IMMUCOR

PreciseType™ HEA Molecular BeadChip Test

Package Insert IVD

PreciseType™ | HEA

Transfuse | Transplant | Transform a **life**

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I. INTRODUCTION

A. Intended Use

The HEA BeadChip Kit is an *in vitro* diagnostic test intended for the molecular determination of 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 in human genomic DNA. The test also detects the HgbS mutation in the beta globin gene. The results from this mutation detection are not intended for diagnosis of sickle cell disease.

B. Summary of the Test

The PreciseType HEA Molecular BeadChip Test uses the proprietary Elongation-mediated Multiplexed Analysis of Polymorphisms (eMAP™) technology to identify the presence or absence of the selected alleles associated with a given phenotype. After multiplex PCR amplification and post-PCR processing using Clean-up Reagent and Lambda Exonuclease, the single-stranded DNA strands are incubated on the BeadChip array, allowing them to anneal with the corresponding blood-group-specific probes. An exact match between the 3' end of the probe and the annealed DNA triggers a subsequent elongation reaction in which the probe will be extended through incorporation of fluorescently-labeled dNTP molecules. If there is not an exact match, elongation does not occur. Elongation products of alleles A and B are detected simultaneously by imaging the entire array.

In this method, each probe is attached covalently to a spectrally distinguishable bead type. A library of individual bead types contains all of the probes of interest. The library is immobilized in the BeadChip array, allowing for the simultaneous detection of the polymorphisms of interest.

BeadChips are read with the BioArray Array Imaging System™ (AIS™ 400), with assay results interpreted, analyzed, and reported through the BioArray Solutions Information System (BASIS™) software. The AIS system captures the fluorescent signal from individual beads in an image of the entire array to determine the identity of the bead by the color of the bead and its position in the array. It also detects the average signal intensity, coefficient of variance standard deviation of the intensities, and number of beads measured for each type of probe. The BASIS software then imports the raw intensity output, assesses the validity of the internal controls, and generates assay results.

Mutations known to result in silencing (non-expression) of Duffy (Fyb) [FY2] and MNS (S) [MNS3] antigens have been incorporated into the test.

C. Product Description

Human erythrocyte blood group antigens – the surface markers located on the membrane of the red blood cell – are polymorphic, inherited protein. If an antigen-negative patient receives blood from an antigen-positive donor, it could trigger an immune reaction (alloimmunization), where the blood recipient's immune system develops antibodies that can attack and reject the donor RBCs. 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, chronic blood transfusion therapy, such as sickle cell disease, autoimmune hemolytic anemia, and aplastic anemia, increased opportunity for alloantibody product occurs. In such cases, studies demonstrate the utility of bolstering serological phenotyping with DNA analysis to identify the presence of blood-group antigens [2][3].

Perinatal or postnatal management of hemolytic disease of the fetus and newborn (HDFN) may be assisted by identification of human erythrocyte antigens. Minor blood group incompatibility occurs in approximately 0.8% of pregnant women and may be associated with Kell, Kidd or Duffy (among others). Anti-K disease may be severe due to hemolysis or erythroid suppression [4][5].

The International Society for Blood Transfusion (ISBT) Committee on Terminology for Red Cell Surface Antigens maintains and updates a database of known alleles for 35 blood-group systems. It provides information regarding the prevalence and significance of alleles in a pan ethnic population as well as standards for nomenclature and terminology in transfusion medicine [6].

Twenty-four polymorphisms associated with 35 Human Erythrocyte Antigens are included in the PreciseType HEA Molecular BeadChip Test and are listed in the following table (Table 1) [7]. One polymorphism in the beta-globin gene associated with hemoglobinopathies (HgbS) is also included.

Table 1: Genetic Markers for Red Blood Cell Antigens in the PreciseType HEA Test

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*
	VS	733 C>G, 1006 G>T	RH20	*RHCE*01.20.01, RHCE01.20.02, RHCE*01.20.04, RHCE*01.20.05*
	V		RH10	
Kell	K/k	698 T>C	KEL1, KEL2	*KEL*01, KEL*02*
	Js^a/Js^b	1910 C>T	KEL6, KEL7	*KEL*06, KEL*07*
	Kp^a/Kp^b	961 T>C	KEL3, KEL4	*KEL*03, KEL*04*
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 (Uvar, Uneg)	230C>T	MNS-3, 5W, MNS-3,-4,-5	*GYPB*03N.01 or GYPB*03N.02*
		In5 g>t		*GYPB*03N.03 or GYPB*03N.04*
Lutheran	Lu^a/Lu^b	230 A>G	LU1, LU2	*LU*01, LU*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*
Diego	Di^b/Di^a	2561 C>T	DI2, DI1	*DI*02, DI*01*
Colton	Co^a/Co^b	134 C>T	CO1, CO2	*CO*01, CO*02*
Scianna	Sc1/Sc2	169 G>A	SC1, SC2	*SC*01, SC*02*

** The GATA mutation listed here has been previously reported at -33 and -46 (ISBT Working Party)[8].

II. BEADCHIP KIT CONTENTS, EQUIPMENT AND SUPPLIES REQUIRED

A. Contents of the PreciseType HEA Kit

Part Number	Description	Quantity *
800-00194	HEA 1.2 PCR Mix	2 x 900 µL
800-00191	Clean-up Reagent	1 x 330 µL
800-00195	Lambda Exonuclease	1 x 330 µL
800-00193	eMAP™ Elongation Mix	2 x 600 µL
800-10242	HotStarTaq® DNA Polymerase	1 x 155 µL
800-00287	Negative Control**	1 x 1000 µL
830-00056 **or** **830-00055**	HEA 8-BeadChip™ Carrier or HEA 96-BeadChip™ Carrier	12 carriers x 8-BeadChip Arrays or 1 carrier x 96-BeadChip Arrays
800-20100	PreciseType BeadChip Test | HEA Data CD	1

* The liquid reagents have been overfilled to ensure total recovery of stated quantity.
** PCR grade water – No-DNA control

B. Equipment Required

Description	Catalog Number
AIS 400 Array Imaging System	(BioArray) 790-20006, 790-20016
Defrost-free freezer (capable of maintaining temperatures of -20°C or colder)	-
Hybridization (incubation) oven (Boekel InSlide Out™)	(Boekel) 241000
Refrigerator (capable of maintaining 2 to 8°C)	-
Thermal cycler (Applied Biosystems Veriti® Dx)	(Applied Biosystems) 4452300

C. Equipment Recommended

Description	Catalog Number*
Cryoblock (recommend Denville or equivalent)	(Denville) R6670
Microplate centrifuge (recommend Eppendorf Model 5430 or equivalent)	(Fisher) 05-400-017
PCR tube racks (recommend Fisher Scientific or equivalent)	(Fisher) 05-541-50
PCR workstation hood with UV light (recommend CBS Scientific or equivalent)	(CBS Scientific) P-030-02
Precision pipettes – multi-channel - capable of delivering 0.5 to 10 µL (recommend Fisherbrand® or equivalent) • Accuracy +/- 12 to 2.4% • Precision < 8 to 1.6%	(Fisher) 21-377-825
Precision pipettes – multi-channel - capable of delivering 5 to 50 µL (recommend Fisherbrand® or equivalent) • Accuracy +/- 5.0 to 1.5% • Precision < 2.0 to 0.7%	(Fisher) 21-377-827
Precision pipettes - single channel - capable of delivering 0.5 to 10 µL (recommend Eppendorf or equivalent) • Accuracy ± 2.5 to 1% • Precision ≤ 1.8 to 0.4%	(Fisher) 13-684-250
Precision pipettes - single channel - capable of delivering 10µL to 100 µL (recommend Eppendorf or equivalent) • Accuracy ± 3.0 to 0.8% • Precision ≤ 1.0 to 0.2%	(Fisher) 13-684-250
Precision pipettes - single channel capable of delivering 100 to 1,000 µL (recommend Eppendorf or equivalent) • Accuracy +/- 3.0 to 0.6% • Precision ≤ 0.6 to 0.2%	(Fisher) 13-684-250
QIAGEN® QIAcube®	(Qiagen) 9001292
Tube centrifuge (recommend Denville MiniMouse II™ or equivalent)	(Denville) C0801
Vortex mixer with tube and flat adaptors (recommend Denville or equivalent)	(Denville) S7030

* Pipette manufacturer part numbers are subject to change, please default to the descriptive language when ordering supplies if catalog numbers are in question.

