

HLA EZ TYPE®
(A/B/C Low, A/B/DR Low, A Low, B Low, DR Low, DR/DQ Low)

INTENDED USE

HLA EZ TYPE® is an assay for the molecular determination of HLA Class I and Class II alleles at low resolution, using PCR amplification of human genomic DNA.

A/B/C Low types for Class I A, B, and C loci

A/B/DR Low types for Class I A and B loci, and Class II DR locus

A Low types for Class I A locus.

B Low types for Class I B locus.

DR Low types for Class II DR locus.

DR/DQ Low types for Class II DR and DQ loci.

For *In Vitro* Diagnostic Use

SUMMARY AND EXPLANATION OF THE TEST

The established method for determination of HLA antigens was for many years the lymphocytotoxicity assay. However, the advent of PCR technologies has made DNA-based typing of HLA antigens routine in the laboratory. Compared to serological methods, PCR-based assays offer simplified interpretation of results due to the ability to discriminate single nucleotide changes. Also, the synthetic reagents of PCR-based assays lead to improvements in both component stability and lot-to-lot variation.

Most HLA genotyping assays are multi-step methods; amplification of HLA regions is followed by a separate discrimination step (RFLP or SSOP methods) and a final detection step. In contrast, PCR-SSP analysis 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 PCR. Detection by agarose gel electrophoresis completes the assay. This inherent assay utility is further enhanced by the inclusion of precast agarose gels in the test system.

PRINCIPLE OF THE PROCEDURE

This assay is based on the *Polymerase Chain Reaction* (PCR), which enables an enrichment (amplification) of defined DNA sequences. 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. Therefore, PCR-SSP analysis requires a number of amplifications to be carried out in parallel. In the “touch down” program described in the Test Procedure, an initial denaturing period is followed by 10 two-temperature PCR cycles at a high annealing temperature (65°C) that guarantees specific amplification of all the SSP reactions. This is followed by 20 cycles of a three-temperature PCR with a lower annealing temperature at which further amplification of specific PCR products is favored. Amplification of internal control primers targeted to the gene for Human Growth Hormone (HGH) demonstrates acceptable reaction conditions for each PCR tube. 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 the recording of allele-specific bands from the gel image on the enclosed Recording Sheet. The pattern of allele-specific bands is then compared to the band patterns on the Interpretation Tables.

REAGENTS

Maximum number of tests per kit:

- 8 typings per kit (A/B/C Low, A/B/DR Low), 96 tubes per assay
- 16 typings per kit (B Low), 48 tubes per assay
- 24 typings per kit (DR/DQ Low), 32 tubes per assay
- 32 typings per kit (A Low, DR Low), 24 tubes per assay

PP

1. **Primer Plate:** 96-tube PCR trays with HLA allele-specific primers and Internal control (HGH) primers dried on the inner surface of tubes sealed with foil covers. Ready for use.

HLAPR

2. **HLA PCR Reagent:** A buffer containing magnesium chloride, free deoxynucleotide triphosphates (dNTPs), glycerol, cresol red, and other proprietary components. Dilute before use.

E-Gel[®] 3. **E-Gel[®]**: precast agarose gels (96 well, 2%) 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 HLA 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 are not to 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 foil sealer during PCR.
- Amplification steps can be set up on the bench top at standard laboratory temperatures (20 – 25°C). Setup at temperatures higher than this may result in PCR artifacts. If these are encountered, the master mixes and Primer Plates should be kept on cold blocks or an ice bath during amplification setup.
- Do not isolate the DNA sample from heparinized blood. Heparin may interfere with the PCR amplification.
- PCR is covered by patents held by Roche Molecular Systems and F. Hoffmann-La Roche Ltd. For information on licenses to practice PCR, contact (in the United States) the Director of Licensing at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501, or (outside the United States) the PCR Licensing Manager, F. Hoffmann-La Roche Ltd, Grenzacher Str. 124, CH-4070 Basel, Switzerland.
- 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 test (NC, PCR tube no. 1 for the DR/DQ Low, A Low, B Low, DR Low kits; tubes 1, 25, 73 for A/B/DR Low and A/B/C Low) 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[®]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 manufactured for that purpose.
- 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 (as long as six years) without affecting results.
- DNA samples should be in the range of 12.5 - 100 ng/μL, and a 260 nm/280 nm ratio ≥ 1.60.
- Presence of excess contaminating protein, RNA, heparin, EDTA, or other chelating agents may interfere with PCR amplification of the purified DNA.

