Immucor PreciseType™ | HEA

MOLECULAR BEADCHIP TEST

Briefing Document for the Blood Products Advisory Committee

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THE CONTENTS OF THIS BRIEFING PACKAGE ARE FULLY RELEASABLE

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1 Introduction

The Immucor PreciseType™ HEA Molecular BeadChip Test is a multiplexed molecular assay that tests for a wide range of genetic variants affecting red blood cell antigen expression. The PreciseType HEA BeadChip Test detects 24 red blood cell polymorphisms associated with 38 antigens and phenotypic variants in a single test. The PreciseType HEA BeadChip Test has proven to be more accurate than serology while simultaneously providing:

- Extended antigen donor typing that can help identify samples as rare or weak
- The capability to detect altered phenotypes
- The power to more deeply characterize a donor registry
- The ability to type recently and polytransfused patients
- A more comprehensive donor/patient phenotype than serology
- Ultimately, a better donor-patient match

By collaborating with industry leaders, the antigens and phenotypic variants that were selected for the PreciseType HEA BeadChip Test were identified as: (1) clinically relevant, (2) variable in the population or (3) having limited-to-no available licensed serological tests. With this in mind, the PreciseType HEA BeadChip Test was developed to meet an unmet need in the transfusion community.

A series of studies have been completed demonstrating the performance of the PreciseType HEA BeadChip Test. These studies verified and validated the design of PreciseType HEA BeadChip Test as:

- A robust, accurate and precise test to type human erythrocyte antigens
- More accurate than serology for human erythrocyte antigen testing
- More comprehensive results than serology
- Less operator error compared to serology
- More timely than it would take to type individual available antigens serologically

Through these studies, we have proven that the product is safe and effective and supports the intended use.

2 Intended Use

The PreciseType HEA Molecular BeadChip Test is an *in vitro* diagnostic test intended for the molecular determination of allelic variants that indicate erythrocyte antigen phenotypes in the **Rh** (C [RH2], c [RH4], E [RH3], e [RH5], V [RH10], VS [RH20]), **Kell** (K [KEL1], k [KEL2], Kpa [KEL3], Kpb [KEL4], Jsa [KEL6], Jsb [KEL7]), **Duffy** (Fya [FY1], Fyb [FY2], GATA [FY-2], Fyx [FY2W]), **Kidd** (Jka [JK1], Jkb [JK2]), **MNS** (M [MNS1], N [MNS2], S [MNS3], s [MNS4], Uvar [MNS-3,5W], Uneg [MNS-3,-4,-5]), **Lutheran** (Lua [LU1], Lub [LU2]), **Dombrock** (Doa [DO1], Dob [DO2], Hy [DO4], Joa [DO5]), **Landsteiner-Wiener** (LWa [LW5], LWb [LW7]), **Diego** (Dia [DI1], Dib [DI2]), **Colton** (Coa [CO1], Cob [CO2]), and **Scianna** (Sc1[SC1], Sc2 [SC2]) blood group systems in human genomic DNA. The test also detects a mutation (HgbS 173A>T) in the Beta Globin gene. The results from this mutation detection are not for diagnosis of Sickle Cell Disease.

3 Description of the Test

Variations in red cell antigen expression can be determined by analyzing the DNA sequences of the genes governing the antigen. These genes can carry polymorphisms, which are mutations of the genetic sequence, resulting in different versions of the gene identified as alleles. Using various techniques, alleles that cause variations in red cell antigen expression can be directly identified by their polymorphisms. Techniques to detect polymorphisms in DNA have been employed in the clinical setting for decades and are already used in transfusion for the detection of blood pathogens.

The BioArray PreciseType HEA BeadChip Test utilizes Elongation mediated Multiplexed Analysis of Polymorphisms (eMAP) technology to identify the presence or absence of these polymorphisms via multiplex Polymerase Chain Reaction (PCR). On the BioArray BeadChip system, genomic DNA targets isolated from whole blood are amplified, captured and fluorescently labeled by elongation on allele specific probes immobilized on synthetic microparticles. The fluorescence of each bead is analyzed on the Array Imaging System (AIS) to determine positive and negative reactions. BioArray Solutions Information System (BASIS) software calculates the adjusted intensity of every reaction to assign a genotype and predicted phenotype for each polymorphism.

The Array Imaging System (AIS 400C), which is under review in a separate, 510(k) submission, is an automated image acquisition system designed for use with BioArray Solutions BeadChip kits. Image information is transmitted by the AIS 400C to BASIS where the data from each chip are analyzed and the resulting phenotype is reported.

Positive and negative controls are included in each run to assess run validity. The positive controls, which are artificial plasmid constructs designed to assess the performance of each probe, are under review in a separate 510(k) submission.

4 Background

Human erythrocyte (red blood cell, RBC) blood group antigens are surface markers located on the membrane of the red blood cell. They are polymorphic, inherited protein and/or carbohydrate structures attached to either lipid or protein structures within or on the cell membrane. Exposure to erythrocytes (RBCs) carrying antigens not expressed on the red cells of the recipients of the transfused cells may produce an immune response in some individuals. These responses vary in degree of severity from immediate and severe to none at all [1]. Once an alloantibody is produced, lifelong immunization occurs, even if the antibody is not detectable. In certain medical conditions requiring frequent, ongoing blood transfusion therapy, increased opportunity for alloantibody production occurs:

- Reported 19-47% alloimmunization rate in sickle cell disease patients [31, 37]
- Reported 12-40% alloimmunization rates in patients with warm autoantibodies [32, 38]
- Reported 11% alloimmunization rate in patients with aplastic anemia [33]
- Reported 29% alloimmunization rate in all hemoglobinopathy patients (including sickle cell disease) [33]
- Reported 11% alloimmunization rate in patients with gastrointestinal bleeding [33]
- Reported 14% alloimmunization rate in patients with renal failure [33]
- Reported 15-59% alloimmunization rate in myelodysplastic syndrome patients [35, 36]

In such cases, the identification of the presence of blood group antigens is usually performed through serological phenotyping; however, analysis of DNA, rather than serological phenotyping, is becoming increasingly useful [2, 3, 4-13].

Currently the methodology for the determination of human erythrocyte antigens is by hemagglutination, serological testing using anti-sera with known antigen specificity, a technology in use for over 100 years [19, 21]. While manual methods are mostly used to perform this testing, most hospitals and testing laboratories also utilize automated instruments that were first introduced in the 1980s and are available from companies including Immucor, Ortho-Clinical Diagnostics, Bio-Rad, and Beckman-Coulter. These instruments automate test processing, result interpretation and data management functions. Automated process controls and error detection mechanisms are often

included to reduce or eliminate opportunities for user error and invalidate suspect results. There are different platform designs used to automate standard immunohematology assays including microwell plates, microscopic slides, micro-tubes and micro-wells. Assays include, but are not limited to, ABO grouping and Rh (D) typing, RHC, c, E, e and Kell antigen typing and detection/identification of IgG red blood cell antibodies.

While serological testing is clearly a proven technology, technical advances and new methods continue to be developed, for example the introduction of techniques utilizing monoclonal antibodies to improve the availability and consistency of the "source" materials and the automation of equipment to process the blood samples and reagents to obtain the required results (automated instruments as described). In spite of these advances there are limitations to serological methods.

4.1 Limitations of Serology

The need for a more comprehensive and patient-specific method of antigen typing is evident by the persistence of alloimmunization. Serology has historically provided a solid foundation for transfusion medicine, but its limitations are evident:

- Patients are typically only matched for ABO and RhD then screened for historical antibodies
- Declining antibody titers, even in the previously alloimmunized, limit serology's ability for detection
- Limited availability of select anti-sera
- Phenotyping (multiple, extensive typing) across all patients and donors is impractical
- The quality of the reagents which can prove challenging or are weakly reactive [18, 20, 21].
- Extensive serological testing to build a complete profile of the donor or patient requires several days of testing
- Multiple sera specific to different antigens are required for extended phenotypes of patients on more than one antigen

This testing is often performed in a serial manner. Due to the need for multiple tests, the processing, even if it's automated, can take several hours with each test interpreted individually and then compiled over several days to build a donor or patient profile. With current serological methods, additional matching for rare antigens is performed only when one or more clinically significant antibodies is detected or when the patient is part of a group or special study protocol designed to reduce the incidence of alloimmunization (e.g., chronic transfusion recipients such as in sickle cell disease) [18, 19, 22].

Lastly, the production of blood typing reagents either from polyclonal or monoclonal based raw materials is fundamentally limited by requiring a biological source (whether actual antibody containing sera or a cell line developed for monoclonal production) as a starting material for large scale production of reagents. Since they are biological in nature, the quality of these reagents can be variable despite quality measures and regulatory review [20, 21, 22].

The limitations of serological testing are summarized below in the following sections.

4.1.1 Technical Limitations to Serology

Technical limitations in serological typing include:

- Subjective interpretation when performing manual serological tests [21]
- Serology often utilizes manual tests that can be labor intensive and involve manual data entry [21]
- Automated typing only exists for RhCcEe and Kell
- Requires use of reliable antisera [19, 20, 21]
- Many rare antisera are not FDA licensed and or are only available in limited volumes, weakly reactive or simply unavailable, e.g. Lutheran (Lu^a) and Dombrock (Do^a/Do^b) [19, 20, 22].

4.1.2 Clinical Limitations to Serology

Clinical limitations to serology include:

- Difficulty in typing recently transfused patients whose circulating donor RBCs can persist for weeks, complicating the determination of an individual's phenotype by hemagglutination methods [19, 20, 22].
- Due to the time, complexity and cost, only a small number of donors are typed for a few antigens, limiting antigen-negative or rare donor registries [22].
- Minor antigens are not routinely tested on a regular basis possibly resulting in alloimmunization and complicating future transfusions [22].
- In patients with warm autoantibodies, red blood cells with a positive direct antiglobulin test are often difficult to type due to the presence of bound immunoglobulin G [19, 21]
- The need to identify, separate and purify polyclonal reagents prior to large scale manufacturing

4.1.3 Impact of Alloimmunization

Red Blood Cell alloimmunization occurs when a patient develops an immune response to antigens expressed on donor erythrocytes. Although alloimmunization occurs in 2% to 6% of transfusion patients, the rate can be much higher in polytransfused patients such as Sickle Cell Disease patients where the rate may be as high as 36% [21]. Alloimmunization from transfusions can range in severity and occur immediately or as a delayed hemolytic transfusion reactions (DHTRs) leading to hemolysis of donor red blood cells. Pregnant women are also at risk of developing alloantibodies against the fetus. Up to 0.8% of pregnancies show some form of minor blood group incompatibility and hemolytic disease of the fetus and newborn (HDFN) can also occur. In all cases of alloimmunization, once the patient develops an antibody, lifelong immunization occurs. This may lead to complications upon future blood transfusions, especially in polytransfused patients [1, 2, 3, 4, 13].