D. Supplies Required

Description	Catalog Number*
1.5 mL centrifuge tubes (recommend Eppendorf™ or equivalent)	(Fisher) 05-402-24B
2.0 mL centrifuge tubes (recommend Eppendorf™ or equivalent)	(Fisher) 05-402-24C
8-Tube strip 0.2 mL thin-wall thermal cycler tube caps (recommend Applied Biosystems MicroAmp® 8-Cap Strip or equivalent)	(Applied Biosystems) N8010535
8-Tube strip 0.2 mL thin-wall thermal cycler tubes (recommend Applied Biosystems MicroAmp® 8-Tube Strip, 0.2 mL or equivalent)	(Applied Biosystems) N8010580
96 Well, PP, clear 0.3 mL non-skirted PCR Plate – (recommend Fisherbrand® or equivalent)	(Fisher) 14230232
Water for BeadChip wash – recommend Invitrogen	(Invitrogen) 10977023
Compressed/canned air, oil free (recommend Fisherbrand® or equivalent)	(Fisher) 23-022523
Decontaminant (recommend Molecular BioProducts™ DNA AWAY™, or equivalent)	(Fisher) 21-236-28
DNA extraction kit (recommend QIAGEN® QlAamp® DSP DNA Blood Mini Kit or equivalent)	(Qiagen) 61104
Filtered (aerosol-resistant) disposable pipette tips covering the range 0.1 µL to 1,000 µL (recommend Eppendorf™ epTIPS™ Filtered or equivalent)	(Fisher) 05-403-14 (Fisher) 05-403-18 (Fisher) 05-403-20
PCR plate seals (recommend Applied Biosystems® MicroAmp® Clear Adhesive Film or equivalent)	(Applied Biosystems) 4306311
Multi-fold deluxe paper towels (recommend Uline brand or equivalent)	(Uline) 5-7127
PreciseType™ HEA BeadCheck® Positive Control Kit	(BioArray) 800-20236

* Manufacturer part numbers are subject to change, please default to the descriptive language when ordering supplies if catalog numbers are in question.

III. DEFINITION OF SYMBOLS

The following special symbols may be found on the components of PreciseType HEA Kit:

Symbol	Definition
	Batch code
	CD, containing data files and instructions for use
	Contents
	In vitro diagnostic medical device
	Manufacturer
	Contains sufficient for <n> tests
	Consult instructions for use
	Catalogue number
	Temperature Limit
	Use-by date
	Authorized representative in the European Community

IV. WARNINGS AND PRECAUTIONS

- In samples of human origin, there is still a potential risk of infection even after DNA extraction. Handle samples using universal precautions. Use appropriate personal protective equipment throughout the test procedure, including gloves, eye protection and lab coat. In the event of contact with eyes, rinse immediately with plenty of water and seek medical advice. For additional safety information please refer to the website: http://extranet.immucor.com
- Never pipette by mouth. Avoid contact of reagents and specimens with skin and mucous membranes.
- Dispose of used materials in accordance with the institution's and local regulations for disposal of potential biohazardous materials. Spillage of potentially infectious material should be cleaned and disposed of immediately in accordance with the institution's policy and procedure for the handling and disposal of potentially biohazardous materials.
- PCR technology is susceptible to contamination, especially from its own product. Aerosols of PCR amplicons that are generated during the post-PCR steps are a frequent source of contamination.
- Take care to prevent excessive splashing and generation of aerosols.
- Follow good laboratory practices for molecular laboratories when using the kit, including wiping of work surfaces before processing or preparing PCR samples with a freshly prepared 10% bleach (or equivalent), use of ultraviolet (UV) light in hoods or biosafety cabinets in between use, space and time separation of pre- and post-PCR activities, use of aliquoted PCR reagents, use of Positive and Negative Controls, etc.
- Consistent, careful technique coupled with liberal incorporation and monitoring of controls will ensure a vigilant, proactive approach to control and monitoring of PCR contamination. (See section VII).
- Operators must participate in the PreciseType HEA Molecular BeadChip Test Training Program prior to performing this assay in order to assure consistent and accurate test results.
- Laboratories should validate their own cleaning procedures.
- Contamination of reagents or specimens may cause erroneous results; therefore, take care to avoid contaminating this product during use. Do not use reagents if you suspect that they may have been contaminated.
- Microbial contamination of reagents or specimens may lead to incorrect results.
- Use the kit reagents and HEA BeadChip Carriers as supplied. Dilution or alteration may generate erroneous results.
- Do not mix reagents or HEA BeadChip Carriers between different lots.
- Do not use leaking or unlabeled vials.
- Previously frozen samples or reagents should be mixed thoroughly and then centrifuged after thawing prior to testing. Avoid generating foam and bubbles in the samples.

- Keep all enzymes and master mixes on ice or cryoblock (2 to 8°C) during use.
- Ensure that sample tubes ae properly sealed prior to amplification to prevent evaporation.
- Due to inherent differences in the mechanisms of thermal cycler performance, variation in results can occur when set thermal profiles are transferred between different makes and models of thermal cycler instruments. In some cases, reaction specificity and sensitivity can be compromised, leading to the false interpretation and reporting of data. The FDA-approved PreciseType HEA IVD test requires use of the Applied Biosystems Veriti Dx thermal cycler. Immucor makes no assurance for assay performance with the use of alternate thermal cyclers and profiles, which must be validated by the user.
- Samples must remain in the BeadChip reaction well during testing.
- Incubation times or temperatures other than those specified may give erroneous results.
- On each day of use, prior to operating the AIS 400, users must perform the Exposure Test Carrier (ETC) procedure to verify AIS performance. If the Exposure Test fails, please contact technical support for appropriate instructions. (See AIS User Manual 190-20185).
- Deviation from the recommended directions for use may result in less-than-optimal product performance. Depending upon the nature and severity of the deviation assay failure (individual sample as well as run failures) and/or erroneous results may occur. For example, we have determined that use of insufficient/inactive Clean-up Reagent in the assay may result in high incidence of false Kp(a)+ calls.
- The results from the mutation HgbS in the Beta Globin gene are not for diagnosis of sickle cell disease.

V. SHIPPING, STORAGE AND STABILITY

The PreciseType HEA reagents, including the PCR mix and all enzymes, are shipped on dry ice. When the kit is received, verify that there is dry ice remaining in the package. If no ice is present, do not use the kit and contact Technical Support. In addition, please contact Technical Support if the vacuum-sealed HEA BeadChip Carrier pouch has been opened or damaged during transit.

Store all test reagents, including the PCR mix and all enzymes, at -20°C to -80°C in a defrost-free freezer. Use benchtop cryoblocks when possible. When stored under these conditions and handled correctly, unopened reagents can be used until the expiration date. Once opened, the contents of a properly stored reagent kit may be used for six months or until the labeled expiration date, whichever occurs earlier. When opening reagent kits, users should determine which date would occur earlier; if the six-month use date is earlier than the labeled expiration date, record this earlier date directly on the kit to ensure that reagents are not used beyond their expiration date.

Store the HEA BeadChip Carriers at 2 to 8°C until use. Unused carriers should be returned immediately to storage at 2 to 8°C in their original packaging. HEA BeadChip Carriers cannot be reused.

Refer to the expiration date of all kit components. Do not use beyond the expiration date. The format of the expiry date is YYYY-MM-DD, which indicates allowable usage through the day indicated. Components of this kit can have expiration dating that is greater than the expiration date of the entire kit. The shortest shelf-life (i.e., earliest expiration date) of any component in the kit will be indicated on the outermost kit label.

VI. SPECIMEN COLLECTION AND PREPARATION

Sample: Whole blood samples must be drawn into EDTA anticoagulant tubes (e.g. BD Product Numbers 366643, 368661, 367654). The PreciseType HEA assay has not been tested with cord blood or cadaveric blood. (see also section on Interfering Substances).

