PROCEDURE MANUAL

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DNA LABORATORY

PROTOCOL FOR EXTRACTION AND QUANTITATION OF CASE EVIDENCE

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Richard D. Grady
Laboratory Manager

Technical Leader, DNA Laboratory

EXTRACTION AND QUANTITATION OF DNA FROM BIOLOGICAL MATERIALS

The methods delineated here assume prior training in and familiarity with forensic DNA typing.

Because not all forensic situations may be foreseen, changes may occasionally be made to these methods at the analyst's discretion in order to accommodate a particular sample.

Reagents are listed in Appendix B

A. ORGANIC EXTRACTION OF DNA FROM BIOLOGICAL MATERIALS

There may be cases in which these volumes might be altered proportionately to accommodate the size of the sample.

1. Cut a **stain** into medium-sized pieces and place the pieces into a 1.5 mL tube.
- Biological material deposited on a **hard or bulky substrate** should be scraped or swabbed as appropriate and placed in a 1.5 mL tube.
- Place approximately 0.5-1.0 cm of **hair root** into a 1.5 mL tube. A similar size portion of the adjacent shaft should also be extracted separately.
- Pulp from **teeth** should be scraped out, or if necessary, the tooth may be crushed and placed directly into a 1.5 mL tube. Insoluble material should be spun out prior to phenol extraction.
- **Bone** should be shaved, crushed or sawed and placed directly into a 1.5 mL tube. Insoluble material should be spun out prior to phenol extraction.

Samples not described here should be treated as deemed appropriate by the experience of the analyst.

2. Add 400 µL stain extraction buffer (SEB).
Add 200 µg Proteinase K (Pro K). (10 µL of 20 mg/mL or 20 µL of 10 mg/mL).
For hairs add 3 mg of DTT.
Ensure that the sample is in the liquid.

3. Incubate at 56°C for ~2 hours.

4. Add an additional 200 µg Pro K to the tube.

5. Incubate at 56°C for another 4 to 16 hours. For freshly prepared reference bloodstains, this second incubation may be shortened to ~2 hours.

NOTE: Alternately, perform the first digestion overnight, followed by addition of more Pro K and a short incubation the following day.

6. Recover the maximum amount of clear DNA-containing digest solution by removing any substrate: first agitate, squeeze out as much liquid as possible, then "piggyback" * as necessary. If fine insolubles are present, remember to spin this digest solution and save the supernatant.

*"Piggyback": Place fabric pieces or other substrate material in a spin basket, cap and spin ~5 minutes in microfuge. Remove the cuttings and basket and recap tube. (Alternatively, punch several holes in the lid of the microfuge tube and place the pieces in the lid. Cover the lid and contents tightly with a piece of parafilm. Spin 5 minutes in microfuge. Remove the cuttings and cap and place a new cap on the tube.)

7. To this digest solution add an equal volume (typically 400 µL) phenol/chloroform/isoamyl alcohol. (Draw this organic reagent from the middle of its container, avoiding the separate layer at the meniscus.) Gently invert the tube by hand to achieve a milky emulsion in the tube. Spin the tube to achieve phase separation and compression of the interface (typically 2-5 minutes).

8. Transfer aqueous phase (top layer) to a new tube without disturbing the interface.

9. Do additional organic extractions as in step 7 until the interface is clean and the aqueous phase clear, then do one more extraction (usually 2-3 extractions total is sufficient).

The results of organic extraction may vary depending on the condition of the sample. The analyst should use his/her judgment in continuing to process the sample. Sometimes it is desirable to perform subsequent extractions with pure chloroform or butanol, e.g. to remove residual phenol or reduce the sample volume.

10. Pipette about ~1.7 mL TE^{-4} into each labeled Centricon 100[†]. Add the sample (aq. phase from the last phenol extract) and spin 20 minutes at 1000 x g.

11. Do two additional washes with 2 mL TE^{-4}. The middle wash spin time may be shortened to 10 minutes.

12. Collect retentate into cup by inverting Centricon and spinning 2 minutes at 500 x g. (If a Centricon 100 is not available, the sample may be precipitated with EtOH. See Appendix A).

13. Transfer retentate to microfuge tube, approximating the amount recovered. This sample may be used for both RFLP and PCR analysis.

Proceed to:

D. TEST GEL FOR ASSESSING THE QUALITY AND QUANTITY OF DNA ISOLATED FROM BODY FLUID STAIN or

E. SLOT BLOT FOR ASSESSING THE QUANTITY OF HUMAN DNA

[†]Another microfiltration device such as a Microcon filtration unit may be substituted for the Centricon 100.

B. DIFFERENTIAL EXTRACTION PROTOCOL FOR BODY FLUID/SEMEN MIXTURES

There may be cases in which these volumes might be altered to accommodate the size of the sample. At times, it may be acceptable to skip the PBS soak and begin with step 5, e.g. where hospital slides are available for evaluation or where additional material is being extracted.

1. Cut the substrate into medium-sized pieces and place the pieces into a 1.5 mL tube or spin basket bottom.

2. Incubate in 400 µL PBS for ~1 hour at 4°C.

3. Vortex 15-30 seconds to loosen cells. Agitate substrate and remove. "Piggyback"* as necessary for maximum fluid and loose cell recovery. Save the substrate for step 5. If no "piggyback" is used, spin the fluid with loosened cells in a microfuge for ~5 minutes. Carefully remove all except ~50 µL of the supernatant (aqueous extract) and store at -20°C.

4. Resuspend the cellular pellet in the ~50 µL of liquid remaining, and remove 3 µL to a microscope slide. Heat dry these slides.

5. Replace the substrate from the sample from step 3 into the tube.
Add: 400 mL stain extraction buffer.
10 μL Pro K (20 mg/mL), or 20 μL of (10 mg/mL).

Incubate ~1 hour at 56°C. Gentle agitation is permissible.

Meanwhile, stain slides as follows and examine microscopically:

Stain sample area with nuclear fast red for 10-15 minutes.
Rinse gently with water.
Stain with picroindigocarmine solution for ~10 seconds.
Rinse with MeOH, air dry.

6. Vortex 15-30 seconds in order to loosen sperm cells. Treat as in Step 3, to remove substrate and spin down sperm cells. Prepare separate tubes to receive e. cell fraction and to save the substrate.

* "Piggyback": Place fabric pieces or other substrate material in a spin basket, cap and spin ~5 minutes in microfuge. Remove the cuttings and basket and recap tube. (Alternatively, punch several holes in the lid of the microfuge tube and place the pieces in the lid. Cover the lid and contents tightly with a piece of parafilm. Spin 5 minutes in microfuge. Remove the cuttings and cap and place a new cap on the tube.)

7. Carefully transfer all except 50 µL of the supernatant (epithelial cell fraction) to a separate labeled tube and reserve. The e. cell fraction may be returned to 56°C incubation while steps 8 and 9 are performed.

8. Add 400-1000 µL PBS, gently resuspend the sperm pellet and microfuge 3-5 minutes. Carefully remove all except ~50 µL of the supernatant and discard.

Repeat this wash step, leaving 50 µL of the supernatant each time.

The number of washes may be varied at the analyst's discretion depending on the condition of the sample. A total of 3 washes is usually adequate.*

*In cases where a high e. cell count is observed upon initial microscopic analysis or the ratio of e. cells to sperm is high, a greater number of washes is generally desirable to remove the free e.cell DNA from the remaining sperm cell pellet. This must be balanced against the potential loss of sperm to lysis during each wash. The number of washes will generally be between 3 and 5 and the volume of each wash can vary from about 400 to 1000 µL, depending on the circumstances described above.

9. After the last wash, mix the remaining ~50 µL as well as possible and remove 3 µL to a microscope slide. Heat dry these slides. Slide staining and examination should be performed as in step 5 above. If e. cells remain, steps may be taken to further digest the residual e.cells in the sperm pellet. See Appendix A.

10. Prepare a master-mix containing the following for each sperm pellet sample plus one:
400 µL stain extraction buffer
200 µg Pro K (20 µL of 10 mg/mL or 10 µL of 20 mg/mL).
3 mg fresh DTT.
Add 410 µL or 420 µL of this master-mix to each sperm pellet tube and incubate at 56°C ~2 hours.

11. Add another 200 µg Pro K to each e. cell fraction tube and sperm fraction tube and continue incubating at 56°C another 4 to 16 hours.

NOTE: Alternately, perform the first digestion overnight, followed by addition of more Pro K and a short incubation the following day.

12. Continue extraction procedure from Section A, Step 7.

C. CHELEX EXTRACTION OF DNA FROM BLOODSTAINS

1. Cut a stain into medium-sized pieces and combine with 1 mL of sterile distilled water in a 1.5 mL microfuge tube. Mix gently.

2. Incubate at room temperature for 15 to 30 minutes. Mix occasionally by inversion or gentle rotation.

3. Spin in a microfuge for 2-3 minutes at 10,000 to 15,000 x g.

4. Carefully remove supernatant (all but 20 to 30 µL or enough to cover the substrate), and discard. Leave the substrate in the tube with the pellet.

5. Add 200 µL of 5% Chelex (w/v in sterile distilled water). Use a P-1000 pipette, stirring with the tip to evenly disperse the Chelex resin before pipetting.

6. Incubate at 56°C for 30 minutes.

7. Vortex at high speed for 5 to 10 seconds.

8. Incubate in a boiling water bath for 8 minutes.

9. Vortex at high speed for 5 to 10 seconds.

10. Spin in a microfuge for 3 minutes at maximum speed (10,000 to 15,000 x g).

Proceed to:

E. SLOT BLOT FOR ASSESSING THE QUANTITY OF HUMAN DNA

In case of extremely minute samples, quantitation may be omitted. The sample may be concentrated using either microfiltration or alcohol precipitation (See Appendix A).

APPENDIX A

ETHANOL PRECIPITATION of DNA

Add 2.5 volumes of cold absolute EtOH to the sample and mix by hand.

Incubate at -20°C for 15-30 minutes.

Spin tube in microfuge 15-30 minutes. Decant the alcohol.

Rinse pellet with an equal volume of room temperature 70% EtOH. Spin for 5 minutes. Remove as much of the EtOH as possible.

Spin tube in Speed-Vac the minimum time necessary to remove remaining alcohol. This should take about 2-5 minutes. (Alternatively, rinse with absolute EtOH and drain over paper towels for 15-30 minutes.)

Add 30 µL TE^{-4} to the tube and place at 56°C for ~1 hour to dissolve the DNA. Be sure that the liquid is distributed around the tube and subsequently spun to the bottom.

OPTIONAL EXTRA DIGESTION OF EPITHELIAL CELLS IN DIFFERENTIAL EXTRACTION

In protocol "B", insert at the end of Step 9, after microscopic examination of the washed sperm pellet:

Repeat the digestion by adding an additional 400µL of SEB and 10 µL of Pro K (20 mg/mL) to the tubes containing sperm pellets.

Incubate ~30 minutes. Microfuge 3-5 minutes and transfer all but ~50 µL of the supernatant (e. cell fraction "2") to a new tube. This e. cell fraction "2" should be reserved, but is likely to contain a mixture of e.cell and sperm DNA, so may not be useful for analysis.

Wash sperm pellet several times as described above, leaving 50 µL after the last wash.

Resuspend the sperm pellet in the 50 µL and repeat the microscopic examination to check for the presence of residual e. cells.

Continue the differential extraction protocol at step 10.

EXTRACTION OF SULFUR DYES FROM DNA ISOLATED FROM BIOLOGICAL MATERIALS ON DENIM

Bead Preparation (sufficient for ~3 samples):

1. Hydrate an ~100 µL equivalent of dry, thiopropyl-activated Sepharose 6B (Sigma T-8387 or equivalent) beads in 1 mL of sterile dH_2O for ~5 minutes at room temperature, mixing occasionally by inversion.

2. Microfuge ~2 minutes to pellet the beads and discard the supernatant. Wash three times with 1 mL of sterile dH_2O discarding the supernatant after each wash.

3. Add an equal volume of sterile dH_2O to the pelleted beads. Resuspend thoroughly and transfer a 200 µL aliquot, using a large bore pipette tip, into a separate microfuge tube for each sample. Microfuge the aliquot for 2 minutes and discard the supernatant. The volume of the hydrated beads should be ~100 μL.

Dye Extraction:

4. Add the ~400 µL aqueous phase (after the last organic extraction) to the pelleted beads (from step 3 above), incubate 15 minutes at room temperature, mixing occasionally by inversion. Note: If dye is being extracted from concentrated sample (i.e. post-Centricon), bring volume up to ~400 µL with TE^{-4}.

5. Microfuge for 5 minutes to pellet the beads. Transfer the supernatant to a clean tube making sure to avoid bead carry-over. Note: If the sample is to be Centricon filtered after dye extraction, the supernatant can be transferred directly to Centricon 100 previously loaded with ~1.5 mL TE^{-4}.

6. Wash the beads with an equal volume (~100 µL) of TE^{-4} for 5 minutes at room temperature, mixing occasionally by inversion. Microfuge for 5 minutes to pellet the beads and combine this bead-free supernatant with the supernatant from step 5.

7. If filtering by Centricon 100, continue from Section A, step 10.

Proceed to:
D. YIELD GEL FOR ASSESSING THE QUALITY AND QUANTITY OF DNA
or
E. SLOT BLOT FOR ASSESSING THE QUANTITY OF HUMAN DNA

CHELEX DIFFERENTIAL LYSIS AND EXTRACTION FROM VAGINAL SWABS AND SEMEN STAINS

1. Dissect swab or fabric into thirds (when possible) using a clean or disposable scalpel. Use a clean cutting surface for each different sample.

2. Pipette 1 mL of sterile distilled water into a sterile 1.5 mL microcentrifuge tube. Add swab or fabric cutting.

3. Incubate at room temperature for 30 minutes.

4. Vortex for 10 seconds, or twirl for 2 minutes with toothpick to agitate the cells off the substrate.

5. Punch holes in the microcentrifuge tube lid, then use a toothpick or sterile pipette tip to move the swab or fabric to the lid. Centrifuge the sample in a microcentrifuge for 1 minute at maximum speed (10,000 to 15,000 g).

6. It is advisable not to discard the substrate until microscopic analysis (Step 8) shows that the sample contains sperm. Store swab or fabric in a sterile tube. If sperm are not visible microscopically, re-extract substrate more vigorously (Step 2-4).

7. Without disturbing the pellet, remove and discard all but 50 μL (or twice the volume of the pellet, whichever is greater) of the supernatant using a sterile Pasteur pipette or the tip of a sterile 1 mL disposable pipette, and discard. Resuspend the pellet in the remaining 50 μL by stirring it with a sterile pipette tip. ***NOTE: This pellet contains epithelial cells and sperm cells, and is called the cell debris pellet.***

8. Remove about 3 μL of the resuspended sample and spot on a glass microscope slide for examination. Perform Christmas tree stain.

NOTE: If epithelial cells are detected, proceed with differential lysis procedure beginning with Step 9. If no epithelial cells are observed, the differential lysis procedure may be omitted, and the sample may be processed beginning with Step 17. If there are no sperm cells present, an option is to extract substrate more vigorously (Steps 2,3 and 4), or use Proteinase K to aid in the initial extraction. (See Below)

Optional treatment of substrate by Cal DOJ: To substrate, add 400 μL digest buffer and 10 μL of 20 mg/ml Proteinase K. Incubate 1 hour at 56°C. Loosen cells, spin using piggy-back arrangement to separate substrate from liquid and cells. Save the supernatant leaving 50 μL on the pellet. Examine 3μL as in step 8. This pellet gets combined with the original pellet at step 13.

9. Add 150 μL TE^{-4} to the cell debris pellet. Add 2 μL Proteinase K(10 mg/mL). Mix gently.

10. Incubate at 37°C for 1 hour to lyse the epithelial cells.

11. Spin in a microcentrifuge for 5 minutes at maximum speed (10,000 - 15,000 g). The resultant pellet is called the sperm pellet.

12. Add 150 μL of the supernatant to 50 μL of 20% Chelex in a fresh 1.5 mL microcentrifuge tube. SAVE FOR EPITHELIAL DNA ANALYSIS, BEGINNING WITH STEP 19.

13. Wash the pellet as follows: Resuspend the pellet in 0.5 mL Digest Buffer. Vortex briefly. Spin in a microcentrifuge for 5 minutes at maximum speed. Remove all but 50 μL of the supernatant and discard the supernatant.

14. Repeat wash Step 13 and additional 1 to 2 times. Additional wash steps are recommended when the ratio of sperm to epithelial cells is low.

15. Wash once with sterile distilled water as follows: Resuspend the pellet in 1 mL water. Vortex briefly. Spin in a microcentrifuge for 5 minutes at maximum speed. Remove all but 50 μL of the supernatant and discard the supernatant.

16. Resuspend the pellet by stirring with a sterile pipette tip. Remove 3 μL of the resuspended sample and spot on a glass microscope slide for examination. Perform Christmas tree stain.

17. Add 150 μL of 5% Chelex to the approximately 50 μL of resuspended sperm pellet. Add 2 μL of Proteinase K(10mg/mL) and 7 μL of 1 M DTT.