Presence of alloantibodies in patients requiring transfusion therapy impairs our ability to supply such patients with compatible red blood cells for transfusion. This is because transfusion under such circumstances requires multiple antigen-negative units that are difficult to find and require phenotyping many more donor units [21]. The risk of alloimmunization can be mitigated through prophylactic matching of donors and patients and although prophylactic matching is recommended to prevent alloimmunization, it is often problematic to perform with serological reagents [40, 41]. Some of the factors preventing the wide spread adoption of prophylactic matching or extended phenotyping of patients utilizing serology include:

- Lack of automation for extended serological typing.
- Loss of sensitivity and specificity of serology for complex cases, including the inability to type recently transfused patients, IgG-coated red blood cells.
- Paucity of licensed reagents to perform extended typing.
- Additional burdens on the donor centers such as loss of laboratory technologist time performing the complex work ups and retention of an adequate red blood cell inventory [21]

4.2 New Molecular Methods

New diagnostic approaches utilizing PCR based methods provide the transfusion community with additional tools to overcome the limitations inherent with serology. These molecular methods do not rely on the availability of sera and can be tailored to detect any antigen with a known genotypic etiology either independently or in multiplex with other antigens, providing the potential for better patient care and faster time to result. Molecular methods can provide:

- Prospective identification of antigens that may cause alloimmunization instead of historical, post-exposure analysis
- Better matching across more antigens
- Superior sensitivity/specificity
- Potential for workflow and process enhancements

The invention of the PCR method in 1983, made rapid, large scale DNA-based test methods considerably easier to develop and led to development of PCR-based (Sequence Specific Primer (SSP) and Restriction Fragment Length Polymorphism (RFLP)) methods in a large variety of fields. The use of PCR-based tests is now commonplace and many are licensed by the FDA. The application of PCR-based tests in blood antigen screening as laboratory developed tests is also prevalent however they do not benefit from the oversight that FDA licensed tests do. These laboratory developed tests for blood antigen screening are necessary due to the lack of or limited availability of sera for antigen testing [14, 17, 19, 20]. Because they are manual and often only test a limited number of antigens, these manual PCR-based laboratory developed tests are not suitable for high throughput donor screening or testing higher volumes of patients.

The PreciseType HEA BeadChip Test was designed specifically to improve the current state of blood antigen typing by addressing the need for a verified and robust method for blood antigen typing that is both multiplexed and clinically validated. The PreciseType HEA BeadChip Test accomplishes this by utilizing established molecular DNA based methodologies to determine human erythrocyte antigens in order to match donated red blood cells with the recipients' blood prior to transfusion.

4.2.1 Advantages of Molecular Testing

Molecular testing provides advantages and addresses many of the limitations in serology discussed above. Advantages include:

Prospective identification of antigens that may cause alloimmunization:

One of the benefits of molecular testing overcomes the barriers present today due to the limitations in serology with respect to polytransfused patients.

- Provide genotypically matched donor units needed for patient transfusion. [14]
- Permit preliminary screening for "special", rare phenotype units which allows centers to identify and retain blood units that are less prevalent so that they may be provided to patients requiring specific antigen combinations when needed. [22]
- Units are characterized in one multiplex test in one shift as opposed to multiple serological tests performed over multiple days.

Better matching than serology across more antigens:

- Molecular methods allow for the detection of multiple targets within the same test.
- Molecular techniques allow for typing of complex patients that are historically difficult to type serologically such as those with multiple transfusions, positive direct antiglobulin test (DAT) or weak antigen expression [21, 22, 23].
- Oligonucleotide probes used in molecular typing can be designed to target any DNA variants that correspond to blood group antigens.
- There is no inherent limit to the number of antigens that can be detected by molecular methods.

Superior sensitivity/specificity to serology:

- Genotype chronically transfused patients who are more susceptible to alloimmunization for a comprehensive number of antigens [15, 17].
- Detect antigens in instances where a serological result may be unattainable such as: recently transfused, positive direct antiglobulin tests (DATs), rare blood group phenotypes, warm auto-antibodies [19, 21]

Better inventory utilization:

- Build deeper blood inventory of units with rare phenotypes enabling blood centers to phenotype their own inventories to minimize the need for purchasing units with rare phenotypes. [14, 16]
- Extended typing of non-ABO blood groups allows donor centers to utilize more A and B blood units, conserving O blood units for use in Trauma
- The availability of molecular testing is not dependent on availability of sera.

Potential FDA licensed testing:

- There are no FDA licensed antisera tests for the Colton, Scianna, Dombrock or Landsteiner-Wiener blood group or the Diego (b), Lutheran (a), Joseph and Holley antigen, all of which are on the PreciseType HEA BeadChip.

When contrasting the advantages of molecular testing with the limitations of serology, molecular testing is highlighted as an ideal complement to, in some cases, a viable replacement of existing serology. This is exemplified in the Duffy system, where the Fy^{x} (Fy^{bw}) can be missed due to its weak reaction with antisera. In such

cases, molecular technologies and serological methods can be used in a complementary fashion to elucidate all of the variants [12, 21-23].

4.2.2 Potential Limitations of Molecular Techniques

There are some limitations associated with molecular technologies, including:

- Molecular methods require that operators utilize molecular laboratory techniques and the ability to handle microliter volumes of reagents; skills that are not necessarily required for routine laboratory technologists but are readily learned.
- Expression of specific antigens require more than their corresponding allele and can be influenced by factors such as mutations not detected by the molecular method in the same gene or other genes that are required for antigen expression.

5 Device Description and Principles of Operation

BioArray Solutions' Human Erythrocyte Antigen Test, (HEA) is a DNA microarray assay that utilizes PCR amplification of genomic DNA coupled with the elongation mediated multiplexed analysis of polymorphisms (eMAP) platform [24] to determine allelic variants of human erythrocyte antigens. The test consists of five steps, followed by genotype analysis and phenotype determination using BioArray Solutions proprietary BASIS™ wHEA software. The process begins with extraction of DNA from whole blood, followed by multiplex polymerase chain reaction (mPCR) amplification of genomic regions of interest simultaneously generating multiple amplicons. Once the mPCR is complete, residual oligonucleotide primers and deoxyribonucleotide triphosphate (dNTPs) from the reaction are digested using ExoSAP (Exonuclease I and Recombinant Shrimp Alkaline Phosphatase) in the Clean-up step, which is then followed by Lambda Exonuclease digestion to generate single-stranded DNA (ssDNA) targets. The ssDNA targets are then applied to the HEA BeadChip, a planar array with ≈4,000 fluorescently-encoded 3.2 µm beads. Each fluorescently-encoded bead contains a unique blend of three primary fluorescent dyes, Ultraviolet (UV), Blue and Green respectively, that generates a distinct fluorescent signature. Each of the different oligonucleotide probes specific for nucleotide polymorphisms in Human Erythrocyte Antigens (HEAs) are conjugated to beads, each with a different and unique fluorescent signature. While the ssDNA targets incubate on the BeadChip surface, only probes with ssDNA complements in solution hybridize and elongate, incorporating a fluorescently-labeled dCTP. The elongation products on the BeadChip surface are then detected using a microscope capable of capturing fluorescent images.

The image of the fluorescence pattern within the array is recorded with an automated Array Imaging System (AIS) equipped with fluorescence optics and a charged coupled device camera. This assay image is then combined with previously recorded identity of the probes on individual beads within the array to record probe specific intensities.

Data interpretation is carried out using the BASIS (BioArray Solutions Information System). Within BASIS, the following functions are performed:

i) Association between BeadChips and sample IDs
ii) Verification of batch run validity and sample validity criteria
iii) Calculation of genotypes and predicted phenotypes
iv) Generation of reports

The user associates BeadChips with the corresponding sample identification. Run-based Negative and Positive Controls are defined and verified. Individual BeadChips are verified based on assay performance specifications. The ratio of assay signal intensities produced by a pair of probes, one probe matching the “A” allele and another matching the “B” allele, provides the basis for genotype determination. Using the recorded intensities and lot specific cut-off values, genotype and predicted phenotypes are determined for each BeadChip. Finally, assay typing results are generated for viewing and reporting.

5.1 Validity Criteria

Run Validity: The batch run is validated based on the validation criteria set with the BAS-provided Negative and Positive Controls.

- A BAS-provided Negative Control is valid if it does not generate sufficient signals above the preset lower cutoff.
- A BAS-provided Positive Control (designed to cover all probes in the panel) is valid if the phenotype pattern observed for the control corresponds with an expected pattern for the control predefined in the HEA analysis software.

For a batch run to be valid, the negative and the positive controls within the batch run must be valid. Invalid runs produce no genotype or phenotype results for all samples contained in that run.

Sample Validity: The validity of each Sample (or BeadChip) is established by tracking:

- The overall background on the BeadChip; a high background or HB flag invalidates a BeadChip
- The CV or ratio between the standard deviation to the average intensity of probes; if the CV is high for a preset percentage of probes, the BeadChip is invalidated
- The number of LS phenotypes; if any of the polymorphisms report an LS phenotype, the BeadChip is invalidated
- The number of IC (Indeterminate Call) phenotypes; if any of the polymorphisms report an IC phenotype, the BeadChip is invalidated

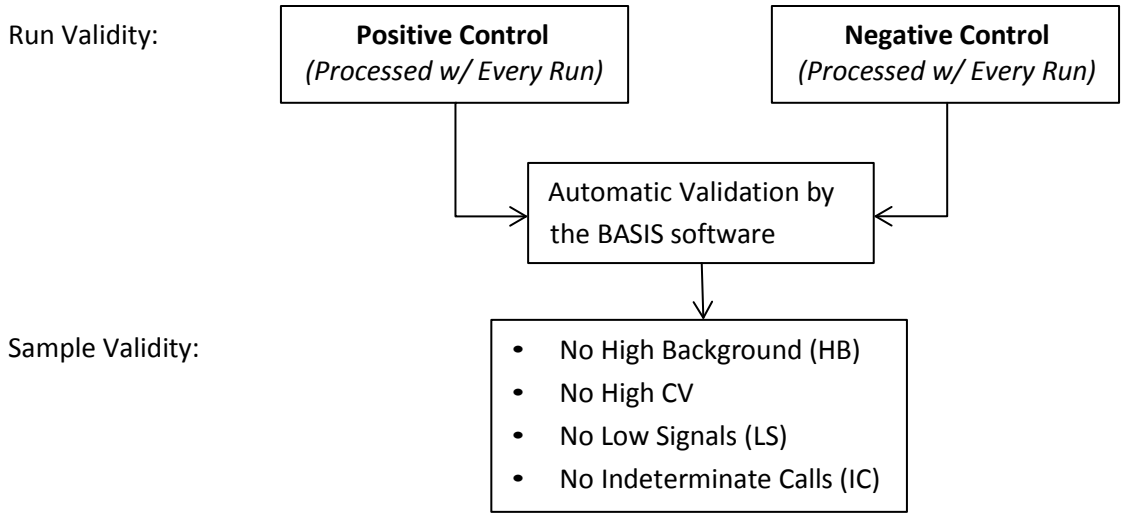


Figure 5-1 Validity Criteria Flowchart

5.2 Phenotypes and Genotypes Determined

The PreciseType HEA BeadChip Test detects the following genotypes and predicted phenotypes:

Table 5-1 Phenotypes and Genotypes by Blood Group

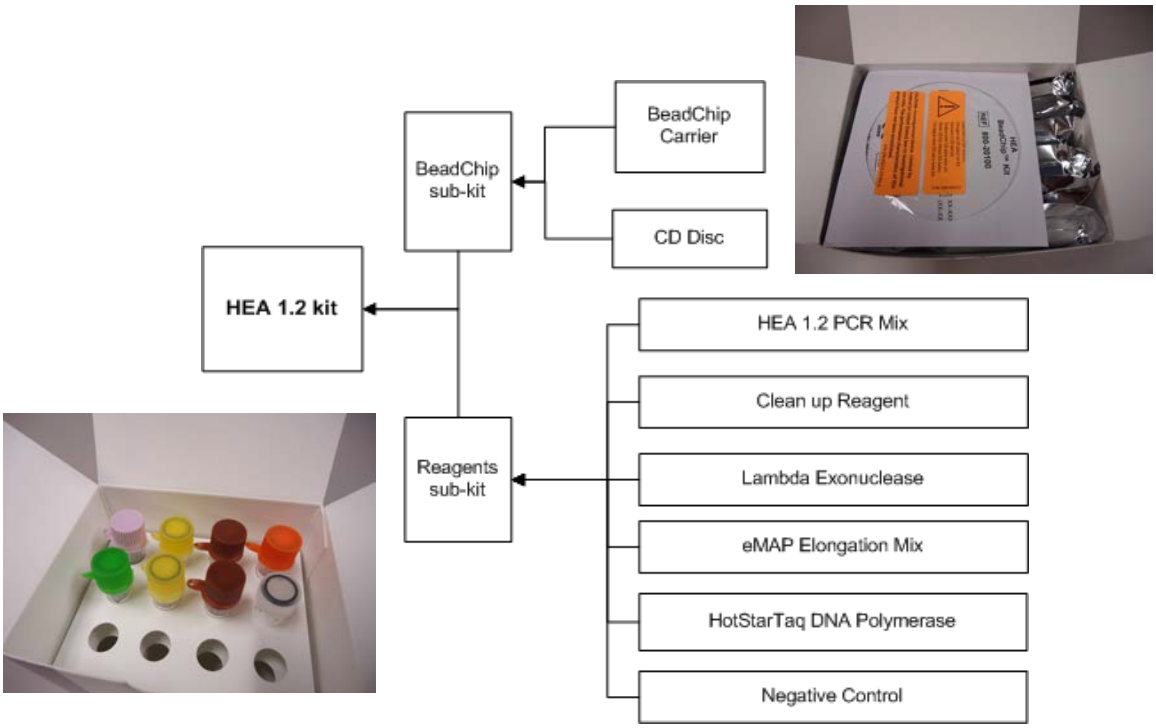
Blood Group System	Analyte	Polymorphism	ISBT Phenotype	*ISBT Genotype*
Rh	c/C	307 C>T	RH4, RH2	*RHCE*4, RHCE*2*
		109 Ins		
	e/E	676 G>C	RH5, RH3	*RHCE*5, RHCE*3*
	V	1006 G>T,	RH10	*RHCE*01.20.01, RHCE01.20.02, RHCE*01.20.04, RHCE*01.20.05*
	VS	733 C>G	RH20	
Kell	K/k	698 T>C	KEL1, KEL2	*KEL*01, KEL*02*
	Kp^a/Kp^b	961 T>C	KEL3, KEL4	*KEL*03, KEL*04*
	Js^a/Js^b	1910 C>T	KEL6, KEL7	*KEL*06, KEL*07*
Duffy	Fy^a/Fy^b	125 G>A	FY1, FY2	*FY*01, FY*02*
	GATA (Silencing *FY)*	-67 T>C	FY-2	*FY*02N.01*
	$Fy^x[Fy(b+^w)]$	265 C>T	FY2W	*FY*02M*
Kidd	Jk^a/Jk^b	838 G>A	JK1, JK2	*JK*01, JK*02*
MNS	M/N	59 C>T	MNS1, MNS2	*GYPA*01, GYPA*02*
	S/s	143 T>C	MNS3, MNS4	*GYPB*03, GYPB*04*
	Silencing S	In5 g>t	MNS-3, 5W MNS-3,-4,-5	*GYPB*03N.01 or GYPB*03N.02*
	Silencing S	230C>T		*GYPB*03N.03 or GYPB*03N.04*
Lutheran	Lu^a/Lu^b	230 A>G	LU1, LU2	*LU*01, LU*02*
Diego	Di^a/Di^b	2561 T>C	DI1, DI2	*DI*01, DI*02*
Colton	Co^a/Co^b	134 C>T	CO1, CO2	*CO*01, CO*02*
Dombrock	Do^a/Do^b	793 A>G	DO1, DO2	*DO*01, DO*02*
	Hy+/Hy	323 G>T	DO4	*DO*04*
	Jo(a+)/Jo(a)	350 C>T	DO5	*DO*05*
Landsteiner-Wiener	LW^a/LW^b	308 A>G	LW5, LW7	*LW*05, LW*07*
Scianna	Sc1/Sc2	169 G>A	SC1, SC2	*SC*01, SC*02*
---	HgbS	173 A>T	---	---

5.3 The PreciseType HEA BeadChip Kit

The PreciseType HEA BeadChip Kit is comprised of two sub-kits (Figure 5-2), one containing the liquid reagents and the other containing BeadChip Carriers. The reagents are stored frozen (-20°C or colder) and the BeadChip carriers are stored refrigerated (2-8 °C).

- The BeadChip sub-kit contains one CD-ROM and either 12 pouches of BeadChip slides or one pouch containing one BeadChip plate, enough to perform 96 tests.
- The HEA Reagent sub-kit contains six reagents.

Figure 5-2: PreciseType HEA BeadChip kit components



5.4 Steps in the HEA Test

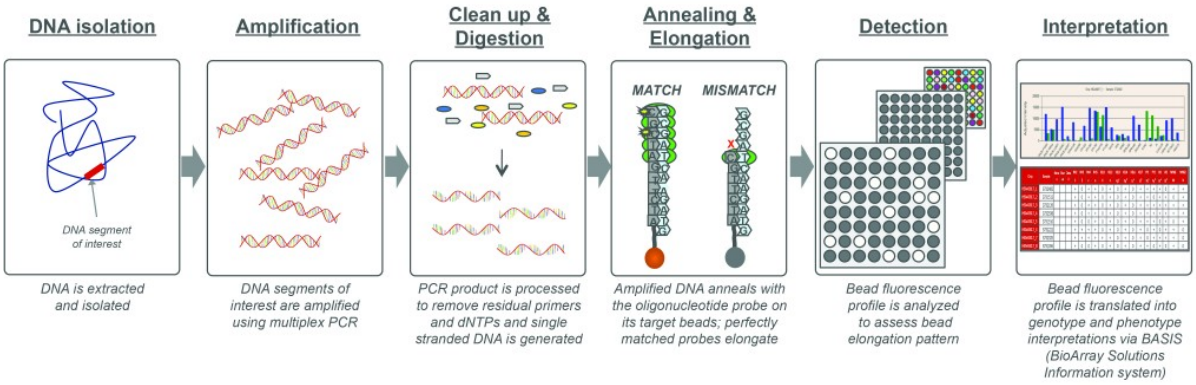


Figure 5-3 Steps in the HEA Test

Step 1: DNA Extraction and Isolation from Whole Blood

The process starts with extracting DNA from whole blood obtained via venous collection and stored up to 30 days. The DNA is extracted using the Qiagen QIAamp DSP DNA Blood Mini Kit following the manufacturer's instructions for manual or automated extraction on the QIAcube. Extracted DNA may be used immediately or stored at -20°C until ready for further processing.

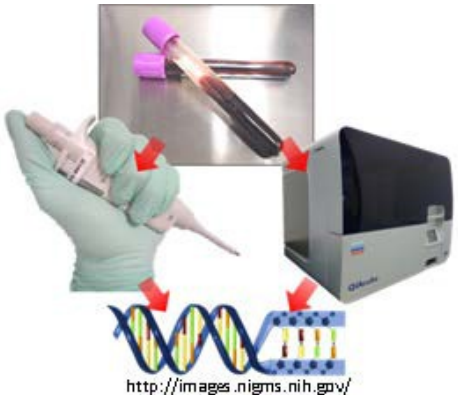


Figure 5-4 DNA extraction from whole blood

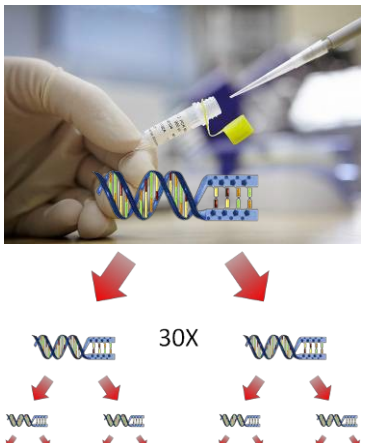


Figure 5-5 HEA PCR

Step 2: mPCR Amplification of DNA Segments of Interest

Prior to PCR amplification of the extracted genomic DNA, a PCR Master Mix is prepared and aliquotted into a PCR plate or tube strip. Extracted genomic DNA is added and mixed by pipette aspiration. The PCR tubes are then sealed using PCR tape or cap strips, vortexed briefly to mix, and centrifuged.

The PCR reactions are placed in the Veriti thermal cycler (Life Technologies) and incubated at 94°C for fifteen minutes to activate the thermostable HotStarTaq® DNA

polymerase enzyme. The samples are then subjected to thirty cycles of denaturation, annealing, and elongation respectively. This is followed by eight minutes at 68°C to ensure any remaining single-stranded DNA is fully extended. The tubes are then held at 4°C until removed from the thermal cycler.

During PCR denaturation, double-stranded DNA (dsDNA) is held at temperatures sufficient to denature or "melt" the complementary strands of dsDNA to produce single-stranded DNA (ssDNA). The PCR solution is then cooled to a temperature favorable for oligonucleotide primer annealing to the genomic DNA. During this annealing step, the abundance of PCR primers in solution preferentially bind to the potential genomic target (gDNA), providing an exposed 3' DNA end and template strand for DNA polymerase elongation. The elongation PCR step produces copies of the gDNA sequence, called amplicons, which exponentially increase the number of target DNA sequences in solution.

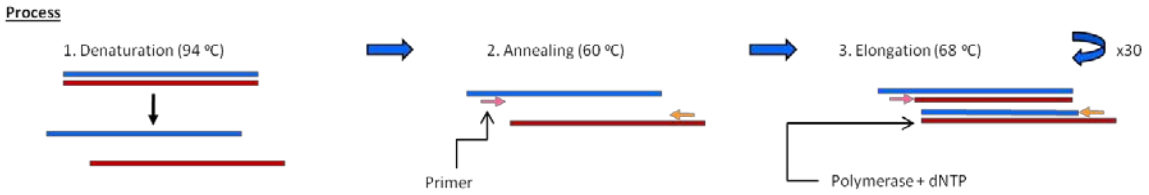


Figure 5-6 PCR Amplification

Step 3: Clean-up Reagent Reaction

Following the mPCR step, the PCR amplification tubes are removed from the thermal cycler and the PCR product is centrifuged. After centrifugation, each PCR product is transferred into a new PCR reaction tube and Clean-up Reagent is added. This mixture is then gently aspirated by pipette, sealed, vortexed, and centrifuged. This Clean-up reaction mixture is placed on the thermal cycler and incubated for twenty-five minutes at 37 °C, then fifteen minutes at 80 °C for complete enzyme denaturation/deactivation. The Clean-up Reagent contains two enzymes designed to digest ssDNA oligonucleotides (remaining primers) and deoxyribonucleotide triphosphates that have not been incorporated into the dsDNA. One of the enzymes in the Clean-up Reagent, Exonuclease I, is derived from Escherichia *coli* and digests ssDNA into deoxyribonucleoside 5'-monophosphates which cannot be incorporated into downstream reaction products.