DNA samples should be extracted using the QIAamp DSP DNA Blood Mini Kit (QIAGEN cat# 61104) following manufacturer's instructions for use. Use of alternative procedures requires validation by the customer.

Storage: Genomic DNA must be stored at -20°C or colder in a defrost-free freezer until use. Avoid multiple freeze/thaw cycles.

Interfering Substances: Presence of PCR inhibitors such as citrate [9], heparin [9], hemoglobin, ethanol, etc. can interfere with the PCR reaction.

DNA Quantity: A concentration ≥ 15 ng/µL of extracted genomic DNA is required for optimal performance.

VII. PROCEDURE

A. Verify the Veriti Dx thermal cycler programs

The Veriti thermal cycler will come pre-programmed for new customers. Verify the following settings for your Veriti Dx thermal cycler prior to running the assay. In the event that programming is required, please contact Technical Support .

Ensure heated lid option is selected for all three programs below.

HEA PCR Program		
94°C	15 minutes	
94°C	30 seconds, 60% ramp	30 cycles
60°C	30 seconds, 50% ramp	
68°C	50 seconds, 35% ramp	
68°C	8 minutes	
4°C	up to 72 hours	

HEA Post-Clean-up Program	
37°C	25 minutes
80°C	15 minutes
4°C	up to 3 hours

HEA Post-Lambda Program	
37°C	25 minutes
80°C	15 minutes
4°C	up to 3 hours

B. Procedural Notes

- To reduce or eliminate the chances of carryover contamination, users should assign three (3) separate laboratory areas, including: (1) pre-PCR /set-up activities, (2) DNA Addition, and (3) post-PCR procedures.
    - Steps in Section C, “PCR Master Mix Preparation,” should be performed in the pre-PCR area, within a PCR Workstation hood or clean room, using aerosol-resistant (filtered) pipette tips.
    - Steps in Section D, “DNA Addition Step,” are recommended to be performed in the DNA-Addition area within a dedicated hood or dead air box, using aerosol-resistant (filtered) pipette tips.
    - The remainder of the procedure after Section E, “PCR Amplification,” should be performed in the post-PCR area.
- Prior to use, wipe down the processing area surfaces with 10% Bleach and/or DNA AWAY, including:
    - Bench-tops and inside hood surfaces
    - Supportive equipment
    - All working pipettes
    - Inside mini mouse centrifuge lid, tube racks and covers
    - Thermal cycler and plate centrifuge surfaces
    - Clean inside lid and thermal cycler plate wells using DNA Away (or equivalent) and rinsing with deionized water. Use 10% bleach solution for removing contamination from the Veriti™ instrument sample block(s); excessive use of the solution, however, can corrode the sample block(s) material.
- Prior to using the hood, turn on the UV light for a minimum of 15-20 minutes.
- Remove the required quantities of reagents, samples, and controls from storage and, if frozen, allow them to thaw prior to use. Return unused portions to proper storage immediately.
- Use filtered tips for all pipetting steps in the procedural steps of this assay.
- Multi-channel or single-channel pipettes may be used, depending upon laboratory preference. All pipettes used must be calibrated. The quantity of reagents supplied with each PreciseType test kit is sufficient for pipetting the quantities required within this procedure.
- Precise pipetting of samples and reagents is required for accurate results.
- Take care to mix samples and reagents adequately. Avoid foaming.
- Combine working reagents just prior to use.

- Keep all reagents on ice or in a cryoblock until use when applicable. Ensure that the Veriti Dx Thermal Cycler is pre-programmed for each of the PCR Amplification and Post PCR Processing steps (Clean-Up and Single-Stranded Target Generation). Before each step, confirm that you have selected the proper pre-programmed profile.
- Contaminating DNA was found to have adverse effects on genotype results in the HEA assay at a concentration >10ng per reaction. Contamination in the Negative Control is detectable at a much lower concentration of 0.2 ng per reaction.
- It is recommended to add the DNA after PCR Master Mix preparation in a continuous process.
- Remove HEA BeadChip Carriers from storage and bring to room temperature before use (generally takes 15 to 20 minutes).
- It is extremely important to prevent cross-contamination between BeadChip wells. Exercise care when pipetting, rinsing, and removing fluids.
- The BioArray Solutions AIS 400 and hybridization oven should be turned on at least 30 min prior to operation. Place two paper towels in the tray of the hybridization oven and saturate them with a total of 25 mL of deionized water to maintain humid conditions during incubation.
    - If the hybridization oven has been used previously during the day, discard the old paper towels and insert two new paper towels and saturate as before with a total 25 mL of deionized water.

C. PCR Master Mix Preparation

Precautionary measures

- Always prepare PCR Master Mix inside a PCR workstation hood to prevent cross-contamination (DNA specimen should be added outside of the hood).
- Once made, the Working PCR Master Mix should be used immediately but may be kept in a cryoblock (stored at 2°C to 8°C) or on ice for up to 15 minutes.
- PCR plate may be cut if testing fewer than 96 samples. However, leave an extra column blank to prevent evaporation due to cracked plastic or mis-sealing.

  Note: Arrange samples and controls in the order they will be added to the PCR plate. Record the sample IDs in the sample plate map in the same order. The plate map will be used later for sample association (sample maps can be created in Excel® or directly in the BASIS software).

Note: A Negative Control (no DNA control) supplied with the kit is required for each run. The use of a BeadCheck Reference Panel-A and BeadCheck Reference Panel-B (sold separately) is required as Positive Controls for each run.

Assay Procedure

1. Reagent & sample preparation

   1.1 Remove PCR Mix (yellow cap) from the freezer and thaw at room temperature. Put in cryoblock (2 to 8°C) when fully thawed. Thawing the PCR Mix should take about 15 to 30 minutes depending on volume. Vortex and centrifuge briefly before use (approximately 3 to 5 seconds).

   1.2 Remove HotStarTaq DNA Polymerase (orange cap) from the freezer and place in cryoblock (2 to 8°C) or on ice. Vortex and centrifuge briefly before use (approximately 3 to 5 seconds).

   1.3 Bring DNA specimen to room temperature. Vortex and centrifuge briefly before use (approximately 3 to 5 seconds).

2. PCR Master Mix preparation (in PCR workstation hood or clean room)

   2.1 Determine the number of samples and controls to be run.

   2.2 Label a 1.5 or 2.0 mL microcentrifuge tube for PCR Master Mix.

   2.3 Prepare PCR Master Mix in microcentrifuge tube using the volumes listed in Table 2. Immediately return reagents to proper storage after use.

Table 2: PCR Master Mix Preparation - Reagent Volumes

Sample #	1	8	16	24	32	40	48	56	64	72	80	88	96
PCR Mix (µL)	16	144	296	448	592	736	880	1,008	1,168	1,296	1,456	1,584	1,744
HotStarTaq DNA Polymerase (µL)	1.0	9.0	18.5	28.0	37.0	46.0	55.0	63.0	73.0	81.0	91.0	99.0	109.0

*Table is intended as a guideline, providing approximately 10 to 20% more working reagent than is needed for performing the assay.

   2.3.1 Dispense the appropriate amount of HEA eMAP PCR Mix into the labeled microcentrifuge tubes using an appropriate pipette. Dip filtered pipette tip slowly into PCR mix reagent vial to avoid overflow and spillage.

   2.3.2 Dispense the appropriate amount of HotStarTaq into the tube with PCR mix using an appropriate pipette and **mix three times**. Immediately return reagents to cryoblock (~2 to 8°C), on ice or to the freezer after use.

   2.3.3. Secure the cap of the PCR Master Mix tube, vortex and centrifuge briefly (approximately 3 to 5 seconds). Keep in a cryoblock (~2 to 8°C) or on ice until use, when performing the pipetting manually.