18. Vortex epithelial fractions and sperm samples for 10 seconds. Spin in a microcentrifuge for 3 seconds.

19. Incubate all fractions at 56°C for 1 hour.

20. Vortex tubes for 10 seconds.

21. Incubate samples in boiling water for 8 minutes.

22. Vortex tubes for 10 seconds.

23. Spin in a microcentrifuge for 3 minutes.

24. Estimate the amount of DNA in samples.

25. The samples are now ready for PCR amplification.

26. Store samples at 4°C or frozen. Prior to reuse of these samples for amplification, repeat Steps 22 and 23.

3/97
Adopted:

ORGANIC EXTRACTION OF WHOLE BLOOD/BLOODSTAINS

1. Pipette 0.5 mL Digest Buffer into a sterile 1.5 mL microcentrifuge tube.

   Add one of the following and mix.

   a. 10 to 50 μL whole blood
   b. 2 to 10 μL buffy coat
   c. 1 cm^2 blood stain

   NOTE: ***When working with bloodstains, an initial soak in 1 mL of deionized water is recommended.***

2. Add 15 μL of 10mg/mL Proteinase K solution, to a final concentration of about 0.3 mg/ml. Mix.

3. Incubate at 56°C for at least 1 hour. For evidence samples it is recommended that digestion continue for a minimum of 6 hours. Digestion may be performed overnight, but more than 24 hours is not recommended.

4. If a cloth or other substrate is present, punch several holes in the lid of the tube using a clean needle. Using a disposable pipette tip, or a fresh sterile toothpick, place the substrate in the lid and spin for 5 minutes to collect fluid remaining in the cutting. Remove the cutting, and place a new cap on the tube.

5. Add 0.5 mL buffered phenol-chloroform solution to the 0.5 mL lysed and digested cells.

6. Vortex for 15 seconds. Spin in a microcentrifuge for 5 minutes. Repeat steps 5 and 6 as necessary; aqueous phase should be clear and the interface clean. For each repeat, transfer upper layer to fresh tube.

7. (Optional) Use a sterile pipette tip to transfer the **upper aqueous** phase to a fresh sterile 1.5 mL microcentrifuge tube. Add 0.5 mL Chloroform. Vortex 10 to 15 seconds. Spin in a microcentrifuge for 5 minutes.

8. Transfer the **upper aqueous** phase to the upper chamber of Centricon tube.

9. Bring final volume to the 2 ml mark on the tube with TE^{-4}.

10. Centrifuge tubes at 1000 x g for 20 minutes in the Hermle Centrifuge.

11. Discard the liquid in the lower chamber; add 2 ml TE^{-4} to the upper chamber of the Centricon device.

12. Centrifuge as in Step 10.

13. Repeat TE^{-4} washes (Steps 9-12) two times, for a total of 3 washes. After the last wash cycle, collect the approximately 40μL concentrated DNA sample (as per Centricon instructions) by inverting the upper reservoir into the provided retentate cup and centrifuging at 500 x g for 2 minutes to transfer concentrate into cup. Label the retentate cup. Transfer to 1.5 ml Eppendorf tube estimating volume as you transfer. Should be 15-20μL. (Use 5μL pipette, count as you transfer.) The sample is now ready for amplification. Store the sample at 2 to 8°C or freeze at -20° until ready to perform PCR.

3/97
Adopted:

CHELEX EXTRACTION FROM WHOLE BLOOD/BLOODSTAINS

1. Add 3 μL whole blood or a bloodstain approximately 3 mm by 3 mm to a sterile 1.5 mL microcentrifuge tube. Pipette 1 mL sterile distilled water (or PBS) into the tube. Vortex 2 seconds.

2. Incubate at room temperature for 15-30 minutes. Mix occasionally by inversion or Vortex. Vortex 5 seconds.

3. Spin in a microcentrifuge for 3 minutes at maximum speed (10,000-15,000 g).

4. Without disturbing the pellet, carefully remove and discard the supernatant leaving enough behind to cover the pellet without disturbing it. If the sample is a bloodstain, leave the fabric substrate in the tube with the pellet.

5. Add 200 μL 5% Chelex. (Use P-1000 pipette, use tip to stir Chelex before pipetting).

6. Incubate at 56°C for 30 minutes.

7. Vortex at high speed 5 to 10 seconds.

8. Incubate in a boiling water bath for 8 minutes.

9. Vortex at high speed for 5 to 10 seconds.

10. Spin in a microcentrifuge for 3 minutes at maximum speed (10,000-15,000 g).

11. Quantitate the DNA (in the supernatant).

12. The sample is now ready for the PCR amplification process. Take supernatant, avoiding Chelex beads in bottom.

13. Store the remainder of the supernatant at 2-8°C or frozen. To re-use, thaw and repeat Steps 9 and 10.

3/97
Adopted:

PCR SETUP -- PM DQA1

Turn on the thermal cycler

1. Make appropriate dilutions of samples to total 20 uμL using sterile water. (Sample prep: Chelex ext: vortex and centrifuge 3 min. at max).

2. Remove tubes containing 40 μL of reaction mixture from main serology refrigerator. SPIN TO REMOVE CONDENSATION.

3. Label these tubes with sample numbers plus amplification $\oplus$ and $\ominus$ controls.

4. Add 40 μL of primer reagent (already containing 4% BSA) to the reaction mixture tubes, pipetting against the side to minimize mixing.

5. At this time, you have 20 minutes to complete the sample setup for amplification.

6. Add two drops of mineral oil to the tubes, cap each tube loosely.

7. Add 20 μL of sample, 20 μL of DNA for $\oplus$ amp control, and 20 μL of sterile H_2O for neg. Amp control to the appropriately labeled tubes. (BELOW MINERAL OIL LAYER)

8. Place tubes into cardboard tray, and take into product room for amplification.

9. Place samples into wells, checking that lids are tightly on tubes. Use worksheet for documentation. Push "FILE 40" then "ENTER" and then push "START". Watch the first cycle to ensure instrument is properly functioning. The amplification takes approximately 2.5 hours. When the process is completed, the samples will be held at 4 degrees until they are removed.

Adopted: 5/9-1

QUANTIBLOT HUMAN DNA QUANTITATION KIT

This kit is available through Perkin-Elmer (part no. N808-0114). The procedure is based on the hybridization of a biotinylated oligonucleotide probe to DNA samples immobilized on a nylon membrane. The probe included in this kit is complementary to a primate specific alpha satellite DNA sequence at the locus D17Z1.

A. PREPARATION OF DNA STANDARDS:

1. Label seven 0.5 mL autoclaved GeneAmp PCR Reaction Tubes A through G.

2. Vortex the DNA Standard A to mix it thoroughly.

3. Transfer 120 uL of DNA Standard A into the tube labeled A.

4. Aliquot 60 uL of TE^{-4} Buffer into each of the six remaining tubes labeled B through G.

5. Add 60 uL of DNA Standard A (tube A) to the 60 uL of TE^{-4} Buffer in tube B. Vortex to mix thoroughly.

6. Add 60 uL of diluted DNA Standard B (tube B) to the 60 uL of TE^{-4} Buffer in tube C. Vortex to mix thoroughly.

7. Add 60 uL of diluted DNA Standard C (tube C) to the 60 uL of TE^{-4} Buffer in tube D. Vortex to mix thoroughly.

8. Continue the serial dilution through tube G.

The concentrations of human DNA is listed below:

DNA STANDARD	CONCENTRATION (ng/uL)	QUANTITY DNA/5uL (ng)
A	2	10
B	1	5
C	0.5	2.5
D	0.25	1.25
E	0.125	0.625
F	0.0625	0.3125
G	0.03125	0.15625

Each hybridization reaction must include the following ten control samples: seven DNA Standards, the two DNA Calibrators and one blank (spotting solution only). DNA Calibrators are provided as an internal control for DNA standard performance.

3/97
Adopted:

B. SLOT BLOTTING/IMMOBILIZATION OF DNA

1. Determine the number of samples to be analyzed including the seven Human DNA Standards (A through G), the DNA Calibrators 1 and 2 (provided in Kit), and the one blank (Spotting solution only). Aliquot 150 uL of Spotting solution into a new 0.5 mL GeneAmp PCR Reaction Tube for each sample.

2. Label seven of the tubes containing 150 uL Spotting Solution as follows: A,B,C,D,E,F and G and label two of the tubes containing 150 uL of Spotting Solution as follows: DNA Calibrator 1 and DNA Calibrator 2. Label one tube "blank."

3. Vortex the seven DNA standards and the two DNA Calibrators. Add 5 uL of each solution to the corresponding labeled tube containing 150 uL of Spotting Solution.

4. Add 1 to 5 uL of each test sample DNA to the remaining tubes containing 150 uL of Spotting Solution.

5. Cut a piece of Biodyne B membrane 11.0 x 7.9 cm. Cut a small notch in the upper right corner of the membrane to mark orientation. Place the membrane in approximately 50 mL of Pre-Wetting Solution. Incubate at room temperature for 1 to 30 minutes.

6. Place the membrane on the gasket of the slot blot apparatus, then place the top plate of the slot blot apparatus on top of the membrane. Turn on the vacuum source. Turn off the sample vacuum and turn on the clamp vacuum. Push down on the top to ensure a tight seal.

7. Pipet each sample (approximately 155 uL) into a different well of the slot blot apparatus. Slowly dispense each sample directly into the center of each well of the slot blot apparatus ensuring that the pipet tip is approximately 5 mm above the membrane.

8. After all samples have been pipetted into the wells of the slot blot apparatus, slowly turn on the sample vacuum. Leave the sample vacuum on until all of the samples have been drawn through the membrane (approximately 30 seconds). Inspect each well that contains a sample for a uniform blue band. Turn off the sample vacuum and clamp vacuum.

9. Disassemble the slot blot apparatus and remove the membrane. Proceed without delay to hybridization.

C. DNA HYBRIDIZATION

Warm the hybridization solution and the wash solution to between 37°C and 50°C in the water bath. All solids must be in solution before use. Mix well. Turn on rotating water bath, set to 50°C.

1. **Pre-hybridization:** Transfer the membrane to 100 mL of pre-warmed Hybridization Solution. Add 5 mL of 30% H_2O_2. Place lid on the container. Use a weight to keep tray from floating in the water bath. Rotate in a 50°C (+/-1°) water bath (50 to 60 rpm) for 15 minutes (+/-2 minutes). Pour off the solution.

2. **Hybridization:** Add 30 mL of Hybridization Solution to the tray containing the membrane. Tilt the tray to one side and add 20 uL of QuantiBlot D17Z1 Probe to the Hybridization Solution. Place lid on the tray. Rotate in a 50°C(+/-1°) water bath (50 to 60 rpm) for 20 minutes (+/- 2 minutes). Pour off solution.

3. Rinse membrane briefly in approximately 100 mL of pre-warmed Wash Solution by rocking the tray for several seconds. Pour off the solution.

4. **Stringent Wash/Conjugation:** Add 30 mL of the pre-warmed Wash Solution to the Hybridization Tray. Tilt the tray to one side and add 180 uL Enzyme Conjugate: HRP-SA to the 30 mL of Wash Solution. Place lid on tray. Rotate in a 50°C(+/- 1°) water bath (50 to 60 rpm) for 10 minutes (+/- 1 minute). Pour off the solution.

5. Rinse the membrane thoroughly for 1 minute in approximately 100 mL of pre-warmed Wash Solution by rocking the tray or rotating it on an orbital shaker (100-125 rpm) at room temperature. Pour off the solution. Rinse again for 1 minute. Pour off the solution.

6. Wash the membrane by adding approximately 100 mL of pre-warmed Wash Solution to the tray. Place the lid on the tray. Rotate at room temperature on an orbital shaker (100 to 125 rpm) for 15 minutes. Pour off the solution.

7. Rinse the membrane briefly in 100 mL of Citrate Buffer by rocking the tray. Pour off the solution.

D. DETECTION STEPS

1. Prepare the Color Development Solution no more than 10 minutes before use. Add the following reagents in the order listed to a glass flask and mix thoroughly by swirling. Do not vortex. To 30 mL of Citrate Buffer add 1.5 mL of Chromogen: TMB Solution and 30 uL of 3% H_2O_2 (or 3 uL of 30% H_2O_2).

2. Add Color Development Solution to the membrane in the tray. Cover the tray with lid to protect the membrane from strong light.

3. Shake at room temperature on an orbital shaker (50 to 60 rpm) for 20 to 30 minutes.

4. Remove tray from shaker, pour off liquid.

5. Stop the color development by washing in deionized water (100 mL). Shake for 5 to 10 minutes (50 to 60 rpm) with the lid on the tray. Repeat for a total of three washes.

6. Photograph the membrane when it is wet.

Adopted:

PM + DQA1 PCR AMPLIFICATION PROTOCOL

This analysis is performed using the Amplitype ® PM + DQA1 Amplification and Typing Kit from Perkin-Elmer. Using a dedicated pipetter and working in the biological hood, put aliquots of 40µL of Reaction Mix from the kit into GeneAmp® tubes. Store in the refrigerator.

(Turn on the Thermal Cycler. Work in the biological hood to set up the amplification tubes.)

1. Make appropriate dilutions of samples to total 20 µL using sterile water. (Sample prep: for Chelex extract vortex and centrifuge 3 min. at max g). The samples will typically contain 1-10 ng of DNA.

2. Remove tubes containing 40 µL of reaction mixture from the refrigerator. Pulse spin to ensure all liquid contents in bottom of tube.

3. Label these tubes with sample numbers plus amplification ⊕ and ⊖ controls.

4. Prepare master mix for all tubes plus one :
   40 µL of primer reagent
   0.4 µL of 4 % BSA
   Add 40 µL of this master-mix to each reaction mixture tube, pipetting against the side to minimize mixing.

5. At this time, the amplification set-up must be completed within 20 minutes.

6. Add two drops of mineral oil to the tubes, cap each tube loosely.

7. Add 20 µL of each sample, 20 µL of kit DNA std. for ⊕ amp control, and 20 µL of sterile H_2O for ⊖ amp control to the appropriately labeled tubes. (BELOW MINERAL OIL)

8. Place tubes into cardboard tray, and take into product room for amplification.

9. Place samples into wells, checking that lids are tightly on tubes. Use worksheet for documentation. Start the amplification program: Push "FILE 40" then "ENTER" and then push "START". Watch the first cycle to ensure instrument is properly functioning. The recommended amplification conditions for this kit are 32 cycles as follows:
   94°C denaturation for 1 minute
   60°C annealing for 30 seconds
   72 °C extension for 30 seconds
   After the last cycle, extend at 72°C for 7 minutes, then hold at 4°C

The amplification takes approximately 2.5 hours. When the process is completed, the samples will be held at 4 degrees until they are removed.

Adopted: 10/97

PRODUCT GEL PROTOCOL

1) Remove 5 µL amplified DNA and combine with 2µL loading buffer. The loading buffer is placed into the tube first. Mix the sample and loading buffer with the pipet tip.

2) Add 5µL of 100ng/µL GIBCO BRL 123 ladder to 2µL of loading buffer. (To prepare a 100ng/µL ladder, dilute the 1ng/µL GIBCO BRL product 10µL + 90µL DI H_2O).

3) Prepare a 4% gel

a)Follow directions in reagent preparation book to prepare 4% Nu Sieve agarose gel in 1X TBE buffer. Add EtBr: 5 µL of 10 mg/mL per 100 mL of buffer. Agarose is dispensed into tubes containing 30-35ml each (one tube is used for each product gel run).
b)Melt agarose in microwave on defrost for 2-3 min., checking frequently.
c)Pour a 0.45 cm. thick gel with 8 tooth comb at one end. Allow to solidify about ½ hr.

4) Pour enough .5X TBE mixed with ethidium bromide into the tank to cover gel to about 2 mm, typically about 250 mL. (50µL of 10mg/ml ethidium bromide in 1L of 0.5X TBE).

5) Load the 123 bp ladder and the samples from step 1.

6) Set the voltage at 115v and run for 45-60 minutes or until the dye has gone 2 cm. Run towards the anode (red).

7) Photograph the gel. (Six bands should be present in the control DNA lane and in samples in which the DNA was amplified sucessfully. If all six bands are present, proceed to DNA hybridization.)

Adopted: 11/97

PRODUCT GEL

1) Remove 5μl amplified DNA and combine with 2μl loading buffer. The loading buffer is placed into the tube first. Mix the sample and loading buffer with the pipet tip.

2) Add 5μl of the 100ng/μl GIBCO BRL 123 ladder to the 2μl of loading buffer. (To prepare a 100ng/μl ladder, dilute the 1ng/μl GIBCO BRL product 10μl + 90μl DI H_2O).

3) Prepare a gel

   a) Follow directions in reagent preparation book to prepare product gel. Agarose dispensed into tubes containing a 30-35ml each (one tube is used for each product gel run).
   b) Heat on defrost for 2-3 min. in microwave, checking frequently.
   c) Pour a 0.45 cm. thick gel with 8 tooth comb at one end. Allow to solidify about ½ hr.

4) Place enough .5X TBE mixed with ethidium bromide into the tank to cover gel to about 2 mm (50μl of 10mg/ml ethidium bromide in 1L of 0.5X TBE). Use 250mL.

5) Load the 123 bp ladder in first well and then the samples. 7μl or 7.5μl on P20.

6) Set the voltage at 115v and run for 45-60 minutes or until dye has gone 2 cm. (Alternative parameters from Washoe Co.: 135v for 30 min.) Run towards red - origin on right.

7) Photograph the gel. (Six bands should be present in the control DNA lane and in samples in which the DNA was amplified sucessfully. If all six bands are present, proceed to DNA hybridization.) Suggested settings: 100% UV lamp intensity; f 5.6, 1/8 sec

Check mfg. handbook from kit for specific instructions on preparing reagents, etc.

5/97

Adopted:

D. YIELD GEL FOR ASSESSING THE QUALITY AND QUANTITY OF DNA

A yield gel may be used by itself or in concert with other methods in order to assess the quality and quantity of DNA. DNA at this stage has been through a microfiltration device such as a Centricon 100 or precipitated by EtOH and is in a volume of ~30 µL.

