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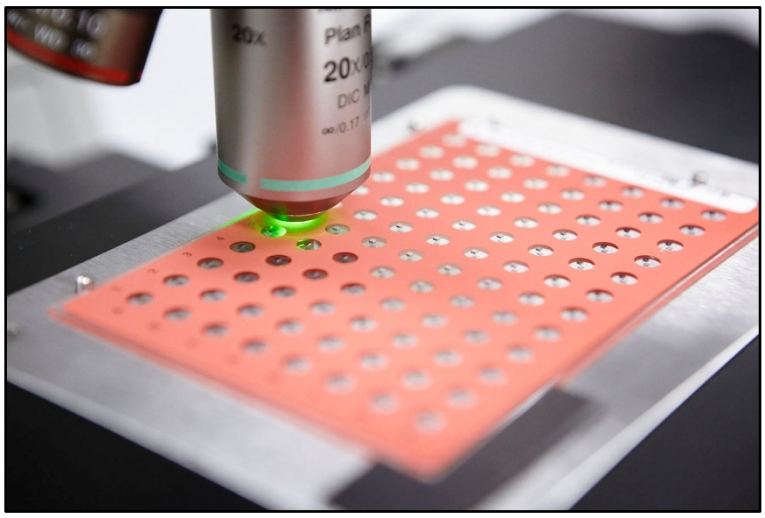
Immucor PreciseType™ | HEA

MOLECULAR BEADCHIP TEST



IVD

Contents

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 DNAs are incubated on the BeadChip array, allowing the annealing with the corresponding probes. The subsequent elongation reaction extends and incorporates fluorescently-labeled dNTP molecules only on those probes where the 3’ end exactly matches the annealed DNA. Elongation products of alleles A and B are simultaneously detected by imaging the entire array.

In this method, each probe is covalently attached 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.

The BioArray Array Imaging System™ (AIS™ 400C) is used to capture the fluorescent signal from individual beads in an image of the entire array, determine the identity of the bead by the color of the bead, its position in the array, and report the average signal intensity, coefficient of variance standard deviation of the intensities, and number of beads measured for each type of probe. The HEA Analysis software in BioArray Solutions Information System (BASIS®) imports the raw intensity output, assesses the validity of the internal controls, and generates assay results.

Mutations known to result in silencing (nonexpression) 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, and/or carbohydrate structures that are attached to lipid or protein. Exposure to erythrocytes containing surface markers not naturally possessed 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, chronic blood transfusion therapy, increased opportunity for alloantibody production occurs. These conditions include, but are not limited to, sickle cell disease, autoimmune hemolytic anemia, and aplastic anemia. In such cases, the identification of the presence of blood group antigens, by analysis of DNA rather than serological phenotyping, is becoming increasingly useful [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 summarizes 33 blood group systems and offers a commonly used standard of terminology. Ongoing efforts offer insight into the incidence of these blood groups throughout a pan ethnic population and their significance, with a wide range of occurrence [6].

Twenty-four polymorphisms associated with thirty-five Human Erythrocyte Antigens are included in the PreciseType HEA Molecular BeadChip Test and are listed in the following table (Table 1) [7]. One polymorphism 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 900µL
800-00191	Clean-up Reagent	1 x 330µL
800-00195	Lambda Exonuclease	1 x 330µL
800-00193	eMAP® Elongation Mix	2 x 600µL
800-10242	HotStarTaq DNA Polymerase®	1 x 155µL
800-00287	Negative Control**	1 x 1000µL
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	Cat #
AIS 400C Array Imaging System	(BioArray) 790-20006
Defrost-free freezer (capable of maintaining temperatures of -20°C or colder)	-
Hybridization (Incubation) Oven (Boekel Inslide-Out)	(Boekel) 241000
Refrigerator (capable of maintaining 2-8°C)	-
Thermal cycler (Applied Biosystems Veriti Dx)	(Applied Biosystems) 4452300

