**INTENDED USE**

**RED CELL EZ TYPE®** kits are intended for the molecular determination of the alleles of blood group antigen systems using PCR amplification of human genomic DNA. These kits are intended as aids to understanding serologic testing results and not to replace serologic typing.

- **ABO Typing (RAB)**
- **Rh: CDE Typing (RRZ)**
- **Rh: Weak D Typing (RWD)**
- **Rh: D Negative Typing (RDN)**
- **Kell-Duffy-Kidd Typing (RKDK)**
- **MNS Typing (RMN)**
- **Rare ID Typing (RRI)**
- **Rare Screen (RRS)**
- **Kell Typing (RKP)**
- **ABO Sub Typing (RABS)**
- **Jk-Fy Typing (RJF)**

*For In Vitro Diagnostic Use.*

**SUMMARY AND EXPLANATION OF THE TEST**

The established method for determination of blood group antigens is serologic typing. Recently the genetic organization of many genes that code for blood group antigens has been elucidated, and the genetic basis for many blood group antigens determined. This knowledge has made possible DNA-based typing for the polymorphisms that encode these antigens. DNA-based typing cannot entirely replace serologic methods. The genetic bases for at least some of the antigens in any system remain unidentified, and unknown genetic variation may cause a predicted antigenic variation to be absent or altered in fact. However, DNA-based typing can resolve questions that have proven to be difficult for serological methods to answer due to lack of appropriate cell panels or typing sera. Further, DNA-based typing can assess zygosity and illuminate variation in antigen expression, both of which are difficult to resolve with serological methods.

DNA-based typing of polymorphisms for blood group antigens following PCR amplification can be performed by a variety of methods: RFLP assays, SSO probe assays, or allele-specific amplification with gel detection (SSP). Of these, PCR-SSP analysis, the basis for **RED CELL EZ TYPE®**, offers improved assay speed and lower hands-on time. The PCR-SSP method only requires two working steps (amplification and detection) because the amplification and discrimination occur during the PCR process. Amplification of the polymorphic sequences of interest is followed by detection with agarose gel electrophoresis. This inherent assay utility is further enhanced by the inclusion of precast agarose gels in the test system. These cassette-enclosed gels eliminate the need for gel casting, buffer preparation, and issues related to mutagenic DNA stains (ethidium bromide) in gels or electrophoresis buffer.

**PRINCIPLE OF THE PROCEDURE**

**RED CELL EZ TYPE®** kits are based on the *Polymerase Chain Reaction* (PCR), which enables amplification of defined target sequences in the genomic DNA. After successful amplification, the sample contains the target DNA sequence in quantities sufficient for detection. Sequence-Specific Priming (SSP) describes a specific type of PCR in which amplification occurs only if the allele is present; samples lacking the target for an allele-specific primer set do not produce that particular PCR product. To be useful, PCR-SSP analysis requires that a number of amplifications be carried out in parallel. Amplification of internal control primers targeted to the Human Growth Hormone (HGH) gene demonstrates acceptable reaction conditions for each PCR tube. If no allele-specific product is present after PCR, the product of this internal control must be clearly detectable. Negative Control tubes detect exogenous DNA contamination if present. PCR products are separated by agarose gel electrophoresis on pre-cast E-Gels®, during which the PCR products are also stained by ethidium bromide. The separated products are then viewed on an ultraviolet (UV) transilluminator. A gel image may be captured by film or digital photography as a record of the assay. Determination of alleles is performed by comparing the pattern of allele-specific bands from the gel image to the patterns identified on the corresponding Recording Sheet.

**ABO BLOOD GROUP SYSTEM AND RED CELL EZ TYPE®**

- **ABO Typing**
- **ABO Sub Typing**

The ABO blood group antigens are comprised of sugar residues transferred from the carbohydrate chains of glycoproteins or glycolipids to terminal fucose sugars on the erythrocyte membrane by specific glycosyl transferases. The A antigen is produced by a terminal N-acetyl-galactosamine, the B antigen by a terminal galactose. Group O actually indicates lack of either of these residues, leaving a terminal fucose sugar termed the H antigen.

The carbohydrates that form the various ABO antigens are not coded directly by DNA sequences. However, the genetic sequences that code for the carbohydrate-specific glycosyl transferases can be readily identified by allele-specific PCR. These glycosyl transferase genes are located on chromosome 9 (9q34). The **ABO Typing** kit detects the genetic variations that result in the most frequent antigens: A, A₂, B, O₁, and O₂. In addition to
these common alleles the ABO blood group system also includes a number of rare alleles whose phenotypic expression is red blood cells (RBCs) that react weakly with the anti-A and –B reagents. Examples include A2, Aε, B3, Bε, Bm, Bε among many others. These rare alleles are not detected by the ABO Typing kit.