1. Remove 2-3 µL of the DNA and combine with 1 µL bromophenol blue (BϕB) loading buffer solution. Or if using a commercial yield gel set of standards, dilute according to package insert.

2. Preparation of test gel: The size used and number of origins depends on the number of specimens that one needs to test. Refer to Appendix B for the appropriate volume of agarose to achieve the appropriate gel thickness for each size tray.

All gels use 1% LE agarose in 1X TBE buffer supplemented with 0.5 µg/mL ethidium bromide (5 µL of 10 mg/mL EtBr per 100 mL of buffer). Alternatively, EtBr may be omitted and the gel stained after the run in a staining tray in the same concentration of EtBr.

Prepare the appropriate volume of 1X TBE buffer.
Weigh out the appropriate amount of LE agarose into a flask or bottle.
Add the 1X TBE.
Heat to dissolve agarose.
Add EtBr (if using).
Place an appropriate size comb into the gel tray.
When the temperature is ~60°C, pour agarose into gel tray. Let stand until solid.

3. Pour 1X TBE buffer into electrophoresis tank. (This buffer does not contain EtBr).

4. Place the gel into the tank with the well comb at the cathodic end. Enough buffer should be present to cover the gel to a depth of ~3 mm (refer to Appendix B). Remove the comb.

5. Each DNA sample or standard mixed with loading solution is pipetted into a separate well with the gel submerged. Well #1 is defined as the well at the upper left side of the gel. Standards to be included once on every origin include 150-300 ng λ*Hind* III (previously heated to 65°C for 5 minutes) and 6 µL each of a range of quantitation standards typically spanning 250-6 ng.

6. Run at constant voltage of 100 volts. When the blue tracking dye has moved ~2 cm from the origin, the run can be stopped.

7. Take a photograph of the gel on a UV transilluminator.

8. From the photograph, visually assess the quality and quantity of DNA in test specimens by comparison with the DNA standards.

PRODUCT GEL PROTOCOL

1) Remove 5 µL amplified DNA and combine with 2µL loading buffer. Mix the sample and loading buffer with the pipet tip.

2) Add 5µL of 100ng/µL GIBCO BRL 123 ladder to 2µL of loading buffer. (To prepare a 100ng/µL ladder, dilute the 1µg/µL GIBCO BRL ladder 10µL + 90µL DI H_2O).

3) Prepare a 4% gel

a)Prepare 4% NuSieve 3:1 agarose gel in 1X TBE buffer.
b)Heat to dissolve agarose in microwave on defrost for 2-3 min., checking frequently.
Add EtBr: 5 µL of 10 mg/mL per 100 mL of buffer.
c)Pour a 0.45 cm. thick gel with 8 tooth comb at one end. Allow to solidify about ½ hr.

4) Remove the comb and pour enough 0.5X TBE into the tank to cover the gel to about 2 mm, typically about 250 mL.

5) Load the 123 bp ladder and the samples from step 1.

6) Set the voltage at 115v and run for 45-60 minutes or until the dye has gone at least 2 cm. Run towards the anode (red).

7) Photograph the gel. (Six bands should be present in the control DNA lane and in samples in which the DNA was amplified sucessfully. If all six bands are present, proceed to DNA hybridization.)

Adopted: 11/97

E. SLOT BLOT FOR ASSESSING THE QUANTITY OF HUMAN DNA USING QUANTITBLOT KIT

PREPARATION OF DNA STANDARDS:

1. Label seven 0.5 mL autoclaved microcentrifuge tubes A through G.

2. Vortex the DNA Standard A to mix it thoroughly, then spin down all fluid.

3. Transfer 20 µL of DNA Standard A into the tube labeled A.

4. Aliquot 10 µL of TE^{-4} Buffer into each of the six remaining tubes labeled B through G.

5. Add 10 µL of DNA Standard A (tube A) to the 10 µL of TE^{-4} Buffer in tube B. Mix thoroughly.

6. Add 10 µL of diluted DNA Standard B (tube B) to the 10 µL of TE^{-4} Buffer in tube C. Mix thoroughly.

7. Continue the serial dilution through tube G.

8. Dilute the K562 human cell line standard using TE^{-4} to 100 ng/µL for storage as a stock solution. For immediate use, dilute in two steps to 0.2 ng/µL (e.g. 5:100, then 4:100)

The concentrations of human DNA are listed below:

DNA STANDARD	CONCENTRATION (ng/µL)	QUANTITY DNA/5µL (ng)
A	2	10
B	1	5
C	0.5	2.5
D	0.25	1.25
E	0.12	0.6
F	0.06	0.3
G	0.03	0.15
K562 cell line	0.2	1.0

Each hybridization reaction must include the following eleven control samples: seven DNA Standards, the two DNA Calibrators, a dilution of K562 cell line standard and one blank (spotting solution only). DNA Calibrators are provided as an internal control for the dilution series of the DNA standard. The K562 solution is an external control on the Quantiblot kit.

SLOT BLOTTING/IMMOBILIZATION OF DNA

1. Determine the number of samples to be analyzed including the seven human DNA standards (A through G), the DNA calibrators 1 and 2 (provided in kit), the K562 standard, and the one blank (spotting solution only). Label sterile 0.5mL centrifuge tubes for these eight standards, two calibrators and blank. Label additional tubes for the samples to be quantitated.

2. To every tube add 150 µL of spotting solution.

3. Mix the seven DNA standards and the two DNA Calibrators. Spin. Add 5 µL of each solution to the corresponding labeled tube containing 150 µL of spotting solution. Add 5 µL of K562 solution to its tube.

4. Add 1 to 5 µL of each test sample DNA to the remaining tubes containing 150 µL of spotting solution.

5. Cut a piece of Biodyne B membrane 11.0 x 7.9 cm. Cut a small notch in the upper right corner of the membrane to mark orientation. Place the membrane in approximately 50 mL of pre-wetting solution. Incubate at room temperature for 1 to 30 minutes.

6. Place the membrane on the gasket of the slot blot apparatus, then place the top plate of the slot blot apparatus on top of the membrane. Turn on the vacuum source. Turn off the sample vacuum and turn on the clamp vacuum. Push down on the top to ensure a tight seal.

7. Pipet each sample (approximately 155 µL) into a different well of the slot blot apparatus. Slowly dispense each sample directly into the center of each well of the slot blot apparatus ensuring that the pipet tip is approximately 5 mm above the membrane.

8. After all samples have been pipetted into the wells of the slot blot apparatus, slowly turn on the sample vacuum. Leave the sample vacuum on until all of the samples have been drawn through the membrane (approximately 30 seconds). Inspect each well that contains a sample for a uniform blue band. Turn off the sample vacuum and clamp vacuum.

9. Disassemble the slot blot apparatus and remove the membrane. Proceed without delay to hybridization.

DNA HYBRIDIZATION

Warm the hybridization solution and the wash solution to between 37°C and 50°C in the water bath. All solids must be in solution before use. Mix well. Turn on rotating water bath, set to 50°C.

1. **Pre-hybridization:** Transfer the membrane to 100 mL of pre-warmed hybridization solution. Add 5 mL of 30% H_2O_2. Place lid on the container. Use a weight to keep tray from floating in the water bath. Rotate in a 50°C (+/-1°) water bath (50 to 60 rpm) for 15 minutes (+/-2 minutes). Pour off the solution.

2. **Hybridization:** Add 30 mL of hybridization solution to the tray containing the membrane. Tilt the tray to one side and add 20 µL of QuantiBlot D17Z1 Probe to the hybridization solution. Place lid on the tray. Rotate in a 50°C(+/-1°) water bath (50 to 60 rpm) for 20 minutes (+/- 2 minutes). Pour off solution.

3. Rinse membrane briefly in approximately 100 mL of pre-warmed wash solution by rocking the tray for several seconds. Pour off the solution.

4. **Stringent Wash/Conjugation:** Add 30 mL of the pre-warmed wash solution to the hybridization tray. Tilt the tray to one side and add 180 µL Enzyme Conjugate: HRP-SA to the 30 mL of wash solution. Place lid on tray. Rotate in a 50°C(+/- 1°) water bath (50 to 60 rpm) for 10 minutes (+/- 1 minute). Pour off the solution.

5. Rinse the membrane thoroughly for 1 minute in approximately 100 mL of pre-warmed wash solution by rocking the tray or rotating it on an orbital shaker (100-125 rpm) at room temperature. Pour off the solution. Rinse again for 1 minute. Pour off the solution.

6. Wash the membrane by adding approximately 100 mL of pre-warmed wash solution to the tray. Place the lid on the tray. Rotate at room temperature on an orbital shaker (100 to 125 rpm) for 15 minutes. Pour off the solution.

7. Rinse the membrane briefly in 100 mL of citrate buffer by rocking the tray. Pour off the solution.

D. DETECTION STEPS

1. Prepare the color development solution no more than 10 minutes before use. Add the following reagents in the order listed to a glass flask and mix thoroughly by swirling. Do not vortex. To 30 mL of citrate buffer add 1.5 mL of Chromogen: TMB Solution and 30 µL of 3% H_2O_2 (or 3 µL of 30% H_2O_2).

2. Add color development solution to the membrane in the tray. Cover the tray with lid to protect the membrane from strong light.

3. Shake at room temperature on an orbital shaker (50 to 60 rpm) for 20 to 30 minutes.

4. Remove tray from shaker, pour off liquid.

5. Stop the color development by washing in deionized water (100 mL). Shake for 5 to 10 minutes (50 to 60 rpm) with the lid on the tray. Repeat for a total of three washes.

6. Photograph the membrane when it is wet.

DNA HYBRIDIZATION: PM + DQA1

The AmpliType PM + DQA1 DNA Hybridization process involves 3 steps performed sequentially as follows: 1) hybridization of amplified DNA to DNA probe strips, 2) binding of HRP-SA to hybridized PCR products, and 3) stringent wash to remove non-specifically bound PCR products. Color development follows the stringent wash step.

N.B. Ensure that 5ul of 200mM disodium EDTA is added to each tube before hybridization step.

Perform these steps in the Amplified DNA Work Areas using reagents and pipets dedicated for use in this area. Do not transport any of these items to the Extraction/PCR Set Up Work Area.

1. Heat a rotating water bath to 55° C. The water level should be 0.5 to 1 cm above the platform. The water level should not be higher than 1 cm, as higher levels may result in water splashing into the wells. The temperature should not go below 54°C or above 56° C. It is essential to check the temperature before each hybridization is performed. Set the bath for 50 to 70 rpm.

2. Warm (37° - 55°C) the Hybridization solution and the Wash Solution (if necessary). All solids must be in solution before use.

3. Allow the tube strips to come to room temperature before opening it. Remove the strips using flat forceps. With a waterproof marking pen, label each probe strip with the designation of the corresponding amplified DNA.

4. Place the tubes of amplified DNA in the thermal cycler after it reaches 95° C. Denature the DNA by incubation for 3 to 10 minutes. Keep each tube at 95° C until use.

5. Add 3 mL of pre-warmed Hybridization Solution to each well at the labeled end of each strip while tilted. **DO NOT WET THE REMAINDER OF THE STRIP.**

6. Perform the following steps for each tube of amplified DNA:

   a. Remove the tube from the 95° C block, open using a tube opener.
   b. Withdraw 20 uL of amplified DNA and immediately (within 20 seconds) add it below the surface of the hybridization solution in the well of the corresponding probe strip.
   c. Add only one sample of amplified DNA at a time. Replace in 95° C block.
   d. If using 2 strips with the same amplified DNA sample, replace the tube in the 95° block after taking the first 20 μL.

   The remaining amplified DNA samples can be stored at 2° to 8° C for two months or at -20° C for 6 months.

7. Transfer the tray to the 55° C water bath. Place a weight on the cover. Rotate the water bath 50 to 70 rpm for 15 minutes (+\- 2 minutes). Record temperature and time of hybe step.

8. Approximately 5 minutes before the end of the hybridization step, prepare the Enzyme Conjugate Solution as follows:
Number of strips X 3.3 mL Hybridization Soln = Volume of Hybridization Soln

Number of strips X 27 uL Enzyme Conjugate = Volume of Enzyme Conjugate

Mix the solution thoroughly and ensure that the solids remain in solution. Leave at room temperature until ready to use. Remove the tray from the water bath, leave the water bath covered and rotating.

9. Aspirate the contents of each well from the labeled end of the strip. Wipe the lid with a clean lab wipe.

10. Dispense 5 mL of pre-warmed Wash Solution into each well. Rock the tray for several seconds with the lid on, then aspirate each well.

11. Dispense 3 mL of the Enzyme Conjugate Solution prepared previously into each well. Transfer to the 55° C water bath and rotate at 50 to 70 rpm for 5 minutes (+\- 1 minute).

12. Aspirate the contents of each well. Wipe the lid with a clean lab wipe. Keep water bath rotating and covered.

13. Dispense 5 mL of pre-warmed Wash Solution into each well. Rinse by rocking the tray for several seconds with the lid on then aspirate the solution from each well.

14. Dispense 5 mL of pre-warmed Wash Solution into each well. Place in the 55° C water bath and rotate at 50 to 70 rpm for 12 minutes (+\1 1 minute). Record the time and temperature of this stringent wash step.

15. After incubation, aspirate the contents out of each well. Wipe the lid with a clean lab wipe.

16. Dispense 5 mL of Wash Solution into each well. Cover and rock the tray gently for several seconds.

17. Aspirate the contents of each well. Wipe the lid with a clean lab wipe.

5/97
Adopted:

COLOR DEVELOPMENT OF STRIPS: PM + DQA1

1. Dispense 5 mL of Citrate buffer into each well. Place on an orbital shaker set at approximately 50 rpm at room temperature for 5 minutes.

2. During this wash step prepare the Color Development Solution as follows:

   Number of strips X 5 mL Citrate Buffer = Volume of Citrate Buffer

   Number of strips X 0.5 uL 30% H_2O_2 = Volume of Hydrogen Peroxide

   Number of strips X 0.25 mL Chromogen = Volume of Chromogen

3. Remove the tray from the orbital shaker and aspirate the contents of each well. Add 5 mL of freshly prepared Color Development Solution to each well.

NOTE: PLACE THE CLEAR PLASTIC LID ON THE TRAY AND COVER THE LID WITH ALUMINUM FOIL DURING STEPS 4, 5 & 6.

4. Develop the strips at room temperature by rotating on an orbital shaker set at approximately 50 rpm for 20 to 30 minutes. Develop until the "C" or "S" dot is visible. Individual strips may develop before 20 minutes and be stopped individually (Step 5).

5. Aspirate the contents of each well. Dispense approximately 5 mL DI water into each well. Place tray on orbital shaker at about 50 rpm for 5 to 10 minutes. Pour off water.

6. Repeat step 5 at least two times for a minimum of three DI water washes. Turn off aspirator, release VAC.

7. Record the pattern of the blue dots from each wet strip prior to photography.

NOTE: KEEP STRIPS WET THROUGHOUT PHOTOGRAPHY.

Adopted: 5/97

STRIP PHOTOS

Use the ½ :1 frame lens assembly (the one with a square frame and 3 tripod feet). If necessary, plug the flash unit power cord into the side of the lens assembly, and attach the upper section (the part with the fat handle and film holder). Use the twist button marked "lock/open" to unlock the upper section and to secure it if you have to move it between lens units.

Make sure type 665 film is loaded. The white pull tab is marked.

Confirm settings of f22 and 125th of a second. Filter is already in place.

Turn flash power unit on, setting control to "+ 1/2".

Arrange strips on black background, blot off any standing water. Click shutter, let develop 30 sec, examine print. If OK*, peel away extra paper from negative and drop it in water. Coat positive print, making sure coating coverage is complete.

*If not OK, adjust for more or less light with flash unit.

Process negative as soon as feasible.

PM + DQA1 PCR AMPLIFICATION PROTOCOL

This analysis is performed using the Amplitype® PM + DQA1 Amplification and Typing Kit from Perkin-Elmer. Using a dedicated pipetter and working in the biological hood, put 40 µL aliquots of Reaction Mix from the kit into sterile GeneAmp® tubes. Store in the refrigerator.

(Turn on the Thermal Cycler. Work in the biological hood to set up the amplification tubes.)

1. Make appropriate dilutions of samples to total 20 µL using sterile water. (Sample prep: for Chelex extract, vortex and centrifuge 3 min. at max. g.) The samples will typically contain 1-10 ng of DNA.

2. Remove tubes containing 40 µL of reaction mixture from the refrigerator. Pulse spin to ensure all liquid contents are in the bottom of the tube.

3. Label these tubes with sample numbers plus amplification ⊕ and ⊖ controls.

4. Prepare a master mix for all tubes plus one:
   40 µL of primer reagent
   0.4 µL of 4 % BSA
   Add 40 µL of the master-mix to each reaction mixture tube.
   At this time, the amplification must be completed within 20 minutes.

5. Add two drops of mineral oil to the tubes, cap each tube loosely.

6. Add 20 µL of sample, 20 µL of kit DNA std. for ⊕ amp control, and 20 µL of sterile H_2O for ⊖ amp control to the appropriately labeled tubes. (BELOW MINERAL OIL LAYER)

7. Place tubes into cardboard tray, and take into product room for amplification.

8. Place samples into wells, checking that lids are tightly on tubes. Use worksheet for documentation. Start the amplification program: Push "FILE 40" then "ENTER" and then push "START". Watch the first cycle to ensure instrument is properly functioning. The amplification conditions for this kit are 32 cycles as follows:
   94°C denaturation for 1 minute
   60°C annealing for 30 seconds
   72 °C extension for 30 seconds
   After the last cycle, extend at 72°C for 7 minutes, then hold at 4 °C
   The amplification takes approximately 2.5 hours. When the process is completed, the program will hold the samples at 4°C indefinitely.

