alloimmunized patients and to patients with positive direct antiglobulin tests.

GCRBC serves more than 170 hospitals and health care institutions in greater Houston and the East Texas and Brazos Valley regions. The consultation and reference laboratory provides compatibility testing services, and thus provides suitable blood and blood components for transfusion to patients in dialysis centers, outpatient surgery centers, oncology treatment facilities, home health care facilities, and small hospitals. In 2010, they expect to distribute 333,000 units of blood, which requires collecting and processing more than 1,000 donations every day. In 2007, Gulf Coast decided to incorporate HEA testing as a tool to screen for antigen-matched red blood cells and to assist in the resolution of complex patient antibody problems. BeadChip™ technology was also introduced as a reliable and cost-effective alternative to serological methods for patient and donor phenotyping.

NYBC is the largest community-based, nonprofit, independent blood center in the United States with operations in the Bronx, the Hudson Valley, Brooklyn, Staten Island, Long Island, Queens, Manhattan, and New Jersey. The center processes up to 2,000 blood donors daily and provides blood products and clinical transfusion services to approximately 200 hospitals in New York and New Jersey. Several clinical facilities have established special programs to provide care to patients with thalassemia and sickle cell anemia. NYBC has developed a special program, PreciseMatch, to recruit donors from diverse ethnic groups to increase the availability of antigen-matched blood for alloimmunized patients. In collaboration with the Immunochemistry Laboratory, a prototype BeadChip™ containing a subset of blood group SNPs was verified with DNA from serologically characterized donors [16]. Later, an expanded

panel consisting of extended set of alleles was evaluated again with a subset of previously tested samples. NYBC revalidated the HEA 1.2 panel by confirming 20 samples provided by BioArray with known typing, confirming a total of 257 donor samples with (partial) phenotypes. The center started donor screening operations using the BeadChip™ system in 2007.

LSCBC is a community blood supplier for local hospitals in Florida, Georgia, and Alabama. The centers consist of over 34 donor centers and 42 blood mobiles, conducting over 1,000 blood drives a month. About 305,000 blood donations are collected per year. The blood serves the needs of patients at over 120 medical facilities throughout the region. In 2007, LifeSouth implemented the BeadChip™ technology in their donor operations for prescreening to assist in the identification of blood for alloimmunized patients.

The CICBC is a nonprofit organization that provides for the blood needs of 19 hospitals in central and southwestern Illinois. The time required for serological testing was limiting their ability to meet the growing demands for phenotyped donor units. In addition, the full phenotypic record for patients afforded by the implementation of BioArray technology promised to improve efficiency and safety.

The BeadChip™ technology along with data analysis is described in Chap. 3 of this volume. This chapter describes the real-world experiences with BeadChip™ technology in blood centers including product performance and proficiency testing as well as the economic impact of implementing DNA testing in blood bank operations.

4.2 Prescreening: Expanding the Extended Phenotyped Donor Pool

In clinical practice at LBC, the number of compatible donor units provided has doubled since the use of DNA testing was introduced to screen for antigen negative and rare phenotypes. The genotyping is performed in the Scientific Support Services Laboratory, which keeps a separate database of all genotyped donors. The genotypes are coded as follows: 1 = rare (occurs <1:1,000), 2 = full phenotype match for SCD patients, 3 = C, E, K, negative, 4 = other genotyped African-Americans, 5 = other genotyped Caucasians, and 6 = match for special patients. This classification system allows for the selection and specific recruitment of needed donors. As shown in Tables 4.1 and 4.2, the blood center has been able to increase the number of antigen compatible units identified, including those with high demand for the ethnic population in the area. The impact of implementing DNA analysis on the identification of antigen negative and rare door units is illustrated in Table 4.2. The percentage of donor units that were found to have antigen negative and rare phenotypes has remained relatively stable, but the larger number of samples screened has resulted in a significant increase in the number of donors identified.

Table 4.1 LifeShare screening statistics by serology

Ethnicity	Black donors		Nonblack donors	
Date	08/2000 to 04/2009	07/26/2005 to 04/10/2009	08/18/2006 to 04/11/2009	12/18/2008 to 03/11/2009
Donors screened	18,384	4,001	14,328	404
Special trait found	213	35	38	1
U–	109	0	0	0
Js(b–)	104	0	0	0
Hy–	0	10	0	0
Jo(a–)	0	25	0	0
Vel–	0	0	11	0
Yt(a–)	0	0	15	0
SC:–1	0	0	0	0
Kp(b–)	0	0	3	0
Co(a–)	0	0	8	0
Lu(a–b–)	0	0	0	1

Table 4.2 LifeShare donor genotyping statistics by year

	2007	2008	2009	2010	Total for all years
Total screened	590	1,951	3,417	635	6,613
Rare	27(4.5%)	103 (5.2%)	114 (3.3%)	31 (4.7%)	275 (4.2%)
Black	553	1,823	2,922	640	59,380
Caucasian	37	128	495	15	675
Co(a–)	4	1	1	1	7
e–	5	23	24	3	55
hr^B–	2	12	13	2	29
Hy–	2	5	7	1	15
Jo(a–)	5	16	17	6	44
Js(b–)	1	8	19	4	33
k–	0	0	1	0	1
Kp(b–)	0	1	0	0	1
Lu(b–)	0	0	2	3	5
MS^u	1	2	0	0	3
U–	8	24	14	4	50
U(var)	0	5	11	7	23

Shading indicates antigens for which molecular testing may be used as the sole source of identification [23]

For example, the Immunohematology Reference Laboratory has screened about 18,384 units by serology over a period of 10 years (Table 4.1) in an effort to find U– units which have resulted in the identification of 109 U– units (0.5%). With DNA analysis an additional 76 U– or U(var) units were identified since 2007. This implementation resulted in reduced serological screening while at the same time providing results for a complete panel of 31 antigens, since DNA analysis allows

simultaneous screening for antigens as compared to individual testing by serology. Similar results were also obtained for other antigen types, such as hr^B–, Hy–, or Jo(a–), where serological typing is unreliable or when antisera are not available. LifeShare, which initiated BeadChip™ analysis in August 2006, was able to reduce/eliminate serological testing for certain antigens such as Hy– or Jo(a–) in December 2006 and for hr^B– in February 2009.

It has been determined that molecular testing may be used as the sole source of identification for certain antigens [23]. This change in process has made it easier for LifeShare to meet American Rare Donor Program (ARDP) requests. Previously, samples provided to ARDP required serological typing twice or one DNA analysis with serological confirmation. Now that DNA screening is used exclusively for Hy/Jo screening, many more units are available. The first year that the ARDP was able to fill all requests for Hy– or Jo(a–) units, many of which came from LifeShare, was 2009.

The center focuses on screening blood from Black donors, since most of the requests for red blood cell units at LBC are for SCD patients. Initially, LifeShare performed DNA analysis on all group O Black donors who had donated 3 times or more; now the testing has been extended to other ethnic groups (90% Black and 10% Caucasian). LifeShare has developed a successful student minority recruitment program where young donors are recruited from high school and colleges, with the hope that these donors are more likely to continue to donate over time.

In June 2007, GCRBC embarked on an effort to expand their inventory of phenotype-matched donor units as a response to increased demand. After getting feedback from others performing HEA testing in their laboratories, the blood center evaluated and implemented the technology. The testing is performed in the molecular laboratory, providing service to both the Rare Donor Program (donor screening for antigen negative/rare donors/units) and the reference laboratory (for the consultation for multiply transfused patients, etc).

Similar to LBC, most requests for blood at GCRBC are for patients with SCD. The implementation of DNA screening for donors has led to a substantially larger pool of donors (>11,000 donors from 2007 to 2009) to fulfill requests for compatible units (Table 4.3). The identification of donors with R_o, Fy(a–b–), etc. increased precipitously with the implementation of DNA testing. Out of 3,077 newly identified antigen-negative donors, 2,922 fell into the category of R_o, Fy(a–b–), and other rare types such as Hy–, Jo(a–), U–, Js(b–), Co(a–) Lu(b–), and Di(b–). Additional benefits realized include:

1. Work flow improvements resulting in decreased labor expenses for screening and maintaining an inventory of antigen-matched blood.
2. Improved service to customers by increasing the percentage of fulfilled special RBC requests.
3. Improved service by decreasing the turnaround time (TAT) to fill special RBC requests.
4. Increased inventory of liquid rare and other antigen-typed units (not having to rely on frozen RBCs to fill these requests with increased efficiency and decreased expenses for the hospitals and, ultimately for, patients).

Table 4.3 Gulf Coast Regional Blood Center donor typing statistics by year

	2007	2008	2009	2010	Total
Total donors screened	1,358	5,848	4,335	2,111	13,652
Black	1,358	5,848	1,535	1,986	10,727
Caucasian	0	0	2,200	125	2,325
Hispanic	0	0	600	0	600
Rare, *n* (% total screened)	41 (3%)	1,882 (32%)	1,154 (27%)		3,077 (27%)
R_o, Fy(a−b−) etc. *n* (% of rare phenotypes)	30 (73%)	1,818 (97%)	1,074 (93%)		2,922 (95%)
Co(a−)	0	1	0		1
Di(b−)	0	1	1		2
Hy−	2	12	15		29
Jo(a−)	6	31	44		81
Js(b−)	0	0	10		10
Lu(b−)	0	2	1		3
U−	3	17	9		29

Source: From BeadChip vs. Serology Summary provided by GCRBC. Shading indicates antigens for which molecular testing may be used as the sole source of identification [23]

All first-time Black donors are tested with DNA as well as repeat Black donors (all O+/O− and a small number of A donors) who have never been phenotyped or only partially phenotyped. In 2009, DNA testing was extended to Hispanic donors for Diego (b–) screening and to Caucasian donors who donate frequently, but have never been completely phenotyped. Results are frequently confirmed by serological methods, especially if a donor is identified as R_o or negative for six or more common antigens. Currently, DNA analysis is used as the test of record only for Dombrock antigens because of the limited availability of the antisera. The BeadChip™ system is also used as a quality control (QC) method to confirm historical phenotypes previously performed by serology.

Due to the ARDP requirements for immunohematology reference laboratories to confirm antigen typing two times, it may cost more at Gulf Coast to verify a rare unit initially identified by DNA analysis. However, DNA testing is economical for overall operations because initial donor screening is now automated and DNA testing determines 32 antigens for multiple samples (up to 96) in a shift versus requiring several days of serologic screening for triaging the same number of samples for a few antigens. The HEA BeadChip™ testing is performed in the molecular testing laboratory. This system frees the most experienced, consultation technologists to resolve complex patient cases and perform cross-matches for patients instead of spending their time screening for requested antigen-negative RBCs and/or rare donors. The HEA testing also allowed for the identification of rare donors who never would have been identified previously because each donor

was not tested for 32 different antigens. For example, a donor could only be identified as Do(a−) by HEA testing since no antisera is available.

The criteria for using BeadChip™ analysis for donor typing at CICBC have evolved to perform testing on blood from all donors who are identified as any other ethnicity than Caucasian, including Blacks, Asians, Hispanics, Polynesians, and Native Americans. Blood from O negative donors is typed by DNA analysis, regardless of the race or ethnicity of the donor. As needed, additional donors are selected for genotyping to manage the CICBC inventory of tested donors.

Table 4.4 shows the total number of donor samples screened and the numbers of rare typings identified at various large donor centers throughout the US, which include CICBC (Central Illinois), NYBC (New York tri-state area), LSCBCs (Florida), and a fourth blood center (Site4, Northern Pacific states region). These represent very different ethnic subgroup mixes in their respective donor population as shown in Tables 4.5 and 4.6.

Table 4.4 Total numbers of antigen-negative units for rare phenotypes identified at various donor centers in 2009

Institution	CICBC	NYBC	LifeSouth	Site4
Total	1,234	3,725	5,904	2,784
Co(a−)	1	4	9	1
e−	24	65	237	122
Hy−	0	1	5	0
Jo(a−)	1	5	14	0
Js(b−)	0	5	9	0
k−	0	5	15	1
Kp(b−)	0	0	0	0
Lu(b−)	0	6	6	0
U−	0	0	0	0
U(var)	8	3	8	1
c−e−	0	0	0	0
Di(b−)	0	0	0	3
LW(a−)	0	0	0	0
SC−1	0	0	0	0
Fy(a−b−)	240	290	939	101
Jk^{b}−S−	227	574	1,067	531

Source: From BASIS™ record

Table 4.5 Ethnic subgroups of donor population in various donor centers estimated from typing data in 2009

Subgroup	LifeShare (%)	Gulf Coast (%)	CICBC (%)	NYBC (%)	LifeSouth (%)	Site4 (%)
Black	89	84	31	10	31	9
Caucasian	11	13	67	84	64	12
Hispanic	0	2	1	0	0	0
Asian and others	0	0	1	6	6	79

[a]Analysis was performed based on the method used in Zhang et al. [24] and allele frequencies from Hashmi et al. [19]

Table 4.6 Antigen-negative subgroups of patients identified at various donor centers in 2009

Institution	Type	Total	Co(a−)	e−	Hy−	Jo(a−)	Js(b−)	k−	Kp(b−)	Lu(b−)	U(var)	c−e−	Di(b−)	Fy(a−b−)	Jk^b−S−
LBC	PT-SCD	94	0	1	0	2	1	0	0	0	1	0	0	66	35
LBC	PT-other	55	0	3	1	2	0	1	0	0	0	0	0	14	12
GCRBC	Patient	102	1	3	0	0	1	0	0	1	1	0	0	15	17
CICBC	Patient	81	1	0	0	0	0	1	0	2	2	0	0	14	15

4.3 Impact of Molecular Testing on Blood Center Operations

4.3.1 Donor Units Testing Process Flow

Donor selection criteria and recruitment processes have evolved with the evolution of the BioArray Solutions assay system, resulting in changes in the overall operations of blood centers which implemented the technology. For example, many of the recipients requiring phenotype-matched units have autoantibodies. At LBC, the system now allows for a genotyped unit with Rh, Duffy, Kell, Kidd, and MNS profiles matched to a specific patient to be the selected unit for that particular patient. All antigen-negative units that are provided for transfusion to alloimmunized patients are also confirmed with serology for the corresponding antigens. A suggested work flow for genotypic matching with donor units is shown in Figs. 4.3 and 4.4.

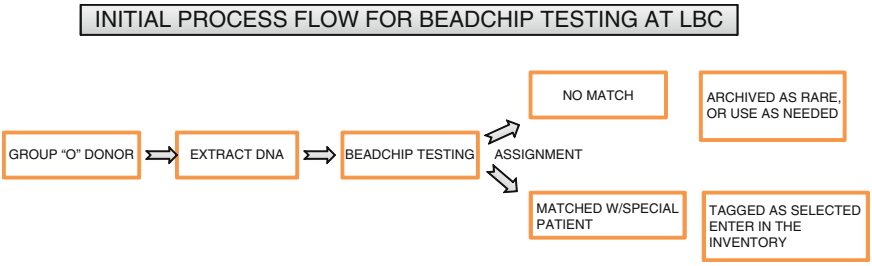

Fig. 4.3 Process flow for BeadChip™ testing at LBC

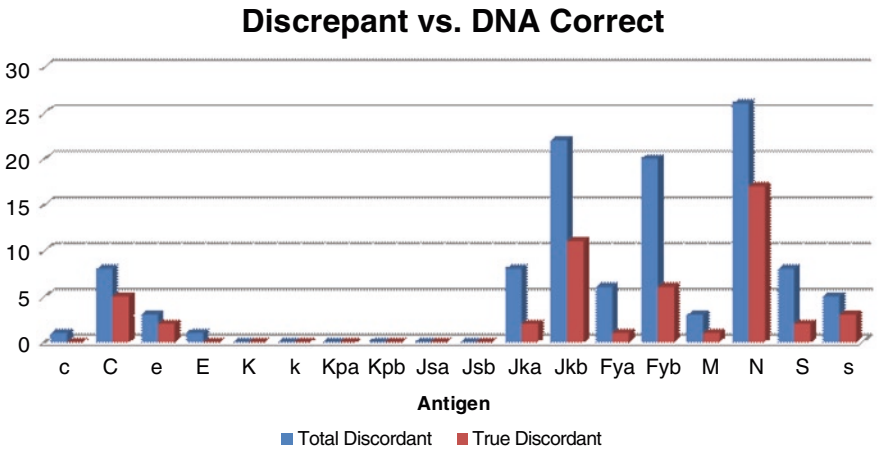

Fig. 4.4 Total number of samples having discordant results between serology and DNA are shown in *blue*. Following repeat serology and/or record checks, the number of true discordant samples is shown in *red*

Initially, results generated with BioArray Solutions Information System (BASIS™) were downloaded and maintained as an Excel-based database at LBC. In the future, the genotyping data will be downloaded to donor records to expand the utility of genotypic matching. In addition, Scientific Support Services personnel maintain a separate database to specifically recruit and track the donations by rare donors. In an effort to increase the available inventory for patients requiring extended matched units, once individuals with required antigen negative and/or rare phenotypes are identified, they are contacted and enlisted as life-long donors. LBC uses ARDP guidelines that allow the use of molecular typing for hr^S, hr^B, V, VS, Do^a, Do^b, Hy, Jo^a, U–, and U(var), but confirm antigen-negative status by serology when possible [23].

GCRBC has long maintained a large inventory of rare liquid and frozen red blood cells. Table 4.7 indicates the total number of rare units exported to blood bank reagent manufacturers, hospitals, and other blood centers. The total numbers increased from 6,436 to 13,433 units after implementation of BeadChip™ analysis from 2007 to 2009, respectively. Similarly, the number of antigen-negative units identified and exported for multiple antigens, such as S–, Jk(b–), Fy(b–), M–, R_o, and the number of units that are negative for >4 antigens, increased significantly. Overall, the number of special requests filled increased from 2,708 units in 2007 (tested by serology only) to 5,787 in 2008, and 5,849 in 2009 when tested by BeadChip™ followed by negative antigen confirmation by serology. The number of donors identified as Fy(a–), Fy(b–), U–, and Hy negative, the most frequently requested antigen types, also increased. The ability to provide >4 antigen-negative blood units has doubled with the implementation of BeadChip™ analysis.

Table 4.7 Number of xHEA-matched unit distribution of rare units

	2007	2008	2009
	Antigen screening by serology only	Antigen screening by BioArray and serology	Antigen screening by BioArray and serology
Reagent red cell manufacturers	106	139	144
Commercial Site 1	46	123	69
Commercial Site 2	76	274	457
Regional Hospitals	6,208	12,656	12,763
Total antigen matched units distributed	6,436	13,192	13,433
Antigen-matched units distributed (by types)			
S–	825	1,811	
Jk(a–)	649	1,326	
Fy(b–)	520	989	
M–	365	708	
R_o	1,276	2,201	2,362
High incidence	55	182	353
Special Ag negative requests	2,708	5,787	5,894
>4 Ag-negative units	1,265	2,802	3,229

At CICBC, a repeat phenotype is done only for Black donors who have been serologically tested in the past. Samples identified as possible r's using BeadChip™ analysis are confirmed with serological methods. During early implementation, serological confirmation was performed for all samples for ce, k−, Fy(a−) or Fy(b−), Jk(b), and S− when identified by BeadChip™. Currently, the antigens are only confirmed if the unit is requested for transfusion. At CICBC, red blood cell donor testing has increased the number of filled orders for antigen-negative units and reduced the need to import units. In addition, in-house testing for HLA and HPA reduces TAT in cases of refractory patients and reduces the expense of outsourcing testing to another laboratory. HEA BeadChip™ results are not used as the test of record unless no antisera are available to confirm the results with serology. For example, many donors who are negative for Scianna 1 and 2, Hy, and Jo^a are historically typed. However, a unit cannot be marked as typed based on donor history. As an alternative, a BeadChip™ result allows these units to be tagged as typed.

4.3.2 Liquid Inventory

An improvement in the donor operations noted at all blood centers resulting from DNA testing for red blood cell antigens is the expanded availability of liquid donor inventory. BeadChip™ users report that TAT for finding rare donors may be reduced from 1 week using serology testing to 24 h using BeadChip™ analysis. Both LBC and GCRBC have leveraged this capability to maintain an inventory of liquid units from extended-HEA (xHEA) BeadChip™-typed donors, reducing the need for frozen units. Liquid units have a shelf life of 42 days, whereas frozen units, once deglycerolized, expire in 24 h. Moreover, frozen units lost to breakage during shipment cost blood centers about $1,000 per unit. GCRBC is able to provide approximately 1,000 Ag negative, xHEA-typed units per month. With the implementation of genotyping, they are able to maintain a rolling inventory of 400 liquid HEA-typed units that are reserved for 40 days. If not distributed within this time, the units are recycled to the general inventory or frozen.

4.3.3 Prescreening for Hemoglobin S Status

Extended phenotype determination by HEA BeadChip™ analysis provides the status of the Hemoglobin S (HbS) marker along with the phenotype results for 32 antigens. Donor centers have established policies in case of a positive identification of a donor sample. The potential to prescreen units for HbS status and exclude HbS+ units from the inventory being used to support patients with SCD is another advantage noted by all blood centers using xHEA typing. Most reference laboratories do not include HbS results on the donor test of record and must repeat the solubility test on each unit that is being transfused to a SCD patient. Using the HEA system, units that are HbS+

(heterozygous) can be separated easily from the inventory for patients with SCD. If a donor is found to be HbS++ (homozygous) with DNA testing, that donor is excluded from future donations and is notified of the need to seek medical treatment. At LBC, one such donor made ten donations and another donated 3 times before being identified as homozygous for HbS by genotyping. Although these donors may have another hemoglobinopathy such as hereditary persistence of fetal hemoglobin, identifying these donors is of potential importance to their health and avoiding the use of their donated blood may be of benefit to transfusion recipients. However, the donors are notified of their HbS test results and it is suggested that they share the information with their physician. They are not excluded from future donations, but are encouraged to donate apheresis plasma or platelets rather than whole blood or apheresis red blood cells. Donors who are HbS positive are flagged in the computer system to prevent inadvertent freezing of these red cells.

4.4 Patient Testing and Providing xHEA-Matched Donor Units

BeadChip™ analysis at LBC is routinely performed for SCD patients requiring chronic transfusion and a database of patient phenotypes is maintained. For those patients who have not yet been immunized, units are matched for Rh and Kell using NIH guidelines [25]. Once a patient shows evidence of alloimmunization, more extended matched units may be selected. The number of patients with SCD who were screened in 2007 increased by nearly fourfold in 2008, and then doubled again from 2008 to 2009. Testing of other alloimmunized patients to provide xHEA-matched units has also increased from 2007 to 2009.

Routine DNA analysis is performed for individuals with multiple antibodies, those with positive DATs and strong warm autoantibodies, and those who cannot be accurately phenotyped due to recent multiple transfusions. Upon receiving a request for patient genotyping from a hospital or laboratory, the LBC Scientific Support Laboratory attaches a unique inventory identification (ID), conducts the BeadChip™ analysis, and sends a report of the HEA results to the hospital and the reference laboratory. If a patient has an alloantibody, matched units are located, confirmed by serology, and labeled for the specific patient. A report (including a disclaimer) is then sent to the hospital with a cover letter. At GCRBC, transfusion patients who have warm autoantibodies, are DAT positive, are positive for all panel cells, or have been transfused within the last 8 h are regularly screened by BeadChip™. Patients with SCD who have had an event suggesting the need for chronic transfusion support, surgical patients, liver transplant candidates, maternity patients, and those for whom it is difficult to obtain valid serology may also be recommended for BeadChip™ analysis.

The number of requests for matching blood (Cc, Ee, and K) for patients with SCD has been declining, whereas requests for units negative for ≥4 antigens are on the rise. Demand for units to transfuse elderly patients has also increased. Elderly patients, particularly those in long-term care facilities, may receive weekly

transfusions to relieve fatigue and anemia. For those who have developed antibodies, such as women who have been exposed to blood cell antigens during pregnancy, testing is performed for additional antigen matching.

DNA analysis is performed at CICBC to identify alloantigens affecting patients with warm-reactive autoantibodies as well as to verify phenotypes in patients suspected of having antibodies to low- or high-frequency antigens. Previously transfused patients are routinely screened regardless of whether they are DAT positive or negative.

4.5 Product Performance

4.5.1 Concordance Statistics

At GCRBC, DNA results generated by BeadChip™ analysis are continuously compared with serology for units provided for transfusion services. Typically, concordance between BeadChip™ and serological phenotyping determinations is >99%. Table 4.8 shows the comparative results at GCRBC from 2007 through 2009. Initially, 2–3% of discrepant results were identified for the RhC antigen using an

Table 4.8 Results obtained using HEA BeadChip vs. serology at GCRBC

Year	July to December 2007	2008	2009
Black donor samples tested with BAS, *N*	1,358	5,848	4,335
No. of R_o identified out of total tested	726	2,459	1,310
Discordant results, *n* (%)	77 (6%)	233 (4%)	32 (<1%)
Technical error, *n* (%)	7	20	1
Nontechnical error, *n* (%)	10	211	31
No resolution, *n* (%)	60	2	0
c, *n* (%)	2 (0.1)	2 (0.0)	(0.0)
C, *n* (%)	41 (3.0)	131 (2.2)	14 (0.3)
e, *n* (%)	1 (0.1)	6 (0.1)	0 (0.0)
E, *n* (%)	4 (0.3)	4 (0.1)	0 (0.0)
K, *n* (%)	1 (0.1)	0 (0.0)	0 (0.0)
Jk^a, *n* (%)	2 (0.1)	2 (0.0)	0 (0.0)
Jk^b, *n* (%)	6 (0.4)	19 (0.3)	2 (0.0)
Fy^a, *n* (%)	7 (0.5)	15 (0.3)	1 (0.0)
Fy^b, *n* (%)	3 (0.2)	15 (0.3)	2 (0.0)
M, *n* (%)	N/A(0.0)	2 (0.0)	2 (0.0)
N, *n* (%)	N/A (0.0)	17 (0.3)	2 (0.0)
S, *n* (%)	4 (0.3)	17 (0.3)	0 (0.0)
s, *n* (%)	1 (0.1)	0 (0.0)	0 (0.0)
Lu^a, *n* (%)	N/A (0.0)	0 (0.0)	0 (0.0)
Lu^b, *n* (%)	N/A (0.0)	1 (0.0)	1 (0.0)
SC, *n* (%)	5 (0.4)	1 (0.0)	2 (0.0)

BAS BioArray Solutions BeadChip Kit

earlier version of HEA (v. 1.1). Less than 1% of discordant results were identified for all other antigens tested. After the introduction of the next version of HEA kit (HEA 1.2), the number of discrepancies between DNA testing and serology decreased from 5.7% of the total samples tested with the BioArray HEA 1.1 kit in 2007 to 0.1% with the HEA 1.2 in 2009. Over the same period, the number of samples processed nearly doubled.

Currently, when discordant results are noted at GCRBC, historical information is reviewed for documentation errors. If none is available, DNA testing is repeated. If the DNA analysis remains the same, serologic testing is repeated using two sources of antisera, when available. Samples that remain unresolved are finally reported to the BAS ID (identification of discordances) program where samples are retested on HEA BeadChip™ followed by analysis with other available resources, including high-resolution panels and sequencing, if needed.

At LBC thus far, 104 (2%) discrepancies have been recorded out of a total of 5,000 comparative samples processed using the HEA 1.2 kit. Initially, the resolution of a discrepant result began with retesting the DNA analysis. If the DNA results were discordant with the serology on the second test, the serology was repeated. Because the errors frequently turned out to be with the serology or manual data entry rather than the genotyping (Fig. 4.5), serological retesting is now done first. This process has reduced the discordant rate to 0.9%. When retesting does not resolve the differences, additional testing with serological reagents from another vendor or another DNA method may be performed in-house followed by sending the samples to the ID program for additional testing. Many of the discordant samples are due to the N, S, or s antigens. Since there are predominantly Black donors, investigations are underway to determine whether these cases represent hybrid alleles that are known to be more common in this ethnic group.

4.5.2 Proficiency Program

Although the Clinical Laboratory Improvement Amendments (CLIA) specify that clinical laboratories participate in outside quality assurance (QA) or proficiency testing programs [26], few such programs exist for molecular testing of red cell blood groups. International molecular genotyping workshops do not occur frequently enough to assist laboratories to determine how to comply with these regulatory requirements. Moreover, genotyping workshops are typically inadequate for users of BeadChip™ assays because they focus on a single antigen or blood group phenotype. Thus, LBC developed a proficiency program for BeadChip™ users.