   2.4 Dispense the appropriate amount of PCR Master Mix into an 8-tube strip using the volumes in the table below and visually inspect each tube:

Table 3: Working PCR Master Mix - Transfer Volumes

Sample #	1-8	16	24	32	40	48	56	64	72	80	88	96
PCR Mix (µL)	Add 17 µL directly to PCR plate	37.0	57.0	76.0	94.0	112.0	128.0	148.0	164.0	188.0	200.0	224.0

   2.5 Prepare a 96-well PCR plate and label plate appropriately.

2.6 Dispense **17.0 µL** of **PCR Master Mix** into the bottom of each well of the labeled PCR plate in a cryoblock (2 to 8°C) or on ice (an appropriate pipette may be used), when performing the pipetting manually. Discard unused PCR Master Mix

D. DNA Sample Addition

Precautionary measures

- Turn on the thermal cycler approximately 10 minutes prior to beginning the PCR cycle.
- Begin to add DNA and controls to working PCR Master Mix outside of the hood as soon as possible, but within 15 to 20 minutes when performing the pipetting manually.
- When performing the pipetting manually, keep samples and working PCR Master Mix in a cryoblock (stored at 2 to 8°C) or on ice during these steps.

  Note: Verify sample order and identification on sample plate map.

Assay procedure

1. Vortex and centrifuge DNA specimen, if not already done.
2. Add **8.0 µL** of required negative control (supplied in PreciseType HEA reagent kit box) to the appropriate PCR plate well using an appropriate pipette.
3. Add **8.0 µL** of required positive controls (Cat. # 800-20236) into appropriate PCR plate wells using an appropriate pipette.
4. Add **8.0 µL** of DNA sample into appropriate PCR plate wells using an appropriate pipette. Mix three times by pipette aspiration to ensure complete transfer of DNA.
5. Seal PCR plate securely with thermal adhesive plate seal. Ensure all wells are sealed completely to prevent evaporation.
6. Gently vortex for approximately **3-5 seconds**.
7. Centrifuge briefly at approximately 1,000 rpm to bring samples to the bottom of wells.

E. PCR Amplification

Precautionary measures

- The centrifuged amplified samples and controls should be used immediately, but may be stored at −20°C (or colder) for up to 4 weeks.

Assay procedure

1. Place the PCR plate in the center of the thermal cycler.
2. Close the lid and push handle down.
3. Log into thermal cycler and select **"Browse/New Methods."**
4. Select "HEA PCR" program and press **"View"** to verify the program is correct:

Cycles	**1**	**30**			**1**	**1**
Temp (C°)	94°C	94°C	60°C	68°C	68°C	4°C
Time	15 min	30 sec	30 sec	50 sec	8 min	Until removal (no longer than 72 hours)
Ramp	100%	60%	50%	35%	100%	100%

5. Press **"Run."** Reaction volume should be **25.0 µL**. Enable the heated lid function with the temperature set at **105°C**.
6. Press **"Start Run Now"** to begin the process and verify that the lid is heating and the program has initiated.
7. Remove PCR plate from the thermal cycler once the program reaches 4°C and centrifuge briefly (approximately 1,000 rpm for 5 seconds). PCR plate must be removed from the thermal cycler at 4°C within 72 hours.

F. Post-PCR Processing: Clean-up

Precautionary measures

- The steps in Section F should be performed continuously without interruption in the post-PCR area when performing the pipetting manually.
- When performing the pipetting manually, the Clean-up Reagent should be added to the post-PCR product within approximately 30 minutes of removing PCR products from the thermal cycler after PCR amplification, or if frozen, within 30 minutes of thawing.
- The PCR plate may be cut if testing fewer than 96 samples. However, leave an extra column blank to prevent evaporation due to cracked plastic or mis-sealing.
- Clean-up Reagent and PCR product should be thawed at room temperature and kept in a cryoblock (~2 to 8°C) or on ice until use.
- When transferring the post-PCR product and Clean-up Reagent into new post-PCR plates, keep in a cryoblock (stored at 2 to 8°C) or on ice during these steps when performing the pipetting manually.
- The centrifuged cleaned-up samples and controls may be used immediately, or stored at -20°C (or colder) for up to 72 hours.

Assay procedure

1. Remove Clean-up Reagent (green cap) from the freezer **10-15 minutes** before use and place in a cryoblock (2 to 8°C) or on ice when performing the pipetting manually.
2. Gently mix and centrifuge (approximately 1,000 rpm for 5 seconds) PCR products before use. Place in a cryoblock (2 to 8°C) or on ice when performing the pipetting manually.
3. Determine the number of samples and controls to be run.
4. Prepare a new PCR plate for post-PCR processing and label it appropriately.
5. Position the PCR Product plate in the same orientation as the Post-PCR plate in a cryoblock (2 to 8°C) when performing the pipetting manually.
6. Transfer **6.5µL** of each PCR product to the bottom of the appropriate well of the new Post-PCR plate using an appropriate pipette.
7. Seal the plate with the remaining PCR products and store in the freezer at **-20 to -80°C** until successful completion of assay run.
8. When performing the pipetting manually, use the table below to determine the appropriate volume, then dispense Clean-up Reagent into an 8-tube-strip with an appropriate pipette and visually inspect each tube. Immediately return reagents to proper storage after use.

Table 4: Clean-up Reagent Volumes

Sample #	8	16	24	32	40	48	56	64	72	80	88	96
Clean-up Reagent (µL)	Add 2 µL directly to Post-PCR plate	6.0	9.0	12.0	14.0	16.0	18.0	20.0	22.0	24.0	26.0	28.0

9. Dispense **2.0 µL** of Clean-up Reagent into each well of the post-PCR plate using an appropriate pipette. Mix each well three times by pipette aspiration.
10. After adding Clean-up Reagent to all sample wells in the Post-PCR plate, discard the 8-tube strip.

11. Seal Post-PCR plate securely with thermal adhesive plate seal.
12. Gently vortex for approximately **3-5 seconds**.
13. Briefly centrifuge at approximately 1,000 rpm to bring samples to the bottom of the wells.
14. Place the **Post-PCR plate** in the center of the thermal cycler.
15. Close the lid and push the handle down.
16. Log into the thermal cycler and select **"Browse/New Methods."**
17. Select the HEA Clean-up program and press **"View"** to verify that the program is correct:

Cycles	**1**	**1**	**1**
Temp (C°)	37°C	80°C	4°C
Time	25 min	15 min	Until removal (no longer than 3 hours)
Ramp	100%	100%	100%

18. Press **"Run."** Reaction volume should be **10.0 µL**. Enable the heated lid function with the temperature set at 105°C.
19. Press **"Start Run Now"** to begin the process and verify that the lid is heating and the program has initiated.
20. Remove PCR plate from the thermal cycler once the program reaches 4°C and centrifuge briefly (approximately 1,000 rpm for 5 seconds). PCR plate must be removed from the thermal cycler at 4°C within 3 hours. The centrifuged amplified samples and controls should be used immediately, but may be stored at -20°C (or colder) for up to 72 hours.

G. Post-PCR Processing: Single-Stranded Target Generation

Precautionary measures

- The steps in Section G should be performed continuously and without interruption.
- Lambda Exonuclease should be removed from the freezer to thaw during the incubation of the post-PCR Clean-up Reagent step in Section F. Once thawed, the Lambda Exonuclease should be kept in a cryoblock (stored at 2 to 8°C) or on ice during transfer to the post- PCR Clean-up Reagent product when performing the pipetting manually.
- The post-PCR Clean-up Reagent product should be kept in a cryoblock (stored at 2 to 8°C) or on ice during these steps once thawed (if previously frozen).
- Add the Lambda Exonuclease to the post- PCR Clean-up Reagent product within approximately 30 minutes of completing the clean-up process when performing the pipetting manually.
- The centrifuged single-stranded samples and controls should be used immediately, but may be stored at -20°C (or colder) for up to 72 hours.

Assay procedure

1. Remove Lambda Reagent (violet cap) from the freezer 10-15 minutes before use. Thaw at room temperature.
2. Briefly mix and centrifuge Clean-up products at room temperature before use. Mix reagents with vortex mixer at mid-range speed for 3-5 seconds. Briefly spin down tubes/plates at approximately 1,000 rpm.
3. Place Post-PCR plate ("Clean-up Products") in a cryoblock (2 to 8°C) when performing the test manually.
4. When performing the pipetting manually, use the table below to determine the appropriate volume, then dispense Lambda Reagent into an 8-tube-strip and visually inspect each tube. Immediately return reagents to proper storage after use.