Adopted: 11/97

APPENDIX B

EXTRACTION REAGENTS

5% Chelex

5 gram Chelex
100 mL sterile H_2O

***0.5 M EDTA**

186.1 g $Na_2EDTA{\cdot}2H_2O$ dissolve in 800 mL of DI H_20
adjust pH to 8.0 with 15-20 g NaOH pellets
adjust volume to 1.0 L

Ethidium Bromide (EtBr)

Purchase pre-prepared (10 mg/mL solution)

***5M NaCl**

292.2 g NaCl
dissolve in DI H20 and adjust volume to 1.0 L

Nuclear Fast Red Stain

5 g aluminum sulfate
0.1 g Nuclear Fast Red
adjust volume with 100 mL hot dH_2O
Refrigerate overnight then filter

Phenol/chloroform/isoamyl alcohol (25/24/1)

Purchase pre-prepared

***PBS** (pH 7.4)

Purchase pre-prepared; prepare according to manufacturer's instructions if concentrated or pre-weighed, OR make according to the following:
8 g sodium chloride
0.2 g potassium chloride
1.44 g Na_2HPO_4, anhydrous
0.24 g KH_2PO_4, anhydrous
adjust pH to 7.4 with HCL
adjust volume to 1.0 L with DI H_2O

*** Solutions that must be autoclaved**

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Picroindigocarmine Stain

0.33 g indigocarmine
100 mL saturated Picric Acid solution
filter

Proteinase K (20 mg/mL)

500 mg proteinase K
dissolve in 25 mL DI H_2O
aliquot into convenient size volumes and freeze

20% SDS (200 g sodium dodecyl sulfate/ 1.0 L H_2O)

Purchase pre-prepared

Stain Extraction Buffer (10 mM Tris; 10 mM EDTA; 100 mM NaCl; 2% SDS)

10 mL of 1M Tris, pH 8.0
20 mL of 0.5M EDTA, pH 8.0
20 mL 5M NaCl
100 mL 20% SDS
adjust volume to 900 mL with DI H_2O
after autoclaving, add 100 mL 20% SDS

***1M Tris**, pH 7.5

121.1 g Tris base
dissolve in 800 mL DI H_2O
adjust pH to 7.5 with concentrated HCl
adjust volume to 1.0 L with DI H_2O

***1M Tris**, pH 8.0

121.1 g Tris base
dissolve in 800 mL DI H_2O
adjust pH to 8.0 with concentrated HCl
adjust volume to 1.0 with DI H_2O

***TE^{-4}** (10 mM Tris; pH 7.5; 0.1 mM EDTA)

10 mL 1 M Tris, pH 7.5
200 µL 0.5 M EDTA, pH 8.0
adjust to 1.0 L with DI H_2O

QUANTITATION REAGENTS (for yield gel and QuantiBlot ™ kit)

NOTE: Some reagents used for quantitation have been described in the previous section.

Citrate Buffer (0.1 M Sodium Citrate, pH 5.0)

Purchase pre-prepared concentrate then dilute according to manufacturer's directions OR make according to the following:
18.4 g trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)
dissolve in 800 mL DI H_2O
adjust pH to 5.0 (±0.2) by the addition of 6g citric acid monohydrate($C_6H_8O_7 \cdot H_20$)
adjust volume to 1.0 L with DI H_20
mix thoroughly

Hybridization Solution (5X SSPE; 0.5% w/v SDS)

250 mL
20X SSPE
25 mL 20% SDS
adjust to 1.0 L with 725 mL DI H_2O
mix thoroughly
NOTE: Hybridization solution solids must be in solution before use; warming may be required to dissolve solids completely. Preparation in a clear glass container is recommended to facilitate visual inspection for solids during warming.

5N NaOH

20 g NaOH pellets
dissolve in 60 mL of DI H_2O
adjust volume to 100 mL

Pre-wetting Solution (0.4N NaOH; 25 mM EDTA)

40 mL of 5N NaOH
25 mL of 0.5 M EDTA
add to 435 mL of DI H_2O
mix thoroughly

QuantiBlot Wash Solution (1.5X SSPE; 0.5% w/v SDS)

150 mL of 20X SSPE
50 mL of 20% SDS
add to 1,800 mL of DI H_20
mix thoroughly
NOTE: Wash solution solids must be in solution before use; warming may be required to dissolve solids completely. Preparation in a glass container is recommended to facilitate visual inspection for solids during warming.

Spotting Solution (0.4 N NaOH; 25 mM EDTA; 0.00008% Bromphenol Blue)

2 mL of 5 N NaOH
1.25 mL of 0.5 M EDTA
50 µL 0.04% Bromphenol Blue (provided in QuantiBlot ™ kit)
add to 22 mL DI H_20
mix thoroughly

20 X SSPE (3.6 M NaCl; 0.2 M NaH_2PO_4; 20 mM EDTA)

Purchase pre-prepared OR make according to the following:
210.4 g NaCl
27.6 g $NaH_2PO_4 \cdot H_20$
400 mL 0.5 M EDTA or 7.4 g of $Na_2EDTA \cdot 2H_20$
adjust pH to 7.4 with NaOH
adjust volume to 1.0 L with DI H_20

1X TBE (0.1 M Tris; 0.09M Boric Acid; 0.001M EDTA; pH 8.4)

Purchase pre-prepared concentrate then dilute according to the manufacturer's instructions

Yield Gel

add .25 g LE agarose to 25 mL of 1X TBE buffer
melt agarose
add 1.25µL of Ethidium Bromide (10 mg/mL)

ORDERING INFORMATION

CTS ARE NOT FOR USE IN DIAGNOSTIC PROCEDURES

In the United States:

For PCR Technical Support call toll-free
1-800-762-4001
FAX: **203-761-2542**

To order PCR consumables and reagents call toll-free
1-800-327-3002

To order PCR instruments or to reach your sales representative call toll-free
1-800-345-7547

For PCR product literature call toll-free
1-800-345-5224

In Europe:

For PCR Technical Support call
Langen, Germany at
49/6103/708301
FAX: **49/6103/708310**

Outside of the United States:

For the number of your local Perkin-Elmer sales representative call:

Australia	61 3 212 8585
Austria	431 1 602 31010
Belgium	32 2 725 9099
Bulgaria	359 2 59 1118
Canada	800 668 6913
Czechoslovakia	42 2 772507
Denmark	45 48 141381
Finland	358 0880 144
France	33 1 4990 1800
Germany	49 6150 1010
Hungary	36 1 251 1116
Italy	39 39 23 831
Japan	814 7380 8500
Latin America-Mexico	52 5 651 7077
Netherlands	31 1820 754 11
Norway	47 22 68 6565
Poland	48 22 33 0936
Portugal	351 1 796 2172
Spain	34 1 803 4210
Sweden	46 87 3300 10
Switzerland	41 42 657 777
United Kingdom	44 192 582 5650

GeneAmp® PCR Instrument Systems from Perkin-Elmer:

N801-0100 DNA Thermal Cycler 480, 120 V, 60 Hz
N801-0101 DNA Thermal Cycler 480, 240 V, 50 Hz
N801-0102 DNA Thermal Cycler 480, 100 V, 50/60 Hz

N801-0001 GeneAmp® PCR System 9600, 120 V, 60 Hz
N801-0002 GeneAmp® PCR System 9600, 240 V, 50 Hz
N801-0003 GeneAmp® PCR System 9600, 100 V, 50/60 Hz

PERKIN ELMER

Perkin-Elmer PCR reagents are developed and manufactured by Roche Molecular Systems, Inc., Branchburg, New Jersey, U.S.A.

Roche

Applied Biosystems Division
850 Lincoln Centre Drive
Foster City, CA 94404

BIO-114
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STORAGE AND STABILITY

1. Store all of the components of the AmpliType® PM PCR Amplification and Typing Kit and the AmpliType PM+DQA1 PCR Amplification and Typing Kit at 2° to 8°C. Isolate the kits from any sources of contaminating DNA, especially amplified PCR product.
2. Store the AmpliType DNA Probe Strips, protected from light (e.g., wrapped in foil), at 2° to 8°C. Store the DNA probe strips with the desiccant in the glass tube and ensure that the screw-cap is securely tightened.
3. Under these conditions, components of the kits are stable through the control date printed on the label.
4. The Chromogen Solution is stable for 6 months after its preparation when stored at 2° to 8°C.

INTRODUCTION

One of the most important developments in the field of human identity testing is the use of DNA typing to analyze biological evidence.[1,2] In particular, the powerful GeneAmp Polymerase Chain Reaction (PCR)[3] is used to analyze samples which cannot be typed by other methods, such as samples containing minute amounts of human DNA and very old and/or degraded DNA.[4-8] The AmpliType HLA DQα PCR Amplification and Typing Kit (AmpliType HLA DQα Kit) was the first PCR-based test applied to forensic casework analysis.[9-12] The AmpliType PM PCR Amplification and Typing Kit (AmpliType PM Kit, **Perkin-Elmer Part No. N808-0057**) was the second commercially available human identity testing product based on the reverse dot blot typing technology[13] for forensic casework analysis.[14-17] The AmpliType PM+DQA1 PCR Amplification and Typing Kit (AmpliType PM+DQA1 Kit, **Part No. N808-0094**) has been added to the product line to enable amplification and typing of all five of the PM loci and the HLA DQA1 locus from the same PCR amplification reaction.

The AmpliType PM Kit and AmpliType PM+DQA1 Kit are optimized for amplification in fixed-volume reactions using the Perkin-Elmer GeneAmp® PCR Instrument System and reaction tube combinations listed in **Table 1.** Typing of the DNA amplified by these kits is performed by hybridization of the amplified PCR products to DNA probe strips for the PM loci or the HLA DQA1 locus. Specifically hybridized amplified DNA is visualized upon enzymatic conversion of a soluble, colorless substrate to a blue-colored precipitate. Descriptions of the amplification and typing protocols for each of these kits are provided in the individual kit summaries below. Additional information can be found in the AmpliType User Guide.[18]

Table 1. Recommended GeneAmp® PCR Instrument System and Reaction Tube Combinations for Amplification.

Instrument	Reaction Tube	Perkin-Elmer Part Number
DNA Thermal Cycler 480	GeneAmp® Thin-Walled Reaction Tubes*	N801-0537
	GeneAmp Autoclaved Thin-Walled Reaction Tubes	N801-0611
	GeneAmp Thin-Walled Reaction Tube with Flat Cap*	N801-0737
GeneAmp PCR System 9600	MicroAmp® Reaction Tube with Cap*	N801-0540
	MicroAmp Autoclaved Reaction Tube with Cap	N801-0612

*Tubes should be autoclaved by the user prior to use in the amplification process.

AMPLITYPE® PM PCR AMPLIFICATION AND TYPING KIT DESCRIPTION

The AmpliType® PM PCR Amplification and Typing Kit (AmpliType PM Kit, **Part No. N808-0057**) includes PCR amplification reagents that direct the simultaneous amplification of specific regions of the following six genetic loci: Human Leukocyte Antigen (HLA) DQA1 (previously referred to as HLA DQα),[19] Low Density Lipoprotein Receptor (LDLR),[20] Glycophorin A (GYPA),[21] Hemoglobin G Gammaglobin (HBGG),[22] D7S8,[23] and Group Specific Component (GC).[24,25] The AmpliType PM Kit also contains detection reagents and DNA probe strips for typing LDLR, GYPA, HBGG, D7S8, and GC but not for typing HLA DQA1 (see AmpliType PM+DQA1 Kit description for information on typing all six loci).

While the AmpliType PM Kit uses the amplification and reverse dot blot typing technologies introduced in the original AmpliType HLA DQα Kit, minor changes to the amplification and typing protocols were made because of the simultaneous analysis of multiple genetic loci. AmpliType PM PCR amplification is performed in a fixed-volume reaction (100 µL total volume) instead of in the variable-volume reaction (102-140 µL total volume) used with the AmpliType HLA DQα Kit. The typing of DNA amplified with AmpliType PM reagents requires a **separate** step to bind the horseradish peroxidase-streptavidin (HRP-SA) enzyme conjugate to the biotinylated PCR product. HRP-SA Enzyme Conjugate is bound to PCR products that have hybridized to the probes only after the excess biotinylated primers and unbound PCR products are removed. When using DNA amplified with the original AmpliType HLA DQα reagents, hybridization of the HLA DQα PCR product to the probes and binding of the HRP-SA Enzyme Conjugate to the biotinylated PCR products are performed simultaneously. **PCR products amplified with the AmpliType PM PCR Reaction Mix and Primer Set should not be typed using the original AmpliType HLA DQα protocol because all dot intensities on the AmpliType PM DNA Probe Strip will be too light.**

...r minor modifications to the original AmpliType® HLA DQα typing protocol are: ...ne addition of disodium EDTA to the PCR product (see **Section 4.0**); 2) the reduction of the volume of the denatured PCR product that is added to the Hybridization Step from 35 µL to 20 µL (see **Section 8.8**); and 3) the reduction of the volume of the Wash Solution, the Citrate Buffer, and the Color Development Solution from 10 mL to 5 mL (see **Sections 8** and **9**).

The AmpliType HLA DQα DNA Probe Strips, supplied in the original AmpliType HLA DQα Kit, should not be used to obtain the HLA DQA1 type of samples amplified with the AmpliType PM PCR Reaction Mix and Primer Set because the "C" dot on these DNA probe strips may not be visible with weakly amplified samples. To obtain both the PM types and the HLA DQA1 type from samples amplified using the AmpliType PM reagents, the AmpliType PM+DQA1 PCR Amplification and Typing Kit should be used (see below).

AMPLITYPE PM+DQA1 PCR AMPLIFICATION AND TYPING KIT DESCRIPTION

The AmpliType PM+DQA1 PCR Amplification and Typing Kit (AmpliType PM+DQA1 Kit, **Part No. N808-0094**) contains all of the amplification and detection reagents for typing the five PM loci and the HLA DQA1 locus from a single aliquot of extracted DNA. PCR amplification is performed using the same amplification reagents that are included in the AmpliType PM Kit. These reagents direct the simultaneous amplification of the HLA DQA1, LDLR, GYPA, HBGG, D7S8, and GC loci. The AmpliType PM+DQA1 PCR setup and amplification protocols are identical to those provided in the AmpliType PM Kit. The AmpliType PM+DQA1 Kit also contains both the AmpliType PM and AmpliType HLA DQA1 DNA Probe Strips. The AmpliType PM DNA Probe Strips are identical to those included in the AmpliType PM Kit. The AmpliType HLA DQA1 DNA Probe Strips contain the nine probes found on the original AmpliType HLA DQα DNA Probe Strips that distinguish six common HLA DQA1 alleles (DQA1 1.1, 1.2, 1.3, 2, 3, 4).[19] The AmpliType HLA DQA1 DNA Probe Strips contain two additional probes that distinguish the DQA1 4.1 allele from the DQA1 4.2 and 4.3 alleles.[26] The AmpliType PM+DQA1 typing protocol is identical to the one used with the AmpliType PM Kit. **PCR products amplified with the AmpliType PM PCR Reaction Mix and Primer Set should not be typed using the original AmpliType HLA DQα protocol because all dot intensities on the AmpliType PM and AmpliType HLA DQA1 DNA Probe Strips will be too light.**

AMPLITYPE PM AND HLA DQA1 GENETIC MARKER CHARACTERISTICS AND POPULATION DATA

The chromosomal location, size of the amplified DNA product, number of alleles distinguished (**Table 2**), and allele frequencies (**Table 3**) for the HLA DQA1, LDLR, GYPA, HBGG, D7S8, and GC genetic markers are listed below. These allele frequencies were obtained by Roche Molecular Systems' scientists by typing unrelated U.S. Caucasian (n = 200), African American (n = 200), U.S. Hispanic (n = 200), and Japanese (n = 89) individuals.[27]

The currently available population data[15] suggest there are no significant deviations from Hardy-Weinberg equilibrium expectations for these six markers. Given the statistical independence of alleles at these loci (linkage equilibrium), the frequency of a multi-locus genotype can be estimated by multiplying the genotype frequencies at each locus.

Table 2. AmpliType® Genetic Marker Characteristics

	HLA DQA1	LDLR	GYPA	HBGG	D7S8	GC
Chromosomal location	6p21.3	19p13.1 - 13.3	4q28 - 31	11p15.5	7q22 - 31.1	4q11 - 13
PCR Product (bp)	239/242	214	190	172	151	138
Number of Alleles	7*	2	2**	3	2	3

*The HLA DQA1 4.2 and 4.3 alleles are detected but not distinguished from each other by the AmpliType HLA DQA1 DNA Probe Strip.

**The GYPA A and A' alleles are detected but not distinguished from each other by the AmpliType PM DNA Probe Strip.

Table 3. Allele Frequencies

Genetic Marker	Allele	U.S. Caucasian (n = 200)	African American (n = 200)	U.S. Hispanic (n = 200)	Japanese (n = 89)
HLA DQA1	1.1	0.158	0.125	0.105	0.084
	1.2	0.190	0.329	0.130	0.118
	1.3	0.073	0.058	0.053	0.236
	2	0.145	0.130	0.115	0.006
	3	0.192	0.090	0.218	0.444
	4.1	0.214	0.185	0.269	0.073
	4.2/4.3	0.028	0.083	0.110	0.039
LDLR	A	0.448	0.235	0.485	0.202
	B	0.552	0.765	0.515	0.798
GYPA	A	0.530	0.527	0.615	0.517
	B	0.470	0.473	0.385	0.483
HGBB	A	0.537	0.439	0.375	0.331
	B	0.450	0.228	0.580	0.669
	C	0.013	0.333	0.045	0.000
D7S8	A	0.610	0.655	0.622	0.612
	B	0.390	0.345	0.378	0.388
GC	A	0.275	0.090	0.203	0.287
	B	0.178	0.720	0.335	0.471
	C	0.547	0.190	0.462	0.242

The power of discrimination (P_d) values calculated from the combined frequencies for the five AmpliType® PM markers and for all six of the markers (including HLA DQA1) are listed in **Table 4.** The P_d value reflects the probability that two unrelated individuals will have different genotypes at one or more of the six loci.