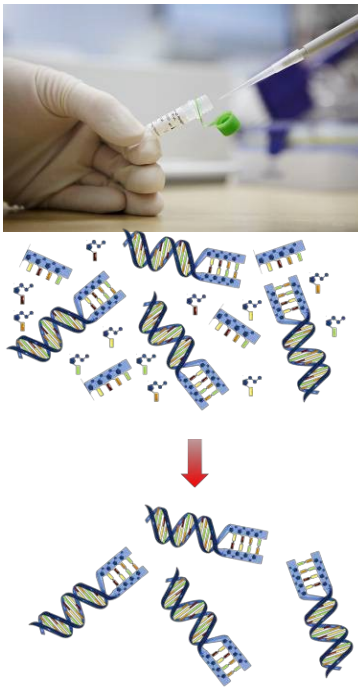


Figure 5-7 Clean-up Reagent Step

Recombinant Shrimp Alkaline Phosphatase (rSAP), an enzyme native to Pandalus borealis shrimp, dephosphorylates the unincorporated dNTPs creating inorganic phosphates and nucleosides that are also not used as substrates in downstream reactions.

Step 4: Lambda Exonuclease Treatment

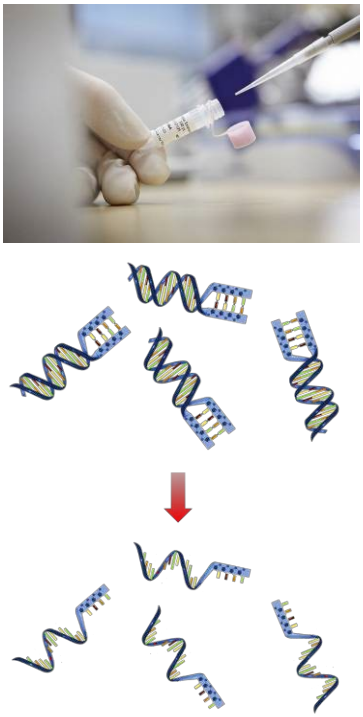


Figure 5-8 Lambda Exonuclease Step

Following the Clean-up Reagent step, the PCR tubes are removed from the thermal cycler and centrifuged briefly. To each of the tubes, Lambda Exonuclease is added, mixed by pipette aspiration, sealed, vortexed, and centrifuged. The tubes are then placed in a thermal cycler and incubated for twenty-five minutes at 37 °C and then fifteen minutes at 80 °C for complete enzyme denaturation/deactivation. Native to Escherichia *coli*, lambda exonuclease digests 5' phosphorylated DNA to yield ssDNA products and deoxyribonucleoside monophosphates. Deoxyribonucleoside monophosphates do not participate as substrates in any downstream reactions.

Step 5: On-BeadChip Elongation

Single-stranded DNA products from the Lambda Exonuclease step are removed from the thermal cycler, centrifuged, and eMAP Elongation Mix is added to each tube. The tubes are then mixed by pipette aspiration and 15 µL is removed and added to individual HEA BeadChips on 8- or 96-BeadChip carriers. Carriers are then incubated at 53 °C in a high humidity hybridization oven for 30 minutes. Each on-BeadChip elongation reaction contains Thermo Sequenase DNA polymerase and 5-carboxytetramethylrhodamine, succinimidyl ester (TAMRA) labeled dCTP. During on-

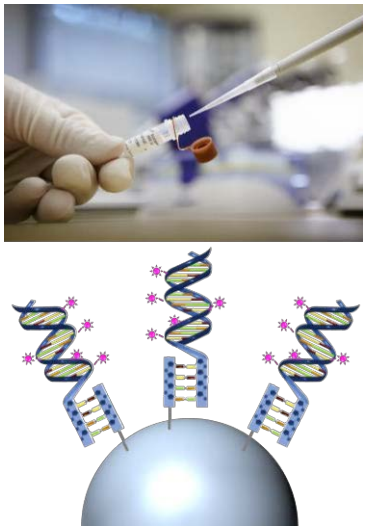


Figure 5-9 On-BeadChip Elongation Step

BeadChip elongation, the ssDNA generated during Step 4 is hybridized to the oligonucleotide probes on the bead surface. After hybridization, the 3' end of the probe is elongated by dNTP incorporation from the elongation mix solution containing the TAMRA-dCTP. Since TAMRA fluoresces after absorbing green light, and each bead in the array contains only one target-specific probe, specific DNA in solution can be detected by fluorescence microscopy after rinsing the BeadChip surface and determining which beads' probes incorporated TAMRA-dCTP. The specificity of each probe is due to the complementary binding site of the 3' end to polymorphisms in the target DNA which are etiological of HEA antigens. Complementary probes indicate the presence of an antigen and thus will elongate and incorporate TAMRA-dCTP during on-BeadChip incubation. Non-complementary probes are unable to elongate off the 3' terminal end by the DNA polymerase and will not incorporate TAMRA-dCTP.

5.5 Probe Discrimination for the Detection of Genotypes

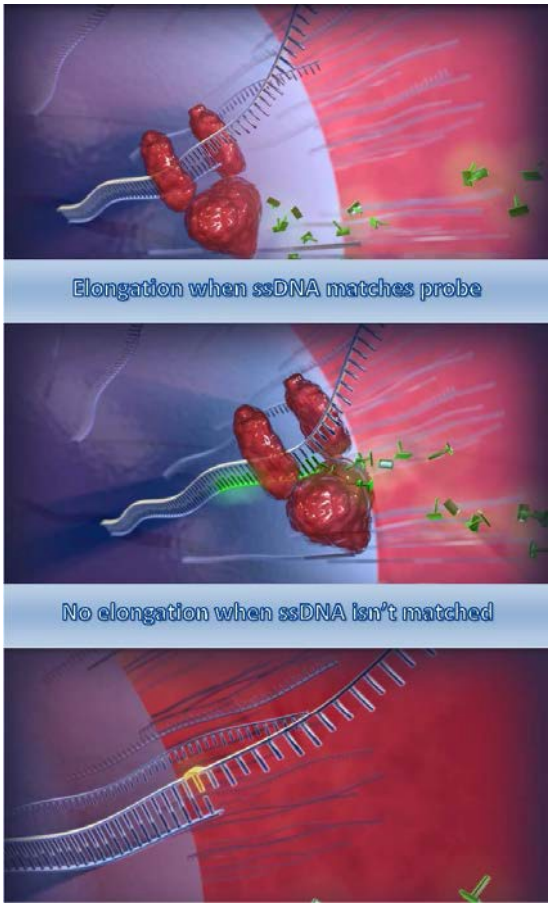


Figure 5-10 On-BeadChip Elongation and Fluorescent Detection

The ability of the PreciseType HEA BeadChip Test to discriminate between four genotypic states (homozygous A, homozygous B, heterozygous AB and homozygous negative) is dependent on the hybridization of homologous probes to ssDNA targets, elongation of the complementary probes to the homologous target ssDNA template, and incorporation of TAMRA-dCTP into the elongation product. This mechanism has been utilized successfully with the Thermo Sequenase DNA Polymerase component of the HEA eMAP Elongation Mix in many applications [26-28] and is able to distinguish single base pair differences. With Single Nucleotide Polymorphisms (SNPs) accounting for almost all of the variations detected within the HEA eMAP panel, this form of probe-template discrimination is effective in reporting the genotype of the assayed sample and subsequently determining the associated phenotype.

The oligonucleotide probes used to determine genotypes in the HEA panel are present, for most antigens, as probe

pairs. Each probe of the probe pair shares complete complementarity along its length apart from the 3' terminal nucleotide. This 3' terminal nucleotide confers the probe's specificity to its corresponding ssDNA target and corresponds directly to the SNP or polymorphism indicative of the antigen or hemoglobinopathy. Fluorescent detection of bound target is limited to probes with 3' complementarity since elongation of the complementary probe is needed for TAMRA-dCTP incorporation, and elongation is greatly favored in complexes with 3' complementarity. Utilizing this characteristic for specific 3' complementary elongation, the HEA probes are designed with the polymorphic region at the 3' terminus so that only the probes with a 3' complementary sequence to the target ssDNA template will be elongated upon and subsequently incorporate the TAMRA-dCTP necessary for fluorescent detection.

Fluorescent signals are mapped back to their corresponding probe identification using a BeadChip map as part of the Array Imaging System Reader (AISR) decoding process. The BeadChip map is chip-specific and provides the coordinates and identification of each bead in the random planar array. Assay intensities are transmitted to the BASIS wHEA software where they are adjusted for background signal intensity and thresholds are applied to determine if the probe intensities represent one of the four genotype states (homozygous A, homozygous B, heterozygous AB and homozygous negative).

5.6 Determination of Phenotypes from Genotypes

Probe intensities are evaluated by the BASIS wHEA software and converted into genotype results. For most antigens, the corresponding genotypes are determined by a pair of probes, A and B, determined to be positive or negative by their relative intensity ratios to each other. Probe pairs that fall into this category produce three genotypes: homozygous A, homozygous B, and heterozygous AB. Since the majority of erythrocyte antigens are determined by one SNP, phenotypes are derived directly from the genotype results, linking the presence of the antigen directly to the status of the SNP.

Genotype	Phenotype	
JKA/JKB	Jka	Jkb
AA	+	0
AB	+	+
BB	0	+

Table 5-2 Simple Genotype to Phenotype

Some antigen systems analyzed by the HEA BeadChip Test are more complex. The U antigen, for instance, is derived from determining the presence or absence of three markers on one gene. The presence of any one of these markers represents the presence of U in a typical or variant form; the absence of all three (determined by the absence of signal for all three probe pairs) introduces a fourth genotype, homozygous negative or simply negative, the U (-), phenotype.

Other negative phenotypes are determined through genotype analysis by the detection of known silencing polymorphisms. These polymorphisms are linked with a particular antigen and are used to determine if the antigen is present in the phenotype. In this

case, the status of the silencing probe pair, which is separate from the probe pair that directly detects the antigen's etiological polymorphism, determines the presence of an antigen. In instances where the presence of an antigen is silenced, the antigen and the associated silencing mutation must both be present in the genotype (i.e., if a mutation silences the presence of the B-antigen, the probe which directly detects the polymorphism of the B-antigen must also be positive). This method is also employed when there is a polymorphism that weakens the expression of an antigen. In either case, if the silenced/weak antigen is found to be a homozygous genotype, the silencing/weakening polymorphism must also be homozygous to silence/weaken the expression. An example would be the Fy^b antigen which is silenced in the presence of a common promoter site mutation affecting gene synthesis and weakened in expression when a common mutation within the coding sequence is present. When the silencing mutation is in-line (in cis) with the Fy^b antigen, that copy of the Fy^b antigen is silenced. Similarly, when the weakening mutation is in-line (in cis) with the Fy^b antigen, that copy of the Fy^b antigen is weakened.

Genotype			Phenotype
GPB-Ss	GPB-230	GPB-Int5	U
AA	AA	AA	+
AA	AA	AA	+
AA	AA	AB	+
AA	AB	AA	+
AB	AA	AA	+
AB	AA	AB	+
AB	AB	AA	+
BB	AA	AA	+
AA	AA	BB	var
AA	AB	AB	var
AA	BB	AA	var
LS	LS	LS	0

Table 5-3 More Complex U Antigen Determination (Summarized)

The determination of the RhC phenotype requires the presence of a SNP as well as a linked insertion elsewhere in the RhC gene sequence.

Probe intensities are converted into genotypes through the application of probe pair intensity delta values (the difference between probe pairs divided by their sum) to lot-specific threshold levels. Probe pair delta values can be classified into several regions which correspond to genotypes. These regions correspond to homozygous AA, Ax (AA or AB, an intermediate delta value between AA and AB), heterozygous AB, xB (AB or BB, an intermediate delta value between AB and BB), and homozygous BB. In instances where a definitive call cannot be made on an allele, it is represented by "x" in the instance of the A-allele (xB) or the B-allele (Ax).