For further detection of weakly expressed A- and B-antigens, ABO Sub Typing kit can be used. This is meant to be a supplementary assay to the ABO Typing kit, and detects 11 different Aweak alleles, Aε, A3, B3, Bε, Bv, Bm, Bel among many others in 8 PCR reactions. For further information on nomenclature see: //www.ncbi.nlm.nih.gov/gv/rbc/xslcgi.fcgi?cmd=bgmut/systems_info&system=abo

ORGANIZATION OF THE RH GENES AND RED CELL EZ TYPE®

- Rh: CDE Typing
- Rh: Weak D Typing
- Rh: D Negative Typing

Two genes located on chromosome 1 in region 1p34 to 1p36, the RHD gene and the RHCE genes, are responsible for expression of the D and CE Rh (Rhesus) antigens. Both genes consist of ten exons and intervening intronic regions. The term Rh d describes the absence of the entire D glycoprotein, due to deletion of entire D genes or to mutations that prevent protein expression (Rh pseudogene, ψ, or Rh d(C)ces). In addition, point mutations also result in the DDEL phenotype, where cell surface expression of the D protein is so low as to be immunologically absent. The CeEe antigens are coded by sequence differences in the RHCE gene. Besides the D-positive and D-negative (Rh d) phenotypes, expression of so-called D variants occurs in rare cases (0.2 to 0.1% in European populations). These may be partial D types (e.g. D/CE hybrids) or weak D types.

CDE
The Rh: CDE Typing kit is designed to distinguish a “normal” D type from the various partial D types by amplification of gene-specific sequences from each of the different exons of the D gene. These partial D antigens, also called hybrids, are produced by exchange of one or more exons of the D gene for the corresponding exons of the CE gene. The most common of these hybrids can be detected with the Rh: CDE Typing kit. In addition, this kit is designed to detect the RHCE alleles that result in the c, c, E, e and C” antigens. Finally, this kit is designed to detect Rh-D pseudogene (psi) and d(C)ces.

Weak D
The weak D phenotypes are the result of point mutations in the RHD gene that lead to single amino acid changes in the expressed protein. These substitutions occur in the intracellular or transmembrane domains of the Rh D protein, and do not alter the immunogenic protein structures (which are extracellular). Instead, these mutations appear to limit D protein expression on the red cell membrane; weak D types all exhibit diminished Rh D protein density on the cell surface compared to D-positive cells. This resulting lower concentration of Rh D epitopes on the erythrocyte membrane, which varies with the particular weak D type, can lead to very great weakening of agglutination in the classic hemagglutination test. Rh: Weak D Typing kit is designed to detect these point mutations. The kit includes specific reactions for the most frequent weak D alleles (types 1, 2, 3, 4.0, 4.1, 4.2, 5, 11, 14, 15, and 17). At least 95% of all observed weak D phenotypes can be classified as weak D alleles type 1 to type 5.

D Negative
The Rh: D Negative Typing kit is designed to detect RH -/- (serological Rh d) positively by siting primers in the 5’ (upstream box) and 3’ (downstream box) flanking regions of the D gene. If the D gene is present (D-positive), the primer sites in the upstream and downstream boxes are too far apart for amplification with this method. The primers fail to amplify, indicating presence of the RHD gene itself. When the D gene is deleted, parts of the upstream and parts of the downstream box produce a so-called hybrid box in which the primer binding sites are now close enough to each other to allow amplification.

RHD gene deletion in the Rhesus box

Thus, amplification of this primer set (tube 1) is a positive indication of the presence of the “hybrid box”, indicating that the RHD gene is absent (RHD -/-, serological d). A second assessment of the presence/absence of RHD is provided by another primer set (tube 2) that targets sequences within the upstream box. Amplification of these primers indicates presence of a complete upstream box, and thus indicates presence of the RHD gene. The primer sets in Tubes 5 and 6 complement this detection of RHD. These primer sets target a polymorphism near the intron 8/exon 9 splice site of the RHD gene. In a true RH -/- (homozygous deleted) individual, reactions 5 and 6 are negative, whereas at least one of the two reactions will be positive with RH D +/- (heterozygous) or D+/+ (homozygous) individuals.

In Caucasians, RHD negativity usually results from the complete loss of the D gene. In people of African descent a non-expressed RHD pseudogene (ψ) or Rh d(C)ces) are the common causes of the d phenotype. Both variants (RHD ψ and Rh d(C)ces) can be identified by positive amplification in tube 4 of the Rh: D Negative Typing kit.

Studies from Southeast Asia confirm the increased incidence of DDEL among RHD-negative individuals in this population group. DDEL antigens are very weakly expressed Rh D, which are difficult to detect serologically. The genetic basis for a number of these DDEL has now been elucidated, and these are detected readily using DNA typing methods. The Rh: D Negative Typing kit is designed to detect the DDEL alleles RHD(M295I), RHD(IVS3+1G>A), and RHD(K409K).
KELL, DUFFY AND KIDD ANTIGENS AND RED CELL EZ TYPE®

- **Kell-Duffy-Kidd Typing**
- **Kell Typing**
- **Jk-Fy Typing**

**Kell**
The Kell system comprises a total of 28 alleles in 5 different gene loci on the KEL gene (chromosome 7q33). The **Kell-Duffy-Kidd Typing** kit (KDK) detects the KEL1 and KEL2 alleles (nt 698 T>C), which code the Kell and Cellano antigens (K/k; Met193Thr) respectively. The **Kell Typing** kit offers a more thorough typing of KEL, detecting the KEL1/KEL2 alleles (K/k); KEL3/KEL4 (nt 961 T>C), which codes the Kpa and Kpb antigens (Trp281Arg); and KEL6/KEL7 (nt 1910 T>C), coding the Jsa/Jsb antigens (Pro597Leu). In addition, the **Kell Typing** kit also detects four of the more frequent Kell null alleles: KEL*02N.06 (IVS3+1G>A) and KEL*02N.12 (IVS8+1 G>A), both splice-site mutations; and KEL*02N.02 (nt382C>T, Arg128X) and KEL*02N.04 (nt1042C>T, Gin348X), both of which code premature stop codons. The KEL3/KEL4 alleles (Kp/Kp) can also be detected with the **Rare Screen** and **Rare ID Typing** kits.