Proficiency testing samples are sent to participating laboratories twice per year. For each test, a set of donors, one Caucasian and one Black, are chosen to reflect various blood group alleles, silencing mutations, or rare blood group phenotypes, including those that are unique to specific races or ethnicities. For the proficiency test samples, genomic DNA is extracted from leukoreduction filters and prepared using PureGene (Gentra). The extracted DNA is validated in the LBC Scientific

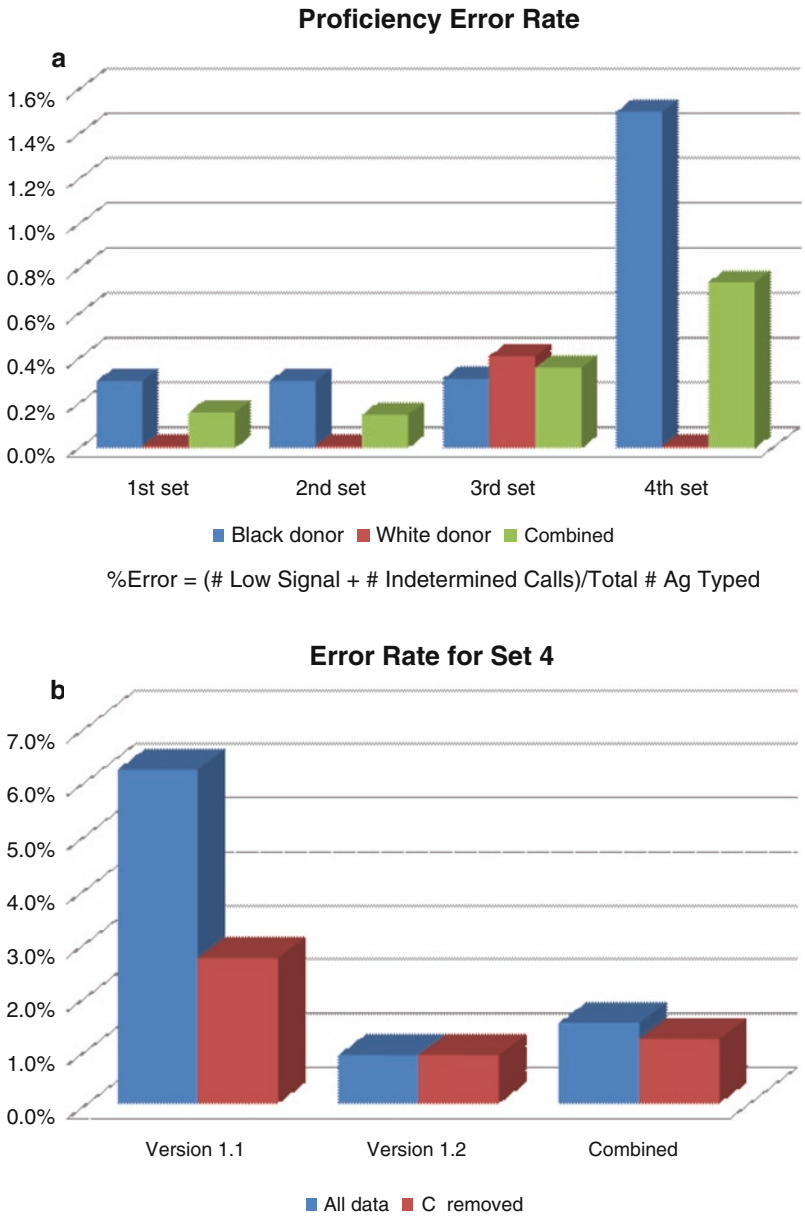

Fig. 4.5 Results of proficiency testing are given showing (**a**) total error rate for all sample sets and (**b**) error rate for sample set 4

Support Laboratory. Serological testing is performed on the red blood cells in parallel using either FDA licensed reagents (when available) or well-characterized rare reagents. In the third set of proficiency tests, one sample was sent as whole blood to test the participants' ability to extract useable DNA [27].

A total of 21, 32, 33, and 38 laboratories participated in four proficiency tests so far, testing for sample sets 1, 2, 3, and 4, respectively (Fig. 4.5a) [27]. Sample set one was assayed for the Duffy alleles, including the GATA mutation at twenty-one sites including seven international laboratories. For set one, these seven laboratories that used PCR-RFLP were 100% concordant with the BeadChip™ results for Duffy genotyping. There was 100% concordance with the serological results for all proficiency samples. Among the participating laboratories, concordance between the genotype and the predicted phenotype was >99.0%. Most of the discrepancies (24/7,432 or 0.3%) were due to either low signal (LS) or indeterminate call (IC) warnings for M (2), Ss (3), C (2), E (1), Lu^a (5), Lub (5), Hy(1), Jo^a(1), Sc1(2), and Sc2 (2). For set three, 13 laboratories used version 1.2 of the HEA BeadChip™ and correctly typed the additional antigens V/VS, $Kp^{a/b}$, and $Js^{a/b}$. All laboratories accurately identified a Co(a−b+), a hemizygous FY*A sample, a GYPB intron 5 silencing mutation for S, a MS^u (deleted GYPB), and the HY/JO genotype that results in a Jo(a−) phenotype. In set four, four of the participants using the HEA 1.1 kit incorrectly identified the Black donor as C negative due to the absence of additional markers for variant typing later included in the HEA 1.2 version. Those laboratories which used HEA 1.2 kit characterized the sample as C positive indicating the presence of possible r^s variant. Figure 4.5b shows the results from set four stratified by the HEA kit used. After eliminating the false negative results associated with use of the HEA 1.1 kit, the error rate was 0.74%, slightly higher than expected. The elevated error rate was from LS and IC warnings that were counted as discordant. Overall, BeadChip™ analysis demonstrates a high degree of reproducibility among users [27].

4.6 Economic Benefits for the Blood Bank Operation

An analysis of the economic impact of red blood cell genotyping was conducted at LBC. Included in the analysis was a time-motion study involving four medical technologists performing manual serological testing and one using DNA typing. Actual hands-on time required for donor samples to be typed serologically for C/c, E/e, K/k, Fy^a/Fy^b, Jk^a/Jk^b, and MNSs using a tube technique and to perform an HbS solubility screen was recorded. Similar logs were kept for the preparation of DNA and performance of the HEA BeadChip™ assay using the HEA 1.2 kit to genotype for HbS and 32 blood group antigens, including those tested for by serology. Reagent costs based on current contract pricing were calculated for a run of 95 donors and appropriate controls were tested on the same day [28].

BeadChip™ analysis resulted in a 60% reduction in hands-on time. On average, it took 10 min to phenotype one donor for 14 common antigens and HbS as compared with 4 min per donor to screen for 32 antigens and HbS using the HEA BeadChip™ assay. The total time required to screen 95 donors for the 14 antigens using serology and to characterize HbS was 15.8 h compared to 6.3 h with DNA typing. In addition to saving the technicians' time, DNA analysis allows identification of donors lacking high-prevalence antigens, such as k, Kp^b, Js^b, U, Lu^b, Di^b, Co^a, Hy, Jo^a, LW^a, and Sc1, who would not be found easily with serology [28].

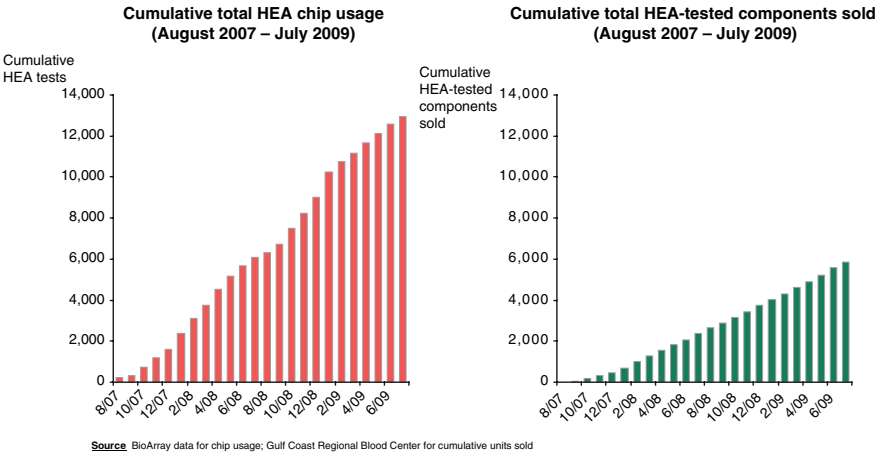

Fig. 4.6 Cumulative BeadChip™ usage and HEA-tested components distributed by GCRBC from August 2007 through July 2009 are shown

Using LBC current contract pricing for antisera, HEA BeadChips™, reagents, and consumables, genotyping resulted in a cost savings of approximately $34.00 per donor tested (a 51% decrease in cost over serology). The average cost savings for new users is estimated to be $18.50 per donor tested [28]. Another opportunity for cost reduction and efficiency is related to a reduced need to hire consultation technologists to perform the initial screening of Black donors. On the other hand, for some cases the full BioArray panel is not necessary. Some donors can be eliminated with one to two serology tests without the need to perform a full HEA panel on 100% of the donors.

Incremental revenue from HEA-tested units and decreased serological cost for HEA screening were observed at GCRBC over the last 3 years. Since 2007, GCRBC has conducted approximately 13,000 HEA tests and distributed over 6,000 xHEA-typed units (Fig. 4.6). An increase in the total revenue was observed due to the identification of large numbers of donor units identified with 5, 6, and 7 antigen-negative phenotypes for c, C, e, E, Fy^a, Fy^b, Jk^a, s, and Kp^a. Additional revenue was realized by the identification of units with other antigens (M, N, Jk^b, and S) and also for high-incidence antigens, such as Jo^a, Do^a, Di^b, Hy, and Lu^b. As a collateral benefit due to the multiplex nature of the test, results for all 32 antigens are provided along with the result for the HbS marker.

4.7 Roundtable

Authors from the highlighted institutions were asked to participate in a roundtable discussion of various aspects of their experiences with implementing BeadChip™ technology at their institutions (LBC, NYBC, CICBC).

4.7.1 Validation and Monitoring

What was involved in validating your set up?

JoAnn Moulds LBC (JM): "Current molecular standards [29, 30] require that at least 20 samples be tested for validation of product performance at each installation (Standard 5.3.2). As an early adopter, LBC chose to perform a more rigorous evaluation and validation protocol for the BeadChip™ HEA 1.1 kit. In phase 1, 81 donor samples with serologically documented rare or null phenotypes were tested for specificity as follows: 3 e–, 4 k–, 1 Jk–3, 12 Fy(a–b–), 12 U–, 3 Lu(b–), 2 Di(b–), 7 Di(a+b+), 8 Co(a–), 2 Co(a+b+), 12 Hy–, 12 Jo(a–), 1 LW(a–), 1 Sc:–1, and 1 Sc:1,2. Only one sample, a presumed Di(a+b–), was discordant. Upon further investigation, the donor was shown to be Di(a+b+) by both PCR-RFLP and SDS-PAGE, confirming the BeadChip™ result.

In phase 2, accuracy and precision were further validated by genotyping 40 Black donor samples in duplicate and comparing the results to historical serological types. The same 40 DNA samples were tested by an outside laboratory and the results were 100% concordant between the laboratories. Concordance between runs were further evaluated with another 30 random samples tested in duplicate. Once again, the concordance between runs was 100%.

In phase 3, samples from 200 Black donors were selected at random for comparison between the BioArray HEA 1.1 kit and serological testing for the following antigens: C/c, E/e, MNSs, Fy^a/Fy^b, Jk^a/Jk^b, and K. In this assessment, 28 samples were identified as being M+N+ by serology but genotyped as M+N–. Investigation by BioArray Solutions identified a new SNP in the *N* gene and the HEA 1.2 assay was adjusted accordingly to detect these variants. In addition, due to the presence of a high number of donors with variant C harboring r^s status, discrepancies with Rh C antigen status with the HEA 1.1 kit prompted the release of the HEA 1.2 kit in October 2008, where the RhC antigen status is determined by three polymorphisms, P103S, L245V, and G336C along with the presence or absence of 109-insert in intron 2.

The new kit was validated at LBC using 20 samples with proven concordance between the HEA 1.1 kit and serology. An additional 50 samples that were reported as V+ using HEA 1.2 kit were confirmed by serology."

Kimberly Nail, CICBC (KN): "To validate the HEA 1.2 kit at CICBC, 11 samples were tested using the samples already tested by HEA 1.1. In addition, serological testing for the four new antigens was performed. With the exception of an IC reported for the Lu^a and Lu^b using HEA 1.1, the results from the 11 donor samples were concordant between the two kits."

Donna Strauss, NYBC (DS): The HEA 1.1 BeadChip™ was initially validated at NYBC by performing BeadChip™ analysis on the DNA extracted from 396 donor samples. "Of the 8,578 successful tests, 62% were predicted to be antigen-positive and 38% were predicted to be antigen-negative. Sixty-seven percent of the negative results were new results (no previous serology testing). Of particular note, the testing revealed six new Sci-1 donors, as well as three e– and many c–, and s– donors who have been confirmed by serology" [12].

A second validation of the HEA 1.1 BeadChip™ was subsequently performed with NYBC personnel. The operational qualification (OQ) consisted of 68 samples with known RBC phenotypes for C/c, E/e, M/N, S/s, K/k, Fy^a/Fy^b, and Jk^a/Jk^b. Included in the OQ were six known samples (cell line) and controls tested in replicate. The cell lines were provided by BioArray Solutions. For the run to be considered valid, all cell line sample results were required to match the panel key and the negative control was required to be negative. Sixty-seven of the 68 samples were found to be concordant with the historic records, though one sample did not correlate with the HEA 1.1 BeadChip test result. Historically, the sample tested c−, E− using serologic testing. The molecular test predicted that the sample would possess c and E antigens. Repeated hemagglutination and DNA testing by LDT confirmed both results. This sample is being investigated further, but the lack of correlation is likely attributable to a silenced *RHCE*cE* allele. For performance qualification, NYBC tested 960 specimens with unknown phenotypes. Once tested, the results were confirmed with serology. There were no discordant results [12].

4.7.2 Current BAS Validation Protocol

Currently, for product performance qualification, BioArray Solutions recommends that each site perform DNA extraction and complete BeadChip™ analysis of 25–30 whole blood samples, which have been previously tested for antigen type by serologic methods. Antigen typing results should reflect a 95% correlation with previous results. In addition, BioArray Solutions typically provides ten gDNA samples extracted from cell line and two reference control samples, all of known HEA phenotypes.

In addition to proficiency testing, how do you monitor acceptable performance?

JM: “LBC-validated positive and negative controls are tested with each new lot of BioArray chips as required by AABB standards [29]. A water control is run with each assay run and the results reviewed by the laboratory director before the results are reported. On a yearly basis, an internal QA review is performed to look for trends in the numbers of IC or LS that are suggestive of discordant results or poor chip performance. The QA team at LBC also performs vendor qualification assessments.”

KN: “As part of the CICBC protocol, sterile water acts as a negative control and previously genotyped DNA acts as a positive control with each run. Following each run, the data are reviewed first by the technologist followed by the laboratory director. If a failure occurs, the sample may be sent for serological testing for antigens for which reagents are available, such as Rh, Kell, Kidd, and Duffy. However, serological testing can be expensive, for example, S reagent costs more than $700 per 5-ml bottle.”

Table 4.9 Suggested CPT codes

Procedure description	CPT code(s)
Isolation or extraction of highly purified nucleic acid	83891
Amplification of patient nucleic acid, multiplex; first two nucleic acid sequences	83900
Amplification of patient nucleic acid, multiplex each additional nucleic acid sequence	83901
Enzymatic digestion	83892
Mutation ID by enzyme ligation or primer extension, single segment	83914
Interpretation and report	83912

Source: Current Procedural Terminology. CPT 2010, Professional Edition, AMA
CPT current procedural terminology

4.7.3 Billing and Reimbursement for HEA Typing

JM: "At LBC, patient sample testing is reimbursed using molecular current procedural terminology (CPT) codes (Table 4.9). The hospital is billed for the genotyping and it is their choice whether they then bill the patient or insurance company. The laboratory business manager works with the individual customer if needed and assists with CPT code information. An informal follow-up with some of the larger hospitals found no problems with patient reimbursement."

4.8 Summary

Over the course of the first 3 years that BeadChip™ technology has been available on the market, blood centers implementing the technology have realized substantial benefits in various aspects of donor center operations. Primarily, among these benefits are the increased pool of xHEA-typed donor inventories of antigen negative and rare donors, reduced TAT for finding matched donors, and the greatly expanded capacity to maintain a rolling inventory of liquid units of xHEA donors. The capability to screen large numbers of donors for multiple antigens provided by BeadChip™ testing is seen as a paradigm shift requiring a new process flow at blood center operations and reference laboratories. The improved capacity for procuring phenotyped donor units, and providing rare donor units as liquid rather than frozen blood, has the potential to improve the safety of transfusion medicine while lowering costs.

The installation validation and proficiency testing protocols have been greatly simplified through the efforts of the early adopters. High rates of concordance between DNA testing with BioArray technology and serology have been established for a wide variety of red cell antigens. At the same time, in response to the experiences of early adopters with discordant results for RhC and N antigen, the panel has been improved by adding additional SNPs for increased specificity in resolution.

While data collection continues, initial cost analysis indicates that the use of genotyping results in a cost savings for materials and reduced hands-on time for technicians of approximately 60%. The considerable time and cost savings realized by using the HEA BeadChip™ assay also allows the reference technologists to perform other work that directly relates to patient care.

In the next phase of implementing DNA testing in donor center laboratories, integration and management of reported data from the BioArray unit with laboratory databases will be important.

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Chapter 5
Implementation of HEA BeadChip System at Medical Centers: Providing Extended Matched Units and Eliminating Complex Workups for Patients

Ellen Klapper, for the BeadChip User Group at Medical Centers*

Abstract Medical centers have long appreciated the need for a reliable system to provide compatible blood for patients. The immediate need to provide antigen-negative blood usually entails getting units from blood providers which delays transfusion and increases cost. Most hospital transfusion services do not have full reference lab capabilities to resolve complex cases and are forced to send out patient samples. The focus of the institutions highlighted in this chapter is to meet the needs of their special patient populations, while some are also providing services to other area hospitals. All institutions were motivated to implement BeadChip™ technology to expand and manage the inventories of antigen-negative units, thereby reducing their dependence on blood centers for Ag (antigen) negative and rare donor units. The patient samples analyzed are complex workups of patients with multiple antibodies, multiple previous transfusions, or patients with a positive direct antiglobulin test. These are usually submitted to the reference laboratories within the hospital or from other area hospitals. Applying the BeadChip™ technology in the hospital can reduce turnaround time for providing phenotype-matched units for alloimmunized patients. The impact of implementing BeadChip™ technology on patient care and on laboratory operations is discussed.

Keywords Alloimmunization • Autoantibodies • Blood group antigens • Blood groups • DNA testing • DNA array

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5.1 Introduction

The use of molecular testing has emerged as a useful means of providing extended phenotype-matched units for patients with a variety of clinical conditions. Some of the patients most likely to benefit from extended phenotype-matched blood are those who are expected to receive multiple transfusions over their lifetime thus increasing their risk for alloimmunization. The resolution of many complex serological problems in recently transfused alloimmunized patients who may also have warm or cold autoantibodies is often aided by characterization of the pretransfusion erythrocyte antigen profile [1]. Separation techniques to isolate and enrich reticulocytes, and adsorption and elution studies using selected cells of known phenotype are helpful in distinguishing alloantibodies from autoantibodies [2]. However, the presence of a positive direct antiglobulin test (DAT) indicative of autoantibodies or a delayed transfusion reaction (alloantibodies) often limits the ability to obtain valid serological phenotyping results. Mouse monoclonal and humanized chimaeric monoclonal antibodies directed against human blood groups are helpful in many situations, but it has not been possible to develop such reagents for all clinically important blood group antigens [3]. These complex serological problems may occur in any transfused patient. However, individuals with sickle cell anemia, thalassemia, and other disorders requiring long-term support are more likely to develop autoimmunity/hyperhemolysis. Prospective antigen matching has been recommended to prevent alloimmunization in patients with warm autoantibodies and hemoglobinopathies [4–7].

Differences in the ethnicity of blood donors and transfusion recipients are a key factor in immunization of some multitransfused patients to blood group antigens. Prospective matching requires recruitment of donors from the same ethnic group as the patient population. Blood centers in collaboration with medical centers have focused on recruiting donors from special ethnic groups to identify matched blood for certain patient populations. The American Red Cross has worked with the Children's Hospital of Philadelphia to create the Blue Tag Program to recruit African-American donors for patients with sickle cell anemia. In Louisiana, LifeShare's program has focused on the recruitment of African-American donors to meet the special needs of patients with sickle cell anemia. They have also recognized that African-American patients who require complex medical treatments will also need transfusion support. The New York Blood Center developed the PreciseMatch Program which has been used primarily to recruit African-American donors was originally created for outreach to the ethnically diverse population of New York City. Finally, one must acknowledge that alloimmunization can still occur when donors and recipients are from the same ethnic group. However, the magnitude of the problem and the antigens involved are quite different [8, 9].

Clinicians are concerned that the recommendation for prospective antigen matching of blood in patients with hemoglobinopathies and possibly other multitransfused patients has not been more widely implemented [10]. Although the

evidence substantiating the value of prophylactic red cell antigen matching is weak or nonexistent for many transfusion indications, the clinical demand may be difficult to deny for many transfusion services. The additional cost to the hospital for providing such units is a barrier to wider implementation. Many transfusion specialists are concerned that using rare blood units on a prophylactic basis makes them less available for patients already alloimmunized. Although hospitals recognize that blood donor centers must recover the costs associated with additional recruiting and testing, the patient's insurance may not provide for reimbursement of costs associated with acquisition of such units.

DNA array technology such as that used in BeadChip™ analysis has been implemented in donor centers and reference laboratories to aid in the resolution of complex serologic workups, to facilitate extended phenotype matching for patients at risk for alloimmunization, and to assure timely provision of antigen-negative blood for alloimmunized patients. DNA typing expedites the identification of rare units lacking multiple antigens and can help direct blood donor recruitment efforts. The cost per test for DNA-based testing is lower than the comparable cost of serological testing when large numbers of donors/patients are typed (see Chap. 4). In this chapter, four academic medical centers, two serving primarily pediatric patients, and two large academic tertiary care medical centers describe their experiences using HEA BeadChip to screen donations (local collections and units purchased from their local blood supplier) and to phenotype patients.

5.1.1 Cedars-Sinai Medical Center

Cedars-Sinai Medical Center in Los Angeles is the largest multispecialty hospital on the West Coast. With over 850 beds, Cedars-Sinai Medical Center offers patients' state-of-the-art care in multiorgan transplantation, hematopoietic stem cell transplantation, comprehensive cancer treatments, cardiac surgery, and a level I trauma center. The medical center serves 55,000 inpatients and 350,000 outpatients annually. Cedars-Sinai Medical Center blood donor services collect over 12,000 units of blood and perform 30,000 red blood cell transfusions annually.

5.1.2 Children's Hospital Boston

Children's Hospital Boston in Boston is one of the largest pediatric medical centers in the United States. Children's Hospital Boston is a 396-bed comprehensive center that offers a complete range of health care services for children from birth through 21 years of age and some adults with congenital diseases. Children's Hospital Boston records approximately 22,600 inpatient admissions each year and 527,500 outpatient visits annually in its 204 specialized clinical programs.

5.1.3 Mayo Clinic at Rochester

Mayo Clinic at Rochester serves 340,000 unique patients and 1.5 million outpatients. St. Marys and Rochester Methodist hospitals have 1,900 licensed beds and Mayo Health System, a network of clinics and hospitals in 64 communities in southern Minnesota, northern Iowa, and western Wisconsin further includes 15 hospitals with more than 907 beds. About 133,700 blood units are transfused at Mayo Clinic annually.

5.1.4 Children's National Medical Center

Children's National Medical Center is a 283-bed nonprofit academic medical center that provides pediatric care in the metropolitan Washington, DC area. Children's National Medical Center cares for more than 360,000 patients each year and is the regional referral center for pediatric emergency, trauma, cancer, cardiac, critical care, neonatology, orthopedic surgery, neurology, and neurosurgery.

This chapter reviews the introduction of DNA testing at the four hospitals' transfusion services to provide extended matched blood units for complex antibody problems and other special patient populations by way of the BeadChip™ system.

5.2 Implementation of Molecular Testing

The use of BeadChip™ technology over the last 3 years at four hospital transfusion services is shown in Fig. 5.1. While patients with sickle cell disease (SCD) make up a significant portion of this patient population, other patient groups who are expected to receive multiple transfusions are included in this category as well.

The ethnic percentages estimated from allele frequencies identified in the contributing medical centers are described in Table 5.1. At CSMC, a high proportion of African-Americans, presumably representing the SCD patient group, is more difficult to match from a donor base with a lower level of matching ethnicity. The Children's National Medical Center donor population more closely matches the patients (a predominantly SCD group). Children's Hospital Boston and Mayo Clinic have patient and donor populations that are predominantly Caucasian, reflecting the demographics and locations they serve.

Statistics of the number of donors and patients tested as well as the rare types identified among those tested in 2009 at the various medical centers is shown in Table 5.2. Following the implementation of BeadChip technology at Children's National Medical Center in 2008, approximately equal numbers of donors and patient samples have been tested. About 1,000 patients with SCD are seen at the hospital, 70 of whom receive regular transfusions. Since the introduction of the

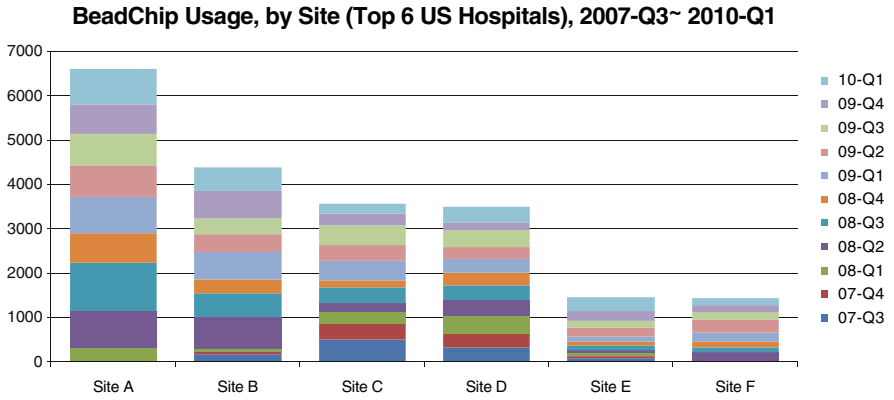

Fig. 5.1 Frequency of HEA BeadChip™ kit utilization for patient testing and donor screening at medical centers

Table 5.1 Ethnic subgroups of donor and patient populations at four medical centers in 2009[a]

	Cedars-Sinai Medical Center		Children's Hospital Boston		Mayo Clinic		Children's National Medical Center	
Subgroup	Donors (%)	Pts (%)	Donors (%)	Pts (%)	Donors (%)	Pts (%)	Donors (%)	Pts (%)
African-American	6	24	4	9	0	6	53	100
Caucasian	72	61	96	63	95	90	39	0
Hispanic	7	0	0	23	0	0	8	0
Asian	15	15	0	5	5	4	0	0

[a]Ethnic percentages estimated from allele frequencies identified in each population

BeadChip™ system, the process to identify compatible units for these patients has evolved and currently all new SCD patients are only tested with BeadChip™ analysis rather than with serology. Typically, patient samples are batched and DNA testing is performed weekly in runs of 24 specimens. Patient samples are given priority and donor samples are used to fill in if fewer than 24 patient specimens are available to screen. Since many of these patients have multiple antibodies that cannot be resolved easily as autoantibody or alloantibody by serology, DNA testing provides a clear analysis of the patient's antigens to predict the antibody status of the patients. The ability to identify the presence of Fy^b negative units also harboring GATA genotype has had a big impact by reducing the need for Fy^b negative units.

At Children's National Medical Center, BeadChip™ analysis is used to screen units from African-American donors for Duffy, Kidd, Kell, and Rh phenotypes to provide compatible units for a large number of SCD patients. As a result of the large number of donors who can be screened with BeadChip™ technology, more matched units can be identified and the need for donor recruitment is reduced. A frozen inventory of patient-matched donor units is maintained. Specific matched

Table 5.2 Rare unit identification in donors and patients genotyped at five medical centers in 2009

Institution	Cedars-Sinai Medical Center		Children's Hospital Boston		Mayo Clinic		Children's National Medical Center		Site A	
Type	Donor	Pt	Donor	Pt	Donor	Pt	Donor (subset)	Pts	Donor	Pt
Total	2,204	246	1,626	28	950	71	171	235	1,698	78
Co(a−)	2	0	2	0	0	0	0	1	24	0
e−	72	13	38	1	29	0	6	3	58	3
Hy−	1	0	0	0	0	0	0	0	0	0
Jo(a−)	2	0	1	0	0	0	0	4	0	0
Js(b−)	1	0	0	0	0	0	1	2	3	0
k−	6	0	2	0	0	0	0		27	0
Kp(b−)	1	0	0	0	3	0	0	0	0	0
Lu(b−)	0	1	4	0	0	0	0	0	16	0
U−	0	0	0	0	0	0	0	0	0	0
U(var)	3	2	2	0	1	0	2	2	0	0
c−e−	0	0	0	0	0	0	0	1	0	0
LW(a−)	0	0	0	0	0	0	0	1	0	0
Sc1−	0	0	0	0	0	0	0	1	0	0
Di(b−)	0	1	0	0	0	0	0	0	0	0
Fy(a−b−)	97	27	25	1	6	3	56	180	44	9
Jkb−S−	334	56	220	6	118	7	41	82	245	9
HbS+/++ (%)	1	5	0	2	0	2	1	100	0	40

donors are actively recruited for future donations. Development of DNA testing for RBC antigens has increased awareness of clinically important phenotypes such as the Diego blood group. Because serology could not be performed easily, the importance of these antigens may have been underappreciated. At Children's National Medical Center, efforts are being made to increase the screening of Hispanic and Asian donors who may express low-prevalence antigens. In particular, there is increasing interest in identifying Di^a and Di^b negative donors.