PROCEDURE

Materials Provided

(Packaged for ≤ -20°C storage)

1. Eight 96-tube color-coded PCR trays sealed with marked foil covers. These contain the HLA allele-specific and the internal control primers.
2. 4 x 700 μL HLA PCR Reagent

TSF 3. 9 x Thermo-sealing foils

(Packaged for 15 to 30°C storage)

1. 8 x 2% E-Gel®

Silicone spacer pad (1 mm). Not included in kit; provided in shipment.

Materials Required (but not provided)

A. For PCR

- 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 HLA EZ TYPE®. Use of other enzymes must be validated by the user.

Table 1

Supplier	Product
Applied Biosystems	AmpliTaq recombinant Taq polymerase
Promega	GoTaq Flexi DNA Polymerase
Genaxis	AxiTaq

- Programmable thermal cycler with block sized to accept 96 x 0.2 mL PCR tubes.
- Adjustable micropipets to deliver 1 - 1000 μL and sterile barrier tips
- Molecular Biology grade water (DNA and DNase free)
- Ice bath or cold blocks to fit 0.6 mL or 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
- Heat seal device
- Sealing roller

B. For Electrophoresis and Analysis

- Mother E-Base™ (EZT-24DEV)
- 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 (EZT-ILLUM)
- 10% bleach or other DNA inactivating agent
- Deionized water
- Gel documentation system (EZT-CAMHOOD)

Optional

- DNA ladder (E-LADDER)
- 8-channel or 12-channel multichannel pipettor capable of delivering 5-20 μL
- Cold block or ice bath to accommodate 0.2 ml PCR tubes (see Precautions)

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 12.5-100 ng/μL and a 260 nm/280 nm ratio ≥ 1.60.
2. Program a thermal cycler with the HLA EZ TYPE® PCR program, which is the same for all HLA EZ TYPE® kits. The program is as follows:

96° C	120 seconds	
96° C	15 seconds	
65° C	60 seconds	10 cycles
96° C	15 seconds	
61° C	50 seconds	20 cycles
72° C	30 seconds	
4° C	Hold	

NOTE: This cycling program has been tested with thermal cyclers from Applied Biosystems (PE9700, PE9600, PE2720), Bio-Rad (PTC-100, PTC-200), and Eppendorf (Mastercycler, Mastercycler EP). Ramping rates on faster cyclers (Mastercycler, Mastercycler EP, PTC-200, PE9700 with gold/silver block) should be adjusted to 1.0 – 1.5°/sec heating and cooling.

B. PCR

- 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.
- Prepare work surfaces and pipettors before use by wiping with 10% household bleach or other DNA-inactivating agent.
- Determine the numbers of samples to be tested. Remove the needed Primer Plates or plate sections, Taq polymerase, and sufficient PCR Reagent from the freezer and thaw at room temperature. Keep DNA samples, PCR Reagent, and Taq polymerase on ice or in a cold block.
- Turn on the heat sealing device and allow it to come to operating temperature (~ 5 – 7 min).
- Use the Master Mix Formulation table (Table 2 below) to prepare a master mix for all samples.

NOTE: The amplification steps can be set up on the benchtop at standard laboratory temperatures (20 – 25°C). Benchtop setup at temperatures greater than this may result in PCR artifacts that can make interpretation complicated. If such artifacts are encountered, the master mixes and Primer Plates should be kept on ice or in cold blocks during setup of the amplification steps.

Table 2

Number of tubes per typing	Preparation of Master Mix			Removal of Negative Control and addition of DNA			Final volume of sample PCR mix (µL)
	Molecular Biology grade water (µL)	Volume of HLA PCR Reagent (µL)	Volume of Taq Polymerase (µL)	Volume of master PCR Mix (µL)	Volume left after adding to negative control tubes (µL)	Volume of DNA Sample (µL)	
For samples in the range of 25 – 100 ng/µL							
24	163	81	2.2	246.2	236.2	26	262.2
32	219	108	3.0	330	320	35	355
48	310	154	4.5	468.5	458.5	50	508.5
96	638	319	8.5	965.5	935.5	103	1038.5
For samples between 12.5 – 25 ng/µL, set up as described below							
24	137	81	2.2	220.2	210.2	52	262.2
32	184	108	3.0	295	285	70	355
48	260	154	4.5	418.5	408.5	100	508.5
96	535	319	8.5	862.5	832.5	206	1038.5

- Remove the foil sealer from the primer plate or plate section and discard. Pipet 10 µL of the PCR mix into the negative control tube(s) (as indicated on the Recording Sheet) PRIOR to adding the required amount of sample DNA to the master mix.