**Fy (Duffy)**
The Duffy (Fy) loci are located on the long arm of chromosome 1, and are controlled by a group of alleles that code for antigens on the Duffy glycoprotein. The **Kell-Duffy-Kidd Typing** kit detects the common FY*A allele (Fya) and the FY*B allele (Fyb), which are expressed as a result of a G to A substitution at position 125 in the Duffy gene. The **Fy (Duffy)** kit detects the FY*A and FY*B alleles or combinations thereof.

**M/N (GYPA) AND S/s (GYPB) ANTIGENS AND RED CELL EZ TYPE®**

- **MNS Typing**
  The M/N and S/s system is a gene complex consisting of two closely adjacent gene loci, GYPA and GYPB, on chromosome 4. Antigens M and N, discovered by Landsteiner and Levine in 1927, are the result of alleles (MNS1/MNS2) on the GYPA gene, which codes for the protein Glycophorin A. Antigens S and s result from reciprocal alleles of a single nucleotide polymorphism on GYPB (MNS3/MNS4, nt239T>C). These antigens were first described in 1947 by Walsh and Montgomery. In central Europe the frequency of the MNS haplotypes is as follows: MS (24.5%), Mn (29.1%), NS (7.9%), Ns (38.5%).

  In addition, a number of extremely rare private antigens are assigned to the MNS system. M1 (MNS11) has a frequency of 0.16% in Switzerland and Sicily, but has not yet been identified in Anglo-Saxon population groups. The MNS9 allele codes the Vw (Verweyst) antigen, which belongs to the Vw antigen system.

**SCREEN / ID FOR RARE ANTIGENS AND RED CELL EZ TYPE®**

- **Rare ID Typing**
- **Rare Screen**

The **Rare Screen** and **Rare ID Typing** kits enable screening and subsequent identification of alleles of the Kell (Kp), Lutheran (Lu), Diego (Di), Wright (Wr), Cartwright (Yt), Colton (Co), Knops (Kn) and Dombrock (Do) blood group systems. These blood group systems are biallelic with one frequent and one rare allele. The Dombrock system is an exception in that both alleles occur with nearly equal frequency.

In the **Rare Screen** kit, one PCR reaction per sample is performed; a positive result indicates the presence of the rare allele for at least one of five antigens: Kp, Lu, Yt, Co, Kn. The rare allele can then be identified using the **Rare ID Typing** kit. This kit detects homozygosity or heterozygosity for all 8 systems: KEL3/KEL4 (Kp/Kp); Lutheran (Lu/Lu); Diego (Di/Di); Wright (Wr/Wr); Colton (Co/Co); Cartwright (Yt/Yt); Knops (Kn/Kn); Dombrock (Do/Do) using 16 different PCR reactions. Table 1 below gives an overview of the blood group systems contained in this assay. The details are derived from “Human Blood Groups” (2002) by Geoff Daniels and refer to the named population groups. The allele frequencies of the rare allele variants are outlined in grey.
### TABLE 1

**Rare Antigens Systems**

<table>
<thead>
<tr>
<th>Name</th>
<th>Short</th>
<th>Chromosome</th>
<th>Sero DNA</th>
<th>Allele Freq</th>
<th>Population Group</th>
<th>Sero</th>
<th>DNA Allele Freq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kell</td>
<td>Kp</td>
<td>7q33</td>
<td>Kp&lt;sup&gt;a&lt;/sup&gt;</td>
<td>KEL3</td>
<td>Europe</td>
<td>Kp&lt;sup&gt;b&lt;/sup&gt;</td>
<td>KEL4 0.9886</td>
</tr>
<tr>
<td>Lutheran</td>
<td>Lu</td>
<td>19q13.2</td>
<td>Lu&lt;sup&gt;a&lt;/sup&gt;</td>
<td>LU1</td>
<td>Europe</td>
<td>Lu&lt;sup&gt;b&lt;/sup&gt;</td>
<td>LU2 0.9610</td>
</tr>
<tr>
<td>Diego</td>
<td>Di</td>
<td>17q12-q21</td>
<td>Di&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DI1</td>
<td>Japan</td>
<td>Di&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DI2 0.9469</td>
</tr>
<tr>
<td>Wright</td>
<td>Wr</td>
<td>17q12-q21</td>
<td>Wr&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DI3</td>
<td>Europe</td>
<td>Wr&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DI4 0.9997</td>
</tr>
<tr>
<td>Colton</td>
<td>Co</td>
<td>7p14</td>
<td>Co&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CO1</td>
<td>Europe</td>
<td>Co&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CO2 0.0439</td>
</tr>
<tr>
<td>Cartwright</td>
<td>Yt</td>
<td>7q22.1</td>
<td>Yt&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Y1</td>
<td>Europe</td>
<td>Yt&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Y2 0.0413</td>
</tr>
<tr>
<td>Knops</td>
<td>Kn</td>
<td>1q32.1-3</td>
<td>Kn&lt;sup&gt;a&lt;/sup&gt;</td>
<td>KN1</td>
<td>Europe</td>
<td>Kn&lt;sup&gt;b&lt;/sup&gt;</td>
<td>KN2 0.0100</td>
</tr>
<tr>
<td>Dombrock</td>
<td>Do</td>
<td>12p13.2-12.1</td>
<td>Do&lt;sup&gt;a&lt;/sup&gt;</td>
<td>DO1</td>
<td>Europe</td>
<td>Do&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DO2 0.5800</td>
</tr>
</tbody>
</table>