Donor screening with DNA testing has improved the turnaround time (TAT). If a unit has been genotyped, it can be readily available in inventory, confirmed with serology, and issued.

At Children's Hospital Boston, the system is located at an independent location at the hospital. Validation is performed by comparison of results from BioArray analysis to serological results for both patients and donors. When serologic testing reagents were unavailable, results were compared with those obtained by BioArray Solutions performing the testing in parallel. The system has been validated for donor whole blood samples, donor segments from preleukoreduced whole blood stored in CP2D, donor segments from prefiltered whole blood stored in CPDA-1, and patients who have been multiply transfused with leukoreduced RBC units. DNA was extracted twice from the validation samples – once within 7 days of

sample collection and again 14 days after sample collection to ensure that blood specimens could be stored up to 14 days. Further storage times were not tested because the medical institution does not expect to store specimens longer than 14 days. All samples passed validation. Implementing BeadChip™ technology has facilitated identification of rare donors at a much higher rate than is possible with serology. At Children's Hospital Boston, initially only group O donors were typed. Subsequently, typing was extended to most group A and O donors and then to donors of all blood groups. The testing has also been extended to first-time donors as well. The usual inventory at the blood bank is small, ranging between 500 and 700 units with a frozen inventory of about 40 units. DNA testing, particularly for C, E, and Kell, is used to prospectively match donor units for patients with SCD. Between 5% and 10% of the inventory is used for approximately 50 patients with SCD, many of whom are chronically transfused. However, no special inventory of HEA matched units has been maintained. The TAT has improved substantially. This has allowed the transfusion service to support increasing numbers of patients on chronic exchange transfusion protocols without increasing the number of units that need to be serologically typed or increasing purchases from community blood centers.

For validation at the Mayo Clinic, DNA analysis of 60 samples using the HEA 1.1 kit was compared with serological results. The BioArray equipment was validated using the protocol recommended by BAS. When the HEA 1.2 kit with improved resolution of the RhC, genotyping was introduced and validation was repeated.

At the Mayo Clinic, 95% of genotyped samples are from Caucasian donors reflecting the ethnicity/race of the general population. For donor testing, all repeat donors are screened (O or A), with most of the O+/− routinely tested. Results of BeadChip™ analysis of donor samples are maintained on file and used as a reference for future donations for that donor. During analysis several "high incidence" antigen-negative samples [Co(a−), k−, Di(b−)] have been identified. Many rare donors with unusual antigen combinations, such as S−, Jk(a−), Fy(a−b−), C−, and e− have been successfully identified.

At Cedars-Sinai Medical Center, the DNA analysis for extended red cell typing was validated using over 100 donor and patient samples, including patients who had been transfused within 3 months and externally derived DNA samples. Once validated, the system was implemented in 2008.

Concordance statistics at donor centers have been described in detail in Chap. 4, and the frequencies would not be expected to be substantially different at medical center labs.

Most of the discordant results observed were for RhC due to the high frequency of r'^{S}. During early implementation, all DNA tests for RhC were confirmed by serology testing per manufacturer's recommendation. The introduction of HEA 1.2 in 2008 has improved the resolution of "C" by having antigen status determined by additional polymorphisms (L245V and G336C) to determine VS and V along with r'^{S} status. Discordant results can usually be resolved internally. In rare instances, when they cannot be resolved, samples are sent to the BAS-ID program.

Donor testing results are confirmed utilizing FDA-approved reagents if available. For patient typing, no confirmation is needed. When FDA approved reagents are not available, then only DNA results are used.

At Cedars-Sinai Medical Center discordant results are well under 1%. When discordance is observed, samples are retested with serology. If samples remain unresolved, the samples are sent to BAS-ID program.

When patients are screened at Children's Hospital Boston, if the serology is antigen negative and DNA is antigen positive, the serological result is assumed to be correct. This is considered the safest practice to ensure that the patient receives antigen-negative units or to ensure that antibodies to that antigen are considered in serologic testing. If discordance arises for a donor unit, the unit is assumed antigen positive. Again, this is considered to be the safest practice to ensure that incompatible antigens are not inadvertently transfused. When a sample is tested by DNA analysis, it is not confirmed by serology unless it is transfused. At that time antigen-negative status is only confirmed by serology. The use of the BeadChip™ system has helped Children's Hospital Boston provide increasing RBC units for more exchange transfusions without having to purchase additional antigen-negative RBC units from blood centers.

At the Mayo Clinic, discordance is observed due to both serology and DNA testing. Repeat serology studies with multiple antisera sources are performed to resolve the discrepancy. In the event serology is inconclusive, samples are sent out for additional molecular testing.

5.3 Impact on Patient Care: Resolving Complex Cases

Screening with extended HEA (xHEA) typing has been particularly useful for resolving complex cases, especially recently transfused patients with positive DAT, with warm autoantibodies, hemolytic disease of the newborn, and autoimmune hemolytic anemia. Medical centers that lack the resources to perform complex serological workups often send samples to an outside reference laboratory, and may incur significant charges along with delays in blood availability [11]. If timely resolution is not possible, incompatible blood must be issued in lieu of fully compatible units.

It has been well recognized that serologic phenotyping of blood group antigens is unreliable or invalid when patients have had a recent transfusion or their DAT is positive for immunoglobulin G. In contrast, DNA analysis may be unaffected by these conditions (Table 5.3). The clinical utility of the BeadChip™ HEA analysis for patients in complex clinical situations was investigated [12]. During the study, a wide range of patient samples were selected for DNA analysis including, pre- and posttransfusion samples, patients with severe leukopenia, and abnormal chemistry from liver or kidney dysfunction. The study concluded that blood group genotyping was reliably achieved in these clinical situations.

Table 5.3 Case 1: 15-year-old female patient with sickle cell disease and pain crisis requiring six RBC units

	K	k	Fy^a	Fy^b	Jk^a	Jk^b	M	N	S	s
Pretransfusion										
Serotype	−	+	−	−	+	−	+	+	−	+
Genotype										
Posttransfusion										
Serotype	−	+	+	+	+	+	+	+	+w	+
Genotype	−	+	−	−	+	−	+	+	−	+

An example demonstrates a case of a patient whose initial work up was positive on antibody screen (GEL); DAT poly 3+; IgG: 2+; complement negative, with an Rh phenotype of R_1R_2. Results of a Gel Panel/Ficin Panel suggested possible anti-Jk^a and anti-K, and an eluate was positive (4+) with all cells in a panel. The autocontrol was positive and EGA stripping failed. The conclusions of the serological workup were that the eluate contained a warm autoantibody (WAA). Additionally, the patient had anti-K and possibly anti-Jk^a antibodies. The presence of antibodies for Fy^a, Jk^b, and S could not be excluded.

The BeadChip™ results confirmed the potential for anti-K antibodies, but indicated the patient was positive for the Jk^a antigen. He was found to be negative for Fy^a, Jk^b, and S antigens, and thus at risk for producing antibodies against those antigens. It is important to emphasize that unlike determination of alloantibodies, the benefit of genotyping for the patient extends beyond the initial screening. Resolution of patient phenotypes with DNA testing allows for future transfusions with phenotype-matched units. For the patient who has a history of bleeding problems requiring transfusion, Fy^a, Jk^b, S, and K negative RBC units can be transfused safely in the future without the need to exclude the development of additional common clinically significant antibodies.

Matching Rh blood group antigens is complicated by the number of variant and unknown antigens. At Children's National Medical Center, the availability of genotyping has aided in the resolution of patient Rh haplotypes. For example, patients who are $R_2r'^S$ or homozygous r'^S will be hr^B- and can produce anti-hr^B. This phenotype also expresses partial C and these patients are at risk to form alloantibody anti-C. Therefore, they should receive C negative blood. Currently, some laboratories interpret a serologic weak D as negative for patient testing and positive for donor units. BeadChip™ systems developed for RhD and CE variant analysis will be of great value in differentiating weak and partial D patients to identify requirement of Rh(D)− RBC units (see Chaps. 10–12).

DNA analysis provides an important window into "antibody potential" that may not be accessed with serology. In at least one situation, DNA testing can eliminate the need for transfusing antigen-negative RBCs where serology cannot. Red cells from patients that are *FY*B* but carry the GATA polymorphism are phenotypically Fy^b negative. Because carriers of this single nucleotide polymorphism do express Fy^b on cell types other than erythrocytes they are not at risk for anti-Fy^b production.

Therefore, they do not require Fy^b negative donor units [13]. Only with DNA typing these patients can be distinguished from those who are truly Fy^b negative, thereby reducing the demand for Fy^b negative blood.

Table 5.4 summarizes ten patient cases with xHEA aligned with alloantibodies. This provides a reference point for potential immunization if incompatible blood has antigen status that does not match the predicted negative characteristics. The extended typing information has value in that those "antibody potentials" may be recorded and deliberately avoided, so future chances of alloimmunization may be reduced. Indeed, Table 5.4 demonstrated a high correlation between those antigen negativities in xHEA and the actual alloantibodies present in those patients.

At Cedar-Sinai Medical Center, patients are genotyped if their RBCs are DAT-positive, warm autoantibody patients or patients who have received RBC transfusions within the past 3 months. Cedar-Sinai Medical Center also uses the technology to aid in the resolution of complex antibody problems (e.g., when antibody specificity cannot be determined, when an antibody to a high-incidence antigen is suspected, or to distinguish allo- from autoantibody)

At Children's Hospital Boston, patients with autoimmune hemolytic anemia are routinely genotyped. This protocol facilitates transfusion of better matched units, when available, and facilitates subsequent serologic workups. By knowing a patient's genotype, the blood bank knows the antibodies the patient is capable of making. This valuable information can be used when interpreting antibody identification panels, deciding when adsorptions are needed, and when interpreting the adsorption results.

5.4 Expanded Inventories of xHEA-Typed Donors

Experience at the medical centers where DNA analysis for extended phenotyping has been implemented demonstrates that maintaining an inventory of phenotyped blood has been an important outcome of HEA typing [14]. Procuring multiple antigen-negative units from donor centers can often take an additional 3–6 h and can cost at least $60 per antigen screened. To improve patient care and reduce costs, some medical centers also run donor centers and maintain an inventory of xHEA-typed units [11].

The demand for rare phenotypes or antigen-negative blood varies from center to center depending on the patient populations. Patients with SCD account for a large percentage of transfusion recipients at Children's National Medical Center, Children's Hospital Boston, and Cedars-Sinai Medical Center. As many as 45% of patients with SCD have alloantibodies for more than one RBC antigen [15]. Thus, the need for multiple antigen-negative units is particularly great at centers that see many of these patients. While centers with fairly small inventories, such as Children's Hospital Boston, are able to find most of the antigen-negative units they need from their inventory and callbacks of previous blood donors, very rare

Table 5.4 Antigen profile determined by BeadChip analysis aligned with antibody profile in ten alloimmunized patients requiring transfusion

BeadChip xHEA phenotype																	Provided by transfusion service		
Sample	c	C	e	E	K	k	Jka	Jkb	Fya	Fyb	M	N	S	s	Hgbs	*ABO*	D	AbProfile	Units needed	Units transfused
SR000001	+	0	+	0	0	+	+	+	0	0	0	+	0	+	++	B	+	Anti-C, anti-E, anti-K	8	8
SR000002	+	0	+	0	0	+	+	0	0	+	+	+	0	+	++	B	+	Anti-C, anti-E, anti-Fya, anti-Jkb, anti-S	2	0
SR000003	+	+	+	0	0	+	+	0	+	0	0	+	0	+	0	AB	+	Anti-E, anti-K	4	2
SR000004	+	0	+	0	0	+	0	+	0	+	+	+	+	0	0	A	−	Anti-c, anti-E, anti-K, anti-Fya, anti-Jka	2	2
SR000005	+	0	+	0	0	+	+	+	0	0	+	+	+	+	++	A	+	Anti-C, anti-E, anti-K	6	4
SR000006	+	+	+	+	0	+	+	+	+	+	+	+	+	+	0	B	+	Anti-K	2	2
SR000007	+	0	+	0	0	+	+	+	+	0	+	+	+	+	0	A	−		2	0
SR000008	+	0	+	0	0	+	+	0	0	+	+	+	+	0	0	O	−	Anti-C	2	2
SR000009	+	0	+	0	0	+	+	+	+	+	+	+	+	+	0	B	−	Anti-C, anti-E, anti-K	2	2
SR000010	+	0	+	0	0	+	+	0	0	+	+	+	+	0	0	AB	−	Anti-K	2	0

Left: xHEA profile of ten alloimmunized patients determined by BeadChip HEA analysis (except for ABO and D). *Right*: Alloantibodies identified by serology (provided by medical center)

phenotypes are still challenging. In these extremely unusual cases, Children's Hospital Boston relies on blood suppliers with large inventories to supply needed units.

As described in Chap. 4, rapid-throughput DNA testing has greatly expedited the identification of donors with rare phenotypes and those patients who are negative for multiple antigens. At Cedar-Sinai Medical Center, BeadChip™ typing has provided greater flexibility and less reliance on local blood suppliers for antigen-negative units. Screening donors locally makes sense because the probability of finding donors with phenotypes that are associated with specific ethnic or racial groups is higher in areas where people linked to those phenotypes are living.

At Cedar-Sinai Medical Center, the number of rare phenotype units in their inventory have grown because BeadChip™ typing has the capacity to detect high- and low-prevalence antigens, such as U^-, U_{var}, and r'^S that cannot be resolved with serology. Cedars-Sinai Medical Center has found that 3.5% of patients and 2.5% of donors have rare phenotypes [k−, Kp(b−), Js(b−), Di(b−), Co(a−), LW(a−), and SC:−1].

At present, Cedars-Sinai Medical Center maintains an inventory of about 1,000 units, of these about 200 units have been genotyped and are stored in labeled drawers for easy access. On an average 150–200 antigen-negative units are transfused each month, and the purchase of antigen-negative units has decreased by an average of 130 units per month. Most of the purchased antigen-negative units are for highly alloimmunized patients with SCD. Nevertheless, the reduction in purchases of antigen-negative units has resulted in an average monthly cost savings of $7,000 per month. The availability of phenotyped units has led to a reduction in TAT for issuing antigen-negative units from 6 h before implementation of DNA typing to approximately 3 h. All antigen-negative units are confirmed serologically prior to issue [11].

All of the medical centers subscribe to the proficiency program developed and implemented through LifeShare Blood Centers. The proficiency program is described in Chap. 4. Briefly, proficiency testing samples that reflect various blood group alleles, silencing mutations, and rare blood groups are sent to participating laboratories twice per year. At the Mayo Clinic, the BeadChip™ results are confirmed with serology prior to crossmatching and the staff investigates any issues or discrepancies. Children's National Medical Center confirms Rh antigens for all new patients with SCD with serology, largely because of Rh system complexity. Water is always used as a negative control. Previously typed samples are used as positive controls in every run or in some cases with each new lot of BeadChips™.

5.5 Billing for Reference Lab Testing

Until recently, the comparative costs for serology and BeadChip™ analysis were similar. The savings generated by shortened technician time offset the increased costs for material. Since the price of antisera has increased recently, HEA typing has become the more cost-effective method at Children's National Medical Center.

Following validation at the Clinical Laboratory Improvement Amendments-certified lab, molecular tests are billed using current CPT codes for molecular testing (College of American Pathologists, CAP, guidelines). At other medical centers that are not described in this chapter, automated consultation is in place. Patients with some specified conditions are tested at diagnosis for BeadChip™ analysis. At Children's National Medical Center, the general cost for a blood unit can cover a portion of the cost of donor typing. The patient is responsible for the remainder. Patient typing is charged by the CPT code (see Chap. 4). The hospital is responsible for securing the payment, not the laboratory.

When Children's Hospital Boston provides HEA testing for other hospitals, they are billed for the services.

At Cedars-Sinai Medical Center, BeadChip™ testing can be billed as a reference lab test, but an automatic consultation process is not in place at this time. As a result, covering the staffing costs and reporting of results remains an issue.

5.6 Outlook

Implementation of DNA analysis in the hospital transfusion service for purposes of predicting an extended red cell phenotype has demonstrated operational and clinical efficiencies when managing patients with complex serologic workups or in those patients who require antigen-matched blood to prevent alloimmunization. The ability to rapidly determine the extended phenotype of both donors and patients in a high-throughput system allows the transfusion service to maintain an inventory of phenotyped blood available for transfusion, once the required antigens have been confirmed serologically. Data management tools are important components of an efficiently applied system to store data for each donor unit as well as to facilitate unit tracking and location. Present system enhancements include the dxBOT™ that operates through an electronic interface to allow the DNA testing results to be reviewed then entered electronically to each donor unit file. This system has improved efficiency by eliminating the need to manually enter multiple data elements. Implementation of an electronic inventory management function as described by Klapper et al. [14] will greatly enhance the practical applications of DNA typing systems in the hospital and donor transfusion services.

5.7 Summary

The use of molecular testing has emerged as a useful means of providing extended phenotype-matched units for patients with a variety of clinical conditions. Some of the patients most likely to benefit from extended phenotype-matched blood are those who are expected to receive multiple transfusions over their lifetime, thus increasing their risk for alloimmunization. Additionally, molecular typing methods

have particular value for pretransfusion testing of recently transfused patients and resolution of complex serologic workups.

Broad application of xHEA typing is predicted to substantially reduce transfusion-related alloantibody production. A retrospective review of SCD patients who received transfusions with major antigen-matched units (ABO and D) found nearly 40% had developed alloantibodies to at least one blood group antigen over a 12-year period [6]. The analysis further revealed that extended phenotype matching for C, c, E, e, K, S, Fy^a, and Jk^b would have prevented alloantibody production in 71% of those patients. As the authors of this 2002 review have indicated, extended phenotype matching was not a practical option at the time these patients were transfused [6]. The development of high-throughput DNA typing provides a practical means of providing extended phenotype-matched red blood cells for many transfusion recipients (see Chap. 4).

DNA analysis using the BeadChip™ system is the first technology to emerge with the potential to revolutionize transfusion services through routine use. The use of BioArray technology for DNA testing has already provided benefits for transfusion services at medical centers that focus primarily on patient services and donor centers that are responsible for providing a large volume of antigen-negative donor units. In both settings, maintenance of screened inventories has greatly reduced the TAT for in-house screening. With regard to transfusion services, there is reduced reliance on donor centers to supply units that are negative for multiple antigens and for those patients with rare blood types.

The user experience described here includes both large and small hospitals as well as small reference laboratories. Valuable feedback and the experience of users have provided information for product enhancements that has led to the development of second generation HEA 1.2 tests. This has eliminated the initial discordance of RhC. Simple key process steps enable the implementation of BeadChip™ in blood centers and transfusion services where molecular testing generally is not performed.

The semiautomated detection process and computerized analysis algorithms provide test results without the need for operator interpretation or data manipulation. These features make molecular testing widely available to both large and small hospital laboratories. This reduces the need for skilled technicians to perform multiple complex serology workups at hospitals and laboratories of all sizes. Costs of in-house tests are significantly lower than the acquisition of test results from workups that are routinely sent out or costs may even be completely eliminated.

In some institutions, current CPT codes have been used successfully for billing (see Chap. 4). This suggests that the BeadChip™ system can provide hospitals and reference laboratories with an additional revenue source while allowing them to better serve their patients with faster and more accurate test results.

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Chapter 6
Human Platelet Antigen Genotyping and Diagnosis of Antiplatelet Alloimmunization

Gerald Bertrand and Cecile Kaplan-Gouet

Abstract Rapid genotyping of human platelets can improve the diagnosis and treatment of neonatal alloimmune thrombocytopenia and minimize the risks of post-transfusion purpura and refractoriness to random donor platelet therapy. Advances in genotyping using microarray technology allow the same sample to be screened for multiple polymorphisms without the need for reference sera or large sample size. At the National Institute of Blood Transfusion in Paris, France, 200 samples were screened using polymerase chain reaction (PCR)-sequence-specific primer or PCR-restriction fragment length polymorphism and BioArray BeadChip™ platform to evaluate the accuracy and liability of human platelet antigen genotyping. The results of this analysis are presented along with methods to minimize the impact of genotypic errors resulting from rare silent mutations.

Keywords Assays • Methods • High-throughput screening

6.1 Discovery of Human Platelet Antigens

Antibodies directed against human platelet alloantigens can result in neonatal alloimmune thrombocytopenia and post-transfusion purpura. These antibodies can also render patients refractory to transfusion therapy with random donor platelets. Despite awareness of the immunological basis of neonatal thrombocytopenia [1] and identification of maternal alloantibodies [2] almost 60 years ago, difficulties exist in the diagnosis and treatment of platelet alloantigen-related clinical conditions.

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Table 6.1 Major human platelet antigens

System	Antigen	Alternative names	Glycoprotein
HPA-1	HPA-1a	Zw^a, PI^{A1}	GPIIIa
	HPA-1b	Zw^b, PI^{A2}	
HPA-2	HPA-2a	Ko^b	GPIbα
	HPA-2b	Ko^a, Sib^a	
HPA-3	HPA-3a	Bak^a, Lek^a	GPIIb
	HPA-3b	Bak^b	
HPA-4	HPA-4a	Yuk^b, Pen^a	GPIIIa
	HPA-4b	Yuk^a, Pen^b	
HPA-5	HPA-5a	Br^b, Zav^b	GPIa
	HPA-5b	Br^a, Zav^a, Hc^a	

von dem Borne and Décary [8] and Metcalfe et al. [9]
HPA human platelet antigen

Laboratory diagnosis and treatment of human platelet antigen (HPA) alloimmunization relies on the detection of the alloantibody and identification of the offending antigen.

In 1953, the first maternal alloantibody was described by Moulinier [2] in France. Then the so-called Zw^a [3] or PI^{A1} or HPA-1a antigen was reported as a serologically and genetically defined platelet antigen [4]. It took until 1980 to find another platelet-specific antigen that could be involved in neonatal alloimmune thrombocytopenia [5]. The molecular basis for HPA-1a/HPA-1b (PI^{A1}/PI^{A2}) alloantigen polymorphism was elucidated in 1989 leading to the development of molecular testing and characterization of platelet antigens [6]. A major breakthrough in platelet immunology was the antigen-capture assays with monoclonal antibodies [monoclonal antibody-specific immobilization of platelet antigens (MAIPA)], leading to the description of new specific platelet antigens [7] and adoption of a platelet nomenclature. Thus far, 24 platelet-specific alloantigens (Table 6.1) [9] have been defined and the number of rare platelet antigens being reported is growing (Table 6.2). Molecular analysis has revealed that single nucleotide polymorphisms (SNPs) in genes encoding platelet glycoproteins account for a substantial proportion of platelet alloantigens.

6.2 Serologic Detection of HPA

Platelet phenotyping with MAIPA was frequently used to identify the culprit alloantigens. The MAIPA method directly exposes platelets to the reference serum as well as monoclonal antibodies directed to glycoprotein of interest. These trimolecular complexes (platelet glycoprotein–HPA–monoclonal antibody) are isolated and analyzed using an immunoabsorbent colorimetric assay. However, the need for reference sera and the time required for test completion are limitations of this method.

Table 6.2 Rare or private human platelet antigens

Antigen	Alternative names	Phenotype frequency* (%)	Glycoprotein	Amino acid change
HPA-6bw	a^a, Tu^a	0.7	GPIIIa	Arginine[489] Glutamine[489]
HPA-7bw	Mo	0.2	GPIIIa	Proline[407] Alanine[407]
HPA-8bw	Sr^a	<0.01	GPIIIa	Arginine[636] Cysteine[636]
HPA-9bw	Max^a	0.6	GPIIb	Valine[837] Methionine[837]
HPA-10bw	La^a	<1.6	GPIIIa	Arginine[62] Glutamine[62]
HPA-11bw	Gro^a	<0.25	GPIIIa	Arginine[633] Histidine[633]
HPA-12bw	Iy^a	0.4	GPIbβ	Glycine[15] Glutamic acid[15]
HPA-13bw	Sit^a	0.25	GPIa	Threonine[799] Methionine[799]
HPA-14bw	Oe^a	<0.17	GPIIIa	Lysine[611] deletion
HPA-15	Gov^a Gov^b	60.2 (81) 80.5 (74)	CD109	Tyrosine[703] Serine[703]
HPA-16bw	Duv^a	<1.0	GPIIIa	Threonine[140] Isoleucine[140]
HPA-17bw	Va^a		GPIIIa	Threonine[195] Methionine[195]

*Phenotype frequencies are reported frequencies in Caucasian populations

Moreover, MAIPA requires 3.0×10^7 platelets to screen for a single antigen, which precludes platelet typing for patients with thrombocytopenia.

6.3 Introduction of Molecular HPA Typing

Genotyping using polymerase chain reaction (PCR) techniques are particularly attractive because no platelets are needed. PCR with sequence-specific primers (PCR-SSP), restriction fragment length polymorphism (RFLP), and real-time PCR are now routinely used in laboratories dealing with platelet alloimmunization. The introduction of BeadChip™ technology (BioArray Solutions, Warren, NJ) has the added benefit of high-throughput screening of a single sample for as many as 17 HPA (Fig. 6.1).

At the National Institute of Blood Transfusion in Paris, France, 200 samples were tested using the BeadChip™ system to evaluate the accuracy and liability of HPA genotyping. These samples had been previously genotyped by PCR-SSP or PCR-RFLP

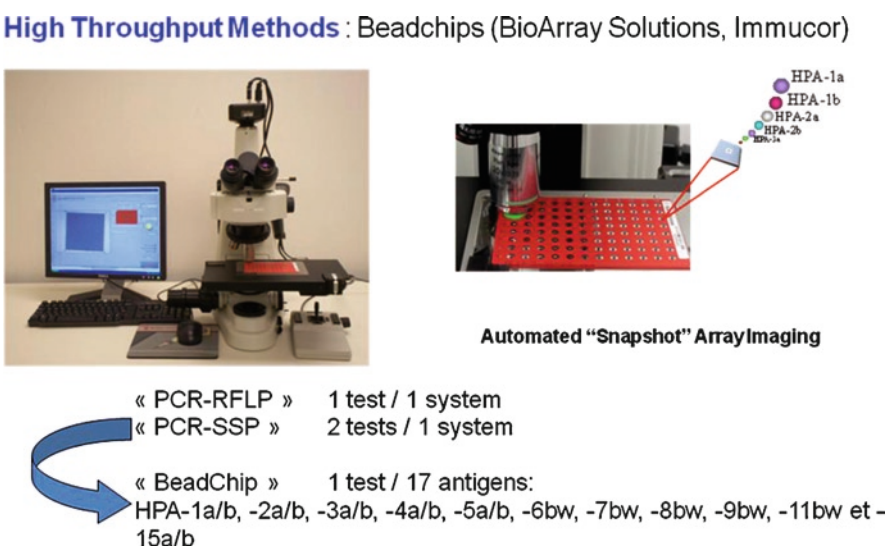

Fig. 6.1 BeadChip™ high-throughput method is described

(December 2005 to February 2009). As HPA-1a is the most frequent platelet antigen implicated on alloimmunization, the selected DNA panel contained an equal proportion of each HPA-1 genotype (HPA-1aa, -1ab, and -1bb). Discrepancies between the genotyping methods were analyzed. Protocols using diverse techniques to reduce false assignment due to unknown polymorphisms have therefore been proposed.

6.4 Performance Study of the BeadChip™

To evaluate the accuracy of HPA genotyping using PCR-SSP, PCR-RFLP, and BioArray technology, 200 samples were analyzed for 17 HPA antigens (Fig. 6.1) and results were compared. Results were 100% concordant for HPA-2, 3, 4, 5, 6, 7, 8, 9, 11, and 15 for all three combinations of AA, AB, and BB genotypes (Table 6.3), while a 97% concordance was observed for HPA-1 AB samples.

The results derived from HPA-1 analysis of DNA samples from patients referred to laboratory for alloimmunization investigation were compared (Table 6.3) using the two methods. Among the 200 DNA samples representing all three possible HPA-1 genotypes in approximately equal proportion, genotyping results were found to be discrepant with the two techniques in six cases (3%). In all discordant cases, samples were found to be heterozygous (HPA-1ab) using PCR-SSP/RFLP plus sequencing compared with homozygous HPA-1bb on the HPA BeadChip™.