Table 4. Power of Discrimination for AmpliType® Genetic Marker Systems.

Combined power of discrimination (Combined $P_d = 1 - [(P_i)_1(P_i)_2...(P_i)_n]$; where n is the number of combined markers and P_i is 1 - P_d for each number)				
	U.S. Caucasian	African American	U.S. Hispanic	Japanese
AmpliType® PM	0.9954	0.9948	0.9961	0.9938
AmpliType PM+DQA1	0.9998	0.9997	0.9998	0.9993

LIST OF KIT COMPONENTS

Caution: Reagents used for amplification must be isolated from any source of contaminating DNA, especially from previously amplified AmpliType PCR products.

Caution: Sodium azide is used as a preservative in several reagents. It can react with lead and copper plumbing to form explosive metal azides. On disposal of the reagent, flush with a large volume of water to prevent azide build-up.

AmpliType PM PCR Amplification and Typing Kit

Reagent	Volume	Description
AmpliType PM PCR Reaction Mix	2.4 mL	1 bottle of PCR Reaction Mix containing AmpliTaq® DNA Polymerase, $MgCl_2$, dATP, dGTP, dCTP, and dTTP, and 0.08% sodium azide in buffer and salt. **Store at 2° to 8°C.**
AmpliType PM Primer Set	1.2 mL/tube	2 tubes each containing twelve biotinylated primers and 0.05% sodium azide in buffer and salt. **Store at 2° to 8°C.**
Control DNA 1	0.2 mL	1 tube containing 100 ng/mL human genomic DNA and 0.05% sodium azide in buffer. AmpliType PM type: LDLR **BB**, GYPA **AB**, HBGG **AA**, D7S8 **AB**, GC **BB**. **Store at 2° to 8°C.**

AmpliType® PM PCR Amplification and Typing Kit (continued)

Reagent (continued)	Volume	Description
Mineral Oil	5 mL	Supplied in dropper bottle. **Do NOT expose to strong ultraviolet light. Store at 2° to 30°C.**
AmpliType® PM DNA Probe Strips	50 strips	The strips are provided in a screw-top tube with a packet of desiccant. **Store the strips at 2° to 8°C in the tightly capped tube with the desiccant and protected from light.**
Enzyme Conjugate: HRP-SA	2.0 mL	1 bottle containing Horseradish Peroxidase-Streptavidin (HRP-SA) Enzyme Conjugate supplied in buffer with preservative. **Store at 2° to 8°C.**
Chromogen: TMB	60 mg	1 bottle containing powdered 3,3',5,5'-tetramethylbenzidine (TMB). Dissolve Chromogen as instructed before use (see **Preparation of Reagents Supplied, Section 1.1**). **Store at 2° to 8°C.**
Package Insert		Containing the AmpliType PM PCR amplification and typing protocols.

AmpliType PM+DQA1 PCR Amplification and Typing Kit

Reagent	Volume	Description
AmpliType PM PCR Reaction Mix	2.4 mL	1 bottle of PCR Reaction Mix containing AmpliTaq® DNA Polymerase, $MgCl_2$, dATP, dGTP, dCTP, dTTP, and 0.08% sodium azide in buffer and salt. **Store at 2° to 8°C.**
AmpliType PM Primer Set	1.2 mL/tube	2 tubes each containing twelve biotinylated primers and 0.05% sodium azide in buffer and salt. **Store at 2° to 8°C.**

Reagent (continued)	Volume	Description
Control DNA 1	0.2 mL	1 tube containing 100 ng/mL human genomic DNA and 0.05% sodium azide in buffer. AmpliType® PM type: LDLR **BB**, GYPA **AB**, HBGG **AA**, D7S8 **AB**, GC **BB**. AmpliType HLA DQA1 type: **1.1, 4.1. Store at 2° to 8°C.**
Mineral Oil	5 mL	Supplied in dropper bottle. **Do NOT expose to strong ultraviolet light. Store at 2° to 30°C.**
AmpliType PM DNA Probe Strips	50 strips	The strips are provided in a screw-top tube with a packet of desiccant. **Store the strips at 2° to 8°C in the tightly capped tube with the desiccant and protected from light.**
AmpliType HLA DQA1 DNA Probe Strips	50 strips	The strips are provided in a screw-top tube with a packet of desiccant. **Store the strips at 2° to 8°C in the tightly capped tube with the desiccant and protected from light.**
Enzyme Conjugate: HRP-SA	2.0 mL/bottle	2 bottles each containing Horseradish Peroxidase-Streptavidin (HRP-SA) Enzyme Conjugate supplied in buffer with preservative. **Store at 2° to 8°C.**
Chromogen: TMB	60 mg/bottle	2 bottles each containing powdered 3,3',5,5'-tetramethylbenzidine (TMB). Dissolve Chromogen as instructed before use (see **Preparation of Reagents Supplied, Section 1.1**). **Store at 2° to 8°C.**
Package Insert		Containing the AmpliType PM+DQA1 PCR amplification and typing protocols.

MATERIALS REQUIRED BUT NOT SUPPLIED

The items listed are those required for PCR amplification, PCR product gel analysis, hybridization, and color development. This list does not include reagents or equipment required for DNA extraction. Many of the items listed below are available from major laboratory suppliers (MLS) such as Baxter Scientific Products (McGaw Park, IL), Fisher Scientific (Pittsburgh, PA), or VWR (So. Plainfield, NJ) unless otherwise noted. Equivalent sources may be acceptable where noted.

Reagents	Source
Use Reagent Grade unless otherwise noted.	
123 Base Pair Ladder	GIBCO BRL, Gaithersburg, MD (Cat. No. 15613-011).
Agarose: buy the following and mix according to protocol (**Section 6.2**):	
NuSieve® GTG® Agarose	FMC, Rockland, ME (Cat. No. 50082).
SeaKem® GTG Agarose	FMC (Cat. No. 50072).
Alcohol 95% ethanol or 70% Isopropanol	MLS
Boric acid	GIBCO BRL (Cat. No. 15583-016), Sigma, St. Louis, MO (Cat. No. B 7901), or equivalent.
Bromophenol blue	Sigma (Cat. No. B 5525) or equivalent.
Citric acid, monohydrate, granular	JT Baker, Phillipsburg, NJ (Cat. No. 0110-05) or equivalent.
Ethanol, 200 proof (100%) in glass container	Quantum Chemical Company, Cincinnati, OH (order by volume) or Gold Shield, Hayward, CA (Cat. No. EL200P24X1).
Ethidium bromide, 10 mg/mL	MLS
Ethylenediaminetetraacetic acid (EDTA), disodium salt, dihydrate	GIBCO BRL (Cat. No. 15575-012), Sigma (Cat. No. E 4884), or equivalent.
Glycerol	Sigma (Cat. No. G 5516) or equivalent.
Hydrochloric acid, concentrated	MLS
Hydrogen peroxide, 30%	Sigma (Cat. No. H 1009) or equivalent.
Sodium chloride	MLS
Sodium dodecyl sulfate (SDS), ultra pure electrophoresis grade	GIBCO BRL (Cat. No. 15525-025), Bio-Rad, Hercules, CA (Cat. No. 161-0302), or equivalent.
Sodium hydroxide (NaOH), 10 N	VWR, South Plainfield, NJ (JT5674-3) or equivalent.
Sodium hydroxide, pellets	MLS
Sodium phosphate, monobasic, monohydrate	Sigma (Cat. No. S 9638) or equivalent.
Trisodium citrate, dihydrate	Sigma (Cat. No. C 8532) or equivalent.
Tris base	GIBCO BRL (Cat. No. 15504-012), Sigma (Cat. No. T 8404), or equivalent.

Equipment	Source
AmpliType™ DNA Typing Tray	**Perkin-Elmer (Part No. N808-0065).**
Aspirator Apparatus (See **Reference 18** for further description)	**Perkin-Elmer (Part No. N930-2697)** or SERI, Richmond, CA (Cat. No. E081).
Balance, accurate to 1.0 mg	MLS
Dispensing Repipet® (5 or 10 mL)	Baxter Scientific Products, McGaw Park, IL (Cat. No. P4965-5 or P4965-10) or equivalent.
Electrophoresis Power Supply	Bio-Rad [Cat. No. 165-4761 (110V) or Cat. No. 165-4762 (220V)] or equivalent.
Filters, cellulose nitrate membrane, 0.2 and 0.45 µm Nalgene®	Fisher, Pittsburgh, PA (0.2 µm filter Cat. No. 127-0020 or 0.45 µm filter Cat. No. 127-0045) or equivalent.
Filter forceps, non-pointed tips	Fisher (Cat. No. 09-753-50).
Filter, orange, Wratten 22 (for use with Polaroid® camera)	Kodak, New Haven, CT (Cat. No. 149 5671).
Freezer (non frost-free, -20°C)	MLS
Horizontal minigel electrophoresis apparatus with Gel combs (teflon, 1.2 mm thick)	Aquebogue Machines, Aquebogue, NY (model 750 Mini Horizontal gel apparatus)
Laboratory glassware	MLS
Laminar flow/biological/biosafety cabinet or designated clean area	Labconco Corp., Kansas City, MO or MLS.
Microcentrifuge†	MLS
Microplate, 96-wells	VWR, So. Plainfield, NJ (Cat. No. 62406-220) or equivalent.
Micro Tube Opener	Robbins Scientific Corp., Sunnyvale, CA (Cat. No. 1012-20-0) or equivalent.
Microtube racks, 1.5 mL†	MLS
Microwave	MLS
pH meter compatible with Tris solutions (need electrodes without silver chloride) and reference buffers	Corning or equivalent.
Pipettors adjustable to deliver 2 - 20 µL†, 10 - 100 µL†, 50 - 200 µL†, and 100 - 1000 µL†	Rainin, Woburn, MA (P20, P100, P200, P1000) or equivalent.
Pipettor, positive displacement repeat pipettor	Fisher (Cat. No. 21-380-8) or equivalent.
Pipette bulbs†	MLS
Polaroid camera and film [either black and white film (type 55, 57, or 667) or color film (type 59 or 559)]	Fotodyne, Hartland, WI, Sigma, or any camera supply store.
Refrigerator (2° - 8°C)†	MLS

† One is needed for pre-PCR sample preparation; a second is needed for post-PCR sample handling.

Equipment (continued)	Source
Shaker, variable speed, orbital platform (needed for color development step)	Bellco, Vineland, NJ [Model No. 7744-00110 (110V) or 7744-00220 (220V)] or Lab-Line, Melrose Park, IL (Model No. 3520 for RPM X 100).
Stir plate and stir bar	MLS
Thermometer, Total Immersion	Recommend: Cole Parmer, Niles, IL (Cat. No. H-08001-34, Graduated to 0.1°C range 49° to 57°C, specify NIST traceable when ordering). Alternative: Scientific Products (Cat. No. T2099-5, range 50° to 80°C).
Timer, 60 minute (± 1 minute)†	MLS
UV transilluminator (and UV eye protection)	MLS
Vacuum pump or Vacuum source	Fisher (Cat. No. 13-875-220) or equivalent.
Vortexer†	MLS
Water bath with cover, rotating, Bellco Hot Shaker Plus (needed for hybridization steps).	Bellco [Model No. 7746-22110 (110V) or 7746-22220 (220V)].
Water bath, stationary **or** incubator (37° to 55°C)	MLS
Weight, approximately 1 kg (e.g., lead ring)	VWR (Cat. No. 29700-048), Cole Parmer (Cat. No. G-06137-06), or equivalent.
PCR Amplification Equipment	Source
Choose one of the following GeneAmp® PCR Instrument Systems:	
DNA Thermal Cycler 480 and equipment:	
DNA Thermal Cycler 480	**Perkin-Elmer [Part No. N801-0100 (120V/60Hz), N801-0101 (230V-240V/50Hz), or N801-0102 (100V/50Hz-60Hz)].**
GeneAmp Thin-Walled Reaction Tubes	**Perkin-Elmer (Part No. N801-0537).**
GeneAmp Autoclaved Thin-Walled Reaction Tubes	**Perkin-Elmer (Part No. N801-0611).**
GeneAmp Thin-Walled Reaction Tubes with Flat Cap	**Perkin-Elmer (Part No. N801-0737).**
Temperature Verification System (for the DNA Thermal Cycler 480)	**Perkin-Elmer (Part No. N801-0434).**
OR	
GeneAmp PCR System 9600 and equipment:	
GeneAmp PCR System 9600	**Perkin-Elmer [Part No. N801-0001 (120V/60Hz), N801-0002 (230V-240V/50Hz), or N801-0003 (100V/50Hz-60Hz)].**

† One is needed for pre-PCR sample preparation; a second is needed for post-PCR sample handling.

PCR Amplification Equipment (continued)	Source
MicroAmp® Autoclaved Reaction Tube with Cap	**Perkin-Elmer (Part No. N801-0612).**
MicroAmp Reaction Tube with Cap	**Perkin-Elmer (Part No. N801-0540).**
MicroAmp Base	**Perkin-Elmer (Part No. N801-0531).**
MicroAmp Tray/Retainer Set	**Perkin-Elmer (Part No. N801-0530).**
Temperature Verification System (for the GeneAmp® PCR System 9600)	**Perkin-Elmer (Part No. N801-0435).**

Supplies	Source
Aluminum foil†	MLS
Calculator†	MLS
Chromatography paper, Whatman® grade 3MM, pure cellulose chromatography and blotting paper	VWR (Cat. No. 21427) or equivalent.
Deionized, glass-distilled, or ultrafiltered water, referred to as DI H_2O in this package insert	
Gloves,† disposable	MLS
Lab bench surface protector	MLS
Lab coat†	MLS
Lab wipes†	MLS
Parafilm®†	MLS
Pen (for labeling probe strips)	Provided with AmpliType™ DNA Typing Trays.
Permanent Ink Marker† (for marking tubes)	Sanford (Sharpie® ultra fine point) or equivalent.
Pipet†, disposable serological (5 - 10 mL)	MLS
Pipet tips† for adjustable pipettors with hydrophobic filter plugged tips (sterile)	GIBCO BRL or equivalent.
Pipet CombiTips® (0.5 mL, sterile)	Fisher (Cat. No. 21-380-8A) or equivalent.
Protective eyewear†	MLS
Protective mask†	MLS
Scissors†	MLS

† One is needed for pre-PCR sample preparation; a second is needed for post-PCR sample handling.

1.0 REAGENT PREPARATION

1.1 PREPARATION OF REAGENTS SUPPLIED

1.1.1 Chromogen: TMB Solution.

Warning: Chromogen: TMB is an irritant. Avoid skin contact and inhalation. Wear lab coat, gloves, mask, and protective eyewear when handling Chromogen: TMB.

Bring the bottle of Chromogen: TMB (TMB) to room temperature (15° to 30°C). Before opening the bottle, tap it on the lab bench to shake the TMB to the bottom of the bottle. Remove the stopper carefully to prevent loss of the powder. Slowly add 30 mL of room temperature reagent grade 100% ethanol to the bottle. **Do NOT use ethanol that has been stored in a metal container. Do NOT use 95% ethanol or other alcohols.** Recap the bottle. Seal the stopper with Parafilm®. Secure the bottle to an orbital shaker and shake in an upright position for 2 hours or until completely dissolved. Protect from heavy metal contamination, especially iron oxide (rust). **Store bottle at 2° to 8°C.** Under these conditions the Chromogen Solution is stable for six months after preparation.

1.1.2 AmpliType® PM PCR Reaction Mix.

This step must be performed either in a biological hood or in a room free from amplified DNA.

All of the AmpliType® PM PCR Reaction Mix should be aliquotted at the same time to decrease the possibility of contamination through repeated opening of the AmpliType PM PCR Reaction Mix bottle.

Upon first use of the AmpliType PM Kit or AmpliType PM+DQA1 Kit, remove the bottle of AmpliType PM PCR Reaction Mix and carefully aliquot 40 µL into autoclaved tubes (GeneAmp® Thin-Walled Reaction Tubes for the DNA Thermal Cycler 480 or MicroAmp® Reaction Tube with Cap if using the GeneAmp PCR System 9600) using a dedicated positive displacement repipettor or a pipettor with hydrophobic filter plugged tips. Ensure that the tubes are capped tightly. Place PCR Reaction Mix tubes in a rack not used for DNA preparation or amplified DNA handling. **Store tubes separated from any source of DNA at 2° to 8°C.**

1.2 PREPARATION OF REAGENTS NOT SUPPLIED

Use reagent grade chemicals unless otherwise noted. Prepare all solutions using deionized, glass-distilled, or ultrafiltered water (identified below as DI H_2O). Wear gloves and follow safety recommendations provided by the manufacturer for handling chemicals. Comply with any and all laws, regulations, or orders with respect to the disposal of any hazardous or toxic chemical, material, substance, or waste. **Store all solutions at room temperature (15° to 30°C) unless otherwise noted.** Reagents are prepared as follows:

1.2.1 Citrate buffer [0.1 M sodium citrate, pH 5.0 (1 L)].

Dissolve 18.4 g of trisodium citrate, dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in 800 mL of DI H_2O. Adjust the pH to 5.0 (± 0.2) by addition of approximately 6 g of citric acid, monohydrate ($C_6H_8O_7 \cdot H_2O$). Adjust to a final volume of 1 L using DI H_2O and mix thoroughly. Autoclave the solution.