C. Equipment Recommended

Description	Cat #
Cryo blocks (recommend Denville or equivalent)	(Denville) R6670
Microplate centrifuge (recommend Eppendorf Model 5430)	(Fisher) 05-400-017
PCR tube racks (recommend Fisher or equivalent)	(Fisher) 05-541-50
PCR Workstation hood with UV light (recommend CBS Scientific or equivalent)	(CBS Scientific) P-030-02
Precision Pipettes - 8 channel - capable of delivering 0.5-10µL (recommend Fisher or equivalent) • Accuracy +/- 24 to 2.4% • Precision < 16 to 1.6%	(Fisher) 21-377-825
Precision Pipettes - 8 channel - capable of delivering 5-50 µL (recommend Fisher or equivalent) • Accuracy +/- 3.0 to 1.0% • Precision < 2.0 to 0.7%	(Fisher) 21-377-827
Precision Pipettes - Single Channel - capable of delivering 0.5-10µL (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 - 100µL (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 100-1000µL (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 Mini Mouse or equivalent)	(Denville) C0801
Vortex mixer with tube and flat adaptors (recommend Denville or equivalent)	(Denville) S7030

D. Supplies Required

Description	Cat #
1.5mL centrifuge tubes (recommend Fisher or equivalent)	(Fisher) 05-402-24B
2.0mL centrifuge tubes (recommend Fisher or equivalent	(Fisher) 05-402-24C
8-Tube strip 0.2mL thin-wall thermal cycler tube caps (recommend Applied Biosystems MicroAmp® 8-Cap Strip or equivalent)	(Applied Biosystems) N80105035
8-Tube strip 0.2mL thin-wall thermal cycler tubes (recommend Applied Biosystems MicroAmp® 8-Tube Strip, 0.2 mL or equivalent)	(Applied Biosystems) N80105080
96 Well, PP, Clear 0.3mL non skirted PCR Plate – (recommend FisherBrand or equivalent)	(Fisher) 14230232
Water for BeadChip wash – recommend: • Applied Biosystems (via Fisher) • Invitrogen	(Invitrogen) 10977023
Canned air (oil free) (recommend Fisher Scientific or equivalent)	(Fisher) 23-022523
Decontaminant (recommend DNA Away, Fisher Scientific 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 0.1µL to 1000 µL (recommend 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
Paper towels (recommend Uline brand or equivalent)	(Uline) 5-7127
PreciseType™ HEA BeadCheck® Positive Control Kit	(BioArray) 800-20236

III. DEFINITION OF SYMBOLS

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

Symbol	Definition
	Batch number
	CD, containing data files and instructions for use.
	Contents
	In Vitro Diagnostic Use
	Manufacturer
	Number of Tests
	Read instructions before use
	Reference number
	Store between temperatures
	Use until

IV. WARNINGS AND PRECAUTIONS

1. 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 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://adextranet.immucor.com/pages/default.aspx

2. Never pipette by mouth. Avoid contact of reagents and specimens with skin and mucous membranes.
3. Dispose of used materials in accordance with the Institution's and or local regulations for disposal of potential bio-hazardous 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.
4. 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. Thus care should be taken to prevent excessive splashing and generation of aerosols. Standard PCR laboratory measures that include 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. should also be followed during the use of the kit. Use of 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, step 14).
5. It is required that operators participate in the PreciseType HEA Molecular BeadChip Test Training Program prior to performing this assay in order to assure consistent and accurate test results.
6. Laboratories should validate their own cleaning procedures.
7. Contamination of reagents or specimens may cause erroneous results; therefore, care should be taken to avoid contaminating this product during use. Do not use contaminated reagents.

8. Microbial contamination of reagents or specimens may lead to incorrect results.
9. Use the kit liquids and HEA BeadChip Carriers as supplied. Dilution or alteration may generate erroneous results.
10. Do not mix reagents or HEA BeadChip Carriers between different lots.
11. Do not use leaking or unlabeled vials.
12. Previously frozen samples or reagents should be thoroughly mixed and then centrifuged after thawing prior to testing. Avoid generating foam and bubbles in the samples.
13. Keep all enzymes and master mixes on ice or cryo block (2 - 8°C) during use.
14. Ensure proper sample tube sealing prior to amplification to prevent evaporation.
15. 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. Alternate thermal cyclers and profiles must be validated by the user.
16. Samples must remain in the BeadChip reaction well during testing.
17. Incubation times or temperatures other than those specified may give erroneous results.
18. On the day of use, prior to using the AIS 400C the Exposure Test Carrier (ETC) procedure must be performed to verify performance of the AIS. If the Exposure Test fails, please contact customer service for appropriate instructions. (See AIS User Manual 190-20185)
19. 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.
20. 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 primer 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. Contact BioArray Customer Service. If the vacuum sealed HEA BeadChip Carrier pouch has been opened or damaged during transit please contact BioArray Customer Service.