Genotype			Phenotype	
FYA/FYB	GATA	FY265	Fya	Fyb
AA	AA	AA	+	0
AB	AA	AA	+	+
AB	AA	AB	+	w
AB	AB	AA	+	0
BB	AA	AA	0	+
BB	AA	AB	0	+
BB	AB	AA	0	+
BB	AA	BB	0	w
BB	AB	AB	0	w
BB	BB	AA	0	0

Table 5-4 Phenotype Determination with Silencing and Weak Phenotypes

6 Non- Clinical Laboratory Studies

The non-clinical laboratory studies conducted to evaluate the performance of the PreciseType HEA BeadChip Test are listed below.

Study	Conclusion
Limit of Detection	The LOD of the test is 15 ng/µL
Interfering Substances	The test is not susceptible to high levels of endogenous or exogenous substances in blood
Cross Hybridization	No cross hybridization is observed between targets and probes
Accuracy	The accuracy for all phenotypes examined by the test are >99% Total Percent Agreement
Lot-to-Lot Reproducibility	The test is reproducible between lots and repeatable across samples
Specimen Stability	Blood is stable for 46 days post draw in EDTA at 2-8°C
DNA Stability	Extracted genomic DNA is stable for at least for 6 months at -20°C
Reagent Stability	Kits are stable for 11 months unopened and 6 months in use
Assay Guard Band	The test is tolerant to variability in all formula and method variations examined
Lot Interchangeability	Different lots of Qiagen DSP extraction kits produce DNA that can be used in any HEA BeadChip Test
Procedural Tolerances	The test performance is tolerant to procedural variations
DNA Cross Contamination	The test does not exhibit wrong or flipped calls when challenged with sample to sample cross contamination
Negative Control Cross Contamination	The test does not exhibit detectable carryover from positive to negative samples
Contamination Titration	DNA contamination is detectable in supplied negative control at a much lower input concentration than in actual samples.

Table 6-1 Non-Clinical Study Summaries

The Accuracy Study, Limit of Detection Study, Lot-to-Lot Reproducibility Study and Stability Study are summarized in the following subsections.

6.1 Accuracy Study (Genotype Detection Study)

This study was conducted to determine the ability of the PreciseType HEA Molecular BeadChip Test to accurately identify the allelic variants that predict erythrocyte antigen phenotypes in the Rh (C [RH2], c [RH4], E [RH3], e [RH5], V [RH10], VS [RH20]), Kell (K [KEL1], k [KEL2], Kpa [KEL3], Kpb [KEL4], Jsa [KEL6], Jsb [KEL7]), Duffy (Fya [FY1], Fyb [FY2], GATA [FY-2], Fyx [FY2W]), Kidd (Jka [JK1], Jkb [JK2]), MNS (M [MNS1], N [MNS2], S [MNS3], s [MNS4], Uvar [MNS-3,5W], Uneg [MNS-3,-4,-5]), Lutheran (Lua [LU1], Lub [LU2]), Dombrock (Doa [DO1], Dob [DO2], Hy [DO4], Joa [DO5]), Landsteiner-Wiener (LWa [LW5], LWb [LW7]), Diego (Dia [DI1], Dib [DI2]), Colton (Coa [CO1],Cob [CO2]), and Scianna (Sc1[SC1], Sc2 [SC2]) blood group systems and a mutation (HgbS 173A>T) in the Beta Globin gene.

The accuracy of the PreciseType HEA Molecular BeadChip Test was evaluated by testing samples that have been well characterized using serology or bi-directional sequencing method.

A total of 914 samples were utilized for carrying out the internal accuracy study. Of the 914 samples 908 generated valid results. In addition, results from a set of 902 additional samples previously run as a part of a performance evaluation study in Europe were used to supplement the accuracy study. Of the 902 samples, 862 generated valid results.

The overall sample invalid rate for the PreciseType HEA Molecular BeadChip Test was 2.5% (46/1816).

The comparator test was well characterized serology (twice tested serologically), where available, and DNA sequencing when serology was not available. DNA sequencing was treated as the gold standard in the event that serology and the HEA BeadChip did not agree, followed by additional investigation if necessary.

	c	C	e	E	K	k	Kp^a	Kp^b	Js^a	Js^b	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	HbS	VS	V	U
TP:	783	578	1277	313	183	865	48	870	117	1334	794	745	601	812	781	735	561	941	132	1374	21	820	1352	99	594	753	625	642	625	4	627	9	118	287	260	305
TN:	363	568	106	1070	966	43	609	5	1041	11	330	377	527	316	272	315	564	185	1088	26	799	0	25	873	386	226	25	8	0	621	0	948	568	362	579	4
FP:	1	0	0	0	0	1	0	0	0	0	0	1	2	0	0	2	0	0	2	14	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2	0
FN:	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0

TP = True Positive. TN = True Negative. FP = False Positive. FN = False Negative.

Table 6-2 Performance of the HEA Test compared with bi-directional sequencing and serology

	Samples	TPA	Lower CI		Samples	TPA	Lower CI
c	1147	99.91%	99.59%	**Lua**	1223	99.75%	99.37%
C	1146	100.00%	99.74%	**Lub**	1414	99.01%*	98.46%
e	1383	100.00%	99.78%	**Dia**	820	100.00%	99.64%
E	1383	100.00%	99.78%	**Dib**	820	100.00%	99.64%
K	1149	100.00%	99.74%	**Coa**	1378	99.93%	99.66%
k	909	99.89%	99.48%	**Cob**	972	100.00%	99.69%
Kpa	657	100.00%	99.55%	**Doa**	980	100.00%	99.69%
Kpb	875	100.00%	99.66%	**Dob**	979	100.00%	99.69%
Jsa	1158	100.00%	99.74%	**Joa**	650	100.00%	99.54%
Jsb	1345	100.00%	99.78%	**Hy**	650	100.00%	99.54%
Jka	1124	100.00%	99.73%	**LWa**	625	100.00%	99.52%
Jkb	1123	99.91%	99.58%	**LWb**	625	100.00%	99.52%
Fya	1131	99.73%	99.32%	**Sc1**	627	100.00%	99.52%
Fyb	1130	99.82%	99.44%	**Sc2**	957	100.00%	99.69%
M	1053	100.00%	99.72%	**HbS**	686	100.00%	99.56%
N	1052	99.81%	99.40%	**VS**	649	100.00%	99.54%
S	1126	99.91%	99.58%	**V**	843	99.53%	98.92%
s	1126	100.00%	99.73%	**U**	309	100.00%	99.04%

Table 6-3 Total Percent Agreement (TPA) for all samples with respective lower one-sided 95% Confidence Interval

The percent correctly identified (accuracy) rate was over 99% for all antigen phenotype tested and the Hemoglobin-S status.
The results of all antigens except Lub and V met the criteria of lower bound of the one-sided 95% confidence interval greater than 99%. Disagreements in Lub between the HEA BeadChip Test and serology were unresolvable, but the HEA Test was consistent with other IVD molecular methods. The lower bound of the confidence interval for V would have benefited from more comparative sample results which were not obtained during the execution of the study. The lower bound for Lub was 98.46% and for V was 98.92%.

6.2 Limit of Detection (Minimum amount of DNA)

This study was conducted to determine the limit of detection (LOD) of the PreciseType HEA Molecular BeadChip Test. The limit of detection was performed with three fully characterized DNA samples extracted from whole blood, run in seven concentrations per sample, on three separate kit lots. Each DNA sample was run twenty times at each concentration on each kit/lot for a total of sixty tests per concentration per lot.

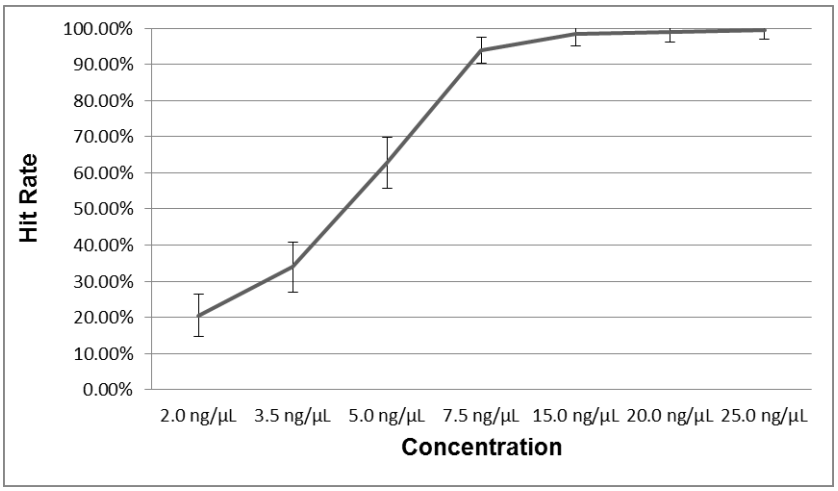


Figure 6-1 Validity at Concentrations Tested

Conc.	Hit Rate
2.0 ng/µL	20.56% (37/180)
3.5 ng/µL	33.89% (62/180)
5.0 ng/µL	62.78% (113/180)
7.5 ng/µL	93.89% (169/180)
15.0 ng/µL	98.33% (177/180)
20.0 ng/µL	98.89% (178/180)
25.0 ng/µL	99.44% (179/180)

The results at 15 ng/µL met the acceptance criteria that the concentration of DNA at which the PreciseType HEA Molecular BeadChip Test detects with 95% hit rate for each marker and lot. The Lower limit of detection of the PreciseType HEA Molecular BeadChip Test is 15 ng/µL, which is below the concentration normally attained by the recommended method of DNA extraction.

Table 6-4 Percent positive results per concentration level

6.3 Lot-to-Lot Repeatability and Reproducibility Study

A single-site, single-operator study was conducted to assess the lot-to-lot reproducibility and repeatability of sample results on the HEA BeadChip Test across different days. Repeatability is defined as the performance of the test under the same study condition. Reproducibility is defined as the performance of the test under different study conditions. Together they represent the precision of the test.

The Lot-to-Lot R&R study was performed by BAS using three HEA BeadChip lots and the fully characterized panel of twenty-two (22) human genomic DNA samples tested by one operator over five (5) days for a total of 330 tests on the human DNA samples.

Validity:

Out of the 330 tests, 1 test (0.3%) failed the sample validation criteria. Eight tests were repeated due to an operator error (not adding reagents to the sample well).

Repeatability:

Concordance in the Lot-to-Lot study was measured for all samples across all lots and all days against the expected results. The test was 100% repeatable within lots, 100% repeatable within days and 100% repeatable within operator for every sample.

Reproducibility:

For reproducibility analysis, phenotype results across all runs within each lot were combined to perform a statistical comparison for lot reproducibility for each lot and each sample. The test was 100% reproducible between lots and 100% reproducible between days across every sample.

Conclusion:

The test is reproducible between lots across all samples and repeatable for every sample across each lot.

6.4 Whole Kit Stability

Two studies were performed to determine kit stability. The first study assessed the stability of the unopened kit and the second assessed the stability of the kit after it was opened.

- Whole Kit Stability (Closed Vial): The purpose of this stability study was to determine the expiration dating of the PreciseType HEA BeadChip Test kit. To demonstrate the shelf life integrity at the intended storage condition a total of 6 lots of kits were tested.