1.2.2 20X SSPE buffer [3.6 M NaCl, 200 mM $NaH_2PO_4 \cdot H_2O$, 20 mM EDTA, pH 7.4 (1 L)].

Warning: EDTA is an irritant. Wear lab coat, gloves, mask, and protective eyewear when handling EDTA.

Warning: NaOH is corrosive and toxic. Wear lab coat, gloves, and protective eyewear. Use caution when handling NaOH.

Dissolve 7.4 g of disodium ethylenediaminetetraacetic acid, dihydrate ($Na_2EDTA \cdot 2H_2O$) in 800 mL of DI H_2O. Adjust the pH to 6.0 (± 0.2) with 10 N sodium

hydroxide (NaOH). Add 210 g of sodium chloride (NaCl) and 27.6 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$). Adjust the pH to 7.4 (± 0.2) with 10 N NaOH (approximately 10 mL). Adjust to a final volume of 1 L using DI H_2O and mix thoroughly.

1.2.3 20% (w/v) Sodium Dodecyl Sulfate SDS (1 L).

Warning: SDS is an irritant. Avoid skin contact and inhalation. Wear lab coat, gloves, mask, and protective eyewear when handling SDS.

Slowly dissolve 200 g of electrophoresis-grade (ultra pure) SDS in 800 mL of DI H_2O. Warming (e.g., in a 37°C water bath) may be required to dissolve solids completely. Adjust to a final volume of 1 L using DI H_2O and mix thoroughly.

1.2.4 Hybridization Solution [5X SSPE, 0.5% w/v SDS (1 L)].

Add 250 mL of 20X SSPE (**Section 1.2.2**) and 25 mL of 20% w/v SDS (**Section 1.2.3**) to 725 mL of DI H_2O and mix thoroughly. Hybridization Solution solids must be in solution and the solution must be well mixed before use; warming (e.g., to between 37° to 55°C in a stationary water bath or an incubator) may be required to dissolve solids completely.

1.2.5 Wash Solution [2.5X SSPE, 0.1% w/v SDS (2 L)].

Add 250 mL of 20X SSPE (**Section 1.2.2**) and 10 mL 20% w/v SDS (**Section 1.2.3**) to 1,740 mL of DI H_2O and mix thoroughly. Wash Solution solids must be in solution and the solution must be well mixed before use; warming (e.g., to between 37° to 55°C in a stationary water bath or an incubator) may be required to dissolve solids completely.

1.2.6 3% Hydrogen peroxide (1 mL).

Add 100 µL of 30% H_2O_2 to 900 µL of DI H_2O and vortex to mix. **Protect from light. Store at 2° to 8°C.** In practice, 3% hydrogen peroxide has a shelf life of approximately 6 months. Alternatively, one-tenth the volume of 30% H_2O_2 can be used directly in the Color Development Step (see **Section 8**).

1.2.7 1 M Tris-HCl, pH 8.0 (1 L).

Warning: Hydrochloric acid (HCl) causes severe burns and is irritating to the eyes. When preparing this reagent, use a fume hood and avoid inhalation and contact with the skin. Wear a lab coat, gloves, and protective eyewear when handling.

Dissolve 121.1 g of Tris base in 800 mL of DI H_2O. Adjust to pH 8.0 (± 0.2) at room temperature by adding approximately 45 mL of concentrated HCl. Adjust the final volume to 1 L with DI H_2O and mix thoroughly. The solution should be autoclaved or filtered through a 0.2 µm Nalgene® filter.

1.2.8 0.5 M EDTA (1L).

Warning: EDTA is an irritant. Wear lab coat, gloves, mask, and protective eyewear when handling EDTA.

Warning: NaOH is corrosive and toxic. Wear lab coat, gloves, and protective eyewear. Use caution when handling. NaOH, when combined with water, results in an exothermic reaction. Dissolve the NaOH pellets in the water

gradually with 3 or 4 additions. Cover and allow the NaOH to dissolve completely and cool between each addition.

Add 186.1 g of disodium ethylenediaminetetraacetic acid dihydrate ($Na_2EDTA \cdot 2H_2O$) to 800 mL of DI H_2O. Stir vigorously on a magnetic stirrer. To dissolve the EDTA powder, adjust the pH to 8.0 (±0.2) by adding approximately 20 g of NaOH pellets. Check the pH and add 10 N NaOH solution if further pH adjustment is needed (for small pH adjustments, pellet addition is not advised). Bring volume up to 1 L with DI H_2O and mix thoroughly. The solution should be autoclaved or filtered through a 0.2 µm Nalgene® filter.

1.2.9 200 mM EDTA (10 mL).

Add 4.0 mL of 0.5 M disodium EDTA (**Section 1.2.8**) to 6.0 mL of DI H_2O and mix thoroughly. The solution should be autoclaved or filtered through a 0.2 µM Nalgene filter. The solution may be aliquotted, 300 µL/tube, into microcentrifuge tubes. **Store at 2° to 8°C.**

1.2.10 10X TBE [890 mM Tris, 890 mM boric acid, 20 mM EDTA (1 L)].

To 40 mL of 0.5 M EDTA, pH 8.0 (**Section 1.2.8**), add approximately 900 mL of DI H_2O. Add 108 g of Tris base and 55 g of boric acid to the diluted EDTA solution. Stir vigorously on a magnetic stirrer. Adjust the volume to 1 L with DI H_2O and mix thoroughly. Filter the mixture using a 0.2 or 0.45 µm Nalgene filter unit to remove particulate matter and prevent the formation of a precipitate. Storage in a glass container is recommended to facilitate visual inspection for precipitates. If a white precipitate is noted in the 10X TBE, the buffer should be discarded and remade.

1.2.11 Running Buffer, 0.5X TBE [44.5 mM Tris-borate, 1 mM EDTA (1 L)].

Add 50 mL of 10X TBE (**Section 1.2.10**) to 950 mL of DI H_2O and mix thoroughly.

1.2.12 Gel Loading Buffer [0.2% bromophenol blue, 50% glycerol, 20 mM Tris (pH 8.0), 2.5 mM EDTA (50.0 mL)].

Dissolve 0.1 g of bromophenol blue in 23.75 mL of DI H_2O. Allow 2 hours for bromophenol blue to go into solution; stir as necessary. Add 1 mL of 1 M Tris-HCl, pH 8.0 (**Section 1.2.7**), 0.25 mL of 0.5 M disodium EDTA (**Section 1.2.8**), and 25 mL of glycerol. Mix thoroughly. **Store at 2° to 8°C.**

Note: Sections 2.0 and 3.0 should be read before preparing AmpliType® PM PCR amplification reactions.

2.0 PRECAUTIONS

The sensitivity of PCR allows minute quantities of DNA to be typed using the AmpliType PCR Amplification and Typing Kits. Contamination of the samples by handling or by exposure to any other source of human DNA is an important concern. **Precautions should be taken to prevent the following three types of contamination: 1) "carryover" of PCR product from one amplification to the next; 2) cross-contamination between extracted DNA samples; 3) contamination with exogenous human genomic DNA.**[18,28] At a minimum, the pre-PCR amplification area must be separated from the post-PCR amplification area.

3.0 PCR AMPLIFICATION PROTOCOLS

The following protocols detail the PCR amplification and typing procedures specific for the AmpliType® PCR Amplification and Typing Kits. Protocols and precautions for DNA extraction are described elsewhere.[18]

Note: Where the use of DI H_2O is indicated below, either deionized, glass-distilled, or ultrafiltered water may be used.

A dedicated area, such as a biological hood or a separate room, should be used for preparing AmpliType PCR amplification reactions whenever possible. All equipment and supplies used to prepare amplification reactions should be kept in this dedicated "clean" area at all times. **Do not use these items to handle amplified DNA. To prevent contamination from other potential sources of genomic DNA, pipet tips plugged with hydrophobic filters must be used.** Trace amounts of amplified DNA, if carried over into other samples before amplification, can lead to results that are not interpretable. **Do NOT bring amplified DNA or equipment and supplies used to handle amplified DNA into the designated clean area.**

Note: Wear clean disposable laboratory gloves while preparing samples for PCR amplification. Change gloves frequently or whenever there is a chance they have been contaminated with DNA.

3.1 Prior to preparing the DNA test samples and PCR amplification reactions, turn on the GeneAmp® PCR Instrument System and program the appropriate PCR profile times and temperatures provided in **Table 5**. Refer to the appropriate GeneAmp PCR Instrument System Users Manual for additional information on programming the instruments. If using a stored file, step through the program to verify the parameters.

If using a GeneAmp PCR System 9600, follow **Sections 3.1.1** and **3.1.2** before proceeding to **Section 3.2**. If using the DNA Thermal Cycler 480, proceed to **Section 3.2**.

3.1.1 AmpliType PM and AmpliType PM+DQA1 amplification, using the GeneAmp PCR System 9600, requires that the block be preheated to 95°C. To preheat the block of the GeneAmp PCR System 9600 instrument, use the following CYCL program flowchart (**Figure 1, page 18**) to bring the block to 95°C. The settings in the Default Mode column will appear automatically when stepping through the CYCL program. The default settings that must be changed to preheat the block are indicated in **bold-type**. The new settings are indicated in **bold-type** in the Program Setting column. Link this preheat program to the beginning of a separate program for 32 cycles using the parameters stated in **Table 5** for the GeneAmp PCR System 9600.

3.1.2 Run the program that was created to preheat the block. The block must be at 95°C before the tubes are placed in the block. It is not necessary to close the heated cover during this step.

3.2 Prepare the DNA test samples for addition to the PCR amplification reactions. It is recommended that the DNA sample be quantitated with the QuantiBlot Human DNA Quantitation Kit (**Part No. N808-0114**). The final DNA concentration should be in the range of 0.1 to 0.5 ng/µL so that 2 to 10 ng of DNA will be added to the PCR reaction in a volume of 20 µL. If the sample DNA concentration is greater than 0.5 ng/µL, dilute a portion of it with autoclaved DI H_2O. For most samples, 2 to 5 ng is sufficient. If the DNA sample contains degraded DNA, it may be appropriate to add >10 ng of DNA.

Table 5. Perkin-Elmer GeneAmp® PCR Instrument Systems PCR Profile Times and Temperatures

GeneAmp® PCR Instrument System	Tube		Time and Temperature									
	Tube Type	Total Reaction Volume in µL/Tube	AmpliType® Kit	Initial Step	Each of 32 Cycles			Final Step				
					Denature	Anneal	Extend					
DNA Thermal Cycler 480	GeneAmp Thin-Walled Reaction Tubes	100 µL plus 2 drops Mineral Oil	PM PM+DQA1	None	STEP-CYCLE			TIME DELAY				
					60 sec 94°C	30 sec 60°C	30 sec 72°C	7 min 72°C				
GeneAmp PCR System 9600	MicroAmp® Reaction Tube with Cap	100 µL (no Mineral Oil added)	PM PM+DQA1	Preheat Block* to 95°C (see 3.1.1)	CYCL			HOLD				
					30 sec 95°C	30 sec 63°C	30 sec 72°C	10 min 72°C				

Note: These programs may be stored as User Files for later use (see Users Manual DNA Thermal Cycler 480 or GeneAmp PCR System 9600 Users Manual).

As with any calibrated piece of laboratory equipment, the GeneAmp PCR Instrument System should have a documented temperature verification test performed on a regular basis [e.g., monthly using the Temperature Verification System (Part No. N808-0434) for the DNA Thermal Cycler 480 or the GeneAmp PCR System 9600 Temperature Verification System (Part No. N808-0435) for the GeneAmp PCR System 9600].

***Preheat Block: The sample block is heated to 95°C prior to placing the tubes into the instrument and the start of amplification cycles (see Section 3.1.1).**

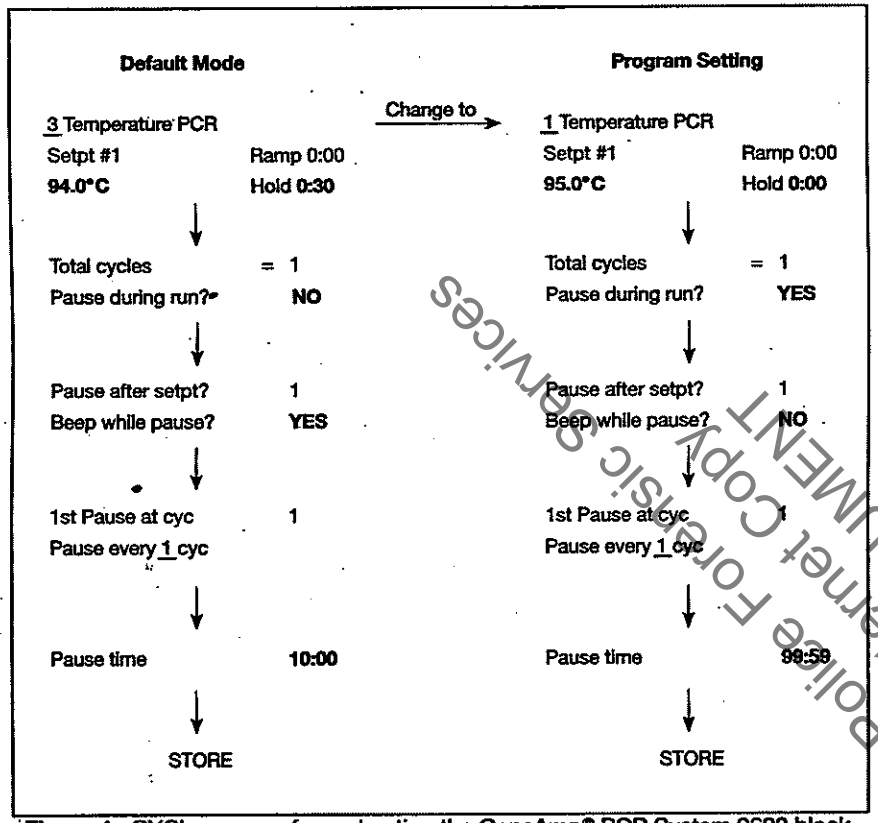

Figure 1. CYCL program for preheating the GeneAmp® PCR System 9600 block.

3.3 Determine the number of samples to be amplified, including positive and negative controls. The Control DNA 1 provided in the kits should be amplified each time the kits are used and run on each PCR product gel (**Sections 5.0** and **6.0**) as a positive control. A negative control consisting of 20 µL of autoclaved DI H_2O in place of the DNA sample should also be included with each set of amplification reactions.

3.4 Transfer the PCR amplification reagents to the designated clean area. Place the required number of reaction tubes containing 40 µL of aliquotted AmpliType® PM PCR Reaction Mix in a rack or MicroAmp® Base not used for the preparation of DNA or the handling of amplified DNA. Use the MicroAmp Base when the AmpliType PM PCR Reaction Mix has been aliquotted into MicroAmp Reaction Tubes. Label the reaction tubes. **Do not label the top of the MicroAmp Reaction Tubes because the heated cover may remove the ink.**

3.5 Ensure that the solution is at the bottom of each tube. Open the caps with a clean Micro Tube Opener (this de-capping tool should not have been used on tubes containing amplified or extracted DNA). **Avoid touching the inside surface of the tube caps.**

3.6 Pipet 40 µL of the AmpliType® PM Primer Set into each tube, including ...ntrol tubes, using a pipettor with hydrophobic filter plugged tips. Pipet carefully against the side of the tube to minimize mixing and to avoid splashing the solution. **It is important to begin the cycling process within 20 minutes after the addition of the AmpliType PM Primer Set to minimize the formation of primer-dimer[29] and other non-specific PCR products.**

3.7 If using the GeneAmp® PCR System 9600, cap each tube loosely and proceed to **Section 3.8.** If using the DNA Thermal Cycler 480, carefully add 2 drops of Mineral Oil from the dropper bottle provided in the kits to all tubes, including the control tubes, before proceeding to **Section 3.8. Be careful NOT to touch the reaction tubes with the dropper.** Cap each tube loosely. **Do NOT vortex, mix, or spin.**

Note: Each AmpliType PM and AmpliType PM+DQA1 PCR amplification is performed in a final volume of 100 µL. The volume allocated for sample addition is 20 µL.

3.8 For each of the following additions, complete the processing of each tube before proceeding to the next tube. **To prevent contamination, no more than one tube should be open at a time.** Use a new, autoclaved pipet tip for each addition. Open the tube and carefully add 20 µL of extracted sample, Control DNA 1, or autoclaved DI H_2O (negative control). If using the DNA Thermal Cycler 480, carefully insert the pipet tip through the mineral oil layer. Discard the pipet tip and re-cap the tube before proceeding to the next sample. **Do NOT vortex, mix, or spin.** Prepare tubes as follows:

3.8.1 DNA Test Sample tubes. Add 20 µL of sample DNA prepared in **Section 3.2** to each labelled DNA test sample tube.

3.8.2 Positive Control tube. Vortex the Control DNA 1 tube and spin briefly in a microcentrifuge before each use to remove any liquid from the cap. Add 20 µL of the 100 ng/mL (2 ng) Control DNA 1 to the labelled positive control tube.