Sequencing of the discordant samples revealed the presence of a silent mutation in intron 2 of the GPIIIa gene (already registered as dbSNP no. 988684) (Fig. 6.2). This SNP is localized in the middle of the forward primer of HPA-1 multiplex PCR,

Table 6.3 Samples identified with HPA antigens: genotype/phenotype concordance

	Set 1 (200 samples)				Set 2 (76 samples)			
	AA	AB	BB	Concordance (%)	AA	AB	BB	Concordance (%)
HPA-1	67	69	66	97	25	20	31	100
HPA-2	151	42	9	100	49	22	5	100
HPA-3	69	104	29	100	25	38	13	100
HPA-4	191	1	0	100	76	0	0	100
HPA-5	149	45	8	100	56	3	17	100
HPA-6	196	4	0	100	74	2	0	100
HPA-7	200	0	0	100	76	0	0	100
HPA-8	200	0	0	100	76	0	0	100
HPA-9	197	3	0	100	75	1	0	100
HPA-11	200	0	0	100	76	0	0	100
HPA-15	52	96	54	100	21	37	18	100

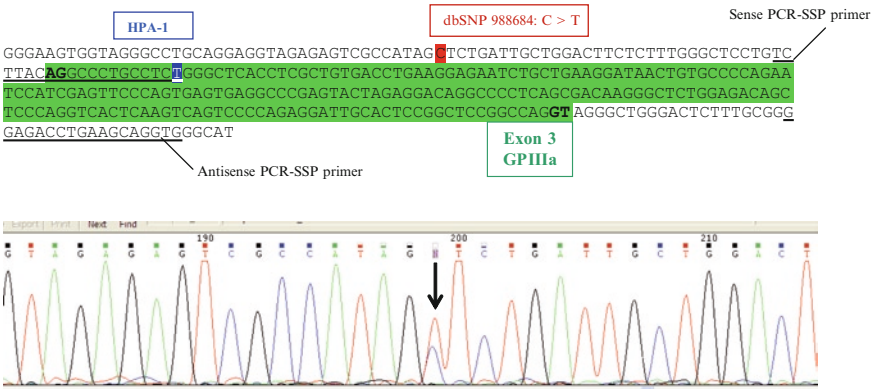

Fig. 6.2 Sequencing of intron 2 and exon 3 (HPA-1 polymorphism) of the gene coding for GPIIIa. PCR-SSP primers for HPA-1 genotyping are underlined. dbSNP 988684 was located outside of one primer of the BioArray kit (first version) and was responsible for erroneous HPA-1 genotyping

and interfered with the proper amplification of the HPA-1a allele. This polymorphism is found to have an allele rate of approximately 9% of the HapMap population of Han Chinese in Beijing (HCB [10]).

As a consequence of these results, the forward primer for HPA-1 was modified with a mixed base at the SNP location to accommodate for the population harboring the mutation by manufacturer and the new version of the kit was used for a second performance study by INTS. The HPA genotyping of the same DNA panel revealed a 100% concordance between PCR-SSP/RFLP results and BeadChip™, allowing the agreement of the European Union for the in vitro diagnostic (IVD) label.

6.5 Experience After 6 Months

BioArray platelet genotyping has been routinely implemented at INTS since September 2009. About 500 DNA samples have been typed with this technology. Genotyping was successful in 95% of the DNAs. For the remaining 5%, one or more antigens were genotyped for individual amplicons by PCR-SSP producing indeterminate calls with BioArray due to sample quality or quantity.

Among the rare HPA, the BioArray technology allowed the identification of two HPA-9abw individuals.

6.6 Advantages of Molecular HPA Typing

Laboratory investigation for common HPA is no longer sufficient to evaluate maternal immunization or the potential for alloantigen sensitization among high-risk patients. HPA-9bw has been shown to be involved in many neonatal alloimmune thrombocytopenia cases [11, 12] and new platelet antigens are still being described [13, 14].

The availability of reliable techniques now makes genotyping possible for routine platelet typing that does not require reference sera or large numbers of platelets. Moreover, the development of BeadChip™ arrays and automation has the potential to routinely screen each sample for multiple polymorphisms. However, because the true genotype can be masked by a small percentage of unknown polymorphisms, diversification of techniques remains important to ensure accurate HPA antigenic system typing.

A case reported by Bertrand et al. [15] illustrates the impact of unknown polymorphisms on platelet genotyping. Mild fetal ventriculomegaly was discovered in a fetus at 21 weeks of gestation following routine ultrasound scan. Parental genotyping using PCR-SSP indicated that the mother was HPA-1b homozygous and the father was HPA-1a homozygous. Phenotypic analysis showed the mother to be heterozygous. PCR-RFLP analysis and subsequent sequencing identified a heterozygous mutation 262T>C in the glycoprotein IIIa exon 3 of the mother (Fig. 6.3). Because the mutation was present in the antisense primer of the HPA-1 PCR-SSP, only the mother's HPA-1b sequence could be amplified. A frequency study showed that the 262T>C polymorphism is not restricted to a single family, but is also found in African populations such as in the Cameroonian population where the frequency is 17.5% [16].

Therefore, national and international collaborations are of importance for further improvement. Platelet immunological investigations due to complexity of the diagnosis for alloimmunization should be performed in a skilled laboratory. Even if a screening may be done locally, the samples should be sent to reference laboratories participating in international wet workshops and proficiency exercises.

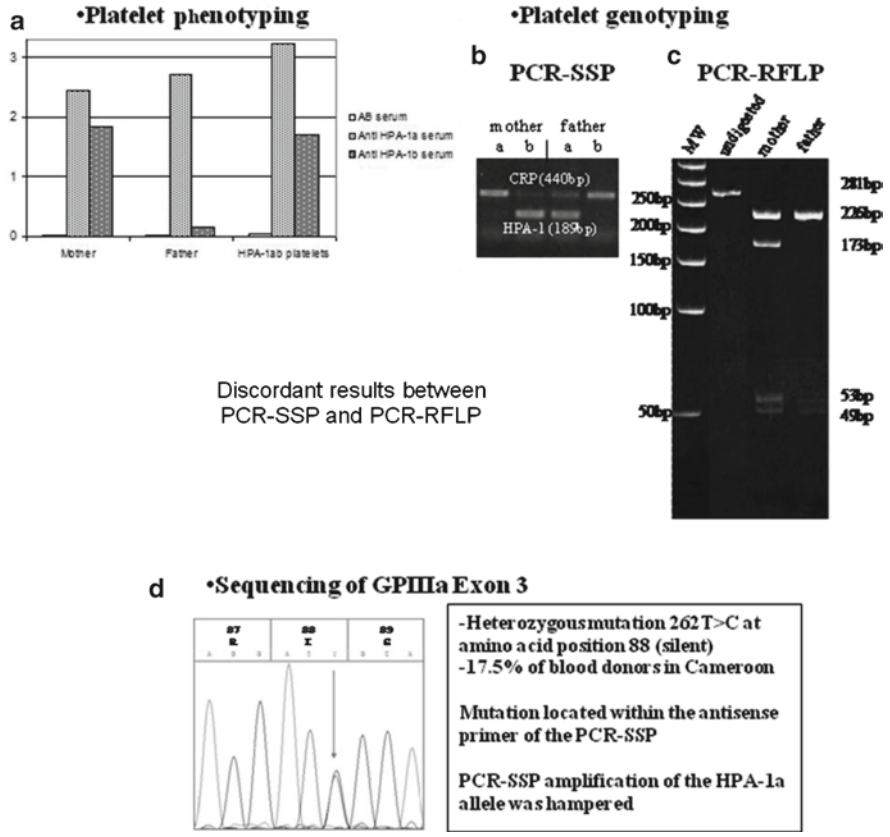

Fig. 6.3 Identification of the 262T>C mutation of the GPIIIa gene. Platelet phenotyping of the parents (**a**) was performed with the MAIPA technique. Genotyping was done by PCR-SSP (**b**, HPA-1bb mother's genotyping) or PCR-RFLP (**c**, HPA-1ab mother's genotyping). (**d**) Sequencing of the GPIIIa exon 3 of the mother

6.7 Materials and Methods

DNA extraction was performed on 400 μL of whole blood for mothers, fathers, or newborns using an extractor (MagnaPure Compact extractor, Roche Diagnostic, Meylan, France). For fetal genotyping, DNA extraction was done on amniotic cells using the QIAamp DNA Blood Mini Kit (Qiagen, Courtaboeuf, France).

6.7.1 Genotyping

PCR-SSP was performed for HPA-1 to -9, -11, and -15 genotyping, as initially described by Kluter et al. [17] and Lyou et al. [18] with modifications [19]. For any

problem of interpretation in PCR-SSP genotyping, PCR-RFLP method was used [16]. Finally, BeadChip™ DNA analysis was performed according to the instructions of the manufacturer (BioArray Solutions, Warren, NJ, USA).

HPA-1a, -3a, and -5b phenotyping were performed with the MAIPA [7] procedure with reference serums containing anti-HPA-1a, -3a, and -5b alloantibodies, respectively.

Sequencing was performed with a cycle sequencing kit, on an automated DNA sequencing machine (BigDye Terminator v3.1 and 3730XL DNA analyzer, Applied BioSystems, Genoscreen, Lille, France).

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Chapter 7
Blood Group Genotyping by High-Throughput DNA-Analysis: Application to the Panel National de Référence du CNRGS

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Abstract The use of blood group genotyping for the prediction of antigen expression has been discussed in clinical transfusion settings, but much less for reagent red blood cells' (RBCs) selection. In France, the Centre National de Référence pour les Groupes Sanguins (CNRGS) produces a reference panel of reagent RBCs, mainly used for red cell antibody identification. The use of high-throughput DNA-analysis has never been applied to blood donors whose RBCs are used as reagents. The aim of this study was to compare the serological phenotype and that predicted from DNA analysis in such donors, and to determine the benefit of DNA analysis in reagent RBC selection strategy.

Keywords Reference panel • Prevalence • Phenotype • RBC • Antigen expression

7.1 Introduction

In France, the Centre National de Référence pour les Groupes Sanguins (CNRGS) produces a reference panel of reagent red blood cells (RBCs) named "the Panel National de Référence du CNRGS." It is mainly used for RBC antibody identification and as positive or negative control for RBC phenotype analysis. It received the CE marking according to the 98/79/EC European directive, and the certification for the EN ISO 13485 standard in 2005. This panel is composed of a minimum of 14 different reagent RBCs, and usually numbers around 15. In France, the phenotypes of reagent RBCs composing a panel for antibody identification are mandatory [1]. All panel cells are group O (ABO: −1, −2, −3).

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The following antigens have to be represented upon the RBCs: RH1(D), RH2(C), RH3(E), RH4(c), RH5(e), RH6(f), RH8(C^w), KEL1(K), KEL2(k), KEL3(Kp^a), KEL4(Kp^b), FY1(Fy^a), FY2(Fy^b), JK1(Jk^a), JK2(Jk^b), MNS1(M), MNS2(N), MNS3(S), MNS4(s), LE1(Le^a), LE2(Le^b), P1, LU1(Lu^a), and LU2(Lu^b). The "homozygous" phenotypic expression is mandatory for FY1, JK1, JK2, and MNS3 antigens, and recommended for FY2 and MNS4 antigens. Finally, at least two RBCs with the following phenotypes are needed: KEL:1 (K+), FY:1,−2 [Fy(a+b−)], FY:−1,2 [Fy(a−b+)], JK:1,−2 [Jk(a+b−)], JK:−1,2 [Jk(a−b+)], MNS:3,−4 (S+s−), MNS:−3,4 (S−s+), and P1:−1 (P_1−). In addition, the CNRGS has documented that RBCs of the Panel National de Référence are negative for the DI3(Wr^a), KEL6(Js^a), MNS9(Vw), and SC2(Sc2) low-prevalence antigens and positive for the GE2(Ge2), GLOB1(P), KEL7(Js^b), and VEL1(Vel) high-prevalence antigens.

7.2 Analysis

We analyzed the concordance between the serological phenotype of RBCs from 356 different blood donors used for the Panel National de Référence du CNRGS and the phenotype predicted from DNA analysis. Samples from 356 voluntary and regular blood donors were collected as part of the production of the standard Panel National de Référence du CNRGS.

The phenotypes were established by agglutination studies using EDTA blood samples. RBCs from each donor were phenotyped by two different technicians with two different reagents: one monoclonal antibody and, when available, one polyclonal antibody for 17 blood group systems and 41 antigens (Table 7.1). Reagents from Diamed (DiaMed, Cressier/Morat, Switzerland), Diagast (Diagast, Loos, France), Ortho BioVue System (Ortho Clinical Diagnostics, Raritan, NJ), Bio-Rad (Bio-Rad, Marnes-la-Coquette, France), and Biotest (Biotest, Dreieich, Germany) were used according to the manufacturers' recommendations.

The molecular analysis was performed using the Human Erythrocyte Antigen 1.2 (HEA 1.2) BeadChips™ from BioArray Solutions Ltd. (Immucor, BioArray Solutions, Warren, NJ) with a previously described protocol.

The HEA 1.2 BeadChips™ genotyping allowed for the analysis of 34 antigens from 11 blood group systems for 356 different panel donors. The comparison between serological phenotype and that predicted from genotype held on 25 antigens from 10 blood group systems for each panel donor. A total of 8,876 antigens were compared. The predicted phenotypes were concordant in 99.95% of cases [2]. Four discordant results were found. In two cases related to the LU and DO system respectively, our data demonstrated that DNA-based analysis could help when RBC antigen expression was weakened to a level where it was undetectable by standard typing yet detectable with an adsorption–elution technique. In two other cases related to the RH and KEL systems, the discrepancies observed between RBC antigen expression determined by the phenotyping method and that predicted from

Table 7.1 Blood group systems and antigens analyzed by serology (CNRGS phenotype) or predicted from DNA analysis by HEA 1.2 BeadChips™ (BioArray Solutions)

Blood group system	ISBT	Common	CNRGS phenotype	HEA 1.2 BeadChip
RH	1	D	+	NT
	2	C	+	+
	3	E	+	+
	4	c	+	+
	5	e	+	+
	8	C^w	+	NT
	10	V	NT	+
	20	VS	NT	+
KEL	1	K	+	+
	2	k	+	+
	3	Kp^a	+	+
	4	Kp^b	+	+
	6	Js^a	+	+
	7	Js^b	+	+
FY	1	Fy^a	+	+
	2	Fy^b	+	+
JK	1	Jk^a	+	+
	2	Jk^b	+	+
LE	1	Le^a	+	NT
	2	Le^b	+	NT
MNS	1	M	+	+
	2	N	+	+
	3	S	+	+
	4	s	+	+
	9	Vw	+	NT
P	1	P1	+	NT
LU	1	Lu^a	+	+
	2	Lu^b	+	+
	19		+	NT
DO	1	Do^a	+	+
	2	Do^b	+	+
	4	Hy	NT	+
	5	Jo^a	NT	+
YT	1	Yt^a	+	NT
	2	Yt^b	+	NT
CO	1	Co^a	+	+
	2	Co^b	+	+
XG	1	Xg^a	+	NT
DI	1	Di^a	NT	+
	2	Di^b	NT	+
	3	Wr^a	+	NT
LW	5	LW^a	NT	+
	7	LW^b	NT	+
SC	1	Sc1	NT	+
	2	Sc2	+	+
GE	2	Ge2	+	NT
GLOB	1	P	+	NT
VEL	1	Vel	+	NT

+ tested, NT not tested

DNA analysis suggested the presence of RH and KEL variants, respectively, leading us to perform ongoing additional molecular biology studies.

The genotyping precision on the Duffy blood group system is of particular interest. In this system, two DNA polymorphisms (*FY*02M* allele and *FY*02N.01* allele) have been described to encode weakened or silenced expression of FY2 antigen [3,4]. Consequently, *FY*A/FY*B* analysis has to include these DNA polymorphisms to predict FY phenotype. Implementation of high-throughput genotyping with the BeadChips™ should help in the selection of reagent RBCs for accurate anti-FY identification by facilitating the systematic identification of these *FY* polymorphisms.

7.3 Conclusion

Systematic blood group system DNA analysis of reagent RBCs supplies important information for the notion of "antigen in double dose," which is specified in several countries by government bodies. At present, this study makes the Panel National de Référence du CNRGS the only commercialized panel of reagent RBCs with a serologic typing and a corresponding molecular characterization, justifying its use as a reference reagent. Considering these results, we think that the genotyping should be used to replace one of the serological studies.

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Chapter 8
Implementation and Assessment of High-Throughput Donor Typing at the Milan, Italy, Immunohematology Reference Laboratory

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Abstract In 2005, the Centro Transfusionale e di Immunomatologia, Dipartimento di Medicina Rigenerativa, an Immunohematology Reference Laboratory in Milan, Italy, instituted a rare donor program to address the transfusion needs of patients with complex immunization to red cell antigens with a rare phenotype. From June 2005 to December 2008, the laboratory used a high-productivity system (Galileo, Immucor, Norcross, GA) for mass-scale antigen screening with profile 1 and 2 antigens for select donors, where 48,715 blood donors were typed with the identification of 6,634 rare blood donors. In April 2009, the laboratory adopted the BeadChip™ platform (BioArray Solutions, Ltd., Warren, NJ) for large-scale DNA typing. The decision to implement was to expand the panel of red blood cell and platelet antigens using the human erythrocyte antigen (HEA) and human platelet antigen (HPA) BeadChip™ formats. As recommended by international guidelines, a validation plan was used to evaluate the sensitivity and specificity of the method. The results of our testing are described in this chapter.

Keywords DNA array • Blood donor • Human erythrocyte antigen • Human platelet antigen • Immunohematology reference laboratory • Platelet • Phenotype

8.1 Introduction

The identification of irregular red blood cell antibodies in patients undergoing transfusion is crucial in the pursuit of finding compatible blood components [1–3]. The Centro Trasfusionale e di Immunoematologia at Policlinico Maggiore Hospital in Milan, which began in 1974, achieved its status as a highly specialized unit

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supplying essential diagnostic services to patients in 1992. The laboratory's accreditation by the American Association of Blood Banks as an immunohematology reference laboratory (IRL) was attained in 2003. As such, our laboratory was expected to provide refined immunohematological methods and specialized diagnostic and advisory services, particularly for patients referred by general practitioners and those whose needs were not met by other institutions [4]. The IRL standards of our institution are set by the American Association of Blood Banks [5–7].

To meet the transfusion needs of critically ill patients with complex immunization to red cell antigens with a rare phenotype, a rare donor program was started in January 2005. The goals of the rare blood donors' bank were to:

1. Identify rare donors in the Lombardy region
2. Establish a regional register
3. Organize a regional bank of liquid and frozen rare units
4. Support activities to ensure IRL status.

The caliber of services provided by the Centro Trasfusionale e di Immunoematologia at Policlinico Maggiore Hospital requires the use of methods based on various technologies, the use of rare reagents, sera, and cells, the maintenance of a donor bank with rare red cell and platelet antigen phenotypes, and collaboration with international centers. The IRL continues to require the adoption, implementation, and evaluation of new techniques as they arise in the field of immunohematology [4].

8.2 BeadChip™ System Implementation

From June 2005 to December 2008, the laboratory used a high-productivity system (Galileo, Immucor, Norcross, GA) for mass-scale antigen screening with profile 1 antigens (Fy^a, Fy^b, Jk^a, Jk^b, S, s, and Lu^b) and profile 2 antigens (Co^a, Js^b, Ge:2, Kp^b, PP_1P^k, U, and Vel/Yt^a) for selected donors of group A or O, K, negative with CCDee (R1R1), ccdee (rr), ccDEE (R2R2), and ccDee (RoRo) phenotype. In April 2009, the laboratory adopted the BeadChip™ platform (BioArray Solutions, Ltd., Warren, NJ) for large-scale DNA typing and decided to use the human erythrocyte antigen (HEA) and human platelet antigen (HPA) BeadChip™ formats to expand our panel of red blood cell and platelet antigens in our reference library. As recommended by international guidelines, a validation plan was used to evaluate the sensitivity and specificity of the method prior to implementation of the technique.

Using the Galileo system (Immucor, Norcross, GA), 48,715 blood donors were typed and 6,634 rare blood donors (13.6%) were identified. Of these, 2,578 (38.9%) were identified as S−s+ group O and 2,266 (34.1%) as S−s+ group A. Additionally, there were 656 (9.9%) S+s− group A and 782 (11.8%) S+s− group O. Three hundred fifty-two (5.3%) donors were identified as rare for high-incidence antigens or Rh phenotype.

Prior to our implementation of HEA and HPA BeadChip™ for genotyping of red cell and platelet antigens, we evaluated the sensitivity and specificity of the method

using the following DNA samples. For the HPA BeadChip™, 202 blood donor samples were tested. Of these, 155 were typed for HPA 1a/b, 2a/b, 3a/b, 4a/b, 5a/b, 15a/b and 47 for HPA 1a/b, 2a/b, 3a/b, 5a/b. Similarly, for the HEA BeadChip™, 257 samples were evaluated. These latter samples included 80 rare donors, 28 donors heterozygous for common antigens, five blood donor samples collected into three different anticoagulants, twenty-three multitransfused patients, twelve patients were HgbS positive, 109 donors of high-incidence rare antigens, and ten samples of known phenotype provided by BioArray Solutions.

The results obtained by BeadChip™ were compared with serology and DNA [sequence-specific primer (SSP) analysis] results to evaluate the reproducibility of the technique.

8.3 Results

8.3.1 HPA BeadChip™

All samples were concordant with polymerase chain reaction (PCR)-SSP for all antigens with the exception of four discrepancies. The HPA BeadChip™ discrepancies included three samples (1.5%) in which BioArray identified HPA-1(a−b+) whereas SSP-PCR indicated 1(a+b+). In one other sample (0.5%), the HPA BeadChip™ identified a sample as HPA-15(a+b−) and the SSP-PCR method showed HPA-15(a+b+).

The three cases with HPA-1a (as indicated in Chap. 6) and one with HPA-15b were resolved with the implementation of mixed base primer designs. This change produced concordant results with SSP-PCR when the same samples were retested.

8.3.2 HEA BeadChip™

Two hundred fifty-seven samples that were previously analyzed with serology and/or another molecular assay based on SSP and Luminiex technology (Luminex Corp., Austin, TX) were selected for BeadChip™ analysis. Of those, a set of samples known to be discordant between serology and SSP were included to challenge the system. Both molecular platforms (BeadChip and SSP) showed complete agreement while discordant with serology indicating either variability of serological reagents (monoclonal vs. polyclonal) or an unknown molecular phenomenon. After BeadChip™ testing, the discordant samples were sequenced for the amplicon and for additional exons known to have silencing mutations (Table 8.1). Out of 257 samples evaluated with the HEA BeadChip™ test, 15 samples were found discrepant. DNA sequencing studies on these samples were performed. For Lu^a/Lu^b, serology versus SSP/BeadChip™ discrepancies were as follows: 12 cases (4.7%) were Lu(a−b−) versus Lu(a−b+) respectively; one sample (0.3%) was Lu(a−b−) versus Lu(a+b+) respectively; and another (0.3%) was Lu(a+b−) versus Lu(a+b+)

Table 8.1 HEA, serology, sequencing, and mutations of patient samples

Sample	HEA	Serology	Sequencing	Mutations identified
1	Jk(a+b+)	Jk(a−b+)	*JK*A/JK*B*	None
2	K+k+	K+k−	*KEL*01.1/KEL*02M.01*	Heterozygous 1088G>A (S363N)
3	K+k+	K+k−	*KEL*01.1/KEL*02*	None
4	Lu(a+b+)	Lu(a+b−)	*LU*A/LU*B*	None
5	Lu(a+b+)	Lu(a+b−)	*LU*A/LU*B*	None
6	Lu(a−b+)	Lu(a−b−)	*LU*B/LU*B*	None
7	Lu(a−b+)	Lu(a−b−)	*LU*B/LU*B*	None
8	Co(a−b+)	Co(a−b−)	*CO*B/CO*B*	None
				Consistent with SSP results

The *EKLF* and the *GATA1* genes have been shown to result in the Lu(a−b−) phenotype. These genes are not currently included in the HEA assay

respectively. For Colton, one sample identified serologically as Co(a−b+) while SSP analysis and BeadChip™ testing showed it to be Co(a+b+) including a newly identified mutation on the COA allele. Additionally, nine other discrepant samples including one Jk^a, two K, five Lu^b, and one Co^b (3.5%) were observed.

The sequencing results of eight samples for red cell antigens were consistent with BeadChip™ testing (Table 8.1) while one sample identified a known silencing mutation.

8.4 Results Following Routine Implementation of HEA and HPA BeadChip™ Analysis

From April to September 2009, there were 1,178 red blood cell donors who were successfully typed with the HEA BeadChip™. An additional 800 donors were typed using the HPA BeadChip™ during this period. Table 8.2 summarizes the number of donors and patients who have been identified with rare typings. Table 8.3 further shows the antigen expression counts and statistics in the donors and patients tested.

Table 8.2 Frequency of donors and patients by type

Type	Donors	Patients
Total screened	1,698	78
Co(a−)	24	0
e−	58	3
Js(b−)	3	0
k−	27	0
Lu(b−)	16	0
Fy(a−b−)	44	9
Jk(b−), S−	245	9

Table 8.3 Antigen expression frequencies in Policlinico Milan from April to September 2009

		Donor		Patient	
System	Antigen	Count	% Expressing Ag	Count	% Expressing Ag
Rh	c	720	44.8	49	64.5
	C	939	58.5	51	67.1
	e	1,377	96.0	71	95.9
	E	81	5.6	19	25.3
Kell	K	36	2.2	5	6.5
	k	1,606	98.3	77	100.0
	Kp^a	40	2.5	10	13.9
	Kp^b	1,625	100.0	76	100.0
	Js^a	11	0.7	1	1.3
	Js^b	1,632	99.8	77	100.0
Kidd	Jk^a	1,196	73.7	57	75.0
	Jk^b	1,130	69.6	57	75.0
Duffy	Fy^a	942	58.3	48	63.2
	Fy^b	1,181	77.2	46	61.3
MNS	M	1,281	79.6	59	77.6
	N	1,121	69.6	53	69.7
	S	781	48.7	48	63.2
	s	1,408	87.7	64	84.2
Lutheran	Lu^a	106	6.6	4	5.3
	Lu^b	1,596	99.0	76	100.0
Diego	Di^a	3	0.2	0	0.0
	Di^b	1,304	100.0	74	100.0
Colton	Co^a	1,606	98.5	76	100.0
	Co^b	108	6.6	2	2.6
Dombrock	Do^a	1,043	64.6	59	77.6
	Do^b	1,337	82.8	61	80.3
	Jo^a	1,614	100.0	76	100.0
	Hy	1,614	100.0	76	100.0
Landsteiner–Weiner	LW^a	1,615	100.0	76	100.0
	LW^b	5	0.3	1	1.3
Scianna	Sc1	1,614	100.0	76	100.0
	Sc2	8	0.5	1	1.4
SCT	HgbS+	3	0.2	22	30.1
	HgbS++	0	0.0	9	12.3

Donors = 1,698; patients = 78

8.5 Conclusion

The BioArray Solutions BeadChip™ is a robust high-throughput method for large-scale genotyping of red blood cell and platelet antigens. This method also provides identification of rare donors who could not be detected due to the paucity or absence of appropriate reagents.

The advancement in molecular technology has allowed for the processing of mass samples and the ability to successfully identify rare or even novel alleles [8–10]. Traditional hemagglutination assays can be used in conjunction with this technique to confirm the findings of both HEA and HPA BeadChip™ assays.

Over the next 3 years, Centro Transfusionale e di Immunoematologia will develop in the following areas. We will continue to provide immunohematological diagnostics for cases of complex red blood cell and platelet immunization and identification of donors with rare phenotypes. We also will focus on large-scale donor typing at the regional level [11, 12]. Specifically, we plan to perform extended phenotyping of 14,500 donors. We intend to identify donors who are IgA-deficient and donors with low levels of anti-T antibodies as well as immunohematological diagnostics for patients with T-activated red blood cells in order to create a registry for these patients [4].

Based on our tests of specificity and sensitivity along with our use of this technique from April 2009 to date, we recommend adding the HEA and HPA BeadChip™ assays to the armamentarium of diagnostic tools in the IRL.

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Chapter 9
Implementation of the BioArray Human Erythrocyte Antigen (HEA) BeadChip™ System: The Spanish Red Cross Blood Centre of Madrid, Spain Experience

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Abstract The Red Cross Blood Centre of Madrid, Spain, is part of the regional organization for blood and plasma donation and typing in Madrid Autonomous Community. To increase the number of patients we serve and identify rare antigens that may be specific to our multiracial population, we implemented the BioArray HEA BeadChip™ system. Herein we describe our experience with the training, installation, and implementation of the HEA BeadChip™ system. We provide information about the blood group antibodies responsible for acute and delayed hemolytic reactions. Future objectives for use are described.

Keywords Alloimmunization • Antigen • DNA array • Blood donor • HEA • HLA • Red Cross • Sickle cell

9.1 Introduction

9.1.1 History and service of the Red Cross Blood Centre of Madrid

The Red Cross Blood Centre of Madrid, Spain (Centro de Transfusión de Cruz Roja Española en Madrid) was created in 1960 and serves more than 20 private hospitals of the Comunidad de Madrid autonomous region. The geographical area served by our Blood Centre has about 6 million inhabitants and is becoming highly multiracial since 2000. That implies the need for expanding the blood donor pool with more individuals of different racial origins. Right now, the blood donor pool is

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composed of 10% of non-Spanish population's in contrast with 14% non-Spanish population out of the total population of Madrid. These demographics make the identification of donors having rarer blood antigens crucial.

Spain has several distinct blood antigens in its varied population. The recent medical literature alone describes the identification of rare blood antigens and chromosomal variation in the Spanish population that present in a variety of disorders, including type I Glanzmann thombasthenia [1], hypercholesterolemia [2], and polycystic kidney disease [3]. In addition to the rare antigens found globally, it is likely that Spain exhibits or does not exhibit antigens specific to its own proband given its history of distinct subpopulations of people within the country and apart from other Hispanic peoples internationally [4–11].

The identification of irregular red blood cell antibodies in patients undergoing transfusion is crucial in the pursuit of finding compatible blood components [12]. Furthermore, with the unique populations found in Spain, additional novel alleles may also be identified such as those found in European nations [13–15].