Table 5: Lambda Exonuclease Volumes

Sample #	8	16	24	32	40	48	56	64	72	80	88	96
Lambda Reagent volume (µL)	Add 2 µL directly to Post-PCR plate	6.0	9.0	12.0	14.0	16.0	18.0	20.0	22.0	24.0	26.0	28.0

5. Dispense **2.0 µL** of Lambda Reagent into each well of the Post-PCR plate using an appropriate pipette. Mix each well three times by pipette aspiration.

6. Discard 8-tube strip after adding Lambda Reagent to all sample wells.
7. Seal Post-PCR plate securely with thermal cycler adhesive plate seal.
8. Gently vortex for approximately **3-5 seconds**.
9. Centrifuge briefly at approximately 1,000 rpm to bring samples to bottom of wells.

10. Load PCR plate to thermal cycler and run “HEA Lambda” program. Press “View” to verify that the program is correct:

Cycles	1	1	1
Temp (C°)	37°C	80°C	4°C
Time	25 min	15 min	Until removal (no longer than 3 hours)
Ramp	100%	100%	100%

11. Press **“Run.”** Reaction volume should be **12.0 µL**. Enable the heated lid function with the temperature set at **105°C**.
12. Press **“Start Run Now”** to begin the process and verify that the lid is heating and the program has initiated.
13. Remove PCR plate from the thermal cycler once the program reaches 4°C and centrifuge briefly (approximately 1,000 rpm for 5 seconds). PCR plate must be removed from the thermal cycler at 4°C within 3 hours. The centrifuged amplified samples and controls should be used immediately, but may be stored at -20°C (or colder) for up to 72 hours.

H. On-BeadChip Array Elongation

Precautionary measures

- The steps in Section H should be performed continuously and without interruption.
- The Elongation Reagent should be taken out of the freezer to thaw during incubation of the Single-Stranded Target Generation step in Section G.
- Once thawed, the Elongation Reagent should be kept in a cryoblock (stored at 2 to 8°C) or on ice when performing the pipetting manually.
- Avoid foaming when pipetting the Elongation Reagent.
- Adding the Elongation Reagent to the single-stranded product should be performed within approximately 30 minutes of completing the Single-Stranded Target Generation process when performing the pipetting manually.
- Do not shake the canned air.

Assay procedure

1. Prepare Boekel oven **at least 30 minutes prior to use.**
   1.1 Turn on Boekel oven and verify set temperature (**53°C**) by holding down the **"*"** button.
   1.2 Remove interior tray. Line tray with two paper towels and saturate with **25mL** of deionized water.
   1.3 Place lid back on tray and insert tray back into the Boekel oven to pre-heat.
2. Remove BeadChip carrier(s) from the refrigerator **15-20 minutes before use.**
3. Remove Elongation Reagent (brown cap) from the freezer **10-15 minutes** before use and thaw at room temperature. Place in a cryoblock (2 to 8°C) when fully thawed when performing the pipetting manually.
4. Briefly mix and centrifuge Lambda products at room temperature before use. Mix reagents with vortex mixer for 3 to 5 seconds. Spin down tubes/plates at approximately 1,000 rpm for 5 seconds.
5. Place **Post-PCR plate** ("Lambda Products") in a cryoblock (2 to 8°C) when performing the pipetting manually.
6. When performing the pipetting manually, use the table below to determine the appropriate volume, then dispense Elongation Reagent) into an 8-tube-strip and visually inspect each tube. Immediately return reagents to proper storage after use.

Table 6: eMAP Elongation Mix Volumes

Sample #	8	16	24	32	40	48	56	64	72	80	88	96
eMAP Elongation Reagent (µL)	Add 10 µL directly to Post-PCR plate	25.0	36.0	47.0	59.0	70.0	80.0	90.0	100.0	114.0	125.0	135.0

7. Dispense **10.0 µL** of Elongation Reagent into each well of the PCR plate using an appropriate pipette. Mix each well three times by pipette aspiration.

8. Discard 8-tube strip after adding Elongation Reagent to all sample wells.
9. Remove BeadChip carrier(s) from foil pouch and number the carriers in numerical order.
10. Carefully place the BeadChip carrier(s) on the BeadChip carrier holder without touching the surface of the BeadChip.
11. Record BeadChip carrier ID(s) to the sample plate map accordingly.
12. Transfer **15.0 µL** of the Elongation Reaction mixture to the corresponding BeadChip using an appropriate pipette. Visually inspect pipette tips during process and ensure that carrier orientation is the same as in the sample plate map.
13. Remove preheated tray from Boekel oven. If there is condensation on the inside of the tray cover, dry it with a lint-free tissue.
14. Carefully place BeadChip holder into Boekel oven tray, placing tray cover completely over the tray.
15. Load tray in the oven, tighten the latch securely, close the Boekel oven door, and set timer for 30 minutes. Incubate the BeadChip carriers at **53±1°C for 30 minutes**. *(Please note: if you intend to read the carrier immediately following elongation, turn on the BioArray AIS 400 Array Imaging System and load the CD when starting the incubation process.)*
16. Remove BeadChip holder from the oven after 30 minutes.
17. Wash the elongation mixture from BeadChip surfaces using deionized water wash bottle:
    - 17.1 Hold the BeadChip carrier so that its surface is vertical over a sink or catch basin.
    - 17.2 Rinse each BeadChip individually for approximately **3 seconds**. The water stream should be directed perpendicular to the BeadChip surface approximately one-inch away.
18. Remove excess water from BeadChip surfaces using compressed/canned air. **Caution! Do not shake canned air!**
19. Remove any remaining water from the back of the carrier(s) with a lint-free tissue.
20. If you cannot read the washed and dried HEA BeadChip carriers immediately following incubation, they may be stored protected from light up to 72 hours at room temperature before reading with the BioArray AIS 400 system.

I. BeadChip Image Acquisition

Precautionary measures

- Turn on the BioArray AIS 400 light source and computer at least 30 minutes prior to use.
- Load PreciseType BeadChip Test | HEA Data CD once per lot.

Assay procedure

1. Open and initialize AISR program on the desktop.
2. Run the ETC (Refer to AIS User Manual 190-20185). Contact Technical Support if results are out of specifications to adjust the exposure time prior to proceeding.
3. Remove PreciseType BeadChip Test | HEA Data CD from BeadChip carrier box and load files from CD onto the computer for each new lot.
4. Read the HEA BeadChip carrier(s) using the BioArray AIS 400 Array Imaging System. Process BeadChip data using the HEA Analysis software in BASIS.
5. Properly shutdown AISR and the light source after use.
6. Continue to BASIS to perform sample association and generate BeadChip reports.

VIII. EXPECTED RESULTS

A. Evaluation - Quality Control

The BASIS software determines run and sample validity automatically.

Run Validity: Two Positive and one Negative Control as supplied, are required for each run. The results for all controls must meet the Run Validity criteria. If any one of the controls does not meet any one criterion, the run is invalid and must be repeated.

If the results for Low Signal (LS) is <32 in the phenotype report for the Negative Control sample, it may indicate contamination by gDNA in a quantity that may impact test results. When this occurs, all sample results in the run are invalid.

The phenotype pattern of the two positive control samples must match the expected phenotype pattern. If either one of the control results do not meet any one criterion, all sample results in the run are invalid. See PreciseType BeadCheck Package Insert (P/N 190-20229) for more details.

See the BASIS AM 4G User Manual (190-20331) for more information on the BASIS software and for examples of how sample results are displayed for valid and invalid runs.