Store all test reagents, including the primer mix and all enzymes, at –20°C to –80°C in a defrost-free freezer. Utilize bench top cryo racks when possible. When stored under these conditions and handled correctly, unopened reagent can be used until the expiration date. Once opened the contents of a properly stored reagent kit maybe used for 6 months or until the labeled expiration date whichever occurs earlier. It is recommended that at the time of opening, users determine which date would occur earlier and if the 6 month use date is earlier than the labeled expiration date, record this earlier date to ensure reagents are not used beyond their expiration date.

Store the HEA BeadChip Carriers at 2–8°C until use. Unused carriers should be returned immediately to storage at 2–8°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: It is required that whole blood samples be drawn into EDTA anticoagulant tubes (BD Product Numbers 366643, 368661, 367654). Not tested with cord blood or cadaveric blood. (see also section on Interfering Substances).

It is recommended that DNA samples 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: It is recommended that genomic DNA 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. Programming the Veriti Thermal cycler

Ensure heated lid option is selected.

HEA PCR Program:

94°C	15 min	
94°C	30 sec, 60% ramp	30 cycles
60°C	30 sec, 50% ramp	30 cycles
68°C	50 sec, 35% ramp	30 cycles
68°C	8 min	
4°C	up to 72 hours	

HEA Post Clean Up Program:

37°C	25 min
80°C	15 min
4°C	up to 3 hours

HEA Post Lambda Program:

37°C	25 min
80°C	15 min
4°C	up to 3 hours

B. Procedural Notes

1. Accurate pipetting of samples and reagents is required for accurate results.
2. Combine working reagents just prior to use.
3. Prior to using the hood, turn on the UV light for a minimum of 15-20 minutes.
4. Ensure that the Veriti Thermal Cycler is pre-programmed for each of the PCR Amplification and Post PCR Processing steps, Amplicon Treatment and Single-Stranded Target Generation. Before each step, ensure the proper pre-programmed profile is selected.
5. It is extremely important to prevent cross-contamination between BeadChip wells. Exercise care when pipetting, rinsing, and removing fluids.
6. Contaminating DNA was found to impact 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.
7. Keep all reagents on ice or in cryo block until use.

8. Multi-channel or single-channel pipettes may be used, depending upon laboratory preference. All pipettes used must be calibrated. Reagent fill volume is sufficient for pipetting quantities suggested within this procedure.
9. Remove HEA BeadChip Carriers from storage and bring to room temperature before using.
10. Remove the required quantities of reagents, samples, and controls from storage and, if frozen, allow them to thaw prior to use. Immediately return unused portions to proper storage.
11. Take care to adequately mix samples and reagents. Avoid foaming.
12. The BioArray Solutions AIS 400C 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 25mL of 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 25mL of water.
13. Wipe down the processing area surfaces with 10% Bleach or DNA Away, including:
    a. Bench-tops and inside hood surfaces
    b. Supportive equipment
    c. All working single and multi-channel pipettes
    d. Inside mini mouse centrifuge lid, tube racks and covers
    e. Thermal cycler and plate centrifuge surfaces
        - Clean inside lid and thermal cycler plate wells using DNA Away (or equivalent) and rinsing with deionizied water.
14. It is recommended that three (3) separate laboratory areas be assigned as a precaution against carryover contamination, including: (1) pre-PCR /set-up activities, (2) DNA Addition, and (3) post-PCR procedures.
    a. 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.
    b. Steps in Section D, DNA Addition Step, should be performed in the DNA Addition area within a dedicated hood or dead air box, using aerosol-resistant (filtered) pipette tips.
    c. The remainder of the procedure after Section E, PCR Amplification, should be performed in the post-PCR area.