### REAGENTS

Maximum number of tests per kit:

- **RAB, RRZ, RWD, RDN, RKDK:** 12 typings per kit
- **RMN, RRI, RKP, RABS, RJF**
- **RRS:** 96 tests per kit

- **PP** Primer Plate: 96-tube PCR trays with allele-specific primers and internal control (HGH) primers, dried on the inner surface of tubes. Sealed with foil covers. Ready for use.
- **PT** Primer Tubes (Rare Screen): 1 x 8 PCR tube strips (12) with allele-specific (Kp<sup>a</sup>, Lu<sup>a</sup>, Yt<sup>a</sup>, Co<sup>a</sup>, Kn<sup>a</sup>, Kn<sup>b</sup>, Kn<sup>a</sup>) primers and internal control (HGH) primers dried on the inner surface of tubes. Each tube is sealed by an individual cap. Ready for use.
- **PR** PCR Reagent: A buffer containing magnesium chloride, free deoxynucleotide triphosphates (dNTPs), glycerol, cresol red, and other proprietary components. Dilute before use.
- **RNCT** Negative Control Tubes: PCR tubes containing dried primers, sealed with tube caps. Ready for use.
- **E-Gel®** Precast agarose gels (2% 16 well, 2% 48 well, or 2% 96 well) used for separation of the PCR products. Ready for use. Supplied separately.

### PRECAUTIONS

- Do not use reagents or gels beyond their expiration date.
- Do not use reagents that are turbid or contaminated.
- Do not use gels that appear cracked, dried out, or show evidence of having been frozen.
- PCR tubes and reagents contained in the kit should not be used in conjunction with any other test system.
- Due to variations in the performance of different thermal cyclers, it may be necessary for the laboratory to establish adjusted parameters for the thermal cycling program in order to achieve valid results. It may also be necessary to determine the appropriate spacer pad to insure complete closure of the reaction tubes during PCR.
- The ABO Sub Typing kit should be electropheresed only on the E-Gel 16.
- Do not isolate DNA samples from heparinized blood. Heparin may interfere with the PCR amplification.

### PCR Laboratory Practice

- PCR is an extremely powerful method of amplifying even the smallest amounts of DNA. Extraordinary precautions must be adhered to in order to avoid contamination with spurious genomic DNA or PCR product. Of particular importance is the avoidance of contamination by PCR product from previous amplifications. The following precautions are of special importance:
  - Spatial separation of the pre-PCR area (DNA isolation and storage, PCR set-up) from the post-PCR area (thermal cycler, gel loading and electrophoresis, evaluation). Instruments and consumables from post-PCR areas must not be taken into the pre-PCR area.
  - Use of pipettes with aerosol protection (sterile barrier tips) in both the pre and post-PCR areas.
  - The Negative Control assay is an environmental control designed to detect DNA contamination and should always be run in parallel with sample amplifications.

The results of these assays should always be interpreted in conjunction with available serological results.
CAUTION

- When finished with the assay, dispose of waste materials as biohazardous waste and decontaminate non-disposable materials with 10% household bleach or other DNA inactivating agent.
- The UV transilluminators used to visualize the PCR product bands emit powerful ultraviolet light which can damage eyes and skin. Always wear protective clothing and UV-blocking glasses or face shield when operating the UV transilluminator.
- E-Gel®s contain a small amount of ethidium bromide as a DNA stain. Ethidium bromide is a known human mutagen. Do not open the gel cassettes. Dispose of gels according to local hazardous waste regulations.

SPECIMEN COLLECTION

- Isolate DNA using a published method or a commercial kit that can deliver DNA samples with a 260 nm/280 nm ratio of 1.60-1.90 and concentration in the range of 25-50 ng/µL.
- Resuspend the DNA in sterile water or 10 mM Tris, pH 7.0-8.0. Samples should not be rehydrated in solutions containing greater than 1 mM EDTA or other chelating agents. These may interfere with PCR.
- DNA samples may be assayed immediately after isolation or stored in a non-defrosting freezer at or below -20ºC.
- Occasional DNA isolates (for example, isolates processed by automated DNA isolation devices) have concentrations < 25 ng/µl. These samples can be assayed by adjusting the volume of sample DNA added to the master mix.
- Presence of excess contaminating protein, RNA, heparin, EDTA, or other chelating agents may interfere with PCR amplification of the purified DNA.