Interpretation of validity of the Positive and Negative Controls is described in Table 7:

Table 7: Run Validity Criteria

Control	BASIS Analysis	Result Reported	Interpretation
Negative	LS ≥ 32	Valid NC	Valid Negative Control
Negative	LS < 32	Invalid NC	Invalid Negative Control; No results reported for all samples in the run
Positive	Phenotype pattern matches pattern expected for the two positive controls from the BeadCheck kit	Valid HEA Ref-pA and Valid HEA Ref-pB	Valid Positive Control
Positive	Phenotype pattern does not match expected pattern for either one of the two positive controls from the BeadCheck kit	Invalid HEA Ref-pA and/or Invalid HEA Ref-pB	Invalid Positive Control; No results reported for all samples in the run

Sample Results Validity: For sample results to be valid, the phenotype results for all antigens must be valid (for a listing of causes of invalid sample results see Table 8 below). See the BASIS AM 4G User Manual (190-20331, Sections 6 and 7) for more information on the BASIS software and for examples of how valid and invalid sample results are displayed.

If a sample result shows an antigen phenotype of either Indeterminate Call (IC) or Low Signal (LS), the sample is invalid, except for S and s where LS is an expected phenotype in conjunction with U negative results. If the sample has a status of High Background (HB) or High Coefficient of Variation (CV), the sample is invalid (for a list of causes for invalid sample see Table 8 below, for a more detailed explanation see section XII Possible Warning Messages Found in BASIS Report).

Table 8: Causes for Invalid Sample Result

Cause	Interpretation
IC ≥ 1	Indeterminate Call
LS ≥ 1	Low Signal
HB	High Background
CV	High Coefficient of Variance

For samples that are part of an invalid run or have invalid phenotype(s) themselves (sample results invalid), all antigen phenotype results are reported as No Type Determined (NTD).

B. Analysis of Results

This is a qualitative test. BASIS computes BeadChip array signal intensity data on each oligonucleotide detecting specific alleles to determine the presence or absence of each allele or the genotype result. The genotype results are then used to compute the predicted antigen phenotype results.

The HEA-analysis software in BASIS performs all calculations automatically. Please refer to the BASIS User Manual (P/N 190-20331) for more details. Expected genotype results are shown in Table 10 below (for a more detailed explanation of Ax and xB results see section XII on Troubleshooting).

For samples that are part of an invalid run or have invalid genotype(s) themselves (sample invalid), all genotype results are reported as No Type Determined (NTD).

C. Phenotype and Genotype

For samples with valid results, the expected phenotype results are shown below in Table 9 and the expected genotype results are shown below in Table 10.

Table 9: Expected Phenotype Results

Result Reported	Interpretation
+	Positive
0	Negative
(+)*	Possible (C)ce^{s} haplotype
(0)*	Fyb variant
PV	Possible Variant
var	U variant (S silencing mutation)
w	Fyb Weak
++	HbS homozygous

Table 10: Expected Genotype Results

Result Reported	Interpretation
AA	Homozygous for A
AB	Heterozygous
Ax	Indeterminate call on B
BB	Homozygous for B
xB	Indeterminate call on A
IC	Indeterminate call on A and B

Note: For the RhCE-109Insert, a positive amplicon control corresponds to probe “A” and a 109-bp insertion specific probe corresponds to probe “B”, therefore:

- ☐ If the RhCE-109Ins = AA, the 109-bp insertion is absent, indicating C-
- ☐ If the RhCE-109Ins = AB, the 109-bp insertion is present in one of the alleles, indicating C+
  - There is no RhCE-109Ins = BB since the positive control is always present
- ☐ When P103 is positive (RhCE-P103S = AA, Ax, or AB), the RhCE-109Ins probe is used for prediction of C phenotype:
  - If the RhCE-109Ins = AA, the phenotype = cc
  - If the RhCE-109Ins = AB, the phenotype = cC
- ☐ When RhCE-P103S = BB, the phenotype = CC, regardless of RhCE-109Ins status

IX. LIMITATIONS OF PROCEDURE

- False negative and/or invalid results may be generated when unanticipated rare mutation(s) affecting the primer or probe binding cause allele and/or amplicon dropout.
    - Presence of RH hybrids and variant mutations in exons 2, 5 and 7 as well as introns 1, 2, 4, 5, 6, and 7 of the RHCE gene can interfere with the detection E/e and C/c antigen. Mutations in RHCE gene leading to the ceMO phenotype[12],[13], which expresses as a weak Rhe, can cause a direct suppression of the Rhe probe and may cause an invalid or false negative result . In select populations, such as Afro-Caribbean patients with sickle cell disease, the prevalence for ceMO phenotype has been reported to be up to 2 % [18].
    - Presence of a rare +3g>a change in intron 5 of GYPB interferes with the detection of the S antigen and may lead to a false negative typing of the S antigen [16].
    - The mutation HgbS in the beta globin gene should not be used for determination of sickle cell disease. Presence of HbSC disease interferes with the detection of the HgbS mutation in the beta globin gene mutation and may result in invalid or inaccurate HbS phenotype call (HbS (++) instead of HbS (+)). In the United States, HbSC disease has a prevalence of 0.017% among African Americans [19]. Presence of some beta thalassemia disorders may interfere with the detection of the HgbS mutation in the beta globin gene and may result in invalid HbS phenotype call.
    - Presence of Mit+(GYPB 161G>A) mutation may result in an invalid or false negative typing of the S antigen. The mutation has a prevalence of 0.1% in western Europeans [6].
    - Presence of a GYPB mutation (c. 137-8C>T) may result in an invalid or false negative typing of the S antigen.
- False positive and/or invalid results may be generated in rare cases where a sample contains examples of molecular events that affect the blood-group antigen expression and phenotypes and the nucleotide changes associated with these events are not explicitly monitored by the assay. Examples include DNA-sequence variations including premature stop codon, SNP leading to missense change in amino acid, hybrid genes, modifying genes; changes at the RNA transcription level including alternative splicing; reduced protein expression, etc. Known phenotypes are Knull, JKnull (JKnull has a prevalence of up to 9% among Polynesians [20]), Rhnull, Rh hybrids, Kmod, Co(a-,b-), In(Lu), Lu(a-,b-) and GP hybrids. Presence of a c.179_180del (Ser60fs) mutation linked with the Fy(b) allele may change the Fy(b) antigen expression and lead to a false positive result.
- The BASIS software is not designed to convert all genotype/allele combinations into phenotype calls. For example, if allele combinations that have not been widely reported in the literature are encountered, the software will display a Possible Variant (PV) result.
- The HEA test uses two point mutations to predict the V and VS antigen phenotypes: 733C>G(L245V) in exon5 and 1006G>T(G336C) in exon7 of the RHCE gene
- The genotype-to-phenotype-prediction conversion rules employed by the HEA test are based on the established fact that the absence of the two mutations are correlated with the absence of V and VS antigens and that the presence of the mutations lead to antigen expression.
- BioArray Solutions is aware of literature [14] that point to certain genotype combinations (involving the two mutations of interest) that do not lead to a unique phenotype. This limitation only affects the V(+)VS(+) call. As per the publication [14], in a small fraction of the cases, the HEA test would report the samples as falsely positive relative to serology when giving the V(+)VS(+) call (4.2% for VS and 1.4% for V). The V(-)VS(-) call is unaffected.
- In the HEA test, the presence/absence of the RhC antigen is reported based on three polymorphisms 307C>T(P103S) in Exon 2, 733C>G (L245V) in Exon 5, 1006G>T (G336C) in Exon 7 and the presence/absence of 109 bp–insert in Intron 2 of the RHCE gene.

The (+)* call on the RHC antigen implies the possible presence of altered C antigen encoded by the (C)ce^{s} haplotype. The (C)ce^{s} haplotype comprises:

i) A hybrid RHD-CE-D allele of the RHD gene, and

ii) ce^{s} allele of the RHCE gene

The (C)ce^{s} haplotype produces weak C, normal c, weak e (also known as e^{s}), and VS (RH20)[15].

- The U antigen (located on the GPB protein) is not polymorphic by itself. The expression of the U antigen is governed by changes that affect the expression of the S antigens. Specifically, the S-s- phenotype is known to be associated with the absence or weak expression of the U antigen. The HEA test monitors three mutations that inform the S-s- phenotype and can call the U(var) and U(neg) phenotype. Occasionally, a U(neg) phenotype call may not be made even if the phenotype call is S-s- due to non-specific residual intensities on the probes governing the silencing of S/s antigen.
- The Fyx allele encodes an amino acid change which causes Fy(b+w) phenotype with varying degrees of weakened Fyb antigen. Licensed serological anti-Fyb reagents may not always react with such a weakened Fyb antigen [17].