3.8.3 Negative Control tube. Add 20 µL of autoclaved DI H_2O to the labelled negative control tube.

3.9 As soon as all samples have been added, place the tubes into the appropriate GeneAmp PCR Instrument System. If using MicroAmp® Reaction Tubes with Caps, place a MicroAmp Tray (not a MicroAmp Base) in the GeneAmp PCR System 9600 block before inserting the tubes. The position of each tube in the block should be recorded. **Use caution when handling tubes since the GeneAmp PCR System 9600 block has been preheated to 95°C.**

3.9.1. If using the GeneAmp PCR System 9600, proceed to **Section 3.9.2.** If using the DNA Thermal Cycler 480, push the tubes down completely into the block and start the 32 cycle amplification file. Verify the cycling parameters by monitoring the first cycle. Check the tubes after the first cycle to ensure they are all still seated tightly in the block. Proceed to **Section 4.0.**

3.9.2 Position and tighten the GeneAmp PCR System 9600 heated cover over the tubes. Immediately press the "run" key to end the preheat program and start the 32 cycle amplification program. Verify the cycling parameters by monitoring the first cycle. Proceed to **Section 4.0.**

4.0 PREPARATION OF PCR PRODUCT FOR DETECTION

After the PCR amplification process, remove the tubes from the GeneAmp® PCR Instrument System. Prior to DNA hybridization (**Section 8.0**), open the tubes one at a time and add 5 µL of 200 mM disodium EDTA (**Section 1.2.9**). Use a new pipet tip for each addition. If using the DNA Thermal Cycler 480, carefully insert the pipet tip through the mineral oil layer. Discard the pipet tip and re-cap the tube before proceeding to the next tube. It may be convenient to remove a 5 µL aliquot for gel electrophoresis (see **Section 7.0**) before recapping the tube.

Note: EDTA must be added prior to heat denaturation of the samples but may be added before or after the aliquot for gel electrophoresis has been removed.

Samples are now ready for analysis by gel electrophoresis, DNA hybridization, and color development. **Amplified samples containing 9.5 mM EDTA may be stored at 2° to 8°C for 2 months or at -20°C for 6 months.** The continued acceptable performance of these samples beyond these periods may vary with the sample. **Store amplified DNA samples separate from all PCR amplification reagents, extracted DNA samples, and casework samples.**

5.0 VERIFICATION OF PCR AMPLIFICATION

It is recommended that the presence and size of the PCR products generated in the AmpliType® PM reactions be verified by agarose gel electrophoresis. This step should be performed **prior** to denaturing the samples for DNA hybridization to ensure sharp product bands on the gel. Alternatively, a portion of the amplified sample can be set aside before proceeding to the typing step. **Do NOT heat denature PCR products prior to gel electrophoresis; additional bands may appear on the gel and the product bands may smear.**

Optimal gel electrophoresis results have been obtained using a 5.5 x 9 x 0.45 cm gel in a minigel electrophoresis apparatus (e.g., Aquebogue model 750 Mini Horizontal Gel Apparatus) with the recommended comb. Other gel sizes and apparatus can give acceptable results if the recommended protocols in **Sections 6.0** and **7.0** are followed.

6.0 PREPARATION AND POURING OF AGAROSE GEL

6.1 Set up a minigel apparatus containing the casting tray on a level surface. If the gel casting tray is separate from the apparatus, tape the ends of the tray to retain the liquefied agarose.

6.2 Prepare a 3% NuSieve®/1% SeaKem® agarose gel solution. Add 3.0 g NuSieve GTG® agarose and 1.0 g SeaKem GTG agarose to 100 mL of 0.5X TBE Gel Running Buffer (**Section 1.2.10**) in a 200 mL heat-tolerant laboratory screw-cap bottle.

6.3 Prior to melting the agarose, weigh the bottle containing the agarose and buffer and record the weight.

6.4 Cover the bottle loosely with the cap during heating. To melt the agarose, heat it in a microwave oven (set at defrost or low) for 3 to 4 minutes or in a boiling water bath until melted. **Do not allow the agarose solution to boil over.** During and after heating, carefully swirl the bottle to aid in dissolving the agarose. Additional heating may be required with some microwave ovens.

6.5 After the agarose is completely dissolved, reweigh the bottle. If the bottle weighs less (volume lost through evaporation), then add a sufficient volume of DI H_2O to return the bottle to its original weight. Swirl the solution to ensure that the solution is homogeneous. If necessary, reheat the bottle (10 to 30 seconds) to ensure that the agarose remains completely dissolved.

Warning: Ethidium bromide is a mutagen. Avoid contact with skin. Wear lab coat, gloves, and safety glasses when handling ethidium bromide.

6.6 After the agarose is completely dissolved, add 5 µL of a 10 mg/mL stock of ethidium bromide to the 100 mL of gel solution to yield a 0.5 µg/mL ethidium bromide concentration. Swirl to mix thoroughly.

6.7 Cool the solution to 55° to 65°C on the bench top or in a water bath prior to pouring the gel. If the gel will not be poured immediately after cooling, maintain the temperature of the agarose at 55° to 65°C to prevent the gel from solidifying.

6.8 It is important that the gel is poured on a level, horizontal surface. Pour enough agarose for a 0.45 cm thick gel into either a gel casting tray or the casting area of the minigel apparatus (e.g., to form a gel 5.5 x 9 x 0.45 cm, 22 mL of liquefied agarose are needed). Avoid formation of air bubbles when pouring agarose. Immediately insert a gel comb. The teeth of the comb used should be 1.0 to 1.2 mm thick and 4.0 to 5.0 mm wide. The comb should sit approximately 1.0 mm above the bottom of the gel.

Note: The remaining agarose solution can be reheated and reused. However, it is important to weigh the bottle containing the gel both before and after heating, and to correct the difference in weight by adding DI H_2O. Additional ethidium bromide is not needed. Do not allow the agarose containing ethidium bromide to boil over; ethidium bromide is a mutagen. If a microwave oven is used to reheat the agarose, it must be dedicated for laboratory use only.

6.9 Allow the gel to solidify completely (approximately 30 minutes) at room temperature (15° to 30°C) before proceeding.

6.10 After the gel has solidified, carefully remove the comb. If the gel was poured in a separate casting tray, place the gel in the horizontal minigel apparatus on a level surface. Add a sufficient volume of 0.5X TBE Gel Running Buffer (**Section 1.2.10**) containing 0.5 µg/mL ethidium bromide (add 50 µL of 10 mg/mL ethidium bromide to 1L of 0.5X TBE Gel Running Buffer) to fill the buffer tanks and cover the gel to a depth of about 2 mm. Check that there are no air bubbles in any of the wells.

7.0 GEL LOADING AND ELECTROPHORESIS

7.1 Add 2 µL of Gel Loading Buffer (**Section 1.2.11**) either to 0.5 mL microcentrifuge tubes or to the wells of a 96-well microplate corresponding to the number of samples to be analyzed. The GIBCO BRL 123 Base Pair (bp) Ladder will also be run in one well of each gel. If a 5 µL aliquot of the amplified DNA sample was removed in **Section 4.0**, add the 2 µL of Gel Loading Buffer directly to the aliquot.

7.2 Dilute a portion of the stock solution of GIBCO BRL 123 bp Ladder to 50 ng/µL with autoclaved DI H_2O.

7.3 Add 5 µL of the 50 ng/µL GIBCO BRL 123 bp Ladder (molecular weight marker) to the 2 µL of loading buffer designated for the molecular weight marker and mix using the pipet tip.

7.4 If 5 µL of each amplified DNA sample has already been mixed with 2 µL of loading buffer (see **Section 7.1**), proceed to **Section 7.5**. If the aliquots have not been previously removed, withdraw 5 µL of the amplified DNA sample from each reaction tube. Avoid pipetting mineral oil along with the sample if amplification was performed in the DNA Thermal Cycler 480 with GeneAmp® Thin-Walled Reaction Tubes. Use a new pipet tip to add each sample to 2 µL of loading buffer. Mix the sample and loading buffer with the pipet tip.

7.5 After all amplified samples have been mixed with the loading buffer, carefully pipet the 123 bp ladder plus loading buffer solution into the first gel well using an adjustable pipet. Add the amplified DNA sample plus loading buffer solutions into the remaining gel wells using a new pipet tip for each sample. **Do NOT produce bubbles or overfill wells. Samples should lie in an even layer at the bottom of each well.**

7.6 Connect the minigel electrophoresis apparatus to the power supply such that the DNA migrates toward the positive electrode. For a minigel apparatus that is 5.5 x 15 cm, run the gel at 115 volts or 7.5 volts/cm (maximum voltage) for approximately 1 hour or until the bromophenol blue dye from the loading buffer has run approximately 7.5 cm down the gel to provide adequate resolution of the six amplified product bands. Electrophoresis run times and voltage settings can vary depending on the gel apparatus used, but the bromophenol blue dye should always be run approximately 7.5 cm from the wells.

7.7 After the minigel has been run, disconnect the power supply and remove the gel. **Wear UV protective eyewear and handle the gel with gloves.**

7.8 To photograph the gel, place it on a UV transilluminator box under a stationary Polaroid® camera with a Kodak™ 22 or 23A Wratten filter. Photograph the gel in the dark under UV illumination with type 55, 57, or 667 black and white Polaroid film.

7.9 The following six bands should be present in the Control DNA 1 lane and in samples in which the DNA was amplified successfully for the AmpliType® PM and AmpliType PM+DQA1 Kits: 242/239 bp (HLA DQA1), 214 bp (LDLR), 190 bp (GYPA), 172 bp (HBGG), 151 bp (D7S8), and 138 bp (GC). Primer-dimer bands and unincorporated primers may appear as broad bands near the bottom of the gel in the region of lower molecular weight. Occasionally, non-specific bands or smearing can be observed above and/or below the six specific bands, but they do not compromise the typing results on the AmpliType PM and AmpliType HLA DQA1 DNA Probe Strips.

7.10 If all six bands are present on the AmpliType PM and AmpliType PM+DQA1 PCR product gel, proceed to **Section 8.0**. If less than six bands are present, see **Troubleshooting Section** before proceeding.

8.0 DNA HYBRIDIZATION

Note: Ensure that 5 µL of 200 mM disodium EDTA has been added to each tube (Section 4.0) before proceeding to the DNA Hybridization step.

The AmpliType DNA Hybridization process involves three steps performed sequentially as follows: 1) hybridization of amplified DNA to DNA probe strips; 2) binding of HRP-SA Enzyme Conjugate to hybridized PCR products; and 3) stringent wash to remove non-specifically bound PCR products. Color Development is performed after the stringent wash step.

Certain steps in the following procedures involve the aspiration of solutions containing amplified DNA. Instructions for the assembly of the aspirator apparatus, its use, and waste disposal are described in the Laboratory Setup section of the AmpliType® User Guide.[18]

Before starting the DNA Hybridization and Color Development procedures, assemble the required reagents and equipment as follows:

Reagents and equipment required for both AmpliType kits.

HRP-SA Enzyme Conjugate (Included in Kit)

Chromogen: TMB Solution (Included in Kit; prepared in **Section 1.1.1**)

Hybridization Solution (prepared in **Section 1.2.4**)

Wash Solution (prepared in **Section 1.2.5**)

Citrate Buffer (prepared in **Section 1.2.1**)

30% Hydrogen Peroxide or 3% Hydrogen Peroxide (prepared in **Section 1.2.6**)

AmpliType™ DNA Typing Trays with pen for writing on strips (**Part No. N808-0065**)

Additional reagents and equipment required for the AmpliType PM Kit.

AmpliType PM DNA Probe Strips (Included in Kit)

Additional Reagents and equipment required for the AmpliType PM+DQA1 Kit.

AmpliType PM DNA Probe Strips (Included in Kit)

AmpliType HLA DQA1 DNA Probe Strips (Included in Kit)

Clean disposable gloves should be worn throughout the DNA Hybridization and Color Development steps to avoid soiling the DNA probe strips and to protect fingers from the 95°C block. Gloves should be discarded when leaving the work area to avoid transport of amplified DNA from the work area.

8.1 A rotating water bath, heated at 55°C (± 1°C), is necessary for the hybridization and wash steps. **The rotating water bath must be on a level surface. Do NOT use a hot air shaker.**

Note: It is essential to check the temperature with a calibrated total immersion thermometer before the hybridization step is performed. To obtain an accurate thermometer reading, minimize the time that the water bath remains uncovered.

8.2 Heat a covered, rotating water bath to 55°C and maintain the temperature between 54°C and 56°C. To ensure that the water bath will be at the correct temperature for the hybridization and wash steps, maintain the rotation at 50 to 70 rpm while performing **Sections 8.3** through **Section 8.9.** The water level should be between 1/4 and 1/2 inch above the shaker platform. The water level should not be higher than 1/2 inch since higher levels may result in water splashing into the wells of the tray. An empty tray can be used to test the water level prior to use (place a 1 kg weight on the cover of the tray; see **Section 8.10**).

8.3 Warm the Hybridization Solution and the Wash Solution in a heated stationary water bath or an incubator (37° to 55°C) to dissolve the solids. **All solids must be in solution and all solutions must be well mixed before use.**

8.4 Remove the tube of AmpliType DNA Probe Strips from the 2° to 8°C storage area. **Allow the tube to equilibrate to ambient temperature before opening to prevent condensation inside the tube.** Using filter forceps, remove the required number of DNA probe strips from the tube. Using the pen included with the AmpliType™ DNA Typing Trays, label each strip in the space at the right edge of the strip. **The use of other pens is not recommended because some inks may affect the quality of the typing results.** Place one DNA probe strip in each well of the clean AmpliType DNA Typing Tray. Strips should all be in the same orientation.

Note: AmpliType® PM and AmpliType HLA DQA1 DNA Probe Strips can be used to type PCR products from the same PM amplification reaction at the same time but the DNA probe strips must be placed in separate wells of the tray.

8.5 Prepare the GeneAmp® PCR Instrument System to denature the amplified DNA by setting the parameter to hold the temperature at 95°C (see appropriate GeneAmp PCR Instrument System Users Manual). Start the file or program.

8.6 Place the tubes in the GeneAmp PCR Instrument System after it reaches 95°C. Press the tubes down tightly in the block. When using the GeneAmp PCR System 9600, position the heated cover over the tubes during the denaturation step to hold down the tubes to avoid refluxing into the cap. Denature the amplified DNA by incubation at 95°C for 3 to 10 minutes. Keep each tube at 95°C until use.

8.7 As shown in **Figure 2**, tilt the DNA Typing Tray towards the labelled end of the strips. The DNA Typing Tray lid turned upside down may be used as the solid support. Add 3 mL of pre-warmed Hybridization Solution to each well at the labelled end of each strip. **Do not wet the remainder of the strip.**

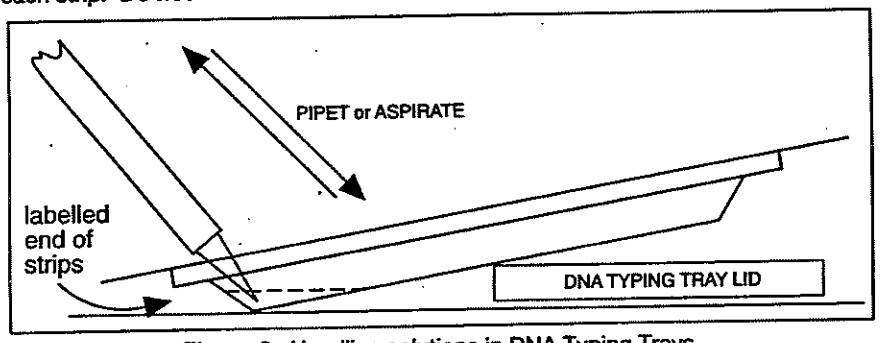

Figure 2. Handling solutions in DNA Typing Trays.

Note: Hybridization solution solids must be completely dissolved before adding to the tray.

8.8 Perform the following steps for each tube of amplified DNA:

NOTE: For each tube, perform 8.8.1 through 8.8.3 within 20 seconds and use a new pipet tip for each addition.

8.8.1 Remove the tube from the 95°C block of the GeneAmp PCR Instrument System. When using the GeneAmp PCR System 9600, it is not necessary to reposition the heated cover over the tubes between each sample removal.

8.8.2 Carefully open the tube using the Micro Tube Opener designated for use only with amplified DNA.

8.8.3 Withdraw 20 µL of amplified DNA and immediately add it below the surface of the Hybridization Solution in the well of the corresponding DNA probe strip (see **Figure 2**). If using the DNA Thermal Cycler 480, be sure to withdraw from the aqueous (bottom) layer.

8.8.4 Cap the tube after adding the denatured amplified DNA.

8.8.4.1 If using both of the DNA probe strips provided in the AmpliType® PM+DQA1 Kit, proceed to **Section 8.8.4.2**. If using the AmpliType PM Kit, or processing only the PM or HLA DQA1 DNA Probe Strips provided in the AmpliType PM+DQA1 Kit, set the capped tube aside. Repeat **Sections 8.8.1** through **8.8.4.1** until each amplified DNA sample has been added to the corresponding well.

8.8.4.2 When both AmpliType PM and AmpliType HLA DQA1 DNA Probe Strips are used to type PCR product from the same tube, add 20 µL denatured amplified DNA to **one** of the two strips designated for this sample (e.g., PM DNA Probe Strip). **Cap and return the tube to the 95°C block.** Add the remaining denatured samples individually to the corresponding DNA probe strips (e.g., PM DNA Probe Strips) and return the capped tubes to the 95°C block. Repeat **Sections 8.8.1** through **8.8.4.2** using the other AmpliType DNA Probe Strips (e.g., HLA DQA1 DNA Probe Strips) until each amplified DNA sample has been added to the corresponding wells. The capped tubes can be set aside instead of returned to the 95°C block after the amplified DNA is added to the second DNA probe strip.