PROCEDURE

Materials Provided

For PCR (≤ -20ºC storage)

1. Primer Plates / Tubes

<table>
<thead>
<tr>
<th>Kit</th>
<th>Qty</th>
<th>Kit</th>
<th>Qty</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABO Typing</td>
<td>1 Plate</td>
<td>Kell-Duffy-Kidd Typing</td>
<td>1 Plate</td>
</tr>
<tr>
<td>ABO Sub Typing</td>
<td>1 Plate</td>
<td>Kell Typing</td>
<td>1 Plate</td>
</tr>
<tr>
<td>Rh: CDE Typing</td>
<td>2 Plates</td>
<td>MNS Typing</td>
<td>1 Plate</td>
</tr>
<tr>
<td>Rh: Weak D Typing</td>
<td>1 Plate</td>
<td>Rare ID Typing</td>
<td>2 Plates</td>
</tr>
<tr>
<td>Rh: D Negative Typing</td>
<td>1 Plate</td>
<td>Rare Screen</td>
<td>96 PCR Tubes</td>
</tr>
<tr>
<td>Jk-Fy Typing</td>
<td>1 Plate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The tubes of each plate contain allele-specific and internal control primers.

2. PCR Reagent
   a. 1 x 500 µL (RAB, RABS, RWD, RDN, RJF, RKDK, RKP, RMN, RRS)
   b. 2 x 500 µL (RRZ, RRI)

3. Twelve (12) Negative Control PCR tubes.

4. Twelve (12) OR Twenty-four (24) cap strips.

Gels (15 to 30ºC storage)

<table>
<thead>
<tr>
<th>GEL16</th>
<th>2% E-Gel® 16 (6 x 2% double-comb E-Gel® 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEL48</td>
<td>2% E-Gel® 48 (8 x 2% E-Gel® 48)</td>
</tr>
<tr>
<td>GEL48-2</td>
<td>2% E-Gel® 48-2 (2 x 2% E-Gel® 48)</td>
</tr>
<tr>
<td>GEL96</td>
<td>2% E-Gel® 96 (3 x 2% E-Gel® 96)</td>
</tr>
</tbody>
</table>

Users who prefer heat sealing of the PCR tubes require the following two additional items, which can be obtained from GTI Diagnostics

9. Thermo sealing foils
10. 1mm silicone spacer pad
Materials Required (but not provided)

A. For PCR

1. Taq Polymerase, either native or recombinant, 5U/µL. Do not use other thermostable polymerases or “hot-start” preparations of Taq polymerase.

The following enzymes have been validated for use with RED CELL EZ TYPE®. Use of other enzymes must be validated by the user.

TABLE 2
Enzyme Suppliers

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied Biosystems</td>
<td>AmpliTaq (Product# N8080172)</td>
</tr>
<tr>
<td>Promega</td>
<td>GoTaq Flexi (M8296)</td>
</tr>
<tr>
<td>Inno-Train</td>
<td>AxTaq</td>
</tr>
</tbody>
</table>

2. Programmable thermal cycler with block sized to accept 96 x 0.2 mL PCR tubes.
3. Adjustable micropipets to deliver 0.5 - 1000 µL and sterile barrier tips
4. Molecular Biology grade water (DNA- and DNase-free)
5. Ice bath or cold blocks to fit 0.6 mL or 1.5 mL tubes
6. PCR (0.2 mL) tube rack
7. Vortex mixer
8. Snap-top conical polypropylene microcentrifuge tubes (0.5 - 2.0 mL), DNA- and DNase free
9. 10% bleach or other DNA-inactivating agent

Optional

10. Electronic pipettor capable of delivering 10 µL
11. Cap roller/setter (if using cap strips to seal PCR tubes)
12. Heat sealing device
13. Sealing roller (if using thermo sealing foil to seal PCR tubes)

B. For Electrophoresis and Analysis

1. E-Gel™ iBase™ or Mother E-Base™ device
2. PCR (0.2 mL) tube rack
3. Adjustable micropipets to deliver 1 - 20 µL, and sterile barrier tips
4. Plastic-backed absorbent paper for lab bench
5. UV transilluminator (GTI cat. #EZT-ILLUM)
6. 10% bleach or other DNA inactivating agent
7. Deionized water
8. Gel documentation system (GTI cat.# EZT-CAMHOOD)

Optional

9. E-Ladder (GTI cat. #ELADDER)
10. 8-channel multichannel pipettor capable of delivering 5-20 µL

Test Procedure

A. In Advance

1. Isolate genomic DNA from all samples to be tested. Each sample should have a DNA concentration in the range of 25-50 ng/µL and a 260 nm/280 nm ratio of 1.60 – 1.90. Samples with concentrations >50 ng/µL should be diluted to the range of 25 – 50 ng/µL. Samples with concentrations <25 ng/µL will require additional sample volume for successful amplification.