Acceptance criteria required that at each time point, the test will generate concordant genotype and phenotype calls for a panel of well characterized human genomic DNA samples that assess each probe in the test along with valid positive and negative controls.

The stability studies for the PreciseType HEA Molecular BeadChip Test kit have been completed and based on 6 lots passing all acceptance criteria for 12 months an expiration date of 11 months has been established.

- Whole Kit Reagent Stability (Open Vial): The purpose of this stability study was to determine the open vial (in-use) expiration dating of the PreciseType HEA BeadChip Test kit. To demonstrate the open-vial integrity two lots of kits were opened and stored at the intended storage condition, and subsequently utilized for testing spanning multiple time points.

  Acceptance criteria required that at each time point, the test will generate concordant genotype and phenotype calls for a panel of well characterized human genomic DNA samples that assess each probe in the test along with valid positive and negative controls.

  The open vial stability studies for the PreciseType HEA Molecular BeadChip Test kit have been completed with two kits and support an open vial stability of 6 months.

7 U.S. Clinical Studies

As part of the clinical studies, the performance of the HEA PreciseType test was compared with serology and / or sequencing. Also as part of the studies, the Reproducibility and Repeatability of the test as well as a validation of the proposed training program also was evaluated across sites.

The R&R study was performed with both the plate and slide BeadChip configurations. In both studies, well characterized DNA samples were used to assess the performance of the HEA Precise Type test. A validation of our training procedure was conducted using both whole blood specimens as well as well characterized DNA samples.

In the comparative study, the four external sites compared results obtained while testing clinical specimens utilizing their approved serology tests with the HEA molecular test from BAS run on the same clinical specimens. The samples were further analyzed by bi-directional DNA sequencing for the antigens not covered by the sites approved serology tests. In addition, any discordant results between the approved serology tests and the HEA molecular test were further analyzed by bi-directional DNA sequencing for the specific antigens in question.

7.1 Method Comparison Study

A prospective, multi-site clinical study was conducted to evaluate the performance of the HEA BeadChip Test compared to serology, the current methodology used for HEA antigen screening for blood. Licensed sera was used to determine the antigen status of c, C, e, E, K, k, Kp^a, Kp^b, Jk^a, Jk^b, Fy^a, Fy^b, M, N, S, s, Lu^b (at two sites), and Di^a.

The study was a paired-evaluator study design in which each serologically-defined sample was also evaluated with the HEA BeadChip Test. For rare alleles for which there was no FDA-licensed serological reagent available, DNA sequencing of the samples was performed as the comparator test. The Sanger sequencing method was utilized for the DNA sequencing, and is considered the reference standard for genotyping variants in DNA templates and is frequently used to identify Single Nucleotide Polymorphisms (SNPs). The following antigen systems were analyzed with DNA sequencing: Do, Hy, Joa, LW, Sc, Js, Di, Co, HgbS, VS, V, Lu as well as Kp at one site.

Study Sites

Four qualified molecular laboratory sites participated in this study from the Southeast, Northeast, Northwest, and Southwest United States.

Site	Dates	Serology	DNA Sequenced
Site 1	Aug 4, 2011 – Aug 26, 2011	c, C, e, E, K, k, Kp^a, Kp^b, Jk^a, Jk^b, Fy^a, Fy^b, M, N, S, s, Lu^b, and Di^a	Do, Hy, Joa, LW, Sc, Js, Di, Co, HgbS, VS, V, and Lu
Site 2	Sep 20, 2011 – Oct 13, 2011 and Oct 28, 2013 – Nov. 18, 2013	c, C, e, E, K, k, Kp^a, Kp^b, Jk^a, Jk^b, Fy^a, Fy^b, M, N, S, s, Lu^b, and Di^a	Do, Hy, Joa, LW, Sc, Js, Di, Co, HgbS, VS, V, and Lu
Site 3	Aug 16, 2011 - Sep 22, 2011	c, C, e, E, K, k, Kp^a, Kp^b, Jk^a, Jk^b, Fy^a, Fy^b, M, N, S, s, and Di^a	Do, Hy, Joa, LW, Sc, Kp (subset), Js, Di, Co, HgbS, VS, V, and Lu
Site 4	Sep 8, 2011 – Oct 26, 2011	c, C, e, E, K, k, Kp^a, Kp^b, Jk^a, Jk^b, Fy^a, Fy^b, M, N, S, s, and Di^a	Do, Hy, Joa, LW, Sc, Js, Di, Co, HgbS, VS, V, and Lu

Table 7-1 Summary of Comparison Study Sites

Study Materials and Methods

Three lots of HEA BeadChip™ Kits were used in the study in total across all four sites.

Study Specimens

Study specimens consisted of whole blood samples collected from blood donors and/or recipients at each of the study sites. Samples used in the study were leftover specimens from each laboratory's routine clinical analysis that normally would be discarded. All samples were de-identified from their laboratory IDs by assigning unique study IDs, and study IDs were exclusively used in this study.

Data Collection and Analysis

The HEA data were compared to the serology data (non-reference standard, current test used for antigen typing) for each antigen.

For those antigens where there was disagreement between HEA and serology, DNA sequencing (the reference standard) was used as the arbiter method (resolved data).

For those antigens with no available licensed serological reagents, HEA results were compared to DNA sequencing.

The results generated by HEA BeadChip test and the comparative methods (serology and sequencing combined) were analyzed for Total Percent Agreement (TPA, percent of all comparable results out of the total). Comparative results between HEA BeadChip and serology were analyzed for Agreement (percent of all comparable results out of the total), Positive Percent Agreement (PPA, percent of comparable positive results out of the total serological positives) and Negative Percent Agreement (NPA, percent of comparable negative results out of the total serological negatives). Comparative results between HEA BeadChip and sequencing were analyzed for Concordance (percent of all comparable results out of the total), Sensitivity (percent of comparable positive results out of the total sequenced positives) and Specificity (percent of comparable negative results out of the total sequenced negatives).

Calculations for TPA, Agreement, PPA, NPA, Concordance, Sensitivity, and Specificity were performed on comparative results generated prior to and following (Initial and Resolved, respectively) discrepant resolution with sequencing as the arbiter method.

Results

Tables Table 7-2 and Table 7-5 contain the total percent agreement for each antigen tested for all comparative methods used in this study. Tables Table 7-3 and Table 7-6 contain the comparison of HEA BeadChip results to serology. Tables Table 7-4 and Table 7-7 contain the comparison of HEA BeadChip results to sequencing. Tables Table 7-5, Table 7-6 and Table 7-7 show the resolved results of the HEA BeadChip Test. Discordant results were resolved by evaluating the sample with the Sanger sequencing method.

Table 7-2 Total Percent Agreement for all Comparative Methods (Initial Data)

	TPA (n)	All Sites TPA	All Site TPA 95% CI	Site 1 TPA (n)	Site 1 TPA	Site 2 TPA (n)	Site 2 TPA	Site 3 TPA (n)	Site 3 TPA	Site 4 TPA (n)	Site 4 TPA
c	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
C	1248	98.48%	97.77%	323	95.67%	436	99.31%	291	100.00%	198	98.99%
e	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
E	1248	99.84%	99.50%	323	99.38%	436	100.00%	291	100.00%	198	100.00%
V	1248	99.76%	99.38%	323	99.69%	436	99.54%	291	100.00%	198	100.00%
VS	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
K	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
k	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Kpa	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Kpb	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jsa	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jsb	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jka	1248	98.64%	97.96%	323	95.05%	436	99.77%	291	100.00%	198	100.00%
Jkb	1248	98.48%	97.77%	323	96.28%	436	100.00%	291	98.28%	198	98.99%
Fya	1248	99.84%	99.50%	323	99.69%	436	99.77%	291	100.00%	198	100.00%
Fyb	1248	98.32%	97.59%	323	100.00%	436	98.39%	291	96.22%	198	98.48%
M	1248	99.12%	98.55%	323	96.90%	436	99.77%	291	100.00%	198	100.00%
N	1248	95.99%	94.96%	323	86.38%	436	99.77%	291	98.63%	198	99.49%
S	1248	99.92%	99.62%	323	100.00%	436	100.00%	291	99.66%	198	100.00%
s	1248	99.84%	99.50%	323	100.00%	436	100.00%	291	99.66%	198	99.49%
U	1248	99.84%	99.50%	323	99.38%	436	100.00%	291	100.00%	198	100.00%
Lua	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Lub	1248	99.92%	99.62%	323	99.56%	436	100.00%	291	100.00%	198	100.00%
Dia	1248	99.92%	99.62%	323	100.00%	436	100.00%	291	99.66%	198	100.00%
Dib	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Coa	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Cob	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Doa	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Dob	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jo(a)	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Hy	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
LWa	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
LWb	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Sc1	1247	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	197	100.00%
Sc2	1247	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	197	100.00%
HgbS	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%

Table 7-3 Comparison to Serology (Initial Data)

	Samples	Agreement	PPA (n)	PPA	NPA (n)	NPA	Site 1 Agreement (n)	Site 1 Agreement	Site 2 Agreement (n)	Site 2 Agreement	Site 3 Agreement (n)	Site 3 Agreement	Site 4 Agreement (n)	Site 4 Agreement
c	1248	100.00%	991	100.00%	257	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
C	1248	98.48%	669	100.00%	579	96.72%	323	95.67%	436	99.31%	291	100.00%	198	98.99%
e	1248	100.00%	1203	100.00%	45	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
E	1248	99.84%	327	99.69%	921	99.89%	323	99.38%	436	100.00%	291	100.00%	198	100.00%
K	1248	100.00%	30	100.00%	1218	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
k	1248	100.00%	1248	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Kpa	1248	100.00%	9	100.00%	1239	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Kpb*	1218	100.00%	1218	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	168	100.00%
Jka	1248	98.64%	1000	99.70%	248	94.35%	323	95.05%	436	99.77%	291	100.00%	198	100.00%
Jkb	1248	98.48%	803	100.00%	445	95.73%	323	96.28%	436	100.00%	291	98.28%	198	98.99%
Fya	1248	99.84%	738	99.86%	510	99.80%	323	99.69%	436	99.77%	291	100.00%	198	100.00%
Fyb	1248	98.32%	488	98.77%	760	98.03%	323	100.00%	436	98.39%	291	96.22%	198	98.48%
M	1248	99.12%	979	99.18%	269	98.88%	323	96.90%	436	99.77%	291	100.00%	198	100.00%
N	1248	95.99%	841	99.29%	407	89.19%	323	86.38%	436	99.77%	291	98.63%	198	99.49%
S	1248	99.92%	413	100.00%	835	99.88%	323	100.00%	436	100.00%	291	99.66%	198	100.00%
s	1248	99.84%	1170	100.00%	78	97.44%	323	100.00%	436	100.00%	291	99.66%	198	99.49%
U	1248	99.84%	1241	100.00%	7	71.43%	323	99.38%	436	100.00%	291	100.00%	198	100.00%
Lub	663	99.85%	662	100.00%	1	0.00%	227	99.56%	436	100.00%	N/A	N/A	N/A	N/A
Dia	1248	99.92%	17	100.00%	1231	99.92%	323	100.00%	436	100.00%	291	99.66%	198	100.00%

** Site 4 did not have enough reagents to completely characterized Kp^b serologically, the remaining samples were supplemented by sequencing*

Table 7-4 Comparison to Sequencing (Initial Data)