C. PCR Master Mix Preparation

I. Precautionary measures

1. The creation of a working Master Mix and the addition of DNA samples is recommended to be performed within approximately 30 minutes, including the thawing steps.
2. Once made, the Working PCR Master Mix should be used immediately but may be kept in a cryoblock (stored at 4°C) or on ice for up to 15 minutes.

II. Assay procedure

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

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 is required for use as Positive Controls for each run.

2. For each Assay Run, prepare a working PCR Master Mix in a hood or clean room using the volumes listed in Table 2.

Table 2: PCR Master Mix Preparation - Reagent Volumes

Number of Samples	1	8	16	24	32	40	48	56	64	72	80	88	96

HEA 1.2 PCR Mix (µL)	16	144	296	448	592	736	880	1008	1168	1296	1456	1584	1744
HotStar*Taq* 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 10-20% more working reagent than is needed for performing the assay.

3. Create an appropriately sized and labeled nuclease-free tube for working PCR Master Mix.
4. Remove HEA 1.2 PCR Mix and HotStarTaq DNA Polymerase from the -20°C freezer.
5. Place the HotStarTaq DNA Polymerase in a cryoblock (stored at 4°C) or on ice once it is removed from the freezer.
6. Thaw the HEA 1.2 PCR Mix at room temperature (generally less than 10-15 minutes). Once thawed, keep the HEA 1.2 PCR mix in a cryoblock (stored at 4°C) or on ice.
7. Mix both the HEA 1.2 PCR Mix and HotStarTaq DNA Polymerase gently by vortexing briefly for 3-5 seconds.
8. Centrifuge both the HEA 1.2 PCR Mix and HotStarTaq DNA Polymerase to bring solutions to the bottom of the tubes (approximately 1000 RPM for 5 seconds).
9. Using a pipette with an aerosol resistant (filtered) pipette tip, aliquot the appropriate volume of HEA 1.2 PCR Mix and HotStarTaq DNA Polymerase into the nuclease free tube.
10. Immediately return unused HEA 1.2 PCR Mix and HotStarTaq DNA Polymerase to storage in the −20°C freezer.
11. Mix Working PCR Master Mix by vortexing briefly for 3-5 seconds.
12. Centrifuge the Working PCR Master Mix to bring solution to the bottom of the tube (approximately 1000 RPM for 5 seconds).

Batch Processing: If processing small sample batches (e.g., 16 or fewer samples and controls) the user may transfer the Working PCR Master Mix directly to the labeled thin-walled PCR tubes described in step 14.

13. Aliquot the appropriate volume of working PCR Master Mix (refer to Table 3) into each tube of an 8-tube strip using a pipette with an aerosol resistant (filtered) pipette tip. The 8 tube strip should be stored in a cryoblock (stored at 4°C) or on ice while performing these steps.

Table 3: Working PCR Master Mix - Transfer Volumes

Up to Sample #	16	24	32	40	48	56	64	72	80	88	96
Volume (µL) per tube in strip	37.0	57.0	76.0	94.0	112.0	128.0	148.0	164.0	188.0	200.0	224.0

14. Using an 8 channel pipette (or if not large batch processing, using a one channel pipette) with aerosol resistant (filtered) pipette tips, pipette **17.0µL** of Working PCR Master Mix from the 8 tube strip into the labeled thin-walled PCR tubes. *Ensure that no bubbles in bottom of 8 tube strip interfere with reagent pipetting volume*. Discard unused Working PCR Master Mix.
15. Seal the thin-walled PCR tubes with the appropriate caps or seal, and transport to DNA addition area, outside of the pre-PCR area workstation hood or clean room.

D. DNA Sample Addition

I. Precautionary measures

1. Adding DNA and controls to working PCR Master Mix should be performed as soon as possible, but within 15-20 min.

2. Keep sample and working PCR Master Mix in a cryoblock (stored at 4°C) or on ice during these steps.
3. Turn on the Veriti approximately 10 minutes prior to beginning the PCR cycle.