2. Program a thermal cycler with the appropriate RED CELL EZ TYPE® PCR program. The kits use a common thermal cycling program except for the Rare ID Typing and Rare Screen kits; these require a unique program. The programs are as follows:
TABLE 3
Thermal Cycling Program
(RAB, RABS, RRZ, RWD, RDN, RJF, RKDK, RKP, RMN)

<table>
<thead>
<tr>
<th>Initial</th>
<th>5 Cycles</th>
<th>10 Cycles</th>
<th>20 Cycles</th>
<th>72°C Hold</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C  2 min.</td>
<td>94°C  20 sec.</td>
<td>94°C  20 sec.</td>
<td>94°C  20 sec.</td>
<td>72°C  5 min.</td>
<td>10°C Hold</td>
</tr>
<tr>
<td>70°C  60 sec.</td>
<td>65°C  60 sec.</td>
<td>61°C  50 sec.</td>
<td>72°C  45 sec.</td>
<td>72°C  45 sec.</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: This cycling program has been tested with thermal cyclers from Applied Biosystems (PE9600, PE9700, Veriti), Biometra (T Professional), Bio-Rad (PTC-100, PTC-200, C1000) and Analytik Jena (Flexcycler). Amplification need not be limited to ~1°C/second (PE9600 mode).

TABLE 4
Thermal Cycling Program (RRI, RRS)

<table>
<thead>
<tr>
<th>Initial</th>
<th>10 Cycles</th>
<th>20 Cycles</th>
<th>72°C Hold</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C  2 min.</td>
<td>94°C  20 sec.</td>
<td>94°C  20 sec.</td>
<td>72°C  5 min.</td>
<td>10°C Hold</td>
</tr>
<tr>
<td>65°C  60 sec.</td>
<td>61°C  60 sec.</td>
<td>72°C  30 sec.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: This cycling program has been tested with thermal cyclers from Applied Biosystems (PE9700, PE9600) and Bio-Rad (PTC-100, PTC-200). For the PE9700 the ramping rate should be limited to ~1°C/second (9600 mode); however, the PTC-200 need not be rate controlled for ramping. Amplification on other thermal cyclers may require optimization of the cycling program.

B. PCR

3. Turn on the thermal cycler in advance of setup to ensure that the heated lid has time to reach its operating temperature. Check the cycling program to be sure it has not been changed.

4. Prepare work surfaces and pipettors before use by wiping with 10% household bleach or other DNA-inactivating agent.

5. If heat sealing will be used, turn on the heat seal device and allow it to come to operating temperature (5-7 min).

6. Determine the numbers of samples to be tested. Remove the required number of primer tube blocks, Taq polymerase, Negative Control Tubes, and sufficient PCR Reagent from the freezer and thaw (PCR Reagent) at room temperature. Keep the Taq polymerase and thawed PCR Reagent on ice or in a cold block. Place the Primer Plate blocks and Negative Control Tube(s) in a tube rack.

NOTE: If more than one DNA sample is being tested with a particular kit, only one Negative Control reaction is required. Similarly, if more than one kit is being run on a DNA sample only one Negative Control reaction is required.

7. Fill out the appropriate PCR Set-up Protocol. The Protocol will indicate the required volumes of reagents for the number of assays being performed.

8. Label a 0.6 mL or 1.5 mL snap-top tube for each DNA sample to be tested plus one 1.5- 2.0 mL snap-top tube if multiple samples are to be assayed. Prepare the master mixes as calculated on the PCR Set-up Protocol. Keep master mixes on ice or in a cold block.

9. Record the kit lot number and expiration date on a copy of the kit Recording Sheet. Remove the foil sealer from the primer tubes being used and discard the foil. Label each block of tubes with an identifier for one of the samples to be tested. Pipet 10 µL of the appropriate DNA-containing master mix into each tube of the corresponding block of primer tubes.

NOTE: It is critical to maintain awareness of the orientation of each primer tube “block”, so that the tube order is known throughout the assay process. For each tube “block” tube 1 is marked by a black dot.

10. Seal the tubes by pressing cap strips into place and proceed to step 12. Heat sealing of the PCR tubes is described in step 11.

11. If heat sealing will be used, place the blocks of primer tubes and the Negative Control Tube into the bottom rack of the heat sealer. Cut a section of thermo-sealing foil large enough to cover the primer tube blocks including the Negative Control Tubes and center the section on top of the primer tubes. Lower the heating element to the plate and press, holding for 3 to 5 seconds. Release the heating element and use a finger to run over the tops of the tubes to aid in sealing. Remove the plate from the sealer.

NOTE: Sealing for longer than 5 seconds or excessive pressure during the sealing operation can narrow the opening of the PCR tubes, making retrieval of the amplified products difficult.

12. Examine the PCR tubes after sealing. Dislodge any bubbles in the bottoms of PCR tubes and force any hanging drops down into the reaction volume. This may be performed by gently tapping tubes on the benchtop or by a brief centrifugation in a swinging-bucket centrifuge equipped with a microwell plate carrier.

13. Start the appropriate program on the thermal cycler. Transfer the primer tube blocks to the block of the thermal cycler.