	Samples	Concordance	Sens. (n)	Sensitivity	Spec. (n)	Specificity	Site 1 Concordance (n)	Site 1 Concordance	Site 2 Concordance (n)	Site 2 Concordance	Site 3 Concordance (n)	Site 3 Concordance	Site 4 Concordance (n)	Site 4 Concordance
V	1248	99.76%	225	98.67%	1023	100.00%	323	99.69%	436	99.54%	291	100.00%	198	100.00%
VS	1248	100.00%	261	100.00%	987	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Kpa	30	100.00%	0	N/A	30	100.00%	N/A	N/A	N/A	N/A	N/A	N/A	30	100.00%
Kpb*	30	100.00%	30	100.00%	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	30	100.00%
Jsa	1248	100.00%	85	100.00%	1163	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jsb	1248	100.00%	1243	100.00%	5	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Lua	1248	100.00%	58	100.00%	1190	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Lub	1248	100.00%	1247	100.00%	1	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Dia	1248	100.00%	18	100.00%	1230	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Dib	1248	100.00%	1248	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Coa	1248	100.00%	1248	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Cob	1248	100.00%	32	100.00%	1216	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Doa	1248	100.00%	592	100.00%	656	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Dob	1248	100.00%	1121	100.00%	127	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jo(a)	1248	100.00%	1237	100.00%	11	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Hy	1248	100.00%	1246	100.00%	2	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
LWa	1248	100.00%	1248	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	198	100.00%
LWb	1248	100.00%	2	100.00%	1246	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Sc1	1247	100.00%	1247	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	197	100.00%
Sc2	1247	100.00%	4	N/A	1243	100.00%	323	100.00%	436	100.00%	291	100.00%	197	100.00%
HgbS	1248	100.00%	48	100.00%	1200	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%

** Site 4 did not have enough reagents to completely characterized Kp^b serologically, the remaining samples were supplemented by sequencing*

Table 7-5 Total Percent Agreement for all Comparative Methods (Resolved Data)

	TPA (n)	All Sites TPA	All Site TPA 95% CI	Site 1 TPA (n)	Site 1 TPA	Site 2 TPA (n)	Site 2 TPA	Site 3 TPA (n)	Site 3 TPA	Site 4 TPA (n)	Site 4 TPA
c	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
C	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
e	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
E	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
V	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
VS	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
K	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
k	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Kpa	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Kpb	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jsa	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jsb	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jka	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jkb	1248	99.44%	98.95%	323	100.00%	436	100.00%	291	98.28%	198	98.99%
Fya	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Fyb	1248	99.92%	99.62%	323	100.00%	436	99.77%	291	100.00%	198	100.00%
M	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
N	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
S	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
s	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
U	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Lua	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Lub	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Dia	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Dib	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Coa	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Cob	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Doa	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Dob	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jo(a)	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Hy	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
LWa	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
LWb	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Sc1	1247	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	197	100.00%
Sc2	1247	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	197	100.00%
HgbS	1248	100.00%	99.76%	323	100.00%	436	100.00%	291	100.00%	198	100.00%

Table 7-6 Comparison to Serology (Resolve Data)

	Samples	Agreement	PPA (n)	PPA	NPA (n)	NPA	Site 1 Agreement (n)	Site 1 Agreement	Site 2 Agreement (n)	Site 2 Agreement	Site 3 Agreement (n)	Site 3 Agreement	Site 4 Agreement (n)	Site 4 Agreement
c	1248	100.00%	991	100.00%	257	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
C	1248	100.00%	688	100.00%	560	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
e	1248	100.00%	1203	100.00%	45	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
E	1248	100.00%	327	100.00%	921	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
K	1248	100.00%	30	100.00%	1218	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
k	1248	100.00%	1248	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Kpa	1248	100.00%	9	100.00%	1239	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Kpb*	1218	100.00%	1218	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	168	100.00%
Jka	1248	100.00%	1011	100.00%	237	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jkb	1248	99.44%	815	100.00%	433	98.38%	323	100.00%	436	100.00%	291	98.28%	198	98.99%
Fya	1248	100.00%	738	100.00%	510	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Fyb	1248	99.92%	496	100.00%	752	99.87%	323	100.00%	436	99.77%	291	100.00%	198	100.00%
M	1248	100.00%	974	100.00%	274	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
N	1248	100.00%	879	100.00%	369	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
S	1248	100.00%	414	100.00%	834	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
s	1248	100.00%	1172	100.00%	76	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
U	1248	100.00%	1243	100.00%	5	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Lub	663	100.00%	663	100.00%	0	N/A	227	100.00%	436	100.00%	N/A	N/A	N/A	N/A
Dia	1248	100.00%	18	100.00%	1230	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%

** Site 4 did not have enough reagents to completely characterized Kp^b serologically, the remaining samples were supplemented by sequencing*

Table 7-7 Comparison to Sequencing (Resolved Data)

	Samples	Concordance	Sens. (n)	Sensitivity	Spec. (n)	Specificity	Site 1 Concordance (n)	Site 1 Concordance	Site 2 Concordance (n)	Site 2 Concordance	Site 3 Concordance (n)	Site 3 Concordance	Site 4 Concordance (n)	Site 4 Concordance
V	1248	100.00%	225	100.00%	1023	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
VS	1248	100.00%	261	100.00%	987	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Kpa	30	100.00%	0	N/A	30	100.00%	N/A	N/A	N/A	N/A	N/A	N/A	30	100.00%
Kpb*	30	100.00%	30	100.00%	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	30	100.00%
Jsa	1248	100.00%	85	100.00%	1163	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jsb	1248	100.00%	1243	100.00%	5	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Lua	1248	100.00%	58	100.00%	1190	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Lub	1248	100.00%	1247	100.00%	1	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Dia	1248	100.00%	18	100.00%	1230	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Dib	1248	100.00%	1248	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Coa	1248	100.00%	1248	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Cob	1248	100.00%	32	100.00%	1216	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Doa	1248	100.00%	592	100.00%	656	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Dob	1248	100.00%	1121	100.00%	127	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Jo(a)	1248	100.00%	1237	100.00%	11	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Hy	1248	100.00%	1246	100.00%	2	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
LWa	1248	100.00%	1248	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	198	100.00%
LWb	1248	100.00%	2	100.00%	1246	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%
Sc1	1247	100.00%	1247	100.00%	0	N/A	323	100.00%	436	100.00%	291	100.00%	197	100.00%
Sc2	1247	100.00%	4	N/A	1243	100.00%	323	100.00%	436	100.00%	291	100.00%	197	100.00%
HgbS	1248	100.00%	48	100.00%	1200	100.00%	323	100.00%	436	100.00%	291	100.00%	198	100.00%

** Site 4 did not have enough reagents to completely characterized Kp^b serologically, the remaining samples were supplemented by sequencing*

Conclusions:

The HEA BeadChip Test demonstrated agreement of at least 99% in comparison to serology for the following HEA antigens:

Di^a, Fy^a, Fy^b, M, N, S, s, Jk^a, Jk^b, Kp^a, Kp^b, Lu^b, C, c, E, e, K, k

High rates of concordance of at least 99%were also observed between the HEA BeadChip Test and DNA sequencing for the following antigens for which no corresponding licensed antisera are currently available:

Co^a, Co^b, Di^b, Js^a, Js^b, Lu^a, LW^a, LW^b, V, VS, Sc1, Sc2, Do^a, Do^b, Jo^a, Hy, and the mutation for HgbS 173A>T

It was observed at the end of testing that most discrepancies between initial results and final results following resolution were due to serology. This was evident in disagreements for Fy^b which were primarily due to limitations in serology in detecting Fyb-weak. This was also apparent in the sensitivity of the method utilized for some licensed sera in disagreements for Jk^a, Jk^b, M and N. At one site, the disagreements attributable to serological methods for M and N alone were over fifty. The majority of the remaining disagreements were attributed to the possible presence of the $(C)ce^S$ haplotype in C and the instance of Jk_{null} in 7 of the 19 Jk^b results in initial disagreement. The instance of Jk_{null} in some populations is a known limitation of the test and is identified in the test's instructions for use.

Additional testing was also required during this study due to the use of unlicensed reagents at one of the study sites. Although only licensed serological reagents were specified for use in this study, the site used their standard procedure for typing Fy, Jk and Ss which allows the use of unlicensed sera. The practice of using unlicensed sera is not uncommon in the labeling of blood units, but it was unacceptable according to the study protocol.

Overall comparative results following discrepant analysis (resolved results), were achieved for all 36 phenotypes assayed in the HEA BeadChip Kit at 99% agreement, all but one at the lower bound of the 95% confidence interval. Limitations of serology in determining the true phenotype upon initial test and transcription errors associated with serology were the primary sources of the 139 out of 23,097 (0.60%) disagreements in this study. The initial serological results obtained in these instances would have been the results reported in normal laboratory practice if not compared with the HEA BeadChip results. Disagreements where the source was a limitation due to the HEA BeadChip occurred in only 8 out of 44,926 results (0.018%) and were all attributable to known silencing mutations that have been noted in the instructions for use. Studies have identified the allele

frequency of the most prevalent of these silencing mutations to be 9% in certain ethnicities and by using phenotype rates from literature and 2010 US Census data, the instance of the phenotype in the general US population ranges from 0.00083% to 0.012% [29][30].

7.2 Training Validation Study

While conducting the R&R Study, it was determined that the BioArray Solutions Training Program required revision. A validation study was designed to demonstrate that the revised HEA Training Program trains operators to produce consistent, accurate results using the HEA BeadChip Test.

This was a multi-site, multi-operator study using human genomic DNA samples and leftover blood specimens from routine laboratory operations at each site. HEA BeadChip kits were provided to the sites in either the Slide or the Plate format, consistent with each site's planned initial implementation of the HEA BeadChip Test. Customers who intended to use the slide format performed the training validation testing on HEA BeadChip Slide Kits, and those who intended to implement the plate format performed the training validation testing on HEA BeadChip Plate Kits.

Operators ran the HEA Test on BioArray Solutions (BAS)-provided blinded panel of DNA samples and residual blood samples provided by each site. Blood and/or extracted DNA from customer-provided samples were sent to BioArray Solutions to confirm the HEA BeadChip results.

<u>Study Sites</u>

Two external laboratory sites with users new to the BeadChip HEA Test and one operator from a third site (who was retrained) participated in this study.

<u>Study Materials and Methods</u>

Both Slide and Plate format HEA BeadChip Kits were used in the study.

Demonstrating validity of the HEA BeadChip Training Program required performing DNA extraction, PCR amplification and HEA analysis as per the Instructions for Use using the samples described in Study Samples below. All operators tested DNA from the BAS-provided Training Validation Panel and blood specimens from each sites' respective donor units. Sites using the HEA BeadChip Test in Slide format performed slide-based validation testing, and sites using the HEA BeadChip Test in Plate format performed plate-based validation testing. Three assays were performed by each

operator trained on the HEA BeadChip Test, with each assay run on a different day.

Study Samples

The study was conducted using a panel of human genomic DNA samples fully characterized for HEA and provided to the study sites by BioArray Solutions. The Training Validation Panel consists of eleven (11) characterized samples composed of extracted human cell line genomic DNA. The DNA Panel members were selected because together, they cover the maximum number of positive phenotypes detectable in the HEA Test.

In addition to the panel provided by BAS, each site performed the test beginning with whole blood so that their ability to extract DNA could be assessed.

Data Collection and Analysis

For repeatability analysis, phenotype results for all sample replicates were compared to the expected values for each antigen. The percentages of concordance with the reference values were calculated.