9.2 Blood Group Classification

The International Society of Blood Transfusion (ISBT), an organization promoting the global standardization and harmonization of blood transfusion, created a system that has provided the genetic classification of 302 antigens. Of these, 67% of the antigens form part of the 30 genetically defined systems that are regulated by 34 genes. Almost all of these genes (33/34) have been cloned and sequenced except for P1. The formation of the Rh, Xg, MNS, and Chido/Rodgers systems are, in each case, defined by two genes. A single gene is responsible for the rest of the 26 systems (ISBT). The genes for ABO, Lewis, H, I, globosides, and system P encode glycosyltransferases for the construction of glycoproteins and oligosaccharides. The remaining genes encode proteins that are expressed in cell membrane.

The larger blood group systems include Rh, with 59 antigens and MNS, with 46 antigens. Eight systems contain only one antigen P, H, Kx, Ok, Raph, I, globoside, and Gill. The classification of blood group systems is designed around the determination of molecular group systems.

Forty antigens that are not included in the 30 antigenic systems are classified into three categories. Collections include antigens that are related serologically, biochemically, or genetically, but do not meet the criteria of a system. The 700 series antigens, which have a very low incidence (<1%), cannot be included in a system or collection. Conversely, the 901 series antigens occur with a high incidence. Yet, these antigens also cannot be included in a system or collection.

With regard to the clinical significance of blood group antibodies, neither acute (AHR) nor delayed hemolytic reaction (DHR) is observed in the Xg, Sciana, LW,

Table 9.1 Clinical significance of blood group antibodies

System	AHR or DHR	HDN
ABO	Severe AHR	Very rarely severe
MNS	Rare active anti-M and anti-N at 37°C can produce AHR and DHR	Anti-M: rare
	Anti-S, s, U, and Mur can cause AHR and DHR	Anti-S, s, U, and Mur can cause severe HDN
P	Rare P active at 37°C can produce AHR and DHR	No
Rh	Severe AHR and DHR	Severe
Lutheran	Moderate DHR	No
Kell	Severe AHR and DHR	Severe
Lewis	Generally not significant	No
Duffy	Anti-Fy^a, Fy^b, and Fy3: AHR and DHR Anti-Fy5: DHR	Anti-Fy^a and Fy^b
Kidd	DHR; anti-Jk^a, and Jk3 can cause AHR	Rare
Diego	Anti-Di^a: one case with DHR; anti-Di^b: no evidence; anti-Wr^a: has caused hemolytic reaction	Severe HDN

AHR acute hemolytic reaction, *DHR* delayed hemolytic reaction, *HDN* hemolytic disease of newborns

Ch/Rg, Cromer, Knops, Raph, and GIL systems. Similarly, hemolytic disease of newborns (HDN) also was not seen in these blood systems.

In blood systems ABO, MNS, P, Rh, Lutheran, Kell, Lewis, Duffy, Kidd, and Diego, acute and DHRs as well as HDN are observed in varying degrees from rare to severe (Table 9.1).

9.3 The BioArray BeadChip™ System

9.3.1 HEA BeadChip™ Assay

The manufacturing process of the BioArray BeadChip™ system (BioArray Solutions, Immucor, Warren, NJ) is described in Chap. 3. Further details of the process have also been described by Hashmi et al. [16, 17]. The HEA BeadChip™ analyzes 32 polymorphisms associated with 11 blood group systems: RH, Kell, Kidd, Duffy, MNS, Dombrock, Colton, Diego, Lutheran, Landsteiner–Wiener, Scianna, and Hemoglobin (HbS).

HEA BeadChip™ provides multiplex analysis of amplified gene fragments of interest from genes defining red blood cell antigens. Annealing of the amplified gene fragments occur with complementary probes (oligonucleotides) that are attached to corresponding colored beads immobilized on the BeadChip™. An elongation reaction extends and fluorescently labels the oligonucleotide pair. Analysis then involves imaging and decoding the BeadChip™ (see Chap. 3).

9.3.2 HEA BeadChip Training

Technicians of Spanish Red Cross Blood Centre of Madrid attended a training program at BioArray Solutions (Warren, NJ) in March 2009. HEA BeadChip™ experts taught the theory and practice of the technique. Technicians learned the fundamentals and development of polymerase chain reaction (PCR) technique as well as concepts related to the BASIS™ software (data analysis software). Technicians demonstrated their understanding of the technique by analyzing batches of 16 samples per person per day.

The HEA BeadChip™ training course required documentation of a list of necessary materials and equipment in addition to operating procedures and work forms. The training manual included sections on DNA extraction using commercial methods, PCR, Clean-up reagent processing, generation of single-stranded DNA (using Lambda exonuclease), eMAP elongation on the BeadChip™, array reading, and BASIS analysis. Other documentation included maintenance and verification procedures, resolution of common problems, and material safety data sheets for reagents.

After the analysis of the 16 samples by each technician, the following results were observed. While the first technical specialist produced batches of 16 samples that were correct and valid, the second technical specialist produced two invalid samples with all other samples correct. We analyzed whether the two invalid samples were an acceptable rate for our department, and both parties determined that the final results were. We concluded that the BioArray course for HEA BeadChip™ training was very well conceived and taught by an expert in the technique.

9.3.3 HEA BeadChip Materials

Equipment for HEA BeadChip™ analysis includes one DNA extractor robot, a laminar flow hood, a thermocycler, two vortexers, two minicentrifuges, a plate centrifuge, an incubator, pipettes (single and multichannel), an AIS 400 reader, a refrigerator at 4°C, and a freezer set at −20°C.

Additional materials include pipette tips with and without filters of various volumes, 96-well plates for PCR, plate seals, 2 ml tubes, eight strip PCR tubes and caps, a cryoblock, reagent racks, and canned air. All of these materials were supplied by BioArray in the starter kit.

Communication requirements for proper HEA BeadChip™ analysis are a broadband internet connection for the transmission of images, data, and results produced by AIS 400 to BASIS™.

The space requirements for the installation of the BeadChip™ system is shelf space of 165 cm wide for the DNA extractor robot, 120 cm wide for the pre-PCR area, and 210 cm wide for the post-PCR area. Red Cross of Spain elected to store the equipment in two rooms as opposed to three – one room for the DNA extraction robot and the other for pre- and post-PCR.

There are two different versions of packaging of the HEA BeadChip™ – one with twelve carriers each with 8-BeadChip™ arrays (for a total of 96 tests) or one carrier with 96 BeadChip™ arrays (96 tests). Both types include the following reagents: HEA PCR mix, Clean-up reagent, Lambda exonuclease, Taq polymerase (CE-marked products only), and eMAP elongation mix. The kit does not include reagents for DNA extraction, however BioArray recommended the Qiagen QIAamp kit. We have chosen to use an automatic DNA extractor and reagents from Roche.

9.3.4 Implementation of HEA BeadChip: DNA Extraction

The automatic extraction robot Magna Pure Compact system kit and Magna Pure Compact nucleic acid isolation kit I (Roche) processed eight samples per batch. The total blood volume used was 200 µl with an elution volume of 100 µl of DNA. The total processing time was 30 min with 10 min needed by the technical specialist.

9.4 Validation of DNA Extraction Technique

In a subsequent training course held in Spain, eight DNA samples were obtained. The DNA samples were required to have a concentration of 10–80 ng/µl and a purity of 1.5–1.95 (A260/A280). The samples were sent to BioArray and met DNA quality requirements with concentrations between 50 and 72 ng/µl.

To validate the performance of the technique, 12 samples were processed for known genotypes sent by BioArray. Ten samples were human DNA and the remaining two were plasmids. Replicates were then analyzed. One DNA sample had three replicates and two other DNA samples had two replicates. The remaining seven DNA samples and the two plasmids were analyzed only once.

We analyzed 35 DNA samples from blood donors of known Rh and Kell phenotypes as well as other defined antigens. The phenotypes were characterized using gel electrophoresis and the WADiana system (Biotech Medical Corporation Sdn Bhd, Kuala Lumpur, Malaysia). The following replicates were then analyzed: 12 samples of four replicates, 18 samples of two replicas, and 5 samples were analyzed only once.

The difficulties that were encountered involved issues not related to the HEA BeadChip™ system itself. They included a 15-day delay on the scheduled date of the training course for acquisition of the DNA extraction Magna Pure Compact system. There was an issue related to the installation of the DSL (Digital Subscriber Line, internet connection) because it had not been configured with physical addresses and the routing of the firewall. Another relatively simple omission was the lack of cotton swabs to clean the chips. There was also difficulty in locating a supplier of compressed air bottles.

Additional DNA extraction validation involved 45 DNA samples and 2 reference control DNA samples used on a total of 112 arrays. The samples were processed

by two technical specialists and a BioArray immunohematology manager. Each technician processed 16 samples per day for a total of 48 samples in 3 days while the BioArray manager processed 16 samples total.

Across the various samples that were analyzed, we obtained the following discrepant validation results: in sample A, an incorrect pipetting error was observed in which two DNA samples were aliquoted into one tube. In sample B, one of three replicates resulted in a very low signal (LS) in all alleles. Sample C produced an IC in Lu^a meaning the results were too close to the cutoff to provide a high-confidence result. Sample D showed a genotype on RHCE that did not match the known phenotype. After two more analyses, the results of the two replicates were observed as identical and consistent with the known phenotype. In our comparison of the results of the three replicas of sample D, we concluded that there was a possible error in pipetting the first sample. The genotype of the replicates (2 and 3) was consistent with the phenotype obtained manually and automatically.

Thirty minutes of technician time was required to extract DNA from 77 DNA samples and 3 controls; 10 min was needed by the technician to extract DNA from eight samples. As such, 80 samples could be processed in 10 h, necessitating only 1 h and 45 min of the specialist's time. The BeadChip™ HEA analysis required 2 h and 15 min for pre-PCR and 3 h for post-PCR assessment. The reading of the arrays and results required 30 min.

9.5 Initial Results of HEA BeadChip™ Array Implementation

We identified eight samples that were misidentified on BASIS™ due to a batch association transcription error through the copy and paste function. Upon reanalysis, we found correct and consistent results with the known phenotype. Only one sample resulted in IC on Lu^a and two samples were ruled invalid due to number of LS and IC. The remaining samples revealed correct and consistent results in concordance with the known phenotype.

9.6 Future Objectives

The 12-month goal of the Spanish Red Cross Blood Centre of Madrid is to genotype 4,000 repeat blood donors. Of these, 3,000 are Spanish donors who are comprised of 1,000 aphresis donors and 2,000 whole blood donors. The remaining 1,000 donors will be of foreign origin. In order to do this we will draw upon the experience of other institutions with large-scale donor typing at the regional level [18, 19]. Additionally, we intend to HPA genotype 1,000 new and/or existing platelet aphresis donors and human lymphocyte antigen (HLA)-I genotype 500 platelet aphresis donors.

Other future objectives include collaboration with BioArray Solutions to obtain the CE mark (the European regulatory conformity standard, received prior

to publication) on the HEA BeadChip™ kit. We intend to increase the availability of blood units that are widely phenotyped for the prevention of alloimmunization in peripubescent children and women of childbearing age. In this group, antibodies against the RhD antigen are not the sole factor in alloimmunization. Risk factors for the presence of other red blood cell antibodies in pregnancy have been documented in the clinical literature by Koelewijn et al. [20]. We also seek to prevent alloimmunization to HLAs in patients who require repeat transfusions of red blood cells in conditions like sickle cell anemia [21, 22] and cancer. We also intend to advance and promote transfusion of compatible blood units to patients who lack a high-frequency antigen (i.e., antigens that occur in >99% of patients). Although infrequent, some patients lack these antigens making transfusion with a matching unit difficult.

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Chapter 10
Looking Beyond HEA: Matching SCD Patients for RH Variants

Marion E. Reid and Christine Halter Hipsky

Abstract Red blood cell (RBC) blood group alloimmunization remains a major problem in transfusion medicine. This is particularly true for chronically transfused patients. Patients with sickle cell disease (SCD) notoriously make more alloantibodies to RBC antigens than any other patient population, especially alloantibodies to Rh antigens. The classical method of testing for blood group antigens and antibodies is hemagglutination. However, this method has certain limitations, some of which can be overcome by testing DNA to predict a blood type. DNA testing allows conservation of antibodies for confirmation by hemagglutination of predicted antigen negativity. High-throughput DNA array platforms allow for the testing of a relatively large number of donors, thereby providing antigen-negative blood to patients in a novel way and possibly preventing immunization. We performed a study to compare the results obtained by using RH BeadChips™ to laboratory-developed tests and to hemagglutination. Two sets of samples were tested: one set chosen had variant Rh types to challenge the BeadChip™ technology. The other set consisted of random samples from African-American donors and SCD patients to estimate the prevalence of RH alleles. The purpose of this testing was to assess the accuracy and efficiency of the BioArray Solutions prototype BeadChip™ system for RH allele determination.

Keywords Blood groups • DNA microarray • DNA testing • Rh blood group system is the correct ISBT term system • Sickle cell disease

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10.1 Background

Red blood cell (RBC) blood group alloimmunization, elicited by antigens on donor RBCs that are absent from the recipient's RBCs, remains a major problem in transfusion medicine. This is particularly true for chronically transfused patients and especially for those with sickle cell disease (SCD) who notoriously make more alloantibodies to RBC antigens than any other patient population. The incident rate of alloimmunization is up to 36% in patients with SCD, compared to 5% for transfusion-dependent patients with thalassemia and approximately 2% in other patient groups who receive RBC transfusion [1–7].

Routine serologic typing and matching of patients and RBC components occurs only for ABO and RhD. Consequently, recipients can develop alloantibodies, notably in the Rh blood group system. For patients who require chronic transfusion therapy, especially where the racial background of the donor pool does not match the racial antigen mix of the recipient population, alloimmunization is a significant problem, e.g., patients with SCD are predominantly of African descent while the donor population tends to be predominantly Caucasian. Some alloantibodies, especially those formed by patients with the so-called partial antigens, are difficult to identify serologically. For example, a patient with RBCs that express a partial D antigen can make anti-D to the 'missing' parts of the antigen and a patient whose RBCs express a partial e antigen can make anti-e. The 'missing' parts of an Rh antigen are most often due to the replacement of amino acids in the RhD protein with those from an RhCE protein, or vice versa, and sometimes to a novel amino acid change. The occurrence of partial antigens in the Rh blood group system is more prevalent in people of African descent than in other populations. In the authors' laboratory, well over half of the samples from African-American donors have at least one variant RH allele. Not infrequently, a variant *RHD* is *in cis* to a variant *RHCE*. The use of high-throughput DNA testing may provide a means to more precisely match donor RBC components to a recipient than is currently possible with hemagglutination. To accomplish this, Hillyer et al. have proposed a scheme for the sequential implementation of the use of RBC components matched to the patient's DNA [8]. The authors suggested to first meet the needs of SCD patients, and then nonsickle cell patients with alloantibodies. This is followed by the use of RBC components with limited DNA-predicted types (namely Rh), and finally to the widespread use of RBC components with more broadly DNA-matching types (e.g., *KEL*, *FY*, *JK*, *S/s*, *DO*). To be successful, implementation of the use of DNA-matched RBC components demands that DNA typing of both donors and recipients be rapid and cost-effective. High-throughput DNA array platforms provide a means to test relatively large numbers of donors. This thereby opens the door to change the way antigen-negative blood is provided to patients and to prevent immunization.

The purpose of this chapter is to review the special needs of patients with SCD and to describe results obtained by using prototype RHD and RHCE BeadChips from BioArray Solutions and to compare the results to those obtained by laboratory-developed tests to predict the Rh phenotype of donors and patients. The results were also

compared to hemagglutination records. Two sets of samples were used for testing. The first set contained variant Rh phenotypes in order to challenge the DNA bead technology. The second set consisted of random samples from African-American donors and from patients with SCD to estimate the prevalence of RH alleles. Data such as these are needed to determine the feasibility of matching the predicted RH types of donor RBC components to transfusion-dependent patients of African ancestry.

10.2 Transfusion Therapy for Patients with Sickle Cell Disease

10.2.1 The Stroke Prevention Trials

Stroke is a devastating complication of SCD and prompted the Stroke Prevention (STOP) trials. The STOP II trial corroborated the efficacy of continuous transfusions to prevent strokes in SCD patients that was established in the STOP I trial [9–11]. So clear were the results of the STOP II trial that the National Heart Lung and Blood Institute (NHLBI) aborted the 6-year trial after only 2 years. Of 79 patients enrolled, 41 were selected to discontinue transfusion. Of these, 14 reverted to high-risk transcranial Doppler ultrasound profiles and resumed transfusion. Two patients suffered a stroke and were transfused while six others resumed transfusion for other reasons. By contrast, none of the 38 patients who continued to receive transfusions had strokes or reverted to a high-risk state. The NHLBI accordingly issued an alert to inform and advise physicians who treat children with SCD that interruption of transfusions for primary stroke prevention is not recommended. Summing up the outcome of the STOP II trial, the Institute's Acting Director, Dr. Barbara Alving, concluded that the study showed, "the value of continuing periodic blood transfusions in preventing the serious and debilitating consequences of strokes. At the same time, there are risks of chronic transfusions and the decision to continue with this treatment must be made on a case-by-case basis." Unfortunately, one major risk of chronic transfusion therapy is alloimmunization and patients with SCD often produce so many alloantibodies to blood group antigens that provision of appropriate antigen-negative blood is problematic. If we are to transfuse these patients effectively and thus prevent strokes and other complications of SCD, we must find more effective ways to reduce the risks of transfusion. The primary method currently used to mitigate RBC alloimmunization is to provide antigen-matched RBC components [12].

10.2.2 Transfusion Therapy in Alloimmunized Patients with SCD

Transfusion management of patients who require chronic transfusion therapy has been the subject of heated debates [13–16]. There is still no consensus as to the best

practical approach although the obvious goal is to provide blood that will survive maximally. Three common approaches used to supply RBC products (ABO and D compatible) to transfusion-dependent patients are to:

1. Give specific antigen-negative RBCs after the patient has made the alloantibody. [This is the traditional approach and performed by 63% of 1,182 participants in a 2003 College of American Pathologists (CAP) survey.]
2. Match for C, E, (c, e), and K antigens (18% in the CAP survey).
3. Match for C, E, c, e, K, Fy^a, Fy^b, Jk^a, and Jk^b antigens (13% in the CAP survey).

Our ability to test a large number of donors for minor antigens has been restricted by labor-intensive hemagglutination-based test procedures and data entry, and limited supplies of typing grade antisera.

Currently, many patients with SCD and alloantibodies to multiple blood group antigens or to an antigen of high prevalence may not be provided with compatible RBC components in a timely manner. While this can lead to an inconvenient delay in routine outpatient transfusions and elective surgeries, the lack of available components can increase morbidity and even be life threatening for patients who require timely management of the severe complications of their underlying disease (e.g., acute chest syndrome, acute hepatic sequestration, stroke, or priapism) or when emergency surgery for trauma is required.

Nowadays, patients with SCD routinely live into their 60s and, therefore, are likely to require several or many transfusions. In the STOP trials, SCD patients were transfused with RBC components matched for highly immunogenic Rh and K antigens. Predictably, this decreased immunization to C, E, c, e, and K antigens. Studies have demonstrated a substantial decrease in RBC alloimmunization with the use of phenotype-matched RBC components – from 35% to 0% and 3% to 0.5% per RBC component transfused [11, 17]. However, even with these precautions, some patients still become immunized to Rh antigens, which underscores the need to provide more precisely matched blood to prevent transfusion reactions and alloimmunization. The ability to accomplish this has been hampered by our incomplete knowledge of the blood group phenotypes in African-Americans, inadequate supplies of reliable antisera, and insufficient inventories of antigen-negative blood. Until we sufficiently understand the immune response to the extent that it becomes feasible to predict the blood transfusion recipients who will be responders or non-responders and/or it is possible to block or subdue the immune response to blood group antigens, transfusion providers have few options available to help chronically transfused immunized patients.

Alloimmunization to antigens in the Rh blood group system, which is thought to be due to their immunogenicity and high-phenotype diversity, is exacerbated by the disparity between the largely Caucasian donor pool and the patients with SCD who are predominately African-American. This fact is illustrated by a 6% rate of RBC alloantibody formation in Uganda, where there is racial similarity in the donor and recipient populations [18] compared to an average rate of 25% in the USA [2, 19]. Similarly, a study that compared antibody production in patients with SCD showed

that 2.6% of 190 patients in Jamaica (where donors and recipients are likely to be ethnically matched) and 76% of 37 patients in England (where there is disparity between donors and recipients) developed antibodies [20].

Most blood donor centers are presently not able to support transfusion with fully phenotype-matched blood. While the hemagglutination technique is simple and requires little in the way of equipment, it is labor-intensive, requiring trained technologists, and manual entry of results. In addition, the special reagents needed are scarce and expensive. As the prevalence of antigens differs in various ethnic groups, phenotype-matched blood for patients with SCD is most likely to be found among African-American donors. For example, the C–, E–, S–, K–, Fy(a–), and Jk(b–) blood type can be found in one in six African-American donors, but in only one in 131 Caucasian donors. In order to increase the number of African-American donors, many blood donor centers have instituted targeted donor recruitment.

As an additional complication, RBCs from African-American donors are more likely to express antigens for which we do not routinely test. Antisera for these is scarce, e.g., V/VS, Go^a, DAK, and Js^a. These antigens are in the Rh and Kell blood group systems and are highly immunogenic. Although these antigens have low prevalence in Caucasians, they occur in up to 20% of African-Americans [21]. Furthermore, because RBCs used for antibody screening panels do not carry these antigens, the corresponding antibodies can go undetected if an antiglobulin crossmatch is not performed. This is now the standard of practice for patients without known alloantibodies in hospitals with validated computer systems [22].

The issues and concerns regarding the transfusion of patients with SCD are still topical and are addressed in an entire issue of *Immunohematology* (22[3]: 2006). Clearly, alternative cost-effective approaches are needed. The availability of high-throughput DNA arrays to test patient and donor samples for the prediction of a blood group phenotype is a novel approach to the way antigen-negative blood is provided to patients and a means by which to prevent immunization. If the number of alleles analyzed is adequate and inventories of antigen-negative (albeit predicted) RBC components are large enough, it should be possible to select appropriate antigen-negative RBC components to eventually prevent alloimmunization.

10.3 DNA Testing for the Prediction of Blood Groups

Historically, a blood group antigen was identified after an immune response (alloantibody) was detected by hemagglutination in the serum of a pregnant or transfused patient. Over the last 60 years, many variants of blood groups have been discovered. The past two decades have seen an astounding pace of growth in the understanding of the molecular basis associated with many blood group antigens and phenotypes [21, 23]. Thus, we are now able to predict the presence or absence of a blood group antigen using genomic approaches.

10.3.1 Genes Encoding Blood Groups

Genes encoding the 30 blood group systems have been cloned and sequenced [24, 25], and over 260 antigens have been defined by alloantibodies [26]. To date, over 1,000 alleles defining blood groups have been described. As the molecular basis of the majority of minor blood group antigens is a single nucleotide change, simple PCR-based assays can be used to predict blood groups. In the authors' laboratory, laboratory-developed tests have been used for over two decades to predict blood group phenotypes as an aid to identify the antibody, to reveal the molecular change of a novel blood group, and to select blood donors [27–36].

Once a gene encoding a blood group system has been cloned and sequenced, information regarding its polymorphisms is obtained by sequencing the defined gene from different people of known phenotype. It has been typical, once the prototype gene is sequenced, to test a few examples of the known polymorphism, e.g., two or three examples each of Jk(a+b−), Jk(a+b+), and Jk(a−b+). This approach will not reveal nucleotide changes in other parts of the gene. To minimize the chance of misinterpreting results obtained by testing DNA, it is important to include assays for nucleotide changes that are known to weaken or silence the expression of encoded antigens, especially for those that are prevalent, e.g., *RHD*ψD* [37], *FY*GATA* [38], *FY*265* [39–41], *GYPB*230*, and GYPB*-i5+5 [42].

DNA array technology is a powerful tool. However, it is important to remember that, regardless of test protocols used, it can only predict a blood type. For example, in blood group null phenotypes, a gene may be present but its expression is silenced as the result of one of several reasons, making it impractical to predict using DNA testing. This is especially true for null phenotypes in the Rh, Kell, and Xk blood group systems. Fortunately, nucleotide changes and gene rearrangements that silence an allele encoding a blood group antigen are rare. Thus, DNA testing is unlikely to completely replace hemagglutination, but it can serve as a valuable adjunct.

10.3.2 Experience with High-Throughput DNA Arrays

DNA array technology has been used for several years and there is now a considerable base of knowledge. The accuracy and effectiveness of predicting a blood group have been shown in several studies [43–47]. Tests of over 4,000 samples with the BioArray Solutions HEA BeadChip™ were concordant with manual DNA test results. The majority of DNA-predicted blood group antigens (4,510) were also in agreement with hemagglutination results. The small number (24) of discrepancies between DNA testing and hemagglutination were likely due to errors in historical data entry into the database. The BeadChip™ analyses produced 19,457 antigen-negative determinations, thereby identifying valuable donors (both useful combinations of antigen negativity and 24 rare donors whose RBCs lacked a high-prevalence antigen) for selection for confirmation by hemagglutination [48, 49].

A prospective study by Klapper et al. was designed to determine the theoretical feasibility of using the BioArray HEA BeadChip™ in conjunction with a Web-based inventory management system to model phenotype matching of donors to recipients. According to the study conducted in four large hospital transfusion services, establishing an inventory of DNA tested donor components from existing hospital inventories resulted in the provision of more extensively matched RBC components than is provided using the current standard of practice [50].

In donor centers, DNA arrays are used to type RBC products and confirm D-negative status [51]. Based on DNA results of 144 SCD patients and 948 donors, Ribeiro et al. were able to provide compatible antigen-matched RBC components for 134 of the patients with SCD [52]. The value of a high-throughput DNA array platform for the prediction of blood groups was demonstrated by testing 2,355 donors for K, k, Jk^a, Jk^b, Fy^a, Fy^b, M, N, S, s, Lu^a, Lu^b, Di^a, Di^b, Co^a, Co^b, Do^a, Do^b, Jo^a, Hy, LW^a, LW^b, Sc1, Sc2, and HgbS. This testing identified 21 rare donors – Co(a−b+), Jo(a−), S−s−, and K+k− [49]. In a study of German blood donors, DNA from 8,442 serologically defined D-negative samples revealed five D-positive donors and one D-positive (6%)/D-negative (94%) chimeric donor. The investigators traced 13 previously donated products from the chimeric donor to two D-negative recipients. Both had formed alloanti-D after transfusions [53].

10.4 DNA Testing for Prediction of RH Alleles

The Rh locus consists of two homologous genes – *RHD* and *RHCE* on chromosome 1p36.11 at respectively 25350k–25400k and 25440k–25490k. *RHD* is flanked by two 9 kbp homologous regions of DNA named *Rhesus boxes*. *RHD* and *RHCE*, in opposite orientation, are separated by an apparently unrelated gene (*SMP1*) of about 30 kbp. The two genes segregate in a single haplotype [54–56] (Fig. 10.1).

The RH genes encode the most immunogenic RBC protein-based antigens. As a consequence of the homology and opposite orientation of the two RH genes, which are features common in duplicated genes, many rearrangements between these two genes have resulted. Extreme complexity has occurred at the *RH* locus and a large number of clinically relevant rare alleles have been identified. This is especially true in black people of African ancestry. *RHD* encodes the D antigen, which encompasses the entire RhD protein rather than single amino acid changes, and, thus, is unique in blood groups. *RHCE* encodes C or c and E or e. Depending on the combination of C/c and E/e, there are several nucleotide differences between *RHCE* and *RHD*. For example, *RHD* and the cE form of *RHCE* differ by 43 nucleotides [54, 57].