II. Assay procedure

1. Carefully remove the caps or seal from the thin-walled PCR tubes. Using a pipette with a separate aerosol resistant (filtered) pipette tip for each sample, pipette **8.0µL** of prepared samples into the appropriate thin-walled PCR tubes and mix by pipetting up and down 3 times. Working PCR Master Mix plus sample should be kept in a cryoblock (stored at 4°C) or on ice during the sample transfer process.
2. For the Negative and Positive Controls, use a pipette with an aerosol resistant (filtered) pipette tip, pipette **8.0µL** of supplied Negative and Positive Controls into the appropriate thin-walled PCR tubes and mix by pipetting up and down 3 times. Working PCR Master Mix plus Negative and Positive Controls should be stored in a cryoblock (stored at 4°C) or on ice.
3. Carefully seal the thin-walled PCR tubes with the appropriate caps or seal, mix gently by vortexing briefly for 3-5 seconds.
4. Centrifuge to bring solution to the bottom of the tubes (approximately 1000 RPM for 5 seconds).

E. PCR Amplification

I. Precautionary measures

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

II. Assay procedure

1. Place the sealed thin-walled PCR tubes in the thermal cycler.
2. Verify and select HEA PCR Amplification Program. Start program.
3. After PCR amplification is complete, remove the thin-walled post-PCR product tubes from the thermal cycler and centrifuge to bring solution to the bottom of the tubes (approximately 1000 RPM for 5 seconds).

F. Post-PCR Processing: Amplicon Clean-up

I. Precautionary measures

1. This step should be performed in the post-PCR Area (see also Section VII B, Step 14).
2. The steps in Section F should be performed continuously without interruption.
3. Adding the Clean-up Reagent to the post-PCR Product should be performed within approximately 30 minutes of removing PCR products from the thermal cycler after PCR Amplification, or if frozen, within 30 minutes of thawing.
4. Keep post-PCR Product and Clean-up reagent in a cryoblock (stored at 4°C) or on ice during these steps.
5. The new thin-walled PCR tubes into which post-PCR product and Clean-up Reagent are transferred should be kept in a cryoblock (stored at 4°C) or on ice.
6. The centrifuged cleaned-up samples and controls may be used immediately, or stored at -20°C (or colder) for up to 72 hours.

II. Assay procedure

1. Appropriately label a new set of thin-walled PCR tubes for all samples and controls.
2. If using frozen post-PCR product, remove the thin-walled PCR tubes from the -20°C freezer and thaw at room temperature (less than 10-15 minutes).
3. Mix the thawed post-PCR product gently by vortexing briefly for 3-5 seconds.
4. Centrifuge to bring solution to the bottom of the tubes (approximately 1000 RPM for 5 seconds).
5. The post-PCR Product should be kept in a cryoblock (stored at 4°C) or on ice.
6. Remove Clean-up Reagent from the -20°C freezer storage and place in a cryoblock (stored at 4°C) or on ice.
7. Transfer post-PCR product to new thin-walled tubes. Carefully remove the caps or seal from the thin-walled PCR tubes, and using a pipette (or 8 channel pipette) with a separate aerosol resistant (filtered) pipette tip for each sample or control, pipette **6.5µL** of each sample and control into the bottom of the new thin-walled PCR tubes. The new thin-walled PCR tubes should be placed in a cryoblock (stored at 4°C) or on ice. Seal and return the original remaining post-PCR product tubes to the freezer.

Batch Processing: If processing small sample batches (e.g. 16 or fewer samples and controls) the user may transfer the Clean-up Reagent directly to the new thin-walled PCR tubes as described in step 9

8. Aliquot the appropriate volume of Clean-up Reagent (refer to Table 4) into each tube of an 8-tube strip using a pipette with an aerosol resistant (filtered) pipette tip. Immediately return unused Clean-up Reagent to storage in the -20°C freezer. Place the 8-tube strip in a cryoblock (stored at 4°C) or on ice.

Table 4: Clean-up Reagent Volumes

Up to Sample #	16	24	32	40	48	56	64	72	80	88	96
Clean-up Reagent volume (µL) per tube in strip	6.0	9.0	12.0	14.0	16.0	18.0	20.0	22.0	24.0	26.0	28.0

