

## **Red Cell EZ TYPE<sup>®</sup>** **(CDE, Weak D, D Negative)**

### **INTENDED USE**

Red Cell EZ TYPE<sup>®</sup> is an assay for the molecular determination of alleles of blood group antigen systems, using PCR amplification of human genomic DNA.

- **CDE** types for a group of polymorphisms in the RHD and RHCE genes that can result in altered antigenicity or weak reactivity in serologic assays. The specific polymorphisms are described in the Principle of the Procedure section below.
- **Weak D** types for a group of point mutations in the RHD gene that result in weak or absent serological reactivity. These point mutations are described more fully in the Principle of the Procedure section below.
- **D Negative** types for a set of genetic variations that result in loss of serologic reactivity for RHD antigens. The specific alleles and deletions are described in the Principle of the Procedure section below.

*For Research Use Only*

### **SUMMARY AND EXPLANATION OF THE TEST**

The established method for determination of the Rh (Rhesus) blood group antigens is serologic typing. Recently the genetic organization of the RHD and RHCE genes has been elucidated, and the genetic basis for many of the Rh antigens determined. This knowledge has made possible DNA-based typing for the genetic variations that encode these Rh antigens. DNA-based typing cannot entirely replace serologic methods. The genetic basis for at least some of the antigens in these systems remains unidentified, and undetected genetic variations may cause a predicted antigen to be absent in fact. However, DNA-based typing can resolve questions that have proven to be difficult for serological methods to answer. For example, DNA-based typing can readily detect the common reasons for Rh seronegativity – RH gene deletion, RH pseudogene ( $\psi$ ), etc. DNA-based typing can also readily explain the altered reactivity caused by RH exon-exchange hybrids (partial D). Finally, weak D typing by DNA-based methods readily identifies the point mutations that lead to low antigen expression on erythrocyte cell surfaces, which in turn causes low or absent serological reactivity.

DNA-based typing of RHD and RHCE polymorphisms 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 the Red Cell EZ TYPE<sup>®</sup> assays, offers improved assay speed and lower hands-on time. The PCR-SSP method requires only two working steps (amplification and detection) because both amplification and discrimination occur during the PCR process.<sup>6</sup> 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<sup>®</sup> assays are based on the *Polymerase Chain Reaction* (PCR), which enables amplification of defined target sequences in the genomic DNA.<sup>2</sup> 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.<sup>3,4</sup> 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<sup>®</sup>, 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.

### **ORGANIZATION OF THE RH GENES**

Two genes located on chromosome 1 in region 1p34 to 1p36, the RH D gene and the CE 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,  $\psi$ , or RHD(C)e<sup>s</sup>). In addition, point mutations also result in the **D<sub>EL</sub>** phenotype, where cell surface expression of the D protein is so low as to be immunologically absent. The CcEe 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 1% in European populations). These may be partial D types (e.g. D/CE hybrids) or weak D types.

## CDE

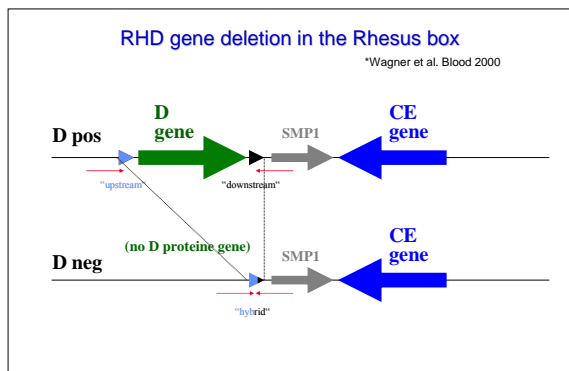
The **Red Cell EZ TYPE® CDE** assay is designed to distinguish a “normal” D type from the various partial D types by amplification of gene-specific sequences from each of the different exons of the D gene. These partial D antigens, also called hybrids, are produced by exchange of one or more exons of the D gene for the corresponding exons of the CE gene. The most common of these hybrids can be detected with the CDE assay. In addition, this assay is designed to detect the RHCE alleles that result in the C, c, E, e and C<sup>w</sup> antigens. Finally, this assay is designed to detect Rh-D pseudogene ( $\psi$ ) and d(C)e<sup>s</sup>.