9. Add the sample DNA to the PCR master mix. Mix well and pipet 10 μL of the sample PCR mix into each tube of the PCR tray which corresponds to the typing format (23 tubes for the A Low and DR Low, 31 tubes for the DR/DQ Low, 47 tubes for the B Low, and 93 tubes for the A/B/C Low or the A/B/DR Low). Be careful not to add additional sample to the negative control tube(s).
10. Place the primer plate into the bottom rack of the heat sealing device. Center a thermo-sealing foil on top of the primer plate. Orient the foil so that its notched corner is over the **bottom left** corner of the plate. If sealing a plate section, cut the foil to fit before sealing. Save the remainder of the foil for use in another assay.

NOTE: *The thermo-sealing foils have a sealable side, which must be oriented down (toward the primer plate) for proper sealing. The notched corner of the foils is a visual cue to proper orientation. If the foil is trimmed to fit a plate section be sure to save the remaining foil piece so that the orientation is known. Sealing with the wrong side down will result in tube leakage and possible assay failure.*

11. Once the thermo-sealing foil is properly positioned, lower the heating element to the plate and press, holding for 3 to 5 seconds. Release the heating element and allow it to rise from the plate. Immediately use the sealing roller to firmly adhere the foil to the plate. 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 the foil sealer is in place. Dislodge any bubbles in the bottoms of PCR tubes. Force any hanging drops down into the reaction volume. This may be accomplished 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 HLA EZ TYPE[®] program on the thermal cycler. Transfer the primer plate(s) from the rack or cold block to the block of the thermal cycler.
14. Place the supplied silicone spacer pad securely over the foil sealer to cover all of the PCR tubes and be certain the pad does not shift when securing the thermal cycler heated lid. Close the lid according to the thermal cycler manufacturer's instructions.
15. When the thermal cycler has reached the last step of the program (the 4°C hold), transfer the primer plate to a PCR rack. Inspect each tube and note any that appear low in volume. Volume loss may result in poor amplification from these tubes. 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.*

16. If PCR tubes were frozen, remove from the freezer and allow to thaw prior to use.
17. Prepare the work area by setting down plastic-backed absorbent paper and setting up the Mother E-Base[™], deionized water, and pipettors at this spot.
18. Remove the E-Gel[®] from its foil pouch and remove the comb. Clean the gel's upper plate with a damp laboratory wipe, if necessary.
19. Label the gel's upper plate using a laboratory marker.
20. Pipet 16 μL of deionized water to each well of the gel except the M wells.
21. If a DNA ladder is desired, load the appropriate volume to each of the M wells. Add water if necessary to bring total volume in each M well to 15 – 20 μL .
22. Puncture the thermo-sealing foil of the first PCR tube (tube A1) and pipet 6 μL into the corresponding well of the E-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 greatly facilitated by the use of an 8-or 12- multichannel pipettor capable of delivering 5-20 μL .*

23. 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).
24. 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.
25. Use the left button to set the timer for 9 minutes. Press the power button (right button) to begin electrophoresis. The red light will change to green while the run is in progress.
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 run. The flashing red light will become a steady red light. Unplug the device and remove the gel from the Mother E-Base™.
27. 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 one of the internal control primer sets (430 bp, 800 bp or 1070 bp, depending on the PCR tube) should be detected in all amplifications except the negative control tubes. However, allele-specific amplification competes with the internal control PCR, so that in amplifications positive for HLA-specific reactions, the internal control band may be suppressed – 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, volume loss from a poorly sealed PCR tube, or failure to load a reactant to the PCR during setup.

The negative control tube should have no detectable product bands. Presence of PCR products in this tube indicates some DNA contamination of the master mix.

For each HLA EZ TYPE® assay, primers for a larger internal control PCR product are included in some PCR tubes. These tubes are spaced in a pattern across the primer plate. This pattern is noted on the assay Recording Sheet and may be compared to the internal control bands on the gel as a check of proper gel loading.