9. Using an 8 channel pipette (or if not large batch processing, using a one channel pipette) with aerosol resistant (filtered) pipette tip, pipette **2.0µL** of Clean-up Reagent from the 8-tube strip into each sample and control in the new thin-walled PCR tubes and mix by pipetting up and down 3 times. *Ensure that no bubbles in bottom of 8 tube strip interfere with reagent pipetting volume.* Use a separate set of aerosol resistant (filtered) pipette tips for each set of 8 samples and controls. Discard the 8-tube strip containing unused Clean-up Reagent.
10. Carefully seal the thin-walled PCR tubes with the appropriate caps or seal, mix gently by vortexing briefly for 3-5 seconds and centrifuge to bring solution to the bottom of the tubes (approximately 1000 RPM for 5 seconds).
11. Place the sealed thin-walled PCR tubes in the thermal cycler.
12. Verify and select HEA Post Clean Up Program. Start program.
13. After Clean Up Program is complete, remove the thin-walled PCR tubes from the thermal cycler and centrifuge to bring solution to the bottom of the tubes (approximately 1000 RPM for 5 seconds).

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

I. Precautionary measures

1. The steps in Section G should be performed continuously and without interruption.
2. 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 4°C) or on ice during its transfer to the post- PCR Clean-up Reagent product.

3. The post-PCR Clean-up Reagent product should be kept in a cryoblock (stored at 4°C) or on ice during these steps once thawed (if previously frozen).
4. Adding the Lambda Exonuclease to the post- PCR Clean-up Reagent product should be performed within approximately 30 minutes of completing the Amplicon clean-up.
5. The new thin-walled PCR tubes into which post-PCR Clean-up Reagent product mix and Lambda Exonuclease are transferred should be kept in a cryoblock (stored at 4°C) or on ice.
6. The centrifuged single-stranded samples and controls are recommended to be used immediately, but may be stored at -20°C (or colder) for up to 72 hours.

II. Assay procedure

1. Remove Lambda Exonuclease from the -20°C freezer and thaw at room temperature.
2. If using frozen post-PCR cleaned-up samples, remove the thin-walled PCR tubes from the -20°C freezer and thaw at room temperature (less than 10-15 minutes).
3. Mix gently by vortexing briefly for 3-5 seconds.
4. Centrifuge to bring solution to the bottom of the tube (approximately 1000 RPM for 5 seconds).

Batch Processing: If processing small sample batches (e.g. 16 or fewer samples and controls) the user may transfer the Lambda Exonuclease directly to the thin-walled PCR tubes as described in step 6.

5. Aliquot the appropriate volume of Lambda Exonuclease (refer to Table 5) into each tube of an 8-tube strip using a pipette with an aerosol resistant (filtered) pipette tip. Place the 8-tube strip in a cryoblock (stored at 4°C) or on ice. Immediately return unused Lambda Exonuclease to storage in the -20°C freezer.

Table 5: Lambda Exonuclease Volumes

Up to Sample #	16	24	32	40	48	56	64	72	80	88	96
Lambda Exonuclease volume(µL) per tube in strip	6.0	9.0	12.0	14.0	16.0	18.0	20.0	22.0	24.0	26.0	28.0

6. Using an 8 channel pipette (or if not large batch processing, using a one channel pipette) with aerosol resistant (filtered) pipette, pipette **2.0µL** of Lambda Exonuclease into each sample or control and mix by pipetting up and down 3 times*. Ensure that no bubbles in bottom of 8 tube strip interfere with reagent pipetting volume*. Use a separate set of aerosol resistant (filtered) pipette tips for each set of 8 samples and controls. Discard the 8-tube strip containing unused Lambda Exonuclease.
7. Carefully seal the thin-walled PCR tubes with the appropriate caps or seal.
8. Mix gently by vortexing briefly for 3-5 seconds.
9. Centrifuge to bring solution to the bottom of the tubes (approximately 1000 RPM for 5 seconds).
10. Place the sealed thin-walled PCR tubes in the thermal cycler.
11. Verify and select the HEA Post Lambda Program. Start program.
12. After HEA Post Lambda program is complete, remove the thin-walled PCR tubes from the thermal cycler and Centrifuge to bring solution to the bottom of the tubes (approximately 1000 RPM for 5 seconds).

H. On-BeadChip Array Elongation

I. Precautionary measures

1. The steps in Section H should be performed continuously and without interruption.

2. The Elongation Reagent should be taken out of the freezer to thaw during the incubation step of the single stranded PCR product step.
3. Once thawed, the Elongation Reagent should be kept in a cryoblock (stored at 4°C) or on ice.
4. Adding the Elongation Reagent to the single stranded product should be performed within approximately 30 minutes of completing the Single-Stranded Target Generation.
5. Do not shake the canned air.