X. SPECIFIC PERFORMANCE CHARACTERISTICS

A. Accuracy Study

BioArray performed a study to demonstrate that the HEA PreciseType Test can accurately identify the phenotypes listed using pre-selected well-characterized samples. Red-blood-cell (RBC) antigen phenotypes were determined using two methods. The RBC antigens characterized using serology (licensed antisera) include 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, and k (U is inferred from S/s typing). The red blood cell antigens characterized using bi-directional sequencing (corresponding licensed antisera are not available) include 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, and Hy (also included is HgbS). Samples were selected for phenotypic diversity to cover all antigen positive statuses and all but three antigen negative statuses (Di^b, LW^a and Sc1).

To assure phenotypic diversity, the accuracy included combined data (unique valid samples) from three different studies: a genotype-detection study, the clinical study described below and the performance evaluation conducted in Europe. Some samples or sample results that were collected from historical sources were not available for subsequent testing for discrepancy resolution.

In order to be accepted, all phenotypes were to meet or exceed 99% at the lower bound of the one-sided 95% confidence interval for accuracy (defined as overall agreement with the comparison method). All antigens met the acceptance criteria with the exception of Lu^b and V, which had lower bounds of 98.46% for Lu^b and 98.92% for V. Subsequent testing showed complete concordance for V on PreciseType compared with bi-directional sequencing. Subsequent testing was not available for Lu^b; the discrepancy for Lu^b between PreciseType and serology may be due to *In(Lu)* or Lu(a-b-) as described in the Limitations of Procedure section.

Table 11: PreciseType Accuracy Study Results

Antigen	Samples	Percent Correct Call	Lower 95% Confidence Limit
c	1147	99.91%	99.59%
C	1146	100%	99.74%
e	1383	100%	99.78%
E	1383	100%	99.78%
K	1149	100%	99.74%
k	909	99.89%	99.48%
Kpa	657	100%	99.55%
Kpb	875	100%	99.66%
Jsa	1158	100%	99.74%
Jsb	1345	100%	99.78%
Jka	1124	100%	99.73%
Jkb	1123	99.91%	99.58%
Fya	1131	99.73%	99.32%
Fyb	1130	99.82%	99.44%
M	1053	100%	99.72%
N	1052	99.81%	99.40%
S	1126	99.91%	99.58%
s	1126	100%	99.73%
Lua	1223	99.75%	99.37%
Lub	1414	99.01%	98.46%
Dia	820	100%	99.64%
Dib	820	100%	99.64%
Coa	1378	99.93%	99.66%
Cob	972	100%	99.69%
Doa	980	100%	99.69%
Dob	979	100%	99.69%
Joa	650	100%	99.54%
Hy	650	100%	99.54%
LWa	625	100%	99.52%
LWb	625	100%	99.52%
Sc1	627	100%	99.52%
Sc2	957	100%	99.69%
HbS	686	100%	99.56%
VS	649	100%	99.54%
V	843	99.53%	98.92%
U	309	100%	99.04%

B. Clinical Overall, Positive and Negative Agreement as Compared with Serology and Clinical Concordance, Sensitivity and Specificity as Compared with DNA Sequencing

From 2011 to 2013, four laboratories across the United States conducted a study entitled "Evaluation of the HEA BeadChip Kit in comparison to established methods for Human Erythrocyte Antigen determination." This study compared the typing results of the PreciseType HEA BeadChip Test with serological and DNA sequencing methodologies. A total of 1,777 samples were tested of which 1,757 could be used for comparison, with 1,684 valid HEA BeadChip test results, bringing the valid rate to 95.85% (1684/1757). Out of the 1,684 valid results, 1,248 paired valid comparative results per phenotype were considered for analysis (SC1 and SC2 have 1,247 valid comparative results). Samples were selected randomly covering all antigen-positive and all but six antigen-negative statuses (k, Kp^b, Di^b, Co^a, LW^a, and SC1 negative).

The RBC antigens characterized using serology (licensed antisera) include $Di^{a,}$ Fy^a, Fy^b, M, N, S, s, Jk^a, Jk^b, Kp^a, Kp^b, Lu^b, C, c, E, e, K, and k (U is inferred from S/s typing). The RBC antigens characterized using bi-directional sequencing (corresponding licensed antisera are not available) include 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, and Hy (also included is HgbS).

Table 12 shows all antigens tested with serological and sequencing methods. For the 18 antigens tested with serological methods, Overall Percent Agreement ranged from 95.99% to 100.00%, Positive Percent Agreement ranged from 98.77% to 100.00%, while the Negative Percent Agreement ranged from 71.43% to 100.00%. For the 21 antigens tested with DNA sequencing methods, Concordance ranged from 99.76% to 100.00%, Percent Sensitivity ranged from 98.67% to 100.00%, while the Percent Specificity was 100.00%.

Table 12: PreciseType Test as compared with Serology and Sequencing

Antigen	Compared to Serology			Compared to Sequencing		
	Samples	Overall Percent Agreement	Lower 95% Confidence Limit	Samples	Concordance	Lower 95% Confidence Limit
c	1248	100.00%	99.76%	N/A	N/A	N/A
C	1248	98.48%	97.77%	N/A	N/A	N/A
e	1248	100.00%	99.76%	N/A	N/A	N/A
E	1248	99.84%	99.50%	N/A	N/A	N/A
K	1248	100.00%	99.76%	N/A	N/A	N/A
k	1248	100.00%	99.76%	N/A	N/A	N/A
Kpa	1248	100.00%	99.76%	N/A	N/A	N/A
Kpb	1218	100.00%	99.75%	N/A	N/A	N/A
Jsa	N/A	N/A	N/A	1248	100.00%	99.76%
Jsb	N/A	N/A	N/A	1248	100.00%	99.76%
Jka	1248	98.64%	97.96%	N/A	N/A	N/A
Jkb	1248	98.48%	97.77%	N/A	N/A	N/A
Fya	1248	99.84%	99.50%	N/A	N/A	N/A
Fyb	1248	98.32%	97.59%	N/A	N/A	N/A
M	1248	99.12%	98.55%	N/A	N/A	N/A
N	1248	95.99%	94.96%	N/A	N/A	N/A
S	1248	99.92%	99.62%	N/A	N/A	N/A
s	1248	99.84%	99.50%	N/A	N/A	N/A
Lua	N/A	N/A	N/A	1248	100.00%	99.76%
Lub	663	99.85%	99.29%	N/A	N/A	N/A
Dia	1248	99.92%	99.62%	N/A	N/A	N/A
Dib	N/A	N/A	N/A	1248	100.00%	99.76%
Coa	N/A	N/A	N/A	1248	100.00%	99.76%
Cob	N/A	N/A	N/A	1248	100.00%	99.76%
Doa	N/A	N/A	N/A	1248	100.00%	99.76%
Dob	N/A	N/A	N/A	1248	100.00%	99.76%
Joa	N/A	N/A	N/A	1248	100.00%	99.76%
Hy	N/A	N/A	N/A	1248	100.00%	99.76%
LWa	N/A	N/A	N/A	1248	100.00%	99.76%
LWb	N/A	N/A	N/A	1248	100.00%	99.76%
Sc1	N/A	N/A	N/A	1247	100.00%	99.76%
Sc2	N/A	N/A	N/A	1247	100.00%	99.76%
HbS	N/A	N/A	N/A	1248	100.00%	99.76%
VS	N/A	N/A	N/A	1248	100.00%	99.76%
V	N/A	N/A	N/A	1248	99.76%	99.38%
U	1248	99.84%	99.50%	N/A	N/A	N/A

C. Overall HEA BeadChip Test Agreement with Serology and Sequencing Post-Discordant Resolution

In the same study mentioned in the section above, all discrepancies observed were further resolved by DNA sequence analysis. Bi-directional sequencing is considered "gold standard" – the reference method for sequence analysis. The term "reference method" refers to a well-validated analytical procedure sufficiently free of systemic or random error to make it useful for validating proposed new analytical procedures for the same analyte [21].