For reproducibility analysis, phenotype results across all analysts and runs were combined in order to perform a statistical comparison for site reproducibility. The Fisher's exact test was used for each polymorphism and sample, and a p-value calculated to determine if there were statistically significant differences in reproducibility.

Results

Repeatability: For each polymorphism, the results were compared to reference values. The percent concordance values (repeatability) were found to be 100% within and across all Operators, Sites, and Days. Repeatability calculations were based on all data (from all Sites and all Operators), and compared against the R&R Panel reference values.

Results from the twenty-one (21) independent samples extracted from blood assayed at each site were compared to results obtained by the Sponsor (21 samples x 3 sites = 63 samples total). All samples extracted from blood were repeatable at 100% agreement.

Reproducibility: Combining data across analysts and runs allowed for a statistical comparison of the reproducibility comparing the study sites. For each polymorphism and sample, the Fisher's exact test (similar results to either Kendall or Fleiss' statistics) was used. A p-value of 1.00 is indicative of perfect correlation. All results produced p-values of 1.00, showing no statistically significant differences in reproducibility across Sites, Operators, and Days.

Results from the twenty-one (21) independent samples extracted from blood assayed at each site were compared to results obtained by the Sponsor (21 samples x 3 sites = 63 samples total). All samples extracted from blood were reproducible at 100% agreement between the site's results and those obtained at BAS.

The individual sample validity rate was 99.58% (959/963). One invalid (no results obtained) run that occurred during the course of the study was repeated.

Conclusions

The data from this validation study demonstrate that the HEA Bead Chip Test is repeatable and reproducible across sites, operators, lots, days and within runs when performed by operators who were trained with the HEA BeadChip Test Training Program. Thus, the results from this study validate that the HEA BeadChip Training program effectively trains operators to produce consistent and accurate results from the HEA BeadChip Test.

The training program was shown to be effective in training the new operators and was successful in retraining the operator from the third site, reducing that operator's invalid rate in the subsequent R&R slide study. All operators showed 100% repeatability and accuracy in results.

7.3 Repeatability and Reproducibility

Following the Training Validation Study a Slide R&R Study was performed. In the study, there were a total of six (6) operators across the three (3) study sites, all operators tested the BAS-provided Reproducibility Panel using the specified lot of the HEA BeadChip Test provided.

The Slide R&R Study was a multi-site, multi-operator study to assess the reproducibility and repeatability of the HEA BeadChip Test. The objective of this R&R study was to demonstrate that the HEA BeadChip™ Test generated reproducible, accurate results on a sponsor-provided panel of samples across sites and operators on different days.

The R&R study was conducted using a fully characterized panel of eleven (11) human genomic DNA samples tested at each of the study sites by six (6) different operators with assigned lots of HEA kits over five (5) days.

Study Sites

Three qualified molecular laboratory sites participated in this study.

Study Materials and Methods

HEA BeadChip™ Kits, Slide Format were used in the study.

In each run, all eleven (11) Reproducibility Panel samples along with BAS-provided external positive and negative controls were tested. All operators ran the same samples in the exact same manner on subsequent days with one day in between each test day (minimum) for a total of 5 days.

Study Specimens

The sample panel used in this R&R study consisted of eleven (11) characterized DNA samples, that when run together, are positive for all antigens in the HEA BeadChip Test.

Data Collection and Analysis

For the repeatability analysis, phenotype results for all sample replicates were compared to the expected values for each antigen. The percentages of concordance with the reference values were calculated.

For reproducibility analysis, phenotype results across all analysts and runs were combined to perform a statistical comparison for site reproducibility. The Fisher's exact test was used for each polymorphism and sample, and a p-value calculated to determine if there were statistically significant differences in reproducibility.

Results

Table 7-8 Slide R&R Results

Repeatability *(Percent Concordance)*	**Site 1**	**Site 2**	**Site 3**	**Total**
Within-Site	100%	100%	100%	100%
Within-Operator	100%	100%	100%	100%
Within-Day	100%	100%	100%	100%
Within-Sample	100%	100%	100%	100%
Reproducibility *(p-value)*	**Total**			
Site to Site	1			
Operator to Operator	1			
Day to Day	1			

Repeatability:

Concordance was measured for all samples at all sites, for all operators, and all days against the expected results. All analyses produced 100% concordance to the expected results for each antigen for each sample;

therefore, the Site to Site, Operator to Operator, and Day to Day repeatability was 100%.

Reproducibility:

For reproducibility analysis, phenotype results across all analysts and runs were combined to perform a statistical comparison for site reproducibility. The Fisher's exact test was used for each polymorphism and sample, and a p-value calculated to determine if there were statistically significant differences in reproducibility. Fisher's Exact Test was used to compare all samples at all sites to determine if the results for each polymorphism were statistically reproducible. All analyses produced p-values of 1, indicating results were reproducible across sites, operators, and days.

The individual sample validity rate was 99.70% (1346/1350).

Conclusions

The results of the Slide R&R study data analyses demonstrated excellent intra-assay and inter-assay repeatability and reproducibility with respect to Site, Operator, and Assay Day.

The HEA BeadChip Test in the slide format is highly repeatable, reproducible and capable of providing reliable information with respect to the antigen status of c, C, e, E, V, VS, K, k, Kp^a, Kp^b, Js^a, Js^b, Jk^a, Jk^b, Fy^a, Fy^b, M, N, S, s, U, Lu^a, Lu^b, Di^a, Di^b, Co^a, Co^b, Do^a, Do^b, Hy, Jo^a, LW^a, LW^b, Sc1, Sc2, and the mutation for HgbS 173A>T.

Results of the study demonstrated that the HEA Test is reproducible between different Sites, on different Assay Days, and between different Operators when using the slide format.

7.4 Plate R&R Study

A multi-site, multi-operator study was conducted to assess the reproducibility and repeatability of the HEA BeadChip Test, Plate format. The objective of this R&R study was to demonstrate that the HEA BeadChip™ Test generated reproducible, accurate results on a sponsor-provided panel of samples across sites and operators on different days.

The R&R study was conducted using a fully characterized panel of eleven (11) human genomic DNA samples tested at each of the study sites by eight (8) different operators with assigned lots of HEA kits over five (5)

days. A single lot of HEA BeadChip kits was used across the four sites performing this study.

Study Sites

Four qualified molecular laboratory sites participated in this study.

Study Materials and Methods

HEA BeadChip™ Kits, Slide Format were used in the study.

In each run, all eleven (11) Reproducibility Panel samples along with BAS-provided external positive and negative controls were tested multiple times. All operators ran the same samples in the exact same manner on subsequent days with one day in between each test day (minimum) for a total of 5 days.

Study Specimens

The sample panel used in this R&R study consisted of eleven (11) characterized DNA samples, that when run together, are positive for all antigens in the HEA BeadChip Test.

Data Collection and Analysis

For the repeatability analysis, phenotype results for all sample replicates were compared to the expected values for each antigen. The percentages of concordance with the reference values were calculated.

For reproducibility analysis, phenotype results across all analysts and runs were combined to perform a statistical comparison for site reproducibility. The Fisher's exact test was used for each polymorphism and sample, and a p-value calculated to determine if there were statistically significant differences in reproducibility.

Results

Table 7-9 Plate R&R Results

Repeatability *(Percent Concordance)*	**Site 1**	**Site 2**	**Site 3**	**Site 4**	**Total**
Within-Site	100%	100%	100%	100%	100%
Within-Operator	100%	100%	100%	100%	100%
Within-Day	100%	100%	100%	100%	100%
Within-Sample	100%	100%	100%	100%	100%
Reproducibility *(p-value)*	**Total**				
Site to Site	1				
Operator to Operator	1				
Day to Day	1				

Repeatability:

Concordance was measured for all samples at all sites, for all operators, and all days against the expected results. All analyses produced 100% concordance to the expected results for each sample; therefore, the Site to Site, Operator to Operator, and Day to Day repeatability was 100%.

Reproducibility:

For reproducibility analysis, phenotype results across all analysts and runs were combined to perform a statistical comparison for site reproducibility. The Fisher's exact test was used for each polymorphism and sample, and a p-value calculated to determine if there were statistically significant differences in reproducibility. Fisher's Exact Test was used to compare all samples at all sites to determine if the results for each polymorphism were statistically reproducible. All analyses produced p-values of 1, indicating results were reproducible across sites, operators, and days.

The individual sample validity rate was 99.17% (3689/3720). Two invalid runs that occurred during the course of the study were repeated.

Conclusions

The results of the study demonstrate excellent intra-assay and inter-assay repeatability and reproducibility with respect to Site, Operator, and Assay Day. The low individual sample invalid result rate of 0.83% from the study demonstrated the consistency in manufacture quality and relative ease-of-use of the HEA Test procedure to trained operators.

In conclusion, the HEA BeadChip Test in the plate format is highly repeatable, reproducible and capable of providing reliable information with respect to the antigen status of c, C, e, E, V, VS, K, k, Kp^a, Kp^b, Js^a, Js^b, Jk^a, Jk^b, Fy^a, Fy^b, M, N, S, s, U, Lu^a, Lu^b, Di^a, Di^b, Co^a, Co^b, Do^a, Do^b, Hy, Jo^a, LW^a, LW^b, Sc1, Sc2, and the mutation for HgbS 173A>T.

Results of the study demonstrated that the HEA Test in the plate format is reproducible between different sites (n = 4), on different days (n = 5), and between different Operators (n = 8).

8 Product History

The initial version of the HEA BeadChip Test was first made available in 2005 as a Research Use Only product. After some preliminary studies and experiences

with the assay and system, in 2007 a project to modify the panel of SNPs available on the chip to detect additional antigens was initiated. This became HEA BeadChip Kit version that is being submitted as part of this PMA application.

Since the initial introduction of the HEA BeadChip Kit there have been over 700,000 tests processed using the BioArray Solutions' BeadChip technology. In May 2010, the HEA BeadChip Kit was CE marked in accordance with the EU IVD Directive. Over 80 sites have used at least one kit, and there are currently 35 centers using BioArray Solutions' technology internationally, primarily in Europe, including some of the largest donor centers and most prominent medical centers in the world.

8.1 Performance Evaluation in Europe

A performance evaluation study for the HEA BeadChip Test was designed and executed in support of the CE marking in Europe. The study was initiated in 2009 at three European reference labs, CNRGS-INTS (Centre National de Référence pour les Groupes Sanguins - Institut National de la Transfusion Sanguine; Paris, France), Ospedale Maggiore Policlinico (Milan, Italy), and Red Cross Blood Donor Centre (Madrid, Spain). A total of 1100 samples were tested in the performance evaluation study including 135 patient samples and 965 donor samples.

The results of the study were an overall concordance rate of >99% and satisfy the requirements for a List A and B device under Annex II of Directive 98/79/EC.

9 Conclusions

The use of serology to determine red blood cell antigen phenotypes has been very successful in providing safer transfusions. However, blood incompatibility remains a significant problem in transfusion medicine due to inherent limitations of serological testing. Furthermore, these limitations remain as barriers to preventing alloimmunization in polytransfused individuals, which significantly impacts patient care. The adoption of molecular methods is critical to increasing blood safety by providing better-matched blood products for transfusion.

The Immucor PreciseType HEA Molecular BeadChip Test was evaluated to demonstrate performance claims for limit of detection, interference, repeatability, reproducibility, accuracy and stability. The results of the non-clinical studies, in conjunction with results from the clinical trial studies, support the intended use statement of the PreciseType HEA Molecular BeadChip Test and can provide the transfusion community a valuable tool to increase blood safety through better-matched blood products.

10 Bibliography/References

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