II. Assay procedure

1. Turn on the hybridization oven and set to 53°C at least 30 min prior to operation. Place two paper towels in the tray and saturate them with a total of 25mL 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 approximately 25mL of deionized water.
2. Remove sufficient HEA 8-BeadChip Carriers from refrigerator and allow to warm up at room temperature (generally takes 15-20 minutes). Once thawed, the Elongation Reagent should be kept in a cryoblock (stored at 4°C) or on ice.
3. Label each HEA BeadChip Carrier appropriately for tracking during and after processing.
4. Centrifuge to bring the single stranded product to the bottom of the tubes (approximately 1000 RPM for 5 seconds).
5. Remove eMAP Elongation Mix Reagent from the -20°C freezer and thaw at room temperature (approximately 10-15 minutes).
6. Mix the Elongation Reagent gently by vortexing briefly for 3-5 seconds. Avoid foaming in the eMAP Elongation Mix.
7. Centrifuge to bring the solution to the bottom of the tube (approximately 1000 RPM for 5 seconds).

Batch Processing: If processing small sample batches (e.g., 16 or fewer samples and controls) the user may transfer the eMAP Elongation Mix directly to the thin-walled PCR tubes with samples and control products, as described in step 9.

8. Aliquot the appropriate volume of eMAP Elongation Mix (refer to Table 6) into each tube in an 8-tube strip using a pipette with an aerosol resistant (filtered) pipette tip. Store the 8-tube strip in a cryoblock (stored at 4°C) or on ice for up to 10 minutes. Immediately return unused eMAP Elongation Mix to storage in the -20°C freezer.

Table 6: eMAP Elongation Mix Volumes

Number of Samples and Controls	16	24	32	40	48	56	64	72	80	88	96
eMAP Elongation Mix Volume per Tube (µL)	25.0	36.0	47.0	59.0	70.0	80.0	90.0	100.0	114.0	125.0	135.0

9. Using an 8 channel pipette (or if not large batch processing, using a one channel pipette) with aerosol resistant (filtered) pipette tips, pipette **10.0µL** of eMAP Elongation Mix into each sample and control and mix by pipetting up and down 3 times. *Ensure that no bubbles in bottom of 8 tube strip interfere with reagent pipetting volume*. Use a separate set of aerosol resistant (filtered) pipette tips for each set of 8 samples and controls. Discard the 8-tube strip containing unused eMAP Elongation Mix.
10. Using a pipette with a separate pipette tip for each tube into which the eMAP Elongation Mix was just added, transfer **15.0µL** of each sample and control to the corresponding array on the labeled HEA BeadChip Carrier. Discard the sample and control tubes.
11. Record the HEA BeadChip Carrier corresponding to the sample on the log sheet.
12. Place HEA BeadChip Carrier(s) in the hybridization oven and incubate for 30 min at 53±1°C.

13. Take HEA BeadChip Carrier(s) out of the oven and wash the elongation mixture from array surfaces by holding the slide(s) or plate so that its surface is vertical over a catch basin. Rinse each BeadChip individually with a constant pressure stream of distilled or deionized H_2O. The water stream should be directed perpendicular to the face of the slide from approximately 1 inch away so the center of each BeadChip is rinsed. Rinse each BeadChip for approximately 3 seconds.
14. Remove any remaining water from the HEA BeadChip Carrier(s) using compressed/canned air.
15. Wipe off any excess water from the back of the HEA BeadChip Carrier with a disposable cleaning tissue.
16. The washed and dried HEA BeadChip Carriers are recommended to be processed immediately, but may be stored protected from light for up to 72 hours at room temperature before being read.

I. BeadChip Image Acquisition

I. Precautionary measures

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

II. Assay procedure

1. Open and initialize AISR program on the desktop.
2. Run the ETC (Refer to AIS User Manual 190-20185). Contact BioArray 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 shut-down of AISR and light source after use.

VIII. EXPECTED RESULTS

A. Evaluation - Quality Control

The determination of the run and sample validity is performed by the BASIS software.