14. If the tubes were heat-sealed, place the supplied silicone spacer pad securely over the foil sealer to cover all of the PCR tubes. If less than a full block of tubes is being amplified, place empty PCR tubes at the unused corners and edges of the block as spacers. Close the heated lid, making certain the silicone pad does not shift.

NOTE: The silicone spacer pad should not be used for tubes sealed with cap strips. Damage to the heated lid of the thermal cycler may result.
15. When the thermal cycler has reached the last step of the program (the 10°C hold), transfer the primer tubes to a PCR rack. Inspect each tube and note any that appear low in volume. Results of these tubes may be questionable. If electrophoresis will not be performed immediately, store the tubes refrigerated (2 to 8°C) for up to 3 days or in a freezer below -20°C for up to 7 days.

C. Detection

NOTE: Detection steps must be performed in an area separate from that used as the pre-PCR area. Devices should not be shared between these areas to avoid carryover contamination.

NOTE: Detection may be carried out in any of the E-Gel® sizes. Each E-Gel®16 may accommodate 1-2 typings. E-Gel®48 or E-Gel®96 gels make possible the processing of multiple samples on a single gel.

16. If the PCR tubes were stored frozen, remove them from the freezer and allow them to thaw prior to use.

17. Prepare the work area by setting down plastic-backed absorbent paper and setting up the iBase™ or Mother E-Base™, deionized water, and pipettors at this spot. Move the PCR tubes and the required number of E-Gels® to this work area.

NOTE: Loading and running the E-Gel®16 gels is described in steps 18-26. Loading and running the E-Gel®48 gels is described in steps 27-35. Loading and running the E-Gel®96 gels is described in steps 36-45.

2% E-Gel®16 Loading and Running

18. Open the pouch of a 2% E-Gel®16 and remove the gel. Insert the gel, right edge first, into the iBase™. Press on the left edge at the top and bottom to seat the gel. Plug in the device.

19. Pre-run the gel. Press and hold the Mode button (M, second from right) until a blinking Program is observed. Use the Up and Down buttons to reach the Pre-run program (program 0). Press the Go button to begin the pre-run. The red LED that signals a properly-inserted gel turns to green when the run begins. The end of the pre-run is indicated by a flashing red light and a beep. Press the Go button to silence the alarm.

20. Label the gel’s upper plate using a laboratory marker.

21. Remove the combs from the gel. Pipet 16 µL of deionized water to each well of the gel except the center half-wells.

22. Pipet the PCR products to the gel.

a. For All Kits Except Rare Screen: Open tube 1 (marked by a black dot) for the first sample, and pipet 6 µL of the contents into the assigned well of the gel. Repeat this process for each PCR tube and its corresponding well on the gel. Change pipet tips after each addition.

b. For Rare Screen: Open each tube and pipet 6 µL of the contents into the assigned well of the gel. Change pipet tips after each addition.

NOTE: This process is made easier by the use of an 8-channel multichannel pipettor capable of delivering 5-20 µL.

23. Pipet 5 µL water to one of the center half-wells. Pipet 5 µL of the negative control reaction to this well.

24. If a DNA ladder is desired as a size marker, pipet 10 µL of E-Ladder to the other half well.

NOTE: Other DNA size markers of the appropriate size range may be used. In this case the particular dilution and loading to the well must be determined by the user.

25. Press the Mode (M) button and use the down arrow to select Program 3. The run time should be 15 minutes; if not, use the Up or Down arrow to adjust. Press the Go button to start electrophoresis. The red LED will change to green and the display will show the remaining time.

26. At the end of electrophoresis the green light will change to a red flashing light and an alarm will sound. Press the Power button to stop. The flashing red light will become a steady red light. Unplug the device and remove the gel from the iBase. Continue to step 46.

2% E-Gel®48 Loading and Running

27. Remove the 2% E-Gel®48 gel from its foil pouch and remove the combs. Clean the gel’s upper plate with a damp laboratory wipe, if necessary. Label the gel’s upper plate using a laboratory marker.

28. Pipet 13 µL of deionized water to each well of the gel except the two M wells on the left side of the rows.

29. Pipet the PCR products to the gel.

a. For All Kits Except Rare Screen: Remove the first cap strip or open tube 1 (marked by a black dot), and pipet 5 µL from the contents of tube 1 for the first sample into the assigned well of the gel. Repeat this process for each PCR tube and its corresponding well on the gel. Do the same for the other samples tested. Change pipet tips after each addition. Be sure that each sample’s PCR tubes are pipetted to the assigned positions on the gel.

b. For Rare Screen: Open each tube and pipet 5 µL of the contents into the assigned well of the gel. Change pipet tips after each addition.

30. Pipet 5 µL of the negative control product to one of the right-side M wells.

31. If a DNA ladder is desired as a size marker, pipet 15 µL of E-Ladder to the two left side M wells.

NOTE: Other DNA size markers of the appropriate size range may be used. In this case the particular dilution and loading to the well must be determined by the user.

32. Plug in the Mother E-Base™. A red light will come on and the time display will light up. Use the power button (right button) to toggle to EG mode (instead of EP mode).

33. Slide the gel under the two electrodes on the Mother E-Base™ and seat the gel so its electrodes make contact with the device electrodes.
34. Use the left button to set the timer for 15 minutes. Press the power button (right button) to begin electrophoresis. The red light will change to green while the run is in progress.