Although some variant RH alleles are due to single nucleotide changes, the majority are due to gene rearrangements that result in hybrid genes. Figure 10.2 shows a selection of gene rearrangements in *RHD* (left panel) and *RHCE* (right panel) that have been found in African-Americans. A patient with a rearranged RHD allele has an altered D antigen, the so-called partial D antigen, and can make anti-D (Table 10.1). No attempt has been made to classify the anti-D, mainly because it has been common

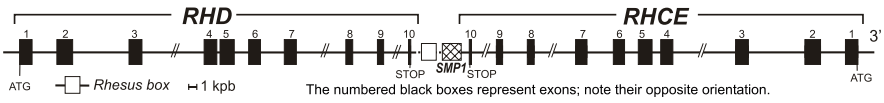

Fig. 10.1 RH gene locus. *RHD* and *RHCE* each have 10 exons and are in opposite orientation on chromosome 1

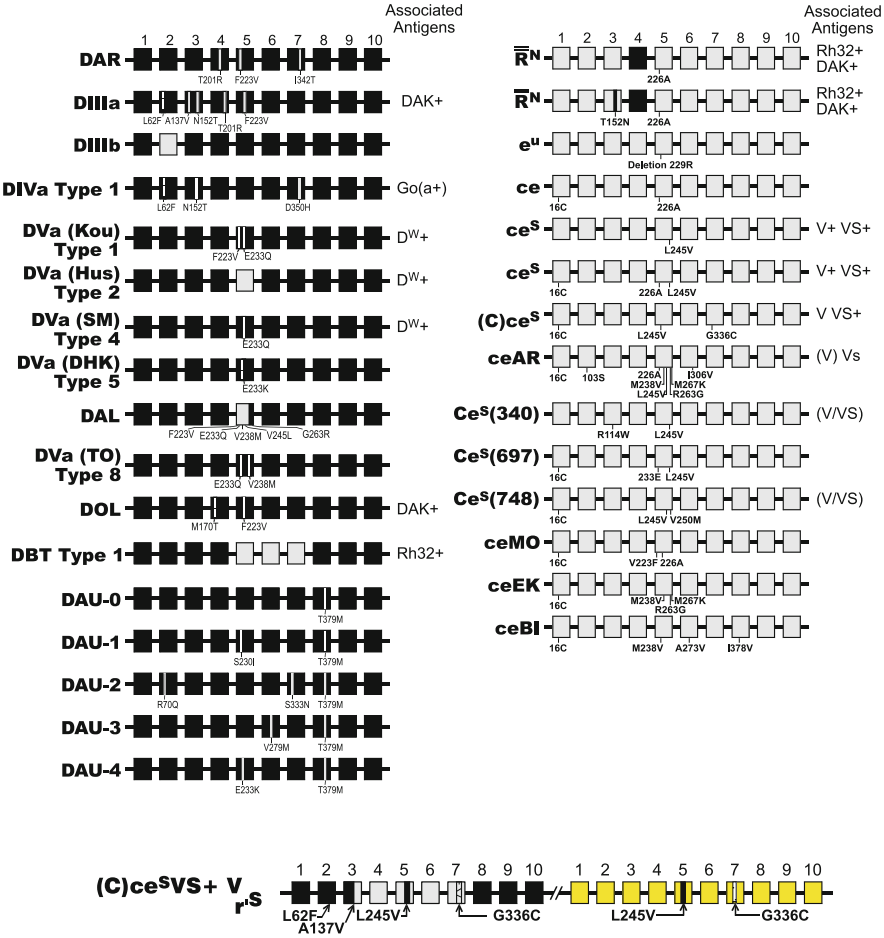

Fig. 10.2 Partial RHD and RHCE alleles in African-Americans. The boxes represent exons. Both genes are shown with exons labeled in the 1–10 order. *Black* = RHD; *gray* = RHCE

practice to provide D-negative RBC components rather than opting for the more complicated process of trying to match the partial D type. By serological testing, most partial D phenotypes usually only are recognized after the production of anti-D or by the detection of a low-prevalence antigen that can serve as a marker (e.g., DAK or Go^a), although certain partial D phenotypes are typed as D– with some anti-D.

Table 10.1 RHD alleles in black African-Americans encoding D partial antigens and low-prevalence antigens associated with altered RhD protein

Allele encoding	Low-prevalence antigen expressed
DIIIa	DAK
DIVa	Go^a
DVa	D^W
DBT	Rh32
DOL	DAK
DAR	None known
DAU	None known

Similarly, a patient with an altered C, c, E, or e antigen can make an alloantibody corresponding to the epitopes missing from the altered (partial) antigen. These are also only identified after the production of anti-C, anti-c, anti-E, or anti-e or by the detection of a marker (e.g., Rh32 or VS). These low-prevalence markers are antithetical to the absent high-prevalence antigen (see Table 10.2). Antibodies to high-prevalence Rhce antigens appear as anti-Rh17. The high-prevalence antigens carried on Rhce have been named because, unlike the partial D/anti-D scenario, provision of D– phenotype RBC components is difficult, if not impossible. Thus, naming the antigens has provided a means of communicating the needs of specific antigen-negative blood when trying to locate compatible blood units.

Many of these variants can be defined by a panel of monoclonal anti-D or anti-e. However, these reagents are not foolproof because they can give different reactions depending on the formulation of the reagent, the condition of the RBCs, and the haplotype *in trans*. DNA testing provides a tool to predict the presence of an altered allele. Obviously, the more nucleotides that are tested, the more accurate the interpretation can be. Even with limited DNA testing, analyses in the authors' laboratory show that variant RH alleles are not uncommon in African-Americans. As an example, for the single nucleotide change *RHCE*733C>G*, we found 120 of 262 random samples had *RHCE*733C/G* or *RHCE*733G/G*, which represents, somewhat surprisingly, 46% of samples with just this one nucleotide change.

Although the molecular bases of many antigens in the Rh system have been reported, it is clear, based on antibodies produced by blacks of African ancestry, that more exist [21, 58]. Different combinations of a few RhCE variants have been described in patients who make anti-Rh-18, an antibody that has caused fatal transfusion reactions [58]. For example, ceAR, ceMO, ceEK, ceBI, and ceMI proteins all lack the related high-prevalence hr^S antigen. However, an antibody made by a patient with one of these variants is not necessarily compatible with RBCs from a donor with a different molecular basis for the hr^S negativity. To elucidate, serum from a ceEK/ceEK person is compatible with ceAR/ceAR RBCs, but serum from a ceAR/ceAR person is not compatible with ceEK/ceEK RBCs. Data about the number, prevalence, and clinical relevance of alleles are required to design the appropriate high-throughput assays to type for the absence of high-prevalence antigens in the Rh blood group system. It is currently a major problem to supply D+ RBCs with certain RHCE phenotypes, let alone RBCs that are D– and C–, E–, hr^S–, or C–, E– hr^B–. Many alloantibodies in transfusion-dependent African-Americans are poorly defined and are against antigens

Table 10.2 RHCE alleles in black African-Americans encoding partial Rhce antigens, low-prevalence antigens associated with the altered Rhce or RhCe protein, and alloantibody

Allele encoding	Low-prevalence antigen expressed	Antibody to antithetical high-prevalence antigen
ceAR; ceBI; ceEK; ceMO	None known	Anti-hr^S (-Rh19); -Rh18
ceS (V– VS+); ceMO	VS (Rh20)	Anti-hr^B (-Rh31); -Rh34
ceS	VS (Rh20)	Anti-hr^B (-Rh31); -Rh34
Rh26–	LOCR (Rh55)	Anti-Rh26
R$^{=N}$	Rh32	Anti-Rh46
C^{W}e	C^W (Rh8)	Anti-MAR (-Rh51)
C^{X}e	C^X (Rh9)	Anti-MAR (-Rh51)
ceS(340)	JAL (Rh48)	Anti-CEST (-Rh57)
ceCF	Crawford (Rh43)	Anti-CELO (-Rh58)
ceAG	None known	Anti-CEAG (-Rh59)
ceBI, ceSM	STEM	None known

in the Rh system. These sensitized patients make complex antibody specificities and present a huge challenge with regard to finding compatible blood for them.

Another level of complexity occurs when the variant *RHCE* is inherited with an altered *RHD*. Thus, in addition to making anti-hr^S or anti-hr^B, the patient also can make anti-D. On initial presentation, such a case would appear serologically to be D+, e+ with anti-D, and anti-e, and most likely with coexisting alloantibodies to other blood groups systems. Such cases are difficult to resolve and it is extremely difficult to find compatible blood. Theoretically, in the African-American population, any *RHD* (left panel, Fig. 10.2) can be *in cis* with any *RHCE* (right panel, Fig. 10.2). Some combinations are more common than others, e.g., *DIVa* with *RHCE*ceTI* [59], DAR with *RHCE*ceAR* or *RHCE*ceEK* [60], DAU0 with *RHCE*ceMO* [61], and *DIIIa*, *DIIIa-CE(4-7)-D*, or *D-CE(4-7)-D* with *RHCE*ceS* [62]. Indeed, we frequently find DIIIa, DAR, DOL, DAU, or DIVa in African-American patients, which historically are not revealed until after the patient makes anti-D or is identified by the presence of a serological marker (e.g., DAK on DIIIa, R$^{=N}$ and DOL, and Go^a on DIVa).

10.5 Testing with RHD BeadChip™ and RHCE BeadChip™

The purpose of this testing was to assess the accuracy and efficiency of the BioArray Solutions prototype RHD and RHCE BeadChip™ systems, which includes software analysis for the interpretation of complex allele "calling." BioArray Solutions RHD BeadChip™ and RHCE BeadChip™ were used to test DNA from two cohorts of samples. One set consisted of known and diverse Rh phenotypes selected to pose a challenge to DNA array technology. The other set consisted of random samples from African-American donors and from patients with SCD. This set was tested to estimate the prevalence of various RH alleles.

Samples tested on the RHD and RHCE BeadChips™ had been analyzed by a combination of laboratory-developed tests, including PCR-RFLP, AS-PCR, sequencing

Table 10.3 Nucleotide markers and corresponding amino acids on RHD BeadChip™ by BioArray Solutions

Amino acid markers	Nucleotide changes	Amino acid markers	Nucleotide changes
S3C	8 C>G	E233K	697 G>A
W16C	48 G>C	V238M	712 G>A
W16X	48 G>A	V245L	733 G>C
L62F	186 G>T	G263R	787 G>A
R70Q	209 G>A	V270G	809 T>G
R114W	340 C>T	V279M	835 G>A
A137V	410 C>T	G282D	845 G>A
A149D	446 C>A	T283I	848 C>T
N152T	455 A>C	M295I	885 G>T
IVS3+1G/A	In3+1G>A	I342T	1025 T>C
psi D	In3 -19 37 bp duplication	D350H	1048 G>C
M170T	509 T>C	G353W	1057 G>T
I172F	514 A>T	G355S	1063 G>A
T201R	602 C>G	G385A	1154 G>C
F223V	667 T>G	E398V	1193 A>T
A226P	676 G>C	733C/G	1227 G>A
S230I	689 G>T	PC1	Positive control 1
E233Q	697 G>C	PC2	Positive control 2

Table 10.4 Nucleotide markers and corresponding amino acids on RHCE BeadChip™ by BioArray Solutions

Amino acid markers	Nucleotide changes	Amino acid markers	Nucleotide changes
W16C	48 G>C	V223F	667 G>T
A36T	106 G>A	A226P	676 G>C
Q41R	122 A>G	Q233E	697 C>G
P103S	307 C>T	M238V	712 A>G
109Ins	109 bp intron 2 ins	L245V	733 C>G
R114W	340 C>T	V250M	748 G>A
L115R	344 T>G	dT744dC	744 T>C
S122L	365 C>T	A273V	818 C>T
T152N	455 C>A	I306V	916 A>G
R154T	461 G>C	G336C	1006 G>T
M167K	500 T>A	T342I	1025 C>T
G180R	538 G>C	Rh r^S	Cde^S 5′ UTR
R201T	602 G>C	PC	Positive control

cDNA RHD and RHCE (exons 1–4 and exons 5–10), sequencing specific exons from gDNA, and cloning. RHD and RHCE BeadChips™ were used according to instructions from BioArray Solutions. For information regarding the design of the RHD and RHCE BeadChip™ system and analysis software, see Chap. 3. For the nucleotides assayed and the corresponding amino acids, see Tables 10.3 and 10.4. The samples also had been tested to varying degrees by numerous technologists over many years via hemagglutination using standard methods appropriate for the selected reagent.

The majority of serotypes were from historical records; however, when appropriate, additional hemagglutination in test tubes or gel cards was carried out.

10.5.1 Test Samples

Two groups of samples were tested:

1. Samples from African-Americans ($n=57$) selected for their Rh variant phenotype, discrepant D, c, e, C, and E antigen typing, the presence of alloanti-D, -C, -c, -E, and -e in an antigen-positive patient, and/or the presence of a low-prevalence antigen. These samples were selected to represent allelic diversity, especially for RHCE alleles.
2. Samples ($n=279$) from random African-American donors ($n=224$) and patients with SCD ($n=55$) with no known indication of having an altered phenotype. These samples were tested to estimate the prevalence (albeit for the small sample size) of variant alleles. Thirteen other samples were excluded from analysis because PCR failed on the RHD BeadChip™, RHCE BeadChip™, or both.

10.5.2 Analyses

Analyses of nucleotide sequences generated using the laboratory-developed tests were performed manually using the Sequencher v4.8 (GeneCodes, Ann Arbor, MI) computer program. Analysis of the BeadChips™ was performed by BioArray Solutions using their tailor-made computer analysis program. For complex allele interpretations, this program uses a set of proprietary algorithms that produce assignment confidence scores along with allele and haplotype assignments (see Chap. 12).

Results obtained by RHD and RHCE BeadChips™ were compared to those obtained with the various laboratory-developed tests and to hemagglutination by using spreadsheets. An enormous quantity of data was generated and only a summarized overview is presented in this chapter.

10.5.3 Interpretation of Set #1: Interpretation of Samples from African-Americans with Known Variant RH Alleles (Diverse Samples)

10.5.3.1 RHD Analysis in Set#1

Several examples of each of the following RHD alleles were included in the testing: D, ψD, DIIIa, DIVa.2, DAR, DOL, DOL-2, partial weak D type 4.0, weak D type 40, DAU0, DAU5, RHD-CE(ex4-7)-D, and RHDIIIa-CE(ex4-6/7)-D. There was good correlation between BioArray Solutions RHD BeadChip™ interpretations and

laboratory-developed test interpretations. Apparent discrepancies were mainly due to the absence of specific probes from the BeadChip™. For example, the *RHD* probes used to detect the nucleotide change associated with DAU0 (*RHD*1136C>T*) were not on the BeadChip™. Likewise, the RHD BeadChip™ does not analyze *RHD* zygosity.

10.5.3.2 RHCE Analysis in Set#1

Several examples of each of the following RHCE alleles were included in the testing: ce, Ce, cE, CE, $C^{w}e$, ce48C, ceAR, ceS, ceS with nt 1006T, ceS(340), ceCF, ceMO, ceEK, ceBI, ceTI, $R^{=N}$, and D^{Har}. In general, there was good correlation between BioArray Solutions RHCE BeadChip™ interpretations and laboratory-developed test interpretations. The BeadChip™ is designed to identify a specific set of clinically relevant alleles that are clearly noted. Apparent discrepancies were mainly due to the absence of specific probes from the BeadChip™, for example, those that are required to detect heterozygous $R^{=N}$ samples, to differentiate *RHCE*ceBI* from *RHCE*ceSM*, or those with certain hybrid RHCE alleles. One heterozygous *RHCE*nt1006T* was missed by BioArray Solutions. Another source of discrepancy is inherent to testing for a series of nucleotide changes vs. cloning. For example, an interpretation of *RHCE*ceTI/RHCE*ce 48C*, *733G* was actually *RHCE*ceTI 733G/RHCE*ce 48C*. One sample, which agreed with laboratory-developed tests but not with hemagglutination, was shown to have a rare silenced RHCE*cE allele. For samples where testing was performed, hemagglutination agreed for D, C, E, c, e, VS/V, hr^{S}, and hr^{B} antigens.

10.5.4 Interpretation of Set #2: Interpretation of Samples from Random African-American Donors and Patients with SCD

10.5.4.1 RHD Analysis in Set #2

To validate BioArray Solutions interpretations, ten samples known to be D+ were compared to manual analysis at the nucleotide locations assayed on the RHD BeadChip™. There was complete agreement. There was also concordance with all samples ($n=22$) with an RHDψ allele (20 heterozygotes, and 2 homozygous or hemizygous). The RHD alleles identified by BioArray Solutions on 279 samples (558 alleles) are listed in decreasing order of their frequency in Table 10.5. Although the number of each allele found provides an estimate of the relative occurrence of the various alleles in the samples tested, the sample size is too small to calculate frequencies and the absence of certain probes would cause inaccuracies. For example, as stated above, the RHD BeadChip™ does not detect DAU0 (*RHD*1136C>T*), which according to laboratory-developed tests is present in 30% of 296 samples (homozygotes/hemizygotes and heterozygotes $n=89$, wild type

Table 10.5 BioArray Solution analysis of RHD alleles in 279 blood samples from random African-Americans

RHD allele	Alleles	Homozygous or hemizygous	Total alleles
D	254	193	447
Dψ	22	4	26
DIIIa-CE(4-7)-D or D-CE(4-7)-D	17	2	19
RHD deletion	8	8	16
DAU-5 or DV type 1	10	2	12
Partial weak D type 4.0 or 4.3	7	0	7
DIIIa	6	1	7
DIVa	7	0	7
DAU-3	5	0	5
Partial weak D type 4.2 or DAR	2	0	2
DOL or DOL-2	2	0	2
DFR or DFR-3	2	0	2
DIV type 4	2	0	2
DAU-4 or DV type 5 (DHK)	1	0	1
DV type 7	1	0	1
DTO	1	0	1
Total			558

Table 10.6 BioArray Solutions analysis of RHCE alleles in 279 blood samples from random African-Americans

RHCE allele	Number of alleles	Homozygous	Total number of alleles
ce, Ce. cE, or CE	225	72	297
ce 48C, 733G	102	12	114
ce 48C	87	12	99
ce 48C, 733G, 1006T	21	0	21
ceTI	12	0	12
ceMO	7	0	7
ceEK	4	0	4
ceAR	2	0	2
ceBI	1	0	1
$C^{w}e$	1	0	1
Total			558

$n=207$). Likewise, in the absence of analysis for *RHD* zygosity, which would be a challenge to any DNA array platform, it is difficult to calculate allele frequencies. The analysis of data performed by BioArray Solutions shows that 17% (94 of 558 alleles) of samples had one or two variant RHD alleles.

10.5.4.2 RHCE Analysis in Set #2

To validate BioArray Solutions interpretations, ten samples with common RHCE alleles (Ccee, $n=4$; ccee, $n=3$; ccEe, $n=2$; and ccEE, $n=1$) were compared to

manual analysis at the nucleotide locations present on the RHCE BeadChip™. There was concordance with all nucleotides. There was concordance with samples ($n=53$) with a RHCE*cE allele: R_2r $n=39$ samples, R_1R_2 $n=10$ samples, and R_2R_2 $n=4$ samples. There was concordance with all nucleotides except for two RHCE*48 (G/C by BAS and C/C by laboratory-developed tests). The RHCE alleles identified by BioArray Solutions on the 279 samples (558 alleles) are listed in order of their decreasing frequency in Table 10.6. The analysis of data performed by BioArray Solutions shows that 47% (253 of 558 alleles) of samples had one or two variant RHCE alleles.

In the samples tested (279 samples, 558 alleles), 125 samples had a total of 137 alleles with *RHCE*733G* [*RHCE*ce 48C*, *733G* ($n=102$ samples; 114 alleles), *RHCE*ce 48C*, *733G*, *1006T* ($n=21$ samples; 21 alleles), and *RHCE*ceAR* ($n=2$ samples; two alleles)]. Thus, a quarter of the samples had at least one allele with the nucleotide 733C>G change. In a comparison of 248 samples for nucleotide *RHCE*733*, there was agreement with manual analysis in 245: 733C/G = 94 samples, 733G/G = 14 samples, and 733C = 133. Three samples were in disagreement at position *RHCE*733*: one sample was 733C/C by BioArray Solutions and 733C/G by laboratory-developed tests; two samples were 733C/G by BioArray Solutions and 733C/C by laboratory-developed tests.

10.6 Conclusions and Perspectives

Laboratory-developed DNA tests have been used for many years as an adjunct to hemagglutination and have added considerable value in terms of patient care. Laboratory-developed tests used by the authors include DNA analysis of the clinically relevant blood group genes. More recently, as illustrated in previous chapters of this book, DNA arrays have proven their value as high-throughput platforms to predict selected clinically relevant blood groups in the area of transfusion medicine. While analysis of DNA to predict a so-called minor blood group antigen status is relatively simple and usually based on testing a single nucleotide change (*KEL*01/KEL*02*, *JK*A/JK*B*, etc.), analysis of *RHD* and *RHCE* is complicated by the fact that multiple nucleotide changes or hybrids of these homologous genes can be involved in encoding a phenotype. Nevertheless, laboratory-developed tests are increasingly being used to analyze blood samples for RH alleles, albeit using complex algorithms. This is particularly true for analysis of RH alleles in black people of African descent, who are known to have a large number of variant RH alleles. As stated above, a higher proportion of patients with SCD make more alloantibodies than any other single category of patients and are most likely to benefit from more precise matching of donor RBC components to recipient. Due to the complexity of serological testing in these patients, more precise matching may only be possible at the DNA level. Analysis of RH alleles is particularly suited for DNA arrays that have software to interpret the combination of nucleotides in specific alleles.

Molecular analyses have revealed subtle differences in RH alleles that encode Rh phenotypes, which cannot easily be defined by standard hemagglutination. This raises the possibility that providing compatible blood based on DNA testing may, in these cases, be superior to hemagglutination. There is a clear need to determine the extent of diversity of alleles encoding Rh phenotypes in African-Americans and to study the alloantibodies these patients can produce to establish their clinical relevance. Once the clinically relevant information has been compiled, appropriate probes should be included in DNA arrays used to screen patients and donors. Probes on the DNA array should be able to detect as many clinically relevant variants as possible, including not only novel nucleotide changes but also the nucleotides that differ between *RHD* and *RHCE*. This will aid in the detection of *RH* hybrids. The DNA arrays also need to predict whether a donor's RBCs possess low-prevalence antigens, such as Go^a, D^W, Rh32, VS, V, and DAK, and lack antigens such as hr^B and hr^S, Rh18, and Rh34. Additionally, haplotypes that encode partial D, C, E, c, or e antigens should be identified. There is a growing body of knowledge that proteins with variant e antigens can lack novel high-prevalence antigens. For example, in African-Americans, RBCs expressing JAL lack CEST [63] and RhceCF lacks CELO [63, 64] (Table 10.2).

The value of DNA arrays is clear for precise matching at the DNA level of donor-to-recipient blood, particularly for patients with complex antibody combinations. For example in this study, the current BeadChip™ design provides rapid results for the complex allele determinations for *RHCE* [ce, Ce, cE, CE, C^We, ce48C, ceAR, ceS, ceS with nt 1006T, ceS(340), ceCF, ceMO, ceEK, ceBI, ceTI, $R^{=N}$ (homo), and D^{Har}] and *RHD* [D, ψD, DIIIa, DIVa.2, DAR, DOL, DOL-2, weak partial D type 4.0, weak D type 40, DAU5, RHD-CE(ex4-7)-D, and RHDIIIa-CE(ex4-6/7)-D]. For complex patients, a well-designed DNA array has the potential to allow economical screening of a large number of donors of any ethnicity for matching RBC components to recipients. Studies need to be performed to address the question of whether it is possible to economically increase inventories of antigen-negative donor blood using genomic approaches, as well as to provide precisely matched donor blood to patients who need it. This would go a long way to ameliorating the clinical course of many transfusion-dependent patients with SCD [65].

As with any technique, DNA arrays have limitations. It is important to know the array's abilities and shortcomings and not to expect too much. As has been discussed elsewhere [66, 67], numerous reasons exist, including technical, medical, and genetic events, for why the genotype is not the phenotype. Given the large number of genetic events that are already known to silence or dramatically weaken expression of antigens encoded by an allele, it will be a long time (if ever) before all relevant nucleotide changes are revealed for all blood group systems in all ethnic groups. While matching alleles of donors to a recipient may improve patient care in certain scenarios, DNA testing will not eliminate the need for hemagglutination. What is expressed on the surface of the RBC is what matters. Even though there are a large number of silencing mutations, some of which are included in the HEA assays, they must not be very common as published studies [49] and data presented in Chap. 3 show >99% concordance between serology and DNA typing.

Determination of which nucleotide changes are present on which allele, i.e., the *in cis* configuration, is not practical by DNA arrays. For example, with a *RHCE*ce 733C/G*, *1025C/T* result, the 733G could equally well travel with the 1025C (*RHCE*ce*^{S}) or with 1025T (*RHCE*ceTI*). Thus, the phenotype could be ceTI/ceS or ceTI type2/ce. In general terms, this is relevant if it affects the antibodies made by a person with one vs. the other phenotype. Fortunately, in most cases the presence of a variant protein encoded by a pair of homozygous alleles, a rare event, is a requirement for antibody production. However, a double heterozygote person, e.g., a variant allele *in trans* to a null allele, can make an antibody to the absent antigen(s). In this scenario, on testing DNA, a valuable donor would not be identified. A patient would be predicted to have RBCs that express antigens and, thus not be identified as a candidate to make alloantibodies. Different combinations of a few Rh variants have been described in patients who make anti-Rh18, an antibody that has caused fatal transfusion reactions [58]. Furthermore, in regard to a potential immune response, the effect of a variant encoded by an allele *in cis* is not fully understood.

As a person's genotype will not change, thought should be given to include a unique simple genoprint on the DNA array, so that on subsequent donations, the donor need only be analyzed for the genoprint profile and not for all the blood group nucleotide changes. This should not only reduce costs in the long run but also speed up the matching process. Various scenarios for screening, confirmation, and labeling RBC components that have been tested for antigen negativity by licensed and unlicensed reagents are given in a published review by Strauss and Reid [68].

RBC transfusions are commonly used to treat patients with SCD. Alloimmunization remains a major complication of their transfusion therapy and there is clearly a need to provide more precisely matched blood than we can currently achieve. DNA arrays have the potential to allow for high-throughput screening to increase antigen-negative inventories and to match alleles, albeit on a limited basis (that is, to the extent of the number of probes on the DNA array) of the donors to a recipient. The physical crossmatch will remain the gold standard for assessing compatibility between recipient's serum/plasma and donor RBCs. It is important to recognize limitations of using the DNA array in the area of transfusion medicine. However, the increasing cost and diminishing supplies of source material of traditional antisera needed for hemagglutination tests make DNA arrays an attractive alternative for screening.

Studies are required to determine the prevalence of clinically relevant RHD and RHCE alleles in different populations and to address cost effectiveness, logistics of implementation, limitations of the process, and medical advantages of DNA matching. While DNA arrays have the potential to revolutionize the way we support patients with blood transfusion by radically changing the way we type antigens (predictotype), consideration should be given to the cost containment. Also, in order to provide adequate numbers of antigen-matched products to chronically transfused patients, an adequate RBC product inventory with appropriate racial/ethnic diversity must be available. The obvious goal is to supply the right blood to the right patient at the right time at a reduced cost.

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Chapter 11
Identification of Altered RHD and RHCE Alleles: A Comparison of Manual and Automated Molecular Methods

Sunitha Vege and Connie M. Westhoff

Abstract RH genotyping is particularly useful in clinical transfusion practice to discriminate partial D from weak D, distinguish alloantibodies from autoantibodies, detect the absence of high-prevalence Rh antigens, and to screen for Rh-compatible donors. Because of the complexity of the RH locus, many areas of RHD and RHCE must be sampled for accurate genotyping; gene sequencing is often required. Manual assays are labor-intensive and complex interpretation is required. Automation is needed to expedite testing and to make a DNA-based approach more accessible for application to clinical transfusion practice. We determined the performance of the automated RHD and RHCE BeadChip™ for detecting RHCE and RHD polymorphisms by testing 149 samples referred for RHD analysis and 168 referred for RHCE genotyping. The majority were assayed in parallel to determine concordance of the BeadChip™ assays with manual polymerase chain reaction-restriction fragment-length polymorphism and gene sequencing. RHD alleles were concordant between manual and automated methods with the exception of seven alleles. For RHCE, all were concordant with the exception of six alleles. This study is significant for the number of samples analyzed by both manual and automated methods. All samples were problem referrals encountered in routine transfusion practice.

Keywords Blood groups • DNA array • Human erythrocyte antigen • RH • RHCE • RHD

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11.1 Background

The Rh blood group system is comprised of two highly homologous genes, *RHD* and *RHCE*, which encode proteins that carry D, and c or C, and e or E antigens, respectively. The Rh system is much more complex than these five common antigens and over 50 Rh serologic specificities have been described. At the genetic level the complexity is even greater. Over 180 *RHD* and approximately 70 *RHCE* are reported. The number of newly identified alleles continues to increase [1, 2]. Rh antigens are clinically significant and the ability to detect RH alleles that differ from the common conventional alleles can be important in a number of clinical situations.

11.1.1 RHD

Distinguishing alleles encoding weak D, partial D, D_{el}, and inactive *RHD* from conventional *RHD* has relevance for transfusion practice. For females, it is important to prevent D alloimmunization to avoid hemolytic disease of the fetus or newborn or costly monitoring of the pregnancy for the possibility of an effected neonate. In contrast to people with RBCs expressing weak D who are not usually at risk for anti-D, those with partial D phenotypes often make anti-D following exposure to conventional D antigen through pregnancy or transfusion. Females with partial D are considered candidates for immune globulin (RhIG) prophylaxis and should receive D− products for transfusion. However, these RBCs cannot be reliably distinguished with serologic testing. Many RBCs with a partial D antigen type strongly D+ with equivalent reactivity to conventional D. Those RBCs with partial D antigens only reactive by the indirect antiglobulin test cannot be distinguished from weak D samples only reactive in the indirect antiglobulin test. A policy to not test pregnant women for weak D, to treat those patients with D reactive 2+ or weaker, or only detected by the indirect antiglobulin test as D− for RhIG and blood transfusion results in unnecessary RhIG administration and strains the already limited D− blood supply. At the same time, women with partial D antigens giving equivalent reactivity to conventional D go undetected and risk sensitization. In our experience, the majority of the latter are African-American with partial *RHD*DIVa*, *DIIIa*, and *DAU5*. In conclusion, *RHD* typing by molecular methods of D+ females of child bearing age as part of pretransfusion testing or prenatal workup allows diagnosis of partial D, thereby allowing informed decisions to prevent alloimmunization and to also avoid unnecessary RhIG injection.