## 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. **Red Cell EZ TYPE® Weak D** assay is designed to detect these point mutations. The **Red Cell EZ TYPE® Weak D** 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 **Red Cell EZ TYPE® D Negative** assay 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.



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 (RH -/-, 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 at the intron 8/exon 9 splice site of the RHD gene. In a true RH -/- (homozygous) individual, reactions 5 and 6 are negative, whereas at least one of the two reactions will be positive with RH D/- (heterozygous) or D/D (homozygous) individuals. Consequently, the specific reaction for d (tube 1) must be negative when tubes 5 or 6 are positive.

In Caucasians, RHD negativity usually results from the complete loss of the D gene. In people of African descent a non-expressed RHD pseudogene ( $\psi$ ) or RHD(C)e<sup>s</sup> are the common causes of the d phenotype. Both variants (RHD  $\psi$  and RHD(C)e<sup>s</sup>) can be identified by positive amplification in tube 4 of the Red Cell EZ TYPE® D Negative assay.

Studies from Southeast Asia confirm the increased incidence of D<sub>EL</sub> among RHD-negative individuals in this population group. D<sub>EL</sub> antigens are very weakly expressed Rh D, which are difficult to detect serologically. The genetic basis for a number of these D<sub>EL</sub> has now been elucidated, and these are detected readily using DNA typing methods. The Red Cell EZ TYPE® D Negative assay is designed to detect the D<sub>EL</sub> alleles RHD(M295I), RHD(IVS3+1G>A), and RHD(K409K).

A very rare RHD variant can be detected by a positive reaction in tube 3. This RHD variant also generates a positive band from tube 1 and would be interpreted incorrectly as RH-negative without tube 3 reaction.

## REAGENTS

Maximum number of tests per kit:

- 12 typings per kit

- |                          |  |
|--------------------------|--|
| <b>PP</b>                | 1. <b>Primer Plate:</b> 96-tube PCR trays with allele-specific primers for RHCE or RHD alleles and Internal control (HGH) primers dried on the inner surface of tubes. Sealed with foil covers. Ready for use. |
| <b>PR</b>                | 2. <b>PCR Reagent:</b> A buffer containing magnesium chloride, free deoxynucleotide triphosphates (dNTPs), glycerol, cresol red, and other proprietary components. Dilute before use.                          |
| <b>NCT</b>               | 3. <b>Negative Control Tubes:</b> PCR tubes containing dried primers, sealed with tube caps. Ready for use.  |
| <b>E-Gel<sup>®</sup></b> | 4. <b>E-Gel<sup>®</sup>:</b> precast agarose gels (2% 16 well, 2% 48 well, or 2% 96 well) used for separation of the PCR products. Ready for use.  |

## PRECAUTIONS

- Do not use reagents or gels beyond their expiration date.
- Do not use reagents that are turbid or contaminated.
- Do not use PCR Reagent that has changed color from red to yellow.
- Do not use gels that appear cracked, dried out, or show evidence of having been frozen or contaminated.
- 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.
- 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 setup) 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 reaction is an environmental control designed to detect DNA contamination and should always be run in parallel with sample amplifications.

## 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<sup>®</sup>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 – 2.0 and concentration in the range of 25 – 100 ng/μL.
- Resuspend the DNA in sterile water or 10 mM Tris, pH 8.0 -9.0. Samples should **not** be rehydrated in solutions containing greater than 0.5 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 -20°C for an extended period (up to 5 years) without affecting results.
- Presence of excess contaminating protein, RNA, heparin, EDTA, or other chelating agents may interfere with PCR amplification of the purified DNA.