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.

Less than thirty two (32) Low Signal (LS) results in the phenotype report for the Negative Control sample indicate possible 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.

For more information on the BASIS software and format of the sample results displayed for valid and invalid runs, including examples of both, see the BASIS AM 4G User Manual (190-20331, Sections 6 and 7).

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). For more information on the BASIS software and format of the sample results displayed, including examples of valid and invalid sample results, see the BASIS AM 4G User Manual (190-20331, Sections 6 and 7).

For a sample, if any antigen phenotype has Indeterminate Call (IC) or Low Signal (LS) result, 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 High Background (HB) or High Coefficient of Variation (CV) status the sample is invalid (for a list of causes for invalid sample see Table 8 below, for a more detailed explanation see section XII on Troubleshooting).

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 utilized to compute the predicted antigen phenotype results.

All calculations are performed by the HEA analysis software in BASIS. 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

1. 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 the Afro-Caribbean Sickle Cell Disease patients, 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.

2. 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, (such as, 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.) and the nucleotide changes associated with these events are not explicitly monitored by the assay. 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 call.

3. 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) call.

4. The HEA test utilizes two point mutations, 733C>G(L245V) in exon5 and 1006G>T(G336C) in exon7 of the RHCE gene to predict the V and VS antigen phenotypes.

   The genotype to phenotype prediction conversion rules employed by the HEA test utilizes 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 unique phenotype. This limitation only affects the V(+)VS(+) call. As per the publication [14], in a small fraction of the cases when giving the V(+)VS(+) call (4.2% for VS and 1.4% for V) the HEA test would report the samples as falsely positive relative to serology. The V(-)VS(-) call is unaffected.

5. 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 of ;

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 called e^s, and VS (RH20)[15].

6. The U antigen (located on the GPB protein), is not polymorphic by itself. The expression of the U antigen is governed by changes which 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 which inform the S-s- phenotype and can call the U(var) and U(neg) phenotype. Occasionally due to non-specific residual intensities on the probes governing the silencing of S/s antigen a U(neg) phenotype call may not be made even if the phenotype call is S-s-.
7. 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

An Accuracy Study was performed to demonstrate that the HEA PreciseType Test could accurately identify the phenotypes listed using pre-selected well-characterized samples. Red blood cell antigen phenotypes were determined using two methods. The red blood cell 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 reach the phenotypic diversity of this study, sample acquisition was compiled from three studies including 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.

Study acceptance criteria for 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). The acceptance criteria for all antigens was met 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; samples in disagreement 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 8: PreciseType Accuracy Study Results

	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

A study entitled “Evaluation of the HEA BeadChip™ Kit in comparison to established methods for Human Erythrocyte Antigen determination” was conducted from 2011 to 2013 at four laboratories across the United States. This study compared the typing results of the PreciseType HEA BeadChip Test with serological and DNA sequencing methodologies. A total of 1777 samples were tested of which 1757 could be used for comparison, with 1684 valid HEA BeadChip test results, bringing the valid rate to 95.85% (1684/1757). Out of the 1684, 1248 paired valid comparative results per phenotype were considered for analysis (SC1 and SC2 have 1247 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 red blood cell 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).

Table 12 shows all antigens tested utilizing serological and sequencing methods. For the 18 antigens tested utilizing 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 utilizing 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 92: PreciseType Test as compared with Serology and Sequencing

	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 the reference method for sequence analysis or the "gold standard". 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 1248)	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 nineteen 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 twenty-one discordant samples, twenty were concordant between PreciseType and sequencing
  - Fifteen were PreciseType positive, serology negative and fourteen 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 nineteen 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 seventeen discordant samples; all PreciseType results were concordant with bi-directional sequencing.
- Samples Discordant for M with serology:
  - There were eleven discordant samples; all PreciseType results were concordant with bi-directional sequencing.
- Samples Discordant for N with serology:
  - There were fifty 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 PreciseType 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 8-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 eleven 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 twenty-four negative phenotypes, there were twelve 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 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 assays were repeated by the same operator 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 HEA PreciseType 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 HEA BeadChip Test.

Microrganisms – 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. TROUBLESHOOTING

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. 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 service 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. 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 service 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 pairs(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 sample

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

Patents

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