Serologic testing can miss RBCs with low levels of RhD antigen resulting in some donor units with weak D or D_{el} (D only detected by adsorption and elution of anti-D) labeled as D−. Although the risk of alloimmunization for D− patients transfused with these is less than if transfused with a unit with normal levels of D antigen [3], the risk is not zero [4]. It would be prudent to identify units with low levels of D antigen and remove them from the D− donor pool, but efforts to increase serologic test sensitivity often results in loss of specificity (false-positives). The potential loss

of D– donors, often in short supply, is a concern. However, *RHD* genotyping on over 150 of our donors with discrepant D typing on previous and current donation and suspected of being weak or "missed D" found 20% of discrepancies were serologic false-positives [5]. Donors with discrepant serologic D typing are often deferred. *RHD* genotyping allowed us to retain these donors in the D– donor pool. In summary, *RHD* genotyping can definitively resolve the donor D status when serologic typing is discrepant or in question to avoid product recalls and aid evaluation for potential FDA-reportable events.

11.1.2 RHCE

Less well recognized than weak or partial D expression, is the fact that RHCE alleles, responsible for expression of C or c and E or e antigens, are also polymorphic and can encode partial antigens. Alleles encoding partial C and e are more prevalent than alleles encoding partial c or E, and are seen most often in African-Americans compared to other ethnic groups. A frequently encountered haplotype in this ethnic group is designated (C)ceS, to convey altered C and e antigens – also referred to as the $r^{'S}$ haplotype. Although many other altered RHCE alleles are found in this ethnic group, (C)ceS has the highest frequency estimated at 8–22% [6, 7]. In our experience, approximately 6.3% of our African-American donors have a (C)ceS haplotypes [8].

Patients with sickle cell disease (SCD) sometimes have partial D, C, and e antigens because alleles encoding partial C or e are often linked to alleles encoding partial D [9]. While many patients with SCD are transfused with units phenotype matched for D, C, and E, some still become sensitized in the Rh system. RH genotyping is helpful to determine patients who are homozygous for altered alleles and are at risk for production of alloantibodies to high-prevalence Rh antigens. Clinically significant anti-hr^B and -hr^S have caused transfusion reactions and fatalities [10, 11]. Transfusion in these situations is not straightforward because not all antibodies that are called anti-hr^B or -hr^S have identical specificity because hr^B- and hr^S-phenotypes are associated with a number of different *RHCE*ce* alleles. Serum from patients with these antibodies may not be compatible with phenotypically matched donor units. Transfusion is further complicated because many altered ce-alleles are linked to alleles encoding partial D. RH genotyping of the patient and donors allows selection of units predicted to be compatible in the Rh system and avoids further Rh alloimmunization.

11.1.3 Automation

Most blood group antigens are encoded by single nucleotide polymorphism that encode amino acid changes. Targeting these with allele-specific-polymerase chain reaction (AS-PCR) or PCR-restriction fragment-length polymorphism (RFLP) has

been relatively straightforward. RH is more challenging because of the large number of different alleles and many have multiple changes. Additionally, hybrid alleles consisting of part of *RHD* inserted into *RHCE*, or part of *RHCE* into *RHD* are also found. Because the genes are highly homologous, it is challenging to optimize assays to ensure *RHD* versus *RHCE* specificity. Numerous areas of the RH genes must be sampled for accurate phenotype prediction, and manual AS-PCR and PCR-RFLP methods are time-consuming, labor-intensive, and require high-complexity interpretation.

In recent years, high-throughput DNA-based array platforms have been applied to blood group determination for minor blood group antigens [12, 13]. Application of this technology to the RH system will expand the availability of testing for altered alleles, reduce cost, and expedite turnaround time for RH genotyping. We determined the performance of the BioArray Solutions automated RH BeadChip™ platform designed to detect polymorphisms associated with *RHD* and *RHCE*. One hundred and eight samples were tested in parallel with manual and automated methods for polymorphisms in *RHD* and 50 samples were assayed in parallel for *RHCE*. Following this initial feasibility study, an additional 41 samples referred for problem workup were assayed for *RHD* polymorphisms and 118 samples tested for *RHCE*. In total, results of testing 149 samples for *RHD* and 168 for *RHCE* are summarized in this chapter. All samples were associated with clinical problem referrals. Many samples assayed for *RHD* had discrepant D typing or RBCs that typed as D+ but anti-D was present in the serum. For *RHCE*, the majority of samples typed as C+ and/or e+, but had anti-C and/or "e-like specificity,"-hr^B or -hr^S, or antibodies to high-prevalence Rh antigens in the serum.

11.2 Methods

Genomic DNA was isolated from WBCs by manual methods with QIAamp Blood Mini kit or semiautomated extraction with QIAcube (QIAGEN, Hilden, Germany).

11.2.1 Manual Assays

PCR-multiplex analysis was performed to detect *RHD* exons 4 and 7, inactivating *RHD* pseudogene, and for C/c status [14]. *RHD* zygosity was determined by assaying for the presence of the hybrid Rhesus box [15]. To detect alleles encoding common partial D and weak D, the following exons were amplified and analyzed by restriction digestion (PCR-RFLP) with the enzymes indicated. Amplification of *RHD* exon 1 and digestion with *Sac*I for the nucleotide (nt) 8C>G change, exon 2 and *Bst*XI for 186G>T; exon 5 and *Hinc*II for 667T >G and *Taq*I for 697G>C; exon 6 and *Apa*LI for 809T>G, *Nla*III for 835G>A, *Kpn*I for 845G>A and 848C>T, exon 7 and *Acu*I for 998G>A and *Hph*I for 1025T>C, exon 8 and *Nla*III for 1136C>T, and exon 9 and *Alu*I for 1154G>C change. In addition to PCR-RFLP, allele-specific (AS)-PCR

was performed for exon 3 nt455A>C. If no changes from conventional *RHD* were found, *RHD*-specific sequencing of exons 1–10 or Rh-cDNA analysis was performed.

Analysis of *RHCE* included PCR-RFLP of exon 1 digested with *Apa*I for 48G>C change, exon 2 with *Hae*II for 254C>G, exon 5 with *Mnl*I for 676G>C (e/E), *Taq*1 for 697C>G, and *Bfa*I for 733C>G, exon 7 with *Hph*I for 1025C>T and AS-PCR were used to detect 1006G>T. Genomic DNA sequencing of *RHCE*-specific exons 1–8 or Rh-cDNA analysis was performed for some samples.

Sequencing was performed by Children's Hospital of Philadelphia sequencing facility. Sequences were aligned and compared using Clustal X. PCR products for manual assays were separated on agarose gels and visualized with ethidium bromide staining.

11.2.2 Automated RHD and RHCE BeadChip™

DNA samples were analyzed on the BioArray/Immucor RHD and RHCE BeadChip™ (Warren, NJ) according to manufacturers' instructions. The RHD BeadChip™ specifications indicate that it detects 36 markers associated with RhD and its altered expression, including two positive controls. Thirty-four polymorphisms (Table 11.2) detect 9 D–, 3 D_{el}, 13 weak D, and 46 partial D alleles and a number of hybrid D-CE-D alleles. The RHCE BeadChip™ targets 26 markers (Table 11.2) associated with Rh antigens that include one positive control. The 25 polymorphisms detect 14 altered *RHCE*ce*, 3 altered *RHCE*cE*, and 5 altered *RHCE*Ce* alleles.

11.3 Results

11.3.1 RHD BeadChip™

BeadChip™ results were validated by testing 108 problem samples, as described above, in parallel by RHD BeadChip™ and manual methods. Table 11.1 summarizes results concordant between manual and BeadChip™ testing in these samples (group A). Parallel testing interrogated 22 of 34 polymorphisms (indicated with a check-mark in Table 11.2). The remaining 12 polymorphisms are less common in our experience and no samples were available to validate those. Discordant alleles, alleles not detected, or not conclusively discriminated, are summarized in Table 11.3. Twenty-six different altered RHD alleles were found and complete concordance for 20 of the 26 altered *RHD* was demonstrated. The BeadChip™ did not discriminate the presence of the *RHD*D-CE(4-7)-D* hybrid in samples also carrying *RHD*DIIIa* (three samples). *RHD*DVI* was detected by RHD BeadChip™; the specific subgroups were discordant. *RHD*DAU0*, *RHD*DVII*, and *RHD**weak

Table 11.1 RHD alleles with concordant results between manual testing and RHD BeadChip™

RHD allele	Group A (no.)	Group B (no.)	RHD* allele	Group A (no.)	Group B (no.)	RHD allele	Group A (no.)	Group B (no.)
D	30	23	DFR	1		wkD type 4.0	7	1
D-CE-D	9	4	Del, M295I	2		wkD type 5	1	
DIIIa	8	5	DIVa	2		wkD type 15	5	2
Ψ	4	3	DV type 7	1		DHK	1	
DAR	7	3	wkD type 1	3	1	DAU-3	2	
DOL	2		wkD type 2	3	1			
DNB	1		wkD type 3	1	1			

Group A: original 108 samples. Group B: alleles diagnosed subsequently in 41 samples. All samples were referred for problem workup

D type 10 and type 18 were not detected by RHD BeadChip™ due to the absence of markers to diagnose these alleles. *RHD*DAU4* and *RHD*DAU5* were not distinguished from *RHD*DHK* and *RHD*DVa*, respectively.

Forty-one samples referred for problem investigation were then screened with RHD BeadChip™ and confirmed with manual assays targeting the alleles discussed above. The results are summarized in Table 11.1 (group B). Nine different altered *RHD* were identified by RHD BeadChip™ in 21 of 41 samples. *RHD*DIIIa*, *RHD*D-CE-D*, and the inactive pseudogene were confirmed by manual methods. Manual methods were used to test for *RHD*DAUO* and for weak D in samples negative for altered alleles when screened by RHD BeadChip™.

11.3.2 RHCE BeadChip

BeadChip™ results were validated with 50 problem samples tested in parallel by RHCE BeadChip™ and manual methods. Table 11.4 summarizes concordant results between manual and BeadChip™ testing in these samples (group A). Parallel testing interrogated 21 of 26 polymorphisms (indicated with a check-mark in Table 11.2). Five markers were not validated as no samples were available. Twenty-one different altered alleles were detected. Complete concordance for 15 of 21 altered *RHCE* was demonstrated. Discordant alleles, alleles detected as polymorphic but not interpreted by the software (one example of ceMO and EIV), and alleles not detected due to the absence of markers on the platform are summarized in Table 11.3. All samples with *RHCE*ceS* were interpreted by RHCE BeadChip™ as (C)ceS, i.e., r^S haplotypes. This led to incorrect diagnosis in 12 samples carrying *RHCE*ceS in trans* to *RHD*DIIIa* or to *RHD* deletion. *RHCE*Ce(JAHK) in trans* to *RHCE*ce* was interpreted as *RHCE*ceSL*. In one sample with *RHCE*ceMO/RHCE*cE*, although the 667G>T change was indicated in the RHCE BeadChip™

Table 11.2 RHD and RHCE BeadChip™ polymorphisms

A. RHD			B. RHCE		
Marker[a]	Nucleotide polymorphism	Assayed	Marker[a]	Nucleotide polymorphism	Assayed
S3C	8C>G	√	W16C	48G>C	√
W16C	48G>C		A36T	106G>A	√
W16X	48G>A		Q41R	122A>G	√
L62F	186G>T	√	N68S	203A>G	√
R70Q	209G>A		P103S	307C>T	√
R114W	340C>T		109ins	109 bp intron 2 ins	√
A137V	410C>T	√	R114W	340C>T	√
A149D	446C>A	√	L115R	344T>G	
N152T	455A>C	√	S122L	365C>T	√
IVS3+1G/A	In3+1g>a		T152N	455C>A	
psi D	In3-19 37 bp duplication	√	R154T	461G>A	√
M170T	509T>C	√	M167K	500T>A	
I172F	514A>T	√	G180R	538G>C	
T201R	602C>G	√	R201T	602G>C	√
F223V	667T>G	√	V223F	667G>T	√
A226P	676G>C		A226P	676G>C	√
S230I	698G>T		Q233E	697C>G	√
E233Q	697G>C	√	M238V	712A>G	√
E233K	697G>A	√	L245V	733C>G	√
V238M	712G>A	√	V250M	748G>A	
V245L	733G>C	√	744T>C exchange	744T>C	√
G263R	787G>A	√	A273V	818C>T	√
V270G	809T>G	√	I306V	916A>G	√
V279M	835G>A	√	G336C	1006G>T	√
G282D	845G>A	√	T342I	1025C>T	√
T283I	848C>T		Rh rS	5′ UTR	√
M295I	885G>T	√			
I342T	1025T>C	√			
D350H	1048G>C	√			
G353W	1057G>T				
G355S	1063G>A				
G385A	1154G>C	√			
E398V	1193A>T				
1227G/A	1227G>A				

Polymorphisms assayed in this study are indicated

[a]Terminology used by manufacturer. Most are actual amino acid changes in the protein

genotype result table, the allele interpretation software did not indicate the presence of the altered allele. In a sample with *RHCE*cEIV in trans* to *RHCE*ceS*, the former allele was not reported by RHCE BeadChip™. Interestingly, the BeadChip™ genotype result table indicated the 602G>C change associated with this allele was detected,

Table 11.3 Summary of discordant results between manual testing and RH BeadChip™

	RHD			RHCE		
	Manual	BeadChip	No.	Manual	BeadChip	No.
Discordant	DIIIa/D-CE-D	DIIIa/DIIIa	3	ceS	(C)ceS $r^{'S}$	12
	DVI type 1	DVI type 2	3	Ce(JAHK)	ceSL	1
	DVI type 2	DVI type 3	3	ceMO	no call	1
				EIV	no call	1
Not detected[a]	DAU0	D	11	R^N	Ce	4
	DVII	D	1	ce(254G)	ce	6
	weak D type 10	D	1			
	weak D type 18	D	1			
Not discriminated	DAU4	DAU4 or DHK	1			
	DAU5	DAU5 or DVa type 1	3			

[a]No markers associated with these alleles on current RHD or RHCE BeadChip

Table 11.4 RHCE alleles with concordant results between manual testing and RHD BeadChip

RHCE allele	Group A (no.)	Group B (no.)	RHCE allele	Group A (no.)	Group B (no.)
ce/cE/Ce/CE	45	64	ceRT	1	
ce48C	9	24	ceMO		4
ce733G[a]	6	69	ceJAL/ceS(340)	2	2
ce48C, 733G[a]	1	18	ceCF	2	3
ceS[b]	9	22	DHAR	1	
ceAR	5	6	ceSL	1	
ceEK	1	1	C^x	1	
ceBI	1	1	C^w	1	
ceTI	5	5	new cE(48C)		1

Group A: original 50 samples. Group B: alleles diagnosed subsequently in 118 samples. All samples were referred for problem workup. ceS = ce48C, 733G, 1006T
[a]Called ceS by BeadChip™
[b]Called (C)ceS by BeadChip™

but 602C/C rather than 602G/C was indicated. *RHCE*CeRN.1*, in which exon 4 of *RHCE*Ce* is replaced with exon 4 of *RHD*, was not detected in any of four samples with this allele. Six samples with ce alleles carrying a nucleotide 254C>G change [16], *RHCE*ce254G*, were not detected. Markers for these later two RHCE alleles are not on the current RHCE BeadChip™.

Following parallel testing, RHCE BeadChip™ was used to screen 118 samples referred for problem investigation. Screening by BeadChip™ was supplemented with manual assays. The alleles identified are summarized in Table 11.4 (Group B). A total of 156 altered RHCE alleles were identified. The results were consistent with manual multiplex PCR and with the serologic presentation.

11.4 Conclusion

RHD and RHCE BeadChip™ showed significant concordance with manual PCR assays and sequencing for diagnosis of RHD and RHCE polymorphisms in parallel testing of 108 samples for *RHD* and 50 samples for *RHCE*. Twenty-two of 34 RHD markers and 21 of 26 RHCE polymorphisms were investigated. This study was performed as a developmental study for the RH BeadChip platform and the information obtained was used for further product development.

For RHD, all samples were concordant between manual and automated RHD BeadChip™ with the exception of seven alleles. No markers for four, *RHD*DVII*, *RHD*DAU0*, and *RHD*weak D* type 10 and type 18, were present on the platform. *RHD*DVI* subgroup types 1 and 2 were identified as types 2 and 3, respectively. This could have some potential clinical relevance because DVI type 1 RBCs are BARC− and DVI types 2, 3, and 4 are BARC+.[19] Lastly, samples with *RHD*DIIIa/*D-CE(4-7)-D* were diagnosed as *RHD*DIIIa/*DIIIa*. It is important to detect *RHD-CE(4-7)-D*, which does not encode D antigen, but encodes altered C, associated with the (C)ce^{s}, i.e., r$^{'s}$ haplotype, and production of allo anti-C. This allele is common in people of African descent [6–8]. Until a polymorphism is identified that can differentiate *RHD*D-CE (4-7)-D* from *RHD*DIIIa*, we suggest the allele interpretation should indicate that confirmation of the RhC status with serologic testing is needed to discriminate *RHD*D-CE(4-7)-D* or *RHD*DIIIa* to correctly identify an r$^{'s}$ haplotype. This problem is not unique to the RH BeadChip™ assay and is also true with manual PCR assays.

For RHCE, all results were concordant between manual and automated RHCE BeadChip™ with the exception of six alleles. The presence of *RHCE*ceS* was always assumed to be with *RHD*D-CE(4-7)-D* resulting in overcall of (C)ceS, i.e., r$^{'s}$ haplotypes in 12 samples. The RHCE BeadChip™ Rh rS 5′ UTR marker is false positive (AB) in samples with *RHCE*ceS in trans to RHD*DIIIa* or deleted *RHD*. *RHCE*Ce(JAHK)* was misidentified as *RHCE*ceSL*. These alleles have the same nucleotide change, 340C>T, and it is not possible to distinguish which allele the change is associated with in samples with both Ce and ce alleles without Rh-cDNA sequencing or serologic evidence of weak C antigen expression. *RHCE*ceMO* was not identified in one of five samples with this allele, but no RHCE BeadChip™ assays were available for repeat testing. We have no explanation for this result. *RHCE*EIV* diagnosis was probably complicated by the presence of *RHCE*ceS in trans* in that sample. No markers for two alleles, *RHCE*CeRN.1* and *RHCE*ce(254G)*, are on the platform. In our experience, *RHCE*CeRN.1* is the more prevalent of the two alleles reported to encode a R^{N} phenotype associated with weak C and e antigen expression and production of allo anti-C, -e, or -Ce (-rh$_{i}$) [17]. *RHCE*ce(254G)* is associated with altered e, production of allo anti-e, and absence of a high in the Rh system designated CEAG [16]. Recent studies by our laboratory in collaboration with the New York Blood Center have found that *RHCE*ce(254G)* has a frequency of 9.6% in African-Americans [18].

In total, results of testing 149 samples for *RHD* and 168 for *RHCE* are summarized in this chapter. All samples were referred for Rh clinical problems. Many samples

Table 11.5 Reported RHD alleles not detected by RHD BeadChip™

Weak D
6, 7, 8, 9, 10, 12, 13, 16, 18, 19, 20, 21–24, 26–28, 30–33, 35–39, 42–46, 48–50, 52–73
Partial D
DII, DAU-6, DAU-7, DCC, DDE, DFL, DFW, DHO, DHR, DIM, DMH, DNAK, DNU, DVL-1, DVL-2, DWI
Del
MII, L18P, 147delA, L84P, A137E, L153P, IVS3+1G>A, IVS3+2T>A, IVS5-38DEL4, W408R, X418L
D−
Q41X, W90X, 325delA, IVS2+1G>A, IVS2-1G>A, 334delC, 449delT, 487del4, W185X, G212V, 711delC, 652delA and 653T>G, 785delA, Y269X, 908instggct,IVS6+1DEL4, G314V, 970del3 and 976del16, Y330X, IVS8+1G>A, Y401X

Table 11.6 Reported RHCE alleles not detected by RHCE BeadChip™

ce
G96S, R114Q, H171R, W217R, P221R, P292L, G385A, X418Y, 5′-UTR-10C>T, R229del
Ce
G96S, H166L, S230T, Y243C, A244T, M267K, L297P, M155R and A373V, RN, H166L, 27DelL, IVS3-5T>G, IVS9-2A>G
cE
R10W, L115P, S119N, I125N, M155R and N159K, L169Q, L303Q, IVS1-1G>A, cEMI
CE
T241I

assayed for *RHD* had discrepant D typing or RBCs that typed as D+ but anti-D was present in the serum. For *RHCE*, most samples typed as C+ and/or e+, but had anti-C and/or "e-like specificity," -hr^B or -hr^S, or antibodies to high-prevalence Rh antigens in the serum. Eighty-one of 108 altered *RHD* alleles and 202 of 227 altered *RHCE* alleles were concordant by RHD and RHCE BeadChip™ and manual PCR-RFLP and sequencing. The majority of discrepancies were due to the absence of markers on the BeadChip™ platform. This study is significant for the number of samples analyzed by both manual and automated methods, and for the fact that all samples were encountered in routine transfusion practice.

RH genotyping is superior to serology for differentiating weak and partial D and determining RH background in samples lacking high-prevalence RHCE antigens. The BeadChip™ assays do not detect all of the polymorphisms reported to date. The alleles that are not detected are summarized in Tables 11.5 and 11.6. The present goals would be to detect the commonly encountered polymorphisms, to act as a screening method, to aid in the diagnosis of altered alleles, and to reduce manual testing and gene sequencing. The RHD and RHCE BeadChip™ assays offer a considerable advantage over manual PCR methods and sequencing, thereby reducing both time and labor and eliminating complex interpretation. As such, this new method will make molecular blood group technology more accessible to the profession.

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Chapter 12
Bayesian Classification Algorithms for Automated Allele Assignment

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Abstract The application of Bayesian analysis to the classification of discretized BeadChip™ signal intensities generated in the context of multiplex allele analysis is described in this chapter. This analysis enables reliable allele assignment for polymorphic genes, notably those encoding the human leukocyte antigens and the variants of RhCE and RhD antigens.

Keywords Allele • Bayesian classification • Bayes' theorem • DNA array • Genotype • Human leukocyte antigen • RHCE • RHD

12.1 Introduction

This chapter describes the application of Bayesian analysis to the classification of discretized BeadChip™ signal intensities generated in the context of multiplex allele analysis in order to enable reliable allele assignment for polymorphic genes, notably those encoding the human leukocyte antigens (HLAs) and the variants of RhCE and RhD antigens. As described in greater detail in Chap. 3, BeadChip™ allele discrimination yields signal intensity distributions reflecting the degree of complementarity between oligonucleotide probes displayed on color-coded microparticles and polymorphic sites within the target sequence(s) of interest. Analysis and interpretation of these continuous signal intensities require their conversion into discrete patterns representing genotypes and the interpretation of such genotypes in terms of a pair-wise superposition of alleles that define phenotypes. In the present context, these phenotypes relate to red blood cell (erythrocyte), platelet, or leukocyte antigen expression. In that sense, the signal intensity pattern, reflecting the interaction of a set of probes with specific molecular target subsequences,

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contains the desired allele or haplotype information. In general, the larger the set of probes, the greater is the design resolution, and consequently, the greater the power to discriminate between similar allele-pairs. As a corollary, design ambiguity reflects the fact that for a given set of probes more than one possible allele-pair may produce the same observed reaction pattern, leaving the candidate allele-pairs unresolved at the given design resolution. Ambiguity that is not design related can also arise when the information implicitly contained in the reaction pattern is insufficient to discriminate between multiple allele-pairs even when their respective reaction patterns are distinct.

Alleles are identified by a multiplicity of probe signal intensities that (to varying degrees) are affected by experimental measurement error that can render probes less informative than they otherwise would be in a noise-free environment. Thus, the signal intensity pattern generated by a finite set of probes may not furnish complete information about the target sequence of interest, and one must ask how to take into account incomplete information and related uncertainty. For example, because the probe-target interaction is not "point-like," it will be affected by polymorphic sites in the sequences flanking the designated site of interest leading to variability in the expected signal intensities – a situation that is not uncommon especially in highly polymorphic targets such as HLA. In addition, signal intensities will contain contributions from nonspecific interactions (even after background corrections). This will display variability that originates in upstream process steps, notably enzyme-catalyzed reactions, such as amplification and elongation, as well as the imaging step itself. On the other hand, multiple and/or overlapping probes within a design may produce redundant information, and one must then ask how to generate a best estimate of the desired information based on partially redundant data.

In some instances, genotypes have a simple (and sometimes a one-to-one) relationship to phenotypes. In such cases, a rule-based approach affords an effective method of phenotype determination by invoking hard-coded logic or look-up tables [1]. However, in the case of allele discrimination of highly polymorphic genes, such as the applications considered here, the analysis is considerably more complex.

Bayesian analysis offers an alternative to a rule-based framework of analysis, allowing reliable allele assignments to be made in the presence of variability and noise. In general, reported algorithms that have been utilized to perform genotype/phenotype analysis often take the form of classifiers that operate on the analysis input data and select assignments/calls from a finite set. The recent literature describes several examples of alternative algorithms applied to automated genotype classification, including Mahalanobis distance [2, 3], linear discriminant analysis [4], support vector machine [5], nearest neighbor [6], Bayesian [7], and variational Bayes [8] classifiers. The approach developed at BioArray Solutions, beyond a classification by genotype, aims to automate the additional classification into allele-pair assignment(s) which, traditionally, has required the manual redaction of assignments by human experts. This proprietary Bayesian allele assignment methodology explicitly takes into account the fact that signal intensities produced by single nucleotide polymorphism defining a genotype may not be equally reliable or informative, and explicitly incorporates allele prevalence rates (often varying

with ethnicity and location) in order to compute a confidence score by which to select the preferred allele assignment among multiple possible candidates. An essential aspect of this classification approach is that of maintaining a supplementary codebook in the form of a hit table, comprising expected genotype patterns for all allele-pairs. It is the description of the methodology underlying this latter classification step, including the training of the classifier and the inferential operation, which is the focus of this chapter.

12.2 Bayesian Classification for Automated Allele Assignment

Bayes' theorem states the relationship between two conditional probabilities $P(C_i|D)$, $P(D|C_i)$ and two marginal probabilities $P(D)$, $P(C_i)$, where C_i and D respectively denote the class and data random variables, as follows:

$$P(C_i \mid D) = \frac{P(D \mid C_i)}{P(D)} P(C_i) \propto P(D \mid C_i)\, P(C_i) \tag{12.1}$$

In general, D represents a data vector of input values, either of a continuous numeric type, as in the genotype classification problem, or of a discrete numeric/string type, as in the allele assignment problem. The marginal probability $P(D)$, sometimes called the evidence, is the probability of the particular data vector being analyzed. However, this generally is not needed in a classification context and usually is absorbed into a normalization factor. In the present context, the class variable C_i represents an allele-pair designation – the desired output of the classifier. The marginal probability $P(C_i)$, also called the class prior probability, is a measure of the probability of the allele-pair i in the general population. This is determined from allele frequencies reported in the literature, or can be estimated from data if a sufficiently large data set of known results is available. $P(D|C_i)$, also called the likelihood, is the joint probability of the observed data, conditioned on the class C_i (allele-pair). Finally, $P(C_i|D)$, also called the posterior probability, is the probability of the allele-pair i, given the observed data vector D and represents the output distribution over possible results in the classification problem. In the context of the classification of allele-pairs, we refer to this posterior probability as the confidence score of the assignment.

For a given input vector D, the distribution of the $P(C_i|D)$ values over n possible allele-pairs must total to 1:

$$\sum_{i=1}^{n} P(C_i \mid D) = 1 \tag{12.2}$$

Because of the information content of the data vector D, in the Shannon sense [9, 10], the application of Bayes' theorem reduces the uncertainty exhibited in the prior distribution $P(C_i)$, resulting in a narrower posterior distribution of $P(C_i|D)$, typically with only a small number of values having any significant probability mass. In many cases, nearly all the posterior probability is concentrated in only one allele-pair.

Thus, by computing posterior probability values (or confidence scores) for all candidate allele-pairs and then ranking candidates in decreasing order by this score, the allele-pair with the highest probability is selected. Equivalently, a maximum posterior criterion can be used to select candidates [10–12]. Confidence scores for reported allele-pairs typically exceed 95%. In cases producing multiple candidate allele-pairs with comparable posterior magnitude, all candidates are reported as an ambiguous set. Such a situation may reflect design ambiguities as well as those due to experimental variability and noise.

For purposes of classification, it is common to further factorize the conditional probability $P(D|C_i)$ by assuming that the components of D are conditionally independent of each other given the class variable C_i. The considerable merit and justification of this approximation has been discussed extensively in the literature [10–13]. For HLA, these components relate to the oligonucleotide probes and can assume values of (0, 1), while for RHCE and RHD, they relate to complementary probe pairs and can assume values of (AA, AB, BB, LS). This factorization produces the following form of Bayes' theorem:

$$P(C_i \mid D) = \frac{P(D \mid C_i)\, P(C_i)}{P(D)} \propto P(C_i) \prod_{j=1}^{d} P(D_j \mid C_i) \tag{12.3}$$

where D_j is the component of the data vector D of dimensionality d. The terms $P(D_j|C_i)$ are called class-conditional probabilities, and measure the likelihood of obtaining one of the possible data values D_j for probe/marker j, given the allele-pair C_i. Multiplication of these factors, as in Eqn. (12.3), produces the overall likelihood $P(D|C_i)$.