## **PROCEDURE**

### **Materials Provided**

#### *PCR Components ( $\leq -20^{\circ}\text{C}$ storage)*

1. Primer Plates - 96-tube, color-coded PCR plates sealed with labeled foil covers
  - o 1 plate (12 – 1x8 primer tubes) for Weak D, color-coded green
  - o 1 plate (12 – 1x8 primer tubes) for D Negative, color-coded clear
  - o 2 plates (12 – 2x8 primer tubes) for CDE, color-coded blue

The plates contain allele-specific and the internal control primers. The plates are cut so that “blocks” of PCR tubes for each assay (1 strip/assay for Weak D, D Negative; 2 strips/assay for CDE) can be readily separated.

2. PCR Reagent
  - o 1 x 500  $\mu\text{L}$  (Weak D, D Negative) or
  - o 2 x 500  $\mu\text{L}$  (CDE)
3. Twelve (12) Negative Control Tubes in a labeled resealable bag.
4. Cap Strips for PCR tubes in a labeled resealable bag
  - o 12 strips (Weak D, D Negative)
  - o 24 strips (CDE)

**RSC**

#### *Gels (ordered separately)(15 to 30°C storage)*

2% E-Gel<sup>®</sup> 16 (6 x 2% double-comb E-Gel<sup>®</sup> per box)

OR

2% E-Gel<sup>®</sup> 48 (8 x 2% E-Gel<sup>®</sup> 48 per box)

OR

2% E-Gel<sup>®</sup> 96 (3 x 2% E-Gel<sup>®</sup> 96 per box)

Users who prefer heat sealing of the PCR tubes require the following 2 additional items, which can be obtained from GTI.

**TSF**

Thermo sealing foils  
Imm silicone spacer pad

### **Materials Required (but not provided)**

#### A. For PCR

- Taq Polymerase, either native or recombinant, 5U/ $\mu\text{L}$ . 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<sup>®</sup>. Use of other enzymes must be validated by the user.

**Table 1**

<b>Supplier</b>	<b>Product</b>
Inno-Train	AxiTaq
Applied Biosystems	AmpliTaq (Product #N8080172)
Promega	GoTaq Flexi (M8296)

- Programmable thermal cycler with block sized to accept 96 x 0.2 mL PCR tubes.
- Adjustable micropipets to deliver 1 - 1000  $\mu\text{L}$  and sterile barrier tips
- Molecular Biology grade water (DNA- and nuclease-free)
- Ice bath or cold blocks to fit 0.6 mL and 1.5 mL tubes
- PCR (0.2 mL) tube rack
- Vortex mixer
- Snap-top conical polypropylene microcentrifuge tubes (0.5 - 1.7 mL), DNA- and DNase free
- 10% bleach or other DNA-inactivating agent

#### *Optional*

- Electronic pipettor capable of delivering 10  $\mu\text{L}$
- Cap roller/setter (if using cap strips to seal PCR tubes)
- Heat sealing device (if using thermo sealing foil to seal PCR tubes)
- Sealing roller (if using thermo sealing foil to seal PCR tubes)

*B. For Electrophoresis and Analysis*

- E-Gel<sup>®</sup> iBase<sup>™</sup> (GTI Cat. # EZT-iBase) or Mother E-Base<sup>™</sup> (GTI Cat. # EZT-24DEV) device
- PCR (0.2 mL) tube rack
- Adjustable micropipets to deliver 1 - 20 µL, and sterile barrier tips
- Plastic-backed absorbent paper for lab bench
- UV transilluminator (GTI cat. #EZT-ILLUM)
- 10% bleach or other DNA inactivating agent
- Deionized water
- Gel documentation system (GTI cat.# EZT-CAMHOOD)