35. At the end of electrophoresis, the green light will change to a red flashing light and an alarm will sound. Press the power button to stop the run. The flashing red light will become a steady red light. Unplug the device and remove the gel from the Mother E-Base™. Continue to step 46.

2% E-Gel®96 Loading and Running

36. Remove the 2% E-Gel®96 gel from its foil pouch and remove the comb. Clean the gel’s upper plate with a damp laboratory wipe, if necessary.

37. Label the gel’s upper plate using a laboratory marker.

38. Pipet 16 µl of deionized water to each well of the gel except the M wells on the right side.

39. Pipet the PCR products to the gel.
   a. For All Kits Except Rare Screen: Open tube 1 (marked with a black dot) for the first sample and pipet 6 µL into the assigned well of the gel. Repeat this process for each PCR tube for the first sample, and then for the remaining samples, with an aliquot of each tube’s contents pipetted to its corresponding well on the gel. Change pipet tips after each addition. Be sure that each sample’s PCR tubes are pipetted to the assigned positions on the gel.
   b. For Rare Screen: Open each tube and pipet 6 µl of the contents into the assigned well of the gel. Change pipet tips after each addition.

NOTE: This process is greatly facilitated by the use of an 8-or 12-channel volume multichannel pipettor capable of delivering 5-20 µL.

40. Pipet 6 µL of the negative control reaction to an unused well. If the plate is full, one of the right-side M wells may be used; in this case add 16 µL deionized water to the well before adding the negative control volume.

41. If a DNA ladder is desired as a size marker, pipet 15 µL of E-Ladder to each of the right-side M wells, except those used for negative controls.

NOTE: Other DNA size markers of the appropriate size range may be used. In this case the particular dilution and loading to the well must be determined by the user.

42. Plug in the Mother E-Base™. A red light will come on and the time display will light up. Use the power button (right button) to toggle to EG mode (instead of EP mode).

43. Slide the gel under the two electrodes on the Mother E-Base™ and seat the gel so its electrodes make contact with the device electrodes.

44. Use the left button to set the timer for 11 minutes. Press the power button (right button) to begin electrophoresis. The red light will change to green while the run is in progress.

45. At the end of electrophoresis the green light will change to a red flashing light and an alarm will sound. Press the power button to stop the run. The flashing red light will become a steady red light. Unplug the device and remove the gel from the Mother E-Base™.

Image Documentation

46. Take the gel to the UV transilluminator for observation of product bands. The gel should be viewed and recorded within 20 minutes. If a record of the assay is desired, a photo of the gel should be taken using a gel documentation system.

QUALITY CONTROL

A PCR product from the internal control primers should be detected in all amplifications except the Negative Control Tubes. Allele-specific amplification does compete with amplification of the internal control, so that in amplifications positive for blood group-specific reactions the internal control product may be faint or absent. This is not cause for concern. However, if DNA was loaded to a PCR tube and no control or allele specific bands are observed, amplification failure is strongly indicated. Such amplification failures can result from deterioration of a reagent, an incorrectly-programmed thermal cycler, poorly purified sample DNA, insufficient quantity of sample DNA loaded to the PCR, a poorly sealed PCR tube, or failure to load a reagent to the PCR during setup.

The negative control reaction should generate no detectable product bands. Presence of PCR products in this tube indicates some DNA contamination of the master mix. Such contamination may occur due to procedural errors or contamination of a reagent or tool used to set up the assay. If a negative control reaction is positive the assay results cannot be relied upon. The assay should be repeated.

INTERPRETATION OF RESULTS

The internal control produces a 434 bp PCR product for all kits except ABO Sub Typing which, instead, produces a 540 bp internal control PCR product. The specific products for some reactions are larger than this internal control product, smaller for others. Some tubes can produce more than one specific product (multiplex reactions). The sizes for all specific products from the reactions are listed on the Recording Sheet.

For each sample note the reaction pattern of positive and negative tubes on the appropriate Recording Sheet. Refer to the patterns on the Recording Sheet to determine the alleles present in the sample. Special attention should be paid to the notes at the bottom of each Recording Sheet; these provide useful information to aid in the interpretation of band patterns.

LIMITATIONS

- RED CELL EZ TYPE® kits contain materials for the amplification of sequences in genomic DNA by the polymerase chain reaction (PCR) and subsequent determination of the specified gene variations for the targeted blood groups as noted on the specific kit’s Recording Sheet. Other variations, including unknown variants in the relevant gene sequences, may adversely affect results.
As part of the production final control, each primer mix is tested for correct positivity and negativity whenever possible. The reactivity of the individual primer mixes was tested using control samples carrying known antigens. The composition of the primer mixes allows reliable identification of the antigens listed in the interpretation tables. The accuracy and reproducibility of the assay results was assessed in the performance assessment studies. Results from all assay systems were compared to results from serological or molecular typing of a set of known samples. For all systems, agreement between methods was 100%.

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**REFERENCES**

**FOR RAB, RABS**


**FOR RRZ, RWD, RDN**


**FOR RJF, RKDK, RKP**


FOR RMN

FOR RRI, RRS