The remaining amplified DNA samples can be stored at 2° to 8°C for 2 months or at -20°C for 6 months. The continued acceptable performance of these samples beyond these periods may vary with the sample. **Store amplified DNA samples separate from all PCR amplification reagents, extracted DNA samples, and casework samples.**

8.9 Put the lid on the tray and mix by carefully rocking the tray. Ensure that each strip is completely wet. **Once hybridization has begun, strips should remain wet through the conclusion of the Color Development and Photography steps.**

8.10 Stop the rotation of the rotating water bath (do not turn off the temperature control). Confirm that the temperature of the water is 55°C and transfer the tray to the water bath. Place a 1 kg weight (e.g., a lead ring) on the covered tray to prevent the tray from sliding or floating. Resume rotation of water bath at 50 to 70 rpm. Check the tray position and confirm that water does not splash into the wells of the tray.

8.11 Replace the water bath cover to maintain the temperature of the water in the bath at 55°C (± 1°C). Hybridize the amplified samples to the DNA probe strips by incubating at 55°C for 15 minutes (± 2 minutes).

8.12 Approximately 5 minutes before the end of the hybridization step, prepare the Enzyme Conjugate Solution in a glass flask using the following equations to determine the volume of each component required:

Number of Strips	x	3.3 mL of Hybridization Solution	=	Volume of Hybridization Solution
Number of Strips	x	27 µL of Enzyme Conjugate: HRP-SA	=	Volume of Enzyme Conjugate: HRP-SA

Mix the solution thoroughly and ensure that the solids remain in solution. **Leave at room temperature (15° to 30°C) until ready to use.**

8.13 After hybridization, stop the rotation of the water bath and remove the tray. **Replace the water bath cover and keep the water bath rotating between incubation steps to maintain the temperature at 55°C (± 1°C).**

8.14 Aspirate the contents of each well from the labelled end of the strip while tilting the tray slightly. Remove condensation from the tray lid with a clean lab wipe. Use of paper towels to wipe the tray lid is not advised because some paper towels contain bleach which can affect the color development.

Note: Wash Solution solids must be completely dissolved and well mixed before use.

8.15 Dispense 5 mL of pre-warmed (37° to 55°C) Wash Solution into each well (a dispensing re-pipet is useful for this purpose). Rinse by gently rocking the tray for several seconds. Aspirate the solution from each well.

8.16 Dispense 3 mL of the Enzyme Conjugate Solution prepared in **Section 8.12** into each well and cover with the lid. Stop the rotating water bath and transfer the tray to the 55°C water bath. Place a 1 kg weight on the covered tray to prevent the tray from sliding or floating. Adjust the rotating water bath to 50 to 70 rpm. Check the tray position and confirm that the water does not splash into the wells of the tray.

8.17 Replace the water bath cover to maintain the temperature at 55°C (± 1°C). Incubate the Enzyme Conjugate Solution with the DNA probe strips at 55°C (± 1°C) for 5 minutes (± 1 minute).

8.18 After incubation, stop the rotation of the water bath and remove the tray. **Replace the water bath cover and keep the water bath rotating between incubation steps to maintain the temperature at 55°C (± 1°C).**

8.19 Aspirate the contents of each well from the labelled end of the strip while tilting the tray slightly. Remove condensation from the tray lid with a clean lab wipe. Use of paper towels to wipe the tray lid is not advised because some paper towels contain bleach which can affect the color development. **Replace the water bath cover and keep the water bath rotating between incubation steps to maintain the temperature at 55°C (± 1°C).**

8.20 Dispense 5 mL of pre-warmed Wash Solution into each well. Rinse by gently rocking the tray for several seconds. Aspirate the solution from each well.

8.21 To perform the stringent wash step, dispense 5 mL of pre-warmed Wash Solution into each well. Cover the tray with the lid and place it in the 55°C water bath. Place a 1 kg weight on the covered tray to prevent the tray from sliding or floating. Adjust the rotation to 50 to 70 rpm and check the tray position to confirm that water does not splash into the wells of the tray.

8.22 Replace the water bath cover to maintain the temperature at 55°C (± 1°C). Incubate the DNA probe strips at 55°C (± 1°C) for 12 minutes (± 1 minute). **The temperature and timing of the stringent wash step are critical.**

8.23 After incubation, remove the tray from the water bath. Take off the lid and aspirate the contents of each well from the labelled end of the strips.

8.24 Dispense 5 mL of Wash Solution into each well. Gently rock the tray for several seconds. Slowly pour off or aspirate the contents from each well.

9.0 COLOR DEVELOPMENT

9.1 Dispense 5 mL of Citrate Buffer into each well. A dispensing re-pipet is useful for this purpose. Cover the tray with the lid and place it on an orbital shaker set at approximately 50 rpm at room temperature (15° to 30°C) for 5 minutes.

9.2 During this wash step, prepare the Color Development Solution. **Do not prepare the Color Development Solution more than 10 minutes before use.** Add the following reagents in the order listed to a glass flask and **mix thoroughly by swirling. Protect from light. Do NOT vortex.** Use the following equations to determine the volumes of each component required:

Number of Strips	x	5 mL Citrate Buffer	=	Volume of Citrate Buffer
Number of Strips	x	5 µL 3% Hydrogen Peroxide **OR** 0.5 µL 30% Hydrogen Peroxide	=	Volume of Hydrogen Peroxide
Number of Strips	x	0.25 mL Chromogen: TMB Solution	=	Volume of Chromogen: TMB

9.3 Remove the tray from the orbital shaker. Remove the lid and slowly pour off or aspirate the contents from each well. Add 5 mL of the freshly prepared Color Development Solution (**Section 9.2**) to each well.

Note: Place the lid on the tray and cover the lid with aluminum foil during Steps 9.4 and 9.5 to protect the DNA probe strips from strong light.

9.4 Develop the strips at room temperature (15° to 30°C) by rotating on an orbital shaker set at approximately 50 rpm for 20 to 30 minutes. Develop until the "S" or "C" dot is visible. Some strips may develop in less than 20 minutes. In this case, the color development may be stopped by proceeding immediately to **Section 9.5**. Color development of the negative control strip should not be stopped until development of all other test strips is stopped.

9.5 Stop the color development by removing the solution from the well. Immediately dispense approximately 5 mL of DI H_2O into the well. Place the tray on an orbital shaker set at approximately 50 rpm for 5 to 10 minutes. Remove the DI H_2O from the well and repeat the wash steps a minimum of three times. Additional 5 to 10 minute washes will reduce the potential for development of background color.

9.6 Record the pattern of blue dots from each **wet** DNA probe strip prior to photography (see **Interpretation of Results**).

Note: Keep strips wet throughout the photography steps.

10.0 PHOTOGRAPHY AND STORAGE

10.1 Photographs should be taken for a permanent record of the results. **Photographs must be taken while the DNA probe strips are still wet.**

10.2 Place **wet** strips on a flat non-absorbent surface (a black background, such as a sheet of exposed X-ray film, is recommended to enhance contrast). Keep the strips wet throughout the photography steps. Minimize exposure to strong light.

10.3 Use a Polaroid® camera with Type 55, 57, or 667 (black and white) film or Type 59 or 559 (color) film.

Note: For black and white photography, an orange filter (Wratten 22 or 23A) will enhance contrast.

10.4 Follow Polaroid film exposure and development instructions.

10.5 After photography, the DNA probe strips may be air-dried on any hard non-absorbent surface or Whatman® 3MM chromatography paper (**do not use paper towels for this step**). Protect from light and oxidizing agents (e.g., acid treated paper, bleach, and nitric acid). The dot intensities fade upon drying.

11.0 DISPOSAL OR REUSE OF TYPING TRAYS

11.1 The AmpliType™ DNA Typing Trays are designed to be disposable but may be reused. If the trays are to be reused, immediately wash the trays and lids according to the following procedure:

Note: Trays which have become discolored due to repeated use should be discarded.

11.1.1 Add approximately 5 to 10 mL of 95% ethanol or 70% isopropanol to each well of the used AmpliType DNA Typing Tray.

Note: Do NOT use detergent or bleach.

11.1.2 Cover the tray with the lid and carefully agitate for 15 to 30 seconds to dissolve any residual Chromogen: TMB.

11.1.3 Remove the lid. Visually inspect each well for a blue or yellow color which will indicate the presence of Chromogen: TMB. If necessary, repeat **Sections 11.1.1** and **11.1.2** until no color is present and then rinse each well in the tray and the tray lid with DI H_2O. If all the color cannot be removed from the walls of the wells, then discard the tray.

11.1.4 Dry tray and lid before reuse.

12.0 INTERPRETATION OF RESULTS

Results are interpreted by observing the pattern and relative intensities of blue dots on the wet AmpliType® PM and AmpliType HLA DQA1 DNA Probe Strips to determine which alleles are present in the DNA sample.

12.1 Reading and Interpreting the AmpliType PM DNA Probe Strips

The AmpliType PM DNA Probe Strips have been spotted with a total of fourteen sequence-specific oligonucleotide probes to distinguish the alleles of five genetic loci (a mixture of two probes is spotted at the GYPA "A" allele position). Under the AmpliType hybridization conditions, the typing probes will bind specifically to PCR product containing the alleles designated on the AmpliType PM DNA Probe Strip.

To read the developed AmpliType PM DNA Probe Strip, the "S" dot is examined first and then each locus is examined separately. The standard probe "S" on the AmpliType PM DNA Probe Strip is identical in sequence to the control probe "C" on the AmpliType HLA DQA1 DNA Probe Strip and detects all of the HLA DQA1 alleles. The "S" dot is designed to be the lightest typing dot on the PM DNA Probe Strip and acts as a minimum dot intensity control for the remaining probes. It is recommended that a DNA probe strip with no visible "S" dot not be typed for any locus.

When a "S" dot is visible on the AmpliType PM DNA Probe Strip, the intensities of the dots at the remaining twelve positions are compared to the intensity of the "S" dot. Those dots that appear either darker than or equivalent to the "S" dot are considered positive. Each positive dot indicates the presence of the corresponding allele. Dots that are lighter than the "S" dot should be interpreted with care.[14,15]

The dots on the AmpliType PM DNA Probe Strip correspond to the following alleles:

The "A" dot for each locus is positive in the presence of the A allele.

Note: The "A" dot for the GYPA locus is positive in the presence of both the A allele and the A' allele. Both the GYPA AB and GYPA A'B heterozygous genotypes have balanced dot intensities, but additional GYPA A and B variant alleles (observed in < 8% of the African American population) may produce a slightly imbalanced heterozygous signal.

The "B" dot for each locus is positive in the presence of the B allele.

The "C" dot for the HBGG and GC loci is positive in the presence of the C allele.

For LDLR, GYPA, and D7S8, three genotypes are possible (AA, BB, and AB). For HBGG and GC, six genotypes are possible (AA, BB, CC, AB, AC, and BC).

An example of a developed AmpliType PM DNA Probe Strip using PCR product amplified from 2 ng of Control DNA 1 is shown in **Figure 3**. A sample from a single individual will produce balanced dot intensities within each locus for which the individual is heterozygous.

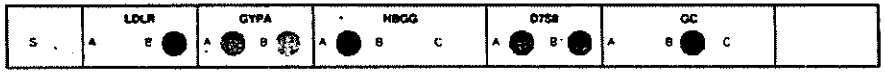

Figure 3. The AmpliType PM types for Control DNA 1 are: LDLR **BB**, GYPA **AB**, HBGG **AA**, D7S8 **AB**, GC **BB**. The dot intensities of the GYPA and D7S8 loci are balanced (i.e., the intensities of the "A" and "B" dots within each locus are similar).

12.2 Reading and Interpreting the AmpliType® HLA DQA1 DNA Probe Strips

The AmpliType® HLA DQA1 DNA Probe Strips have been spotted with a total of eleven sequence-specific oligonucleotide probes to detect eight alleles of the HLA DQA1 locus. Under the AmpliType hybridization conditions, the typing probes will bind specifically to PCR product containing the alleles designated on the AmpliType HLA DQA1 DNA Probe Strip.

To read the developed AmpliType HLA DQA1 DNA Probe Strip, the "C" dot is examined first and then the remaining dots are examined. The control probe "C" on the AmpliType HLA DQA1 DNA Probe Strip detects all of the HLA DQA1 alleles. The "C" dot is designed to be the lightest typing dot on the strip and it indicates that adequate amplification and typing of the HLA DQA1 alleles in the sample have occurred. If the "C" dot is absent, an accurate determination of the type cannot be made. Additional information on the "C" dot can be found in the AmpliType User Guide.[18]

The accurate interpretation of the HLA DQA1 results depends on the presence and intensity of the "C" dot. The intensities of the dots at the remaining ten positions are compared to the intensity of the "C" dot. Those dots that appear either darker than or equivalent to the "C" dot are considered positive. Each positive dot indicates the presence of the corresponding HLA DQA1 allele. Dots with signals less than the "C" dot should be interpreted with care.[9,11,28]

The dots on the AmpliType HLA DQA1 DNA Probe Strip correspond to the following alleles:

The "1" dot is positive in the presence of the HLA DQA1 1.1, 1.2, and 1.3 alleles.

The "2" dot is positive only in the presence of the HLA DQA1 2 allele.

The "3" dot is positive only in the presence of the HLA DQA1 3 allele.

The "4" dot is positive in the presence of the HLA DQA1 4.1, 4.2, and 4.3 alleles.

Four HLA DQA1 sub-typing probes differentiate the HLA DQA1 1.1, 1.2, and 1.3 alleles.

The "1.1" dot is positive only in the presence of the HLA DQA1 1.1 allele.

Note: A faint "1.1" dot will appear with some HLA DQA2 pseudogene alleles.[30]

The "1.3" dot is positive only in the presence of HLA DQA1 1.3 allele.

Note: There is no probe that detects only the HLA DQA1 1.2 allele.

The "1.2, 1.3, 4" dot is positive in the presence of HLA DQA1 1.2, 1.3, 4.1, 4.2, and 4.3 alleles.

Note: The "1.2, 1.3, 4" dot can be lighter than the "C" dot when the genotype has a HLA DQA1 4.2 or 4.3 allele paired with a HLA DQA1 1.1, 2, 3, 4.2, or 4.3 allele because the HLA DQA1 4.2 and 4.3 alleles each have a single partially destabilizing mismatch to the "1.2, 1.3, 4" probe.[18] The partially destabilizing mismatch allows these two alleles to bind to this probe weakly relative to the HLA DQA1 1.2, 1.3, and 4.1 alleles.

The "All but 1.3" dot is positive in the presence of all HLA DQ alleles EXCEPT 1.3. This probe is necessary to differentiate the 1.2, 1.3 genotype from the 1.3, 1.3 genotype.

Note: The "All but 1.3" dot can be equal to or lighter than the "C" dot when the genotype has a HLA DQA1 1.3 allele paired with a HLA DQA1 4.1, 4.2, or 4.3 allele because the HLA DQA1 4.1, 4.2, and 4.3 alleles have a single partially destabilizing mismatch to the "All but 1.3" probe.[18] The partially destabilizing mismatch allows these three alleles to bind to this probe weakly relative to the HLA DQA1 1.1, 1.2, 2, and 3 alleles.

Two additional HLA DQA1 sub-typing probes differentiate the HLA DQA1 4.1 allele from the HLA DQA1 4.2 and 4.3 alleles.

The "4.1" dot is positive only in the presence of the HLA DQA1 4.1 allele.

The "4.2, 4.3" dot is positive in the presence of HLA DQA1 4.2 and 4.3 alleles.

These HLA DQA1 allele designations correspond to the World Health Organization (WHO) nomenclature as shown in **Table 6.**

Table 6. World Health Organization (WHO) Nomenclature for HLA DQA1 alleles

Allele	WHO Designation	Allele	WHO Designation
1.1	0101	3	0301
1.2	0102	4.1	0501*
1.3	0103	4.2	0401*
2	0201	4.3	0601

*Note that the WHO number designations are out of sequence compared to the original allele designations.

An example of a developed AmpliType® HLA DQA1 DNA Probe Strip using PCR product amplified from 2 ng of Control DNA 1 is shown in **Figure 4.**

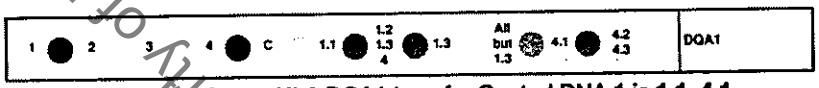

Figure 4. The AmpliType HLA DQA1 type for Control DNA 1 is **1.1, 4.1.**

PERFORMANCE CHARACTERISTICS

The AmpliType® PM and AmpliType PM+DQA1 PCR Amplification and Typing Kits are developed and manufactured by Roche Molecular Systems, Inc. (RMS). Each lot of the AmpliType PM and AmpliType PM+DQA1 PCR Amplification and Typing Kits is carefully tested by RMS to ensure that the kits perform according to specifications and are free from interfering contaminants.

The user of the AmpliType PCR Amplification and Typing Kits will be able to amplify and type a minimum of two nanograms of Control DNA 1 when employing the protocols and reagents provided in the kits.

In the laboratories of RMS, the kit components have been used successfully to type samples containing less than one nanogram of human DNA.

TROUBLESHOOTING

Observation	Possible Cause	Recommended Action
1. No signal or faint signal from both the Control DNA 1 and the DNA test samples at all loci.	No PCR amplification or insufficient PCR amplification of all markers.	Check amplified DNA on agarose gel (see **Observation 1.1**).
	Improper hybridization or assay condition.	Repeat test from Hybridization step (see **Observation 1.2**).
1.1 No amplified product visible on gel.	No DNA added or insufficient DNA added to PCR Reaction Mix.	Quantitate DNA and add 