*Optional*

- DNA ladder (GTI Cat. # E-LADDER)
- 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 25-100 ng/µL and a 260 nm/280 nm ratio of 1.60 – 2.0.
2. Program a thermal cycler with the Red Cell EZ TYPE<sup>®</sup> PCR program, which is used for the CDE, Weak D, and D Negative kits. The program is as follows:

**Table 2 Thermal Cycling Program**

<b>Initial</b>	<b>5 cycles</b>	<b>10 cycles</b>	<b>20 cycles</b>	<b>72°C Hold</b>	<b>End</b>
94°C 2 minutes	94°C 20 seconds 70°C 60 seconds 60 seconds	94°C 20 seconds 65°C 60 seconds 72°C 45 seconds	94°C 20 seconds 61°C 50 seconds 72°C 45 seconds	72°C 5 minutes	10°C Hold

**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 and PTC-200 ramping rates need not be limited to ~1°C/second (9600 mode). 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 Plate 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 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 assay, only one Negative Control reaction is required. Similarly, if more than one assay is being run on a DNA sample only one Negative Control reaction is required.*

7. Fill out the Red Cell EZ Type<sup>®</sup> PCR Setup Protocol, using the appropriate side. 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 Setup Protocol. Keep master mixes on ice or in a cold block.

- Record the kit lot number and expiration date on a copy of the kit Recording Sheet. Remove the foil sealer from the primer plate blocks and discard. Label each block of tubes with an identifier for one of the samples to be tested. Pipet 10  $\mu$ L 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.*

- 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.
- 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 (maximum 11 samples for Weak D and D Negative), 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.*

- 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.
- Start the Red Cell EZ TYPE<sup>®</sup> program on the thermal cycler. Transfer the primer tube blocks to the block of the thermal cycler.
- 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. Slide the heated lid closed, making certain the silicone pad does not shift. Close the lid according to the thermal cycler manufacturer’s instructions.

**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.*

- When the thermal cycler has reached the last step of the program (the 10°C hold), transfer the primer plate 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 – 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 either the E-Gel<sup>®</sup> 16 (2% double-comb E-Gel<sup>®</sup>, 16 wells), E-Gel<sup>®</sup> 48 (2% double-comb gels, 48 wells), or E-Gel<sup>®</sup> 96 (2% gel, 96 wells) gels. Each E-Gel 16 gel can accommodate a single CDE assay or 2 Weak D or D Negative assays. The E-Gel<sup>®</sup> 48 or E-Gel<sup>®</sup> 96 gels make possible the processing of multiple samples on larger gel formats.*

- If the PCR tubes were stored frozen, remove them from the freezer and allow them to thaw prior to use.
- Prepare the work area by setting down plastic-backed absorbent paper and setting up the iBase<sup>™</sup> or Mother E-Base<sup>™</sup>, 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**

- Open the pouch of a 2% double-comb E-Gel<sup>®</sup> 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.
- 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  $\mu\text{L}$  of deionized water to each well of the gel except the center half-wells.
22. Open tube 1 (marked by a black dot) for the first sample, and pipet 6  $\mu\text{L}$  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.

**NOTE: This process is made easier by the use of an 8-channel multichannel pipettor capable of delivering 5-20  $\mu\text{L}$ .**

23. Pipet 5  $\mu\text{L}$  water to one of the center half-wells. Pipet 5  $\mu\text{L}$  of the negative control reaction to this well.
24. If a DNA ladder is desired as a size marker, pipet 10  $\mu\text{L}$  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<sup>®</sup> 48 Loading and Running**

27. Remove the 2% E-Gel<sup>®</sup> 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 12  $\mu\text{L}$  of deionized water to each well of the gel and the two M wells on the right side of the rows.
29. Open tube 1 (marked by a black dot) for the first sample, and pipet 6  $\mu\text{L}$  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. Be sure that each sample's PCR tubes are pipetted to the assigned positions on the gel.
30. Pipet 6  $\mu\text{L}$  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  $\mu\text{L}$  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 switch the device 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 Motherbase. Continue to step 46.