Perform quality control procedures in accordance with local, state, and federal requirements.

INTERPRETATION OF RESULTS

The PCR products observed upon UV transillumination are composed of HLA-specific products, internal control products, and, rarely, non-specific artifacts. For each tube the internal control product is the largest; HLA-specific PCR products and artifacts are always smaller in size than the internal control. Refer to the Comments Table on page 5 of the Recording Sheet of the lot documents for information about non-specific artifact bands, multiplex amplifications, weak reactions, and possible cross-reactivity. These comments are specific to each lot. Record the allele-specific bands on the grid of the enclosed Recording Sheet. Note the positive tubes on the appropriate Interpretation Table. Use the patterns on the Interpretation Tables to determine the alleles present for each locus tested.

LIMITATIONS

- HLA EZ TYPE® contains materials for the amplification of sequences in genomic DNA by the polymerase chain reaction and the subsequent low resolution detection of HLA class I and class II allele specific sequences by agarose gel electrophoresis. Unknown variants in the relevant gene sequences may adversely affect results.
- To ensure optimal results in the HLA EZ TYPE® assay, DNA samples should have a concentration between 12.5 ng/μL and 100 ng/μL with a 260 nm/280 nm ratio of ≥ 1.60. 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), and the Mastercycler and Mastercycler EP (Eppendorf). 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.
- Results from this assay should not be used as the sole basis for clinical decisions.

SPECIFIC PERFORMANCE CHARACTERISTICS

NOTE: *Primer sets for a particular Class I or Class II locus are shared across all the HLA EZ TYPE[®] products. Therefore, the performance characteristics described here for the A/B/C Low and DR/DQ Low assays apply to all of the HLA EZ TYPE[®] low-resolution kits.*

Comparative Methods Studies

The agreement of the HLA EZ TYPE[®] assay to comparative methods was assessed by three different comparison studies:

Comparison of HLA EZ TYPE[®] and DNA based molecular typing assays

Two different studies were performed. In the first, fifty samples covering a wide range of HLA Class I alleles, and 51 samples with a wide range of HLA Class II alleles, were typed using GTI HLA EZ TYPE[®] A/B/C Low or DR/DQ Low. The samples were also tested for Class I or Class II alleles by another PCR-based low-resolution typing method in an independent external evaluation. Agreement between the two methods was 100% for Class I, a 95% confidence lower limit of 0.942 (a 95% probability that agreement is at least 94.2%). For Class II agreement was 100%, a 95% confidence lower limit of 0.943 (a 95% probability that agreement is at least 94.3%).

In the second study, fifty two samples covering a wide range of HLA Class I and Class II alleles were typed using GTI HLA EZ TYPE[®] A/B/C Low or DR/DQ Low. The samples were also tested for Class I or Class II alleles by PCR-based typing methods, low-resolution in some cases, high-resolution in others. Agreement between methods for all samples was 100% for both Class I and Class II loci. The 95% confidence lower limit for both comparisons is 0.944, or a 95% probability that agreement is at least 94.4%.

Comparison of HLA EZ TYPE[®] Results to DNA Sequencing

Thirty samples covering a wide range of HLA Class I or Class II alleles were tested in the HLA EZ TYPE[®] A/B/C Low or DR/DQ Low assays. Results were compared to the results of high-resolution HLA genotyping (sequencing or high-resolution SSP PCR). Agreement was 100% between the two methods.

Assay Precision

The reproducibility of the HLA EZ TYPE[®] assay was determined. Briefly, two samples representing a range of alleles were tested in 20 separate assays for the A/B/C Low assay; for DR/DQ Low two samples were tested in triplicate in 10 separate assays. The results demonstrated 100% agreement between assays and, for DR/DQ, within assays.

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GTi DIAGNOSTICS®

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HLA EZ TYPE®
(A/B/C Low, A/B/DR Low, A Low, B Low,
DR Low, DR/DQ Low)

FOR *IN VITRO* DIAGNOSTIC USE

- STORE AT ≤ -20°C
- STORE AT 15 to 30°C



0459

REF

LR-ABC, LR-ABDR, LR-A, LR-B, LR-DR, LR-DRDQ

Rev. 13 April 2010 (en)

EC REP

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