Antigen	Number of Discordant Samples (Out of 1,248)	PreciseType Concordant with Reference Method (Bi-directional Sequencing)
Jkb	19	12 of 19
Fyb	21	20 of 21
C	19	19 of 19
Jka	17	17 of 17
M	11	11 of 11
N	50	50 of 50
Fya	2	2 of 2
E	2	2 of 2
S	1	1 of 1
s	2	2 of 2
Lub	1 (663)	1 of 1
Dia	1	1 of 1
V	3	3 of 3

- Samples Discordant for Jkb with serology:
  - There were 19 discordant samples; all were PreciseType positive, serology negative.
  - Twelve samples were concordant between PreciseType and bi-directional sequencing.
  - Seven samples did not agree with serology or bi-directional sequencing and were identified as Jk_{null} (see the Limitations of Procedure section).
- Samples Discordant for Fyb with serology:
  - There were 21 discordant samples, 20 were concordant between PreciseType and sequencing
  - Fifteen were PreciseType positive, serology negative and 14 were concordant between PreciseType and bi-directional sequencing (all were Fyb weak).
  - One sample was discordant between PreciseType and bi-directional sequencing and upon further investigation was found to be a novel mutation uncharacterized in literature (see the Limitations of Procedure section).
- Samples Discordant for C with serology:
  - There were 19 discordant samples, all were PreciseType positive, serology negative; all PreciseType results were concordant with bi-directional sequencing.
- Samples Discordant for Jka with serology:
  - There were 17 discordant samples; all PreciseType results were concordant with bi-directional sequencing.
- Samples Discordant for M with serology:
  - There were 11 discordant samples; all PreciseType results were concordant with bi-directional sequencing.
- Samples Discordant for N with serology:
  - There were 50 discordant samples; all PreciseType results were concordant with bi-directional sequencing.
- All other Discrepancies (Fya, E, S, s, Lub, Dia, and V)
  - All other discrepancies for other antigens were found to be in concordance with bi-directional sequencing.

D. Repeatability and Reproducibility

The objective of these studies was to demonstrate that the PreciseType HEA test generates reproducible and repeatable results with a panel of human DNA samples across sites and operators over five days. The studies were performed with both the eight-chip slide and the 96-chip plate.

A total of six operators across three sites participated in the slide study. A total of eight operators across four sites participated in the plate study. Documented training including proficiency testing was completed prior to study initiation. The panel consisted of 11 previously characterized DNA (bi-directional sequencing) samples extracted from immortalized cell lines derived from human whole blood representing all positive phenotypes in PreciseType. The panel also assessed 24 negative phenotypes, there were 12 negative phenotypes that were not assessed (e, k, Kp^b, Js^b, U, Lu^b, Di^b, Co^a, Joa, Hy, LW^a, and SC1).

An assay run was repeated if it was determined to be invalid, (i.e., operator error, apparent equipment failure, or a negative or positive control not valid).

Samples with invalid results (high background, low signal, indeterminate call, high coefficient of variation) were categorized as no type determined (NTD) and were not included in calculations due to study logistics however, the rate of incidence was captured (Slide: 0.3% invalid sample rate, Plate: 0.8% invalid sample rate).

Repeatability results: For both the plate and slide formats, the results showed 100% agreement and the studies showed 100% repeatability.

Repeatability *(Percent Concordance)*	**Total**
Within-Site	100%
Within-Operator	100%
Within-Day	100%
Within-Sample	100%

Reproducibility results: For both the plate and slide formats the results showed 100% reproducibility.

Reproducibility *(Percent)*	**Total**
Site to Site	100%
Operator to Operator	100%
Day to Day	100%

Lot-to-Lot Reproducibility results: A separate lot-to-lot study was performed on a fully characterized panel (n=22) of extracted human genomic DNA samples where the PreciseType HEA test was performed using kits from three different lots to demonstrate the lot-to-lot reproducibility. These 22 samples were blinded to the operator to eliminate bias and selected to represent the broadest ranges of alleles possible that are contained in the PreciseType test. The same operator repeated the same assays on five separate days to demonstrate the repeatability. The results showed 100% agreement and the study shows 100% lot-to-lot repeatability and reproducibility.

Reproducibility *(Percent)*	**Total**
Lot to Lot	100%
Day to Day	100%

Overall Conclusion: On an antigen basis, all sample results (across all samples) were in agreement with their expected results within operator day to day, across operators, across sites and across lots. Therefore, we can conclude that the PreciseType HEA test is 100% repeatable and 100% reproducible.

E. Interfering Substances

The following substances, commonly found on skin and in blood, were not found to interfere with the PreciseType HEA test.

Microorganisms – The following organisms were tested at 10^6 CFU per mL of blood;

Bacillus subtilis, Corynebacterium diphtheria and jeikeium, Escherichia coli, Propionibacterium acnes, Pseudomonas aeruginosa, Salmonella enterica, Staphylococcus epidermidis, haemolyticus and aureus, Streptococcus pneumonia and mitis, Aspergillus niger, Candida albicans. Cytopathic levels of influenza virus were also tested with no interference observed.

Exogenous Substances – Amoxicillin (2.06E+02 µmol/L), Penicillin G Potassium Salt (2.73 µg/mL) Hydroxyurea (3.50 µg/mL), Acetaminophen (1.32E µmol/L), Ibuprofen (2.43 µmol/L), Aspirin (3.62E µmol/L), Naproxen (2.17E µmol/L), Plavix (3.00E µmol/L), Warfarin (3.25E µmol/L), Loratadine (7.80E µmol/L), Atorvastatin (Lipitor) (5.48E+2 µmol/L), Phenylephrine HCl (4.91E µmol/L), Nadolol (3.88E µmol/L), Folic Acid (Vit.B) (1.50E+1 µmol/L), Ascorbic Acid (Vit.C) (3.42E µmol/L)

Endogenous substances – Pathological values of hemoglobin (up to 500g/L), bilirubin (up to 67 mg/dL), triglycerides (up to 1000mg/dL) and total protein (up to 90g/L)

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XII. POSSIBLE WARNING MESSAGES FOUND IN BASIS REPORT

DISPLAY	CAUSE	SOLUTION
Ax (Indeterminate genotype call on B)	Indicates insufficient allelic discrimination present for the affected marker as a result of which the genotype is partially resolved, where a definitive call cannot be made on an allele (represented by "x" call). The sample is confirmed to have the "A" form of the allele.	If the sample result is invalid, repeat the analysis paying particular attention to reagent handling and pipetting technique. If the warning message continues contact technical support or distributor for assistance.
xB (Indeterminate genotype call on A)	Indicates insufficient allelic discrimination present for the affected marker as a result of which the genotype is partially resolved, where a definitive call cannot be made on an allele (represented by "x" call). The sample is confirmed to have the "B" form of the allele.	
CV (High Coefficient of Variance)	Indicates that for the affected sample the variation in measured intensity signal from the individual beads is unacceptably high.	**No results are reported.** Repeat the testing paying particular attention to reagent handling and pipetting technique. If the warning message continues contact technical support or distributor for assistance.
HB (High Background)	Indicates that for the affected sample, the background intensity is unacceptably high.	
IC (Indeterminate Call)	Indicates that for the affected antigen(s) there is insufficient discrimination among the pair(s) of probes used to determine the phenotype or there is higher than expected error associated with the discrimination ratio for the pair(s) of probes used to determine the phenotype.	
LS (Low Signal)	Indicates that the signal intensity for the affected marker is unacceptably low.	
NTD (No type determined)	Indicates that no typing results are provided for the sample due to a failed sample or run criteria.	
PV (Possible Variant)	Indicates that for the affected antigen(s) a genotype pattern was detected for which there is no established phenotype. Hence the genotype results could not be converted to a predicted phenotype result. Possible new allele combination.	Further testing recommended to provide more information about the sample

For troubleshooting regarding the AIS Instrument, refer to the AIS User Manual (190-20185).

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