### **2% E-Gel<sup>®</sup> 96 Loading and Running**

36. Remove the 2% E-Gel<sup>®</sup> 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  $\mu\text{L}$  of deionized water to each well of the gel.
39. Open tube one (marked with a black dot) for the first sample and pipet 6  $\mu\text{L}$  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.

**NOTE: This process is greatly facilitated by the use of an 8-or 12-channel volume multichannel pipettor capable of delivering 5-20  $\mu\text{L}$ .**

40. Pipet 6  $\mu\text{L}$  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  $\mu\text{L}$  deionized water to the well before adding the negative control volume.
41. If a DNA ladder is desired as a size marker, pipet 15  $\mu\text{L}$  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 switch the device 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 Motherbase.
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 (430 bp) should be detected in all amplifications except the negative control tubes. However, allele-specific amplification competes 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 reactant 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. 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 tubes that have produced allele-specific PCR products on the appropriate Recording Sheet (CDE, Weak D, or D Negative), using the row(s) at the bottom of the Results Table. Refer to the patterns on the Recording Sheet to determine the RH alleles present in the sample. Special attention should be paid to the performance notes at the bottom of each Recording Sheet; these provide useful information to aid in the interpretation of band patterns.

## LIMITATIONS

- For Red Cell EZ TYPE<sup>®</sup> CDE: The following very rare D variants cannot be distinguished from D with the present test system: Rh-D<sup>II</sup>, DFW, DHO, DHR, DIM, DMH, DNU, DOL, DWI, weak D type 16-18, 21-25, 26, 31, (W16X), (Q41X), (W90X), (488del4), (G212V), (del711), (906ins TGGCT, IVS6), (G314V), (Y330X), (IVS8+1G>A). Weak D types 1 to 15 can be detected in the Red Cell EZ TYPE<sup>®</sup> Weak D Kit. The D<sub>ELS</sub> Rh-D (IVS3+1G>A), (M295I), (K409K) can be detected using the Red Cell EZ TYPE<sup>®</sup> D Negative kit.
- Red Cell EZ TYPE<sup>®</sup> CDE, Weak D, and D Negative kits contain materials for the amplification of sequences in genomic DNA by the polymerase chain reaction (PCR) and subsequent determination of the specific RHD and RHCE gene variations noted on the specific kit's Recording Sheet. Other variations, including unknown variants in the relevant gene sequences, may adversely affect results.
- The determination of specific RHD or RHCE alleles by Red Cell EZ TYPE<sup>®</sup> CDE, Weak D, or D Negative are intended for use as an adjunct to Rh serology, and cannot be used to replace serologic testing for Rh antigens.
- To ensure optimal results in the Red Cell EZ TYPE<sup>®</sup> assay, DNA samples should have a concentration between 25 ng/μL and 100 ng/μL with a 260 nm/280 nm ratio of 1.60 – 2.0. Poor amplification may result from insufficient DNA added to the PCR reaction, or from poorly-purified sample DNA.
- False-negative results may stem from improperly handled specimens, procedural errors, amplification inhibitors, or from poorly-purified DNA.
- False negative results may stem from use of a DNA polymerase enzyme other than those listed in **Materials Required** (but not provided).
- Contamination from carryover nucleic acids, excessive genomic DNA loaded to amplifications, or failure to use indicated temperatures may result in false-positive amplifications.
- The thermal cycler program has been tested with the thermal cyclers PE 9600, PE 9700, and PE2720 (all Perkin Elmer/Applied Biosystems), the PTC-100 and PTC-200 (BioRad). Some adjustments to the indicated cycling parameters may be required for use of the assay in other thermal cyclers.
- Thermal cyclers should be calibrated and maintained according to the manufacturer's instructions.

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- STORE AT ≤ -20°C for PCR components
- STORE AT 15 to 30°C for Gels

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