An extension to this approach is to treat each probe/marker as an independent information channel [9]. In this model, the input to the channel is the actual state of the marker presented to the channel, encoded as (0, 1) for HLA or (AA, AB, BB, LS) for RHCE and RHD. This information is transferred through the channel via the assay process and is decoded by a discretizing classifier [14, 15]. Because of aggregate noise in the channel, the received output may be imperfectly decoded: misclassifying 0 as 1 or 1 as 0 in HLA, or in RHCE/RHD, mapping the inputs to a larger set of observed states in the set (AA, Ax, AB, xB, BB, xx, LS). These mappings of input to output states, for a given information channel, are shown as channel diagrams in Figs. 12.1 and 12.2.

Considering a single channel, for each possible transition between input X and observed output Y there is an associated transition probability $P(Y|X)$. These can be arranged into a matrix according to the particular states of X and Y, with each row having a sum of unity. These are the forward probabilities of observing the genotype result Y given that the actual input genotype is X. This channel matrix is shown in Table 12.1 for HLA. For HLA, only two independent parameters are required for each channel. We designate these as *FP* and *FN*, which correspond to the false positive and false negative misclassification probabilities. For RHCE/RHD, the classification model leads to a more complex channel diagram and matrix, requiring nine independent parameters per channel. We designate these parameters as double-scripted β values with the lower subscript as the column and the upper

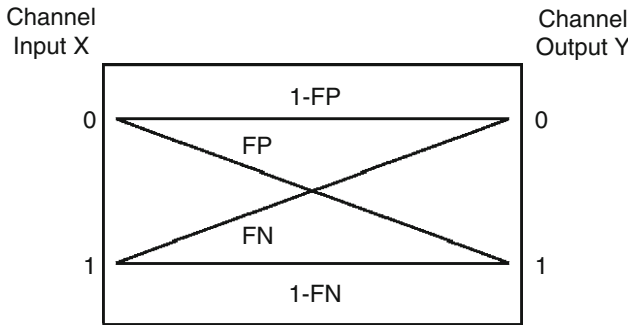

Fig. 12.1 Channel diagram for HLA. Transition probabilities associated with the lines between the inputs and outputs correspond to the channel matrix elements in Table 12.1

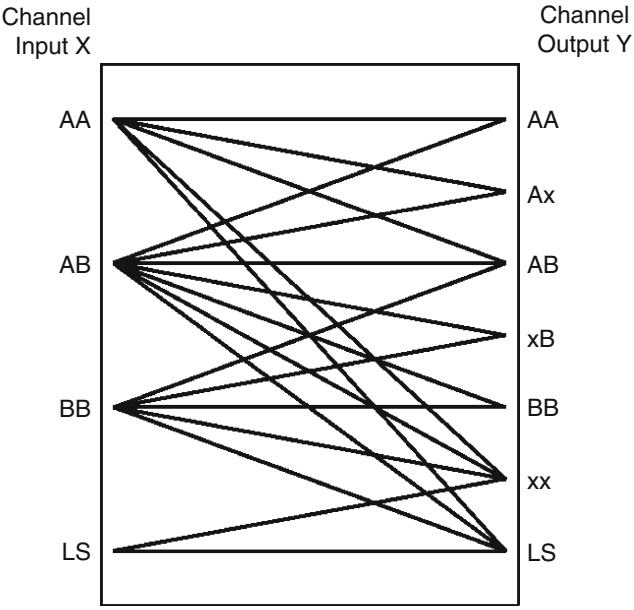

Fig. 12.2 Channel diagram for RHCE and RHD. Transition probabilities associated with the lines between the inputs and outputs correspond to the channel matrix elements

Table 12.1 Channel matrix for HLA

	0	1
0	$1-FP$	FP
1	FN	$1-FN$

subscript as the row. Interpretation of these parameters as probability mass values is indicated in Figs. 12.3 and 12.4. These parameters are determined numerically in the training phase of the discretizing classifier as will be described in later sections.

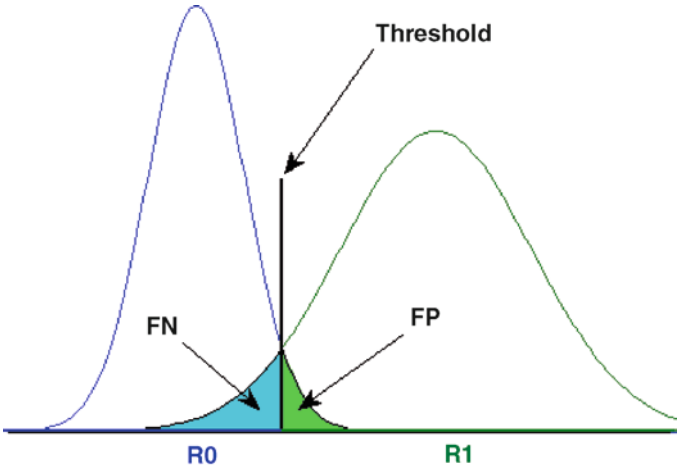

Fig. 12.3 Definition of *FP* and *FN* is given in Eqn. 12.5

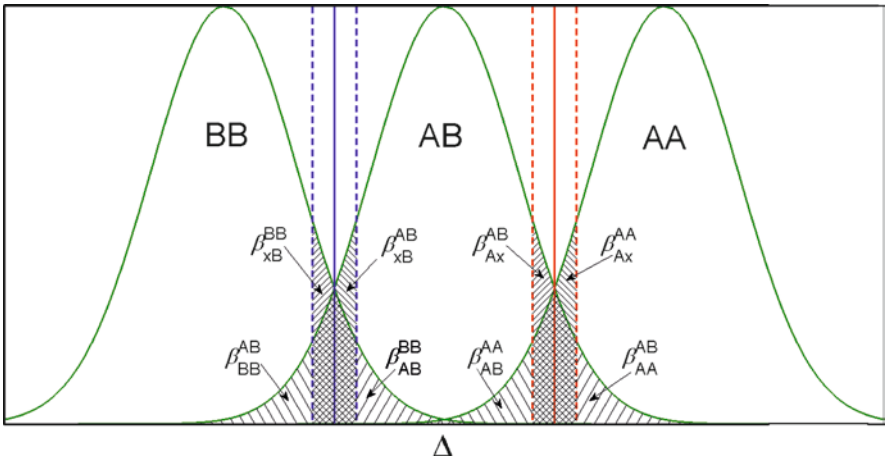

Fig. 12.4 Typical probability density functions of the three genotypes showing threshold lines and areas for corresponding error rates. *Dotted vertical lines* between AB and AA distributions are upper gray-zone boundaries. *Dotted vertical lines* between BB and AB distributions are lower gray-zone boundaries

The hit table or code book contains a row for each class C_i (allele-pair). The jth element of the ith row, h_{ij} represents an allele-pair i and probe marker j, that is the actual input value, $X = h_{ij.}$. For example, h_{ij} assumes the values (0, 1) for HLA and (AA, AB, BB, LS) for RHCE/RHD. The decoded experimentally received value Y, on the other hand, is just D_j for the probe/marker. Therefore, we can identify each of the class-conditional probability factors in Eqn. (12.3) with the value of the channel matrix element where row and column indices are given by D_j and h_{ij}:

$$P(D_j \mid C_i) = P\left(Y = D_j \mid X = h_{ij}\right) = M^{\text{channel}}_{x=h_{ij}, y=D_j} \tag{12.4}$$

Invoking a hit table as a supplementary codebook significantly reduces the number of parameters otherwise required for a Naïve Bayesian classifier when the number of classes is large. The required number of class-conditional probability parameters, normally obtained from training, scales as nc, where c is the number of allele-pairs and n is the number of probes [11, 12]. However, using a hit table and the Gaussian training method described above, the HLA problem only requires two parameters per probe, and thus scales as 2n. The size of the hit table still scales as nc, but this table is readily constructed from first principles given knowledge of the assay design.

12.3 Bayesian Analysis of the HLA Complex

To initiate HLA allele assignment, probe signal intensities recorded for exons 2 and 3 are first normalized by the intensity of the appropriate exon-specific positive control to generate normalized intensities, also referred to as probe ratios. In spite of normalization, variations in probe ratios are observed across sample runs, as illustrated in Fig. 12.5. These are attributed to variations in (1) the concentration of genomic DNA, the starting material for the analysis, (2) the efficiency of PCR-mediated DNA amplification, and (3) operator-to-operator variations.

Across multiple samples analyzed in any assay, we can characterize the distribution of ratio values for each probe conditioned on the probe state (0 or 1). An example of such a distribution, for a probe known as HB111A, is displayed in Fig. 12.6 as a normal probability plot with reference to a Gaussian model.

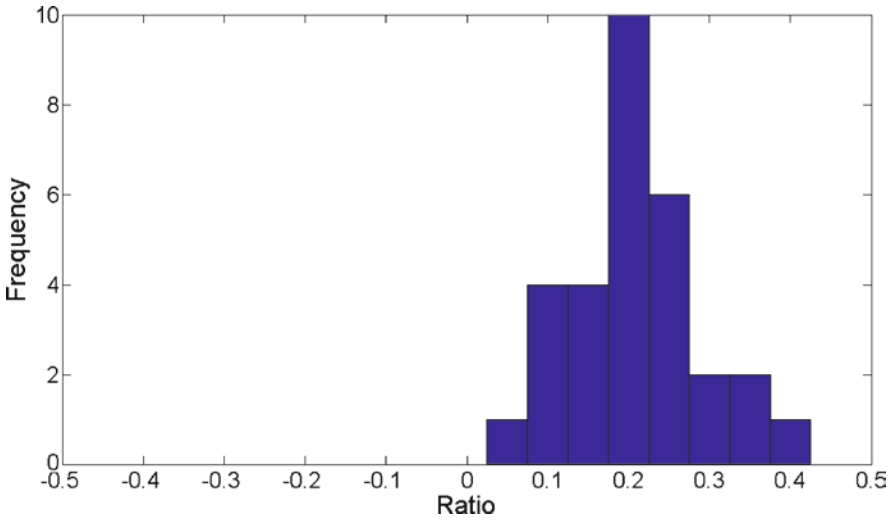

Fig. 12.5 Histogram of probe HB117A ratio values for sample C1083 replicated 30 times

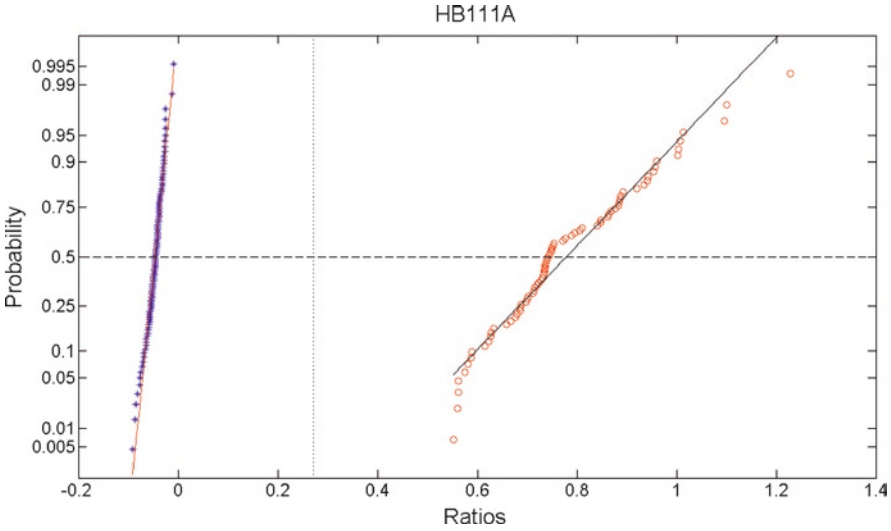

Fig. 12.6 Normal probability plot of 0 (*asterisks*) and 1 (*circles*) states for probe HB111A. The threshold value is indicated by the *vertical dotted line*

As indicated by the reasonable fit of the data to the straight lines in the plot, a Gaussian distribution provides a good representation of the data.

12.3.1 Methodology

The HLA analysis algorithm utilizes two classifiers back-to-back. The first classifier (also referred to as the discretizer) takes the continuously varying ratios for each probe, and classifies the state of the probe as either negative (0) or positive (1) based on probe-specific threshold values. These binary values are collected sequentially into a vector called the reaction pattern which we will designate as *D*. The second classifier, takes the vector *D* as the input, refers to a hit table for existing alleles [16, 17], a set of transition probabilities, and the allele-pair frequencies, then uses Bayes' theorem to estimate the posterior probabilities of all candidate allele-pairs. The allele-pairs with the most significant posterior probabilities are then selected for further processing, namely by "rolling-up" (or marginalizing) selected pairs over allele groups for the final assignments reported. If group ambiguities are discovered during the roll-up, these are also reported.

12.3.2 Classifier Training by Supervised Learning

Each of the above classifiers must be trained on a set of training data – usually produced using samples with independently known allele assignments – in order to

set parameter values ensuring the reliable functioning of the classifier. We use supervised learning to determine the parameters for the first classifier (discretizer). For each manufacturing lot, known samples are collected to provide a representative distribution of 0s and 1s for each probe. The samples are run at different DNA concentrations, and with different operators. Since the correct allele-pair assignment is known for each sample in the training set, the hit table is used to label the class of each of the training results for each probe as either 0 or 1. We use Gaussian discriminant analysis on the continuous ratio space for each probe and utilize maximum likelihood to find best estimates of the mean (μ) and variance (σ^2) for each of the two classes (0, 1). This analysis gives the class-conditional probabilities $P(x|\mu,\sigma^2,S)$ for each probe, where x is the continuous ratio value, and S is the class (0, 1). We assume probe independence, and we also assume that on a given probe, the class-conditional probabilities $P(x|\mu,\sigma^2,S)$ are conditionally independent of the allele-pair. In other words, the Gaussian distributions, given the class 0 or 1, are independent of the particular allele-pair giving rise to the 0 or 1 state. The variations in x expressed by the class-conditional Gaussian distribution for a given state are dominated by process variabilities (noise), not allele-pair differences (Fig. 12.5).

12.3.3 Thresholds and Transition Probabilities

Following the determination of the Gaussian parameters, threshold values for the discretizer are determined by a novel proprietary "minimum-flip" algorithm which finds a unique threshold value based on the labeled training data. These threshold values define the class boundaries that divide the x continuum into two disjoint regions, R0 and R1, corresponding to negative (0) and positive (1) states, respectively.

Subsequent to threshold determination, probability mass values are determined for each probe from the class-conditional probabilities $P(x|\mu,\sigma^2,S)$ and the class boundaries. These are obtained by integrating appropriate class-conditional probability functions over each of the class regions, for each value of the class S as shown in Fig. 12.3 and in the following equations:

$$FP_j = \int_{R1} P\left(x \mid \mu_0, \sigma_0^2, S_j = 0\right) \mathrm{d}x \tag{12.5a}$$

$$FN_j = \int_{R0} P\left(x \mid \mu_1, \sigma_1^2, S_j = 1\right) \mathrm{d}x \tag{12.5b}$$

where the subscript j refers to the probe, but is not shown for all parameters for clarity. Because each class-conditional probability function is normalized to unity over the entire x space, only two parameters, FP_j and FN_j, are required for each probe.

FP and *FN* are the probabilities of misclassifying the 0 and 1 state, respectively, and characterize the discretizer error for each probe. Thus, Eqn. (12.5a) shows *FP* to be the probability that the state of the probe is observed to be equal to 1, given that the actual state of the probe is equal to 0; and (12.5b) likewise shows *FN* to be the probability that the state of the probe is observed to be equal to 0, given that the

actual state of the probe is equal to 1. As mentioned above, because the class-conditional probabilities $P(x|\mu,\sigma^2,S)$ are conditionally independent of the allele-pair, so are the values of *FN* and *FP*.

12.3.4 Allele-Pair Assignment

The second classifier is used to convert the vector of discretized (0, 1) states into allele-pair assignments and uses a discrete Naïve Bayesian classification algorithm. Given the large number of allele-pair classes, which scales as $N(N+1)/2$ where N is the number of known alleles – 505 for HLA-A and 848 for HLA-B [17] – it is not feasible to determine prior probabilities and class-conditional probabilities using training data alone. Instead, allele-pair prior probabilities are estimated from the allele frequencies reported in the literature [18, 19]. The allele-pair prior probabilities are then given by:

$$P\left(A1, A2\right) = 2^{1-\delta(A1,A2)} P(A1)P(A2) \tag{12.6}$$

where $A1$ and $A2$ refer to each member of the allele-pair and $\delta(A1, A2)$ is the Kronecker delta function.

12.4 Bayesian RHCE and RHD Variant Analysis

12.4.1 Methodology

The Bayesian method for HLA typing was extended to BeadChip™ RHCE and RHD variant analysis. Unlike the HLA assay design, the RHCE and RHD assay designs extract information from pairs of analogous probes, one pair per variable site, one member of the pair complementary to the normal (wild-type) allele (A), and the other complementary to the variant (mutant) allele (B). Thus, each marker of interest is characterized by one of the three possible states or genotypes: AA representing homozygous normal; AB representing heterozygous; and BB representing homozygous variant. A possible fourth state, LS, is encountered when neither member of a probe pair matches the actual variant; this is an indication for an unanticipated base or another variation such as a deletion, affecting the target site of interest.

Intensity data obtained from each pair of probes directed to a specific variable site within the target sequence of interest are transformed to obtain the two quantities Δ and σ_Δ. Δ is a measure of the discrimination between intensities recorded from the normal and variant member of each pair and indicates the presence of either one of these alleles ($\Delta \approx 1$ or -1; homozygous configuration) or both of these alleles ($\Delta \approx 0$, heterozygous configuration); σ_Δ gives the error in the estimate of Δ which depends on the averages and variances of the normal, variant, and background probe intensities. The values of Δ and σ_Δ serve to classify signal intensities

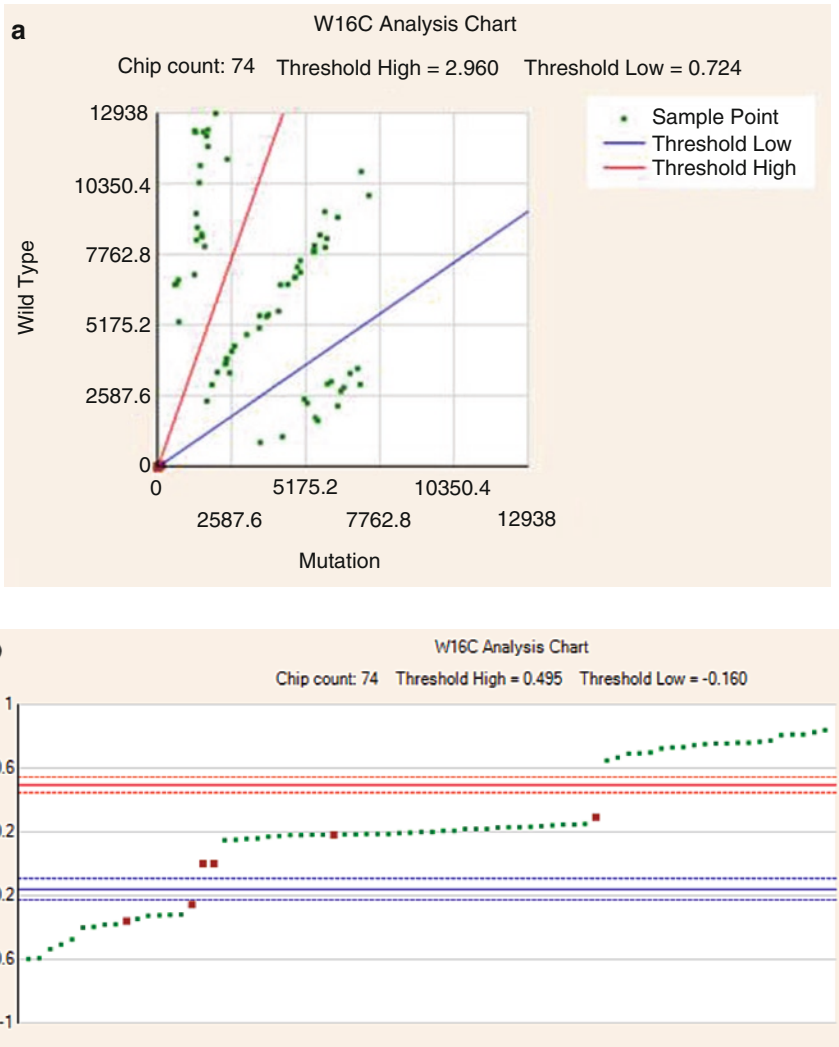

Fig. 12.7 **(a)** Scatter plot for W16C mutation showing distribution of wild-type and mutant intensities. Three clusters are observed for the AA, AB, and the BB genotypes. The *red line* demarcates the threshold between the AA and the AB states and the *blue line* is the threshold between the AB and the BB states. **(b)** Delta plot for the same set of data also show the three regions demarcated by the two thresholds. In addition, the *gray-zones* where one of the genotypes is indeterminate are also shown using the *dotted lines*. In both plots *green square markers* denoted valid samples whereas *brown markers* represent low signal/water samples

recorded for each variable target site into states AA, AB, or BB, defining a genotype, equivalent to the discretization in HLA, or into intermediate states Ax, xB, or xx, where x denotes an indeterminate state (see below). A "Low Signal" (LS) state is indicated when both probe intensities fall below a low intensity cutoff. Figure 12.7a is a depiction of a set of intensities for marker W16C in the RHCE

panel in the classical scatter plot format. The same data set is then shown as a Δ plot in Fig. 12.7b. For each marker, an upper threshold demarcates genotypes AA and AB, and a lower threshold demarcates genotypes AB and BB. On either side of the thresholds are gray-zones of finite width. Samples characterized by Δ values within these gray-zones are classified as Ax or xB. Samples characterized by σ_{Δ} values exceeding a preset cutoff are classified as xx, indicating that a reliable genotype assignment cannot be made. Thresholds, gray-zone widths, and error cutoff parameters are lot-dependent and are determined from training sets before the release of each lot.

Genotype states for all variable target sites are compiled into a binary string defining a reaction pattern which is then analyzed by application of a Bayesian typing algorithm. A hit table is compiled tabulating the theoretical states of all known allele-pairs for the selected set of mutations. Figure 12.8 shows a section of the RHD hit table. Each of the row entries corresponds to the reaction pattern of an allele-pair and each column represents a mutation in the RHD panel. For each recorded reaction pattern, the algorithm iterates through a hit table of all expected allele-pairs. Taking into account the relative frequencies of allele-pairs listed in the hit table and the performance of probe pairs (characterized by error rates; see below), the algorithm identifies the most probable candidate pair(s) yielding the observed reaction pattern and uses Bayes' theorem to compute the posterior probability. The allele-pair with the maximum value of the posterior probability is reported as the final assignment.

12.4.2 Error Rates

To illustrate the significance of error rates, Fig. 12.4 shows the overlapping distributions of the three primary genotypes, along with thresholds and error rates, β,

Mutation	W16C	W16X	S3C	L62F	R70Q	A137V	IVS3+1G/A	A149D	R114W	N152T	M170T	I172F	T201R	pseudoD	F223V	A226P	S230I
Allele																	
D	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
weak D type 1	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
weak D type 2	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
weak D type 3	AA	AA	BB	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
RHD-CE(4-7)-D (C)cde S	AA	AA	AA	BB	AA	BB	AA	AA	AA	BB	LS	LS	LS	LS	LS	LS	LS
RHD-CE(4-7)-D	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	LS	LS	LS	LS	LS	LS	LS
RHD-CE(3-7)-D	AA	AA	AA	AA	AA	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS
RHD-CE(3-9)-D	AA	AA	AA	AA	AA	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS
RHCE(1-3)-D(4-10)	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS	AA	AA	AA	AA	AA	AA	AA

Fig. 12.8 Extract from an RHD hit table showing the various alleles (classes) with their expected genotypes for each mutation in the panel

calculated from the averages and variances of the genotype distributions. For example, the error rate β_{AB}^{AA} or the probability that a sample of actual genotype AA is misclassified as AB, is given by the area under the AA-distribution to the left of the upper gray zone in Fig. 12.4. In addition, error rates relating to transitions between the states LS, xx, and nn also are considered; here, nn denotes all other genotypes combined.

12.4.3 RHCE Variant Analysis

The assay is designed to detect c, C, e, and E antigen-specific polymorphisms and identifies RHCE variants in genomic DNA samples. The classifier incorporates associative linkages between c/C and e/E markers, defining CE antigens, with variant allele markers, reflecting the exclusive associations of certain variants with particular major antigen combinations; this linkage information is stored in a linkage table (Table 12.2). In the first step of the Bayesian analysis, the presence or absence of major antigens is established. The second step of the Bayesian analysis is then performed with a restricted list of variants associated with antigen configurations assigned in the first step as defined in the linkage table.

The Bayesian RHCE classifier was deployed to analyze BeadChip™ assays performed on random African-American samples ($n=224$) and SCD patient samples ($n=55$) by Dr. Marion Reid of the New York Blood Center making automated calls for c, C, e, and E antigens and for RHCE variant alleles. The variant alleles identified in the data set were ce^{S}, ce^{S}(340), V^{S}+V−, ceAR, ceTI, ceMO, ceBI, ceCF, and C^{W}. A comparison of these assignments with those made manually by expert review showed complete agreement. Automated Bayesian assignments also were compared to results obtained with laboratory developed tests (LDT) developed at NYBC wherever available. Discordances were obtained only in situations where (1) samples contained rare alleles, not resolved or targeted by the BeadChip™ design or (2) samples contained known/unknown RHCE–RHD hybrid alleles along with nonhybrid alleles. Details of the assay and analyses are provided in Chap. 9 of this book.

12.4.4 RHD Variant Analysis: Neural Network Classification of Deletions and Hybrids

While RHD variant analysis is designed to detect the RHD gene and identify its variants using a hit table constructed in a manner analogous to that for RHCE variants, it is substantially complicated by deletions of portions or deletion of the entire RHD gene and by the presence of RHCE–RHD hybrids (produced by the exchange of exons between RHD and RHCE genes). As a result, the intensities recorded by

Table 12.2 Linkage table for RHCE analysis with association between major antigens and variant alleles

Major antigens Variants	c	C	e	E
WT	1	1	1	1
16C	1	1	1	1
ceAR	1		1	
ceS	1		1	
ceS (340)	1		1	
ceS (748)	1		1	
ceS (697) (ceCF)	1		1	
ceRT	1	1	1	
ceMO	1	1	1	
ceRA	1	1	1	
ce variant	1	1	1	1
ceEK	1	1	1	
CeVG		1	1	
CeMA		1	1	
ceBI	1	1	1	
ceSL	1		1	
ceTI	1		1	1
ceFV	1	1	1	1
(C)ceS	1		1	
(C)ceCF	1		1	
E type I	1			1
E type III (EFM)	1			1
E type IV	1			1
EKH	1			1
CW		1	1	1
CX		1	1	1
DHAR	1		1	
CeVA		1	1	
ceAR CF	1		1	

1 indicates strong linkage between variant allele and antigen. Blank entries indicate absence of linkage

probes directed to the affected regions will produce low signal intensities that are difficult to distinguish from a noisy background. To address this additional complexity and to ensure the reliable detection of deletions or hybrids, a neural-network-based deletion detector has been developed to differentiate between noise, indicating a nonspecific background, and a valid signal, confirming the presence of RHD-specific polymorphisms in the regions of interest. Raw genotypes obtained from the genotyping classifier are then filtered, by way of the neural network trained on exon-deletion information, to remove extraneous noise. The filtered genotypes are then classified by using Bayesian analysis, in a manner similar to that described for RHCE analysis.

The Baysian RHD classifier was deployed to analyze BeadChip™ assays performed at the New York Blood Center (as described above) for random

African-American donors and SCD patient samples. RHD variant alleles covering D+, weak D, partial D, and D− phenotypes were identified, along with variant alleles RHDψ, DIIIa, DIV, DV, DAU, DHK, DTO, DAR, weak D type 4, RHD deletion, and the hybrid allele DIIIa-CE(4–7). A comparison of these assignments with those made manually by expert review showed complete agreement. Automated Bayesian assignments also were compared to results obtained with "homebrew" or laboratory developed tests developed at NYBC wherever available. Discordances were obtained only in situations where samples contained rare alleles, not resolved or targeted by the BeadChip™ design (such as DAU-0). Zygosity determination was also not included in our assay, so homozygous samples were indistinguishable from hemizygous samples. Details of the assay and analyses appear in Chap. 9 of this book.

12.5 Implementation

All three Bayesian classifiers described above, wHLA, wRHCE, and wRHD, were implemented as ASP.NET web-hosted applications for data analysis and report generation for eventual deployment on the BioArray Solutions Information System (BASIS™).

12.6 Summary

Bayesian analysis, given allele frequencies and the (experimentally determined) error rates for probes in the array design, provides a framework for the quantitative assessment of uncertainty. BioArray Solutions has developed a set of proprietary algorithms that produce, along with allele and haplotype assignments, assignment confidence scores. This approach has proven robust for both hMAP and eMAP formats, the latter being especially relevant to RH variant analysis, and will carry forward to the realization of the eMAP-S format, illustrated by the data presented in several chapters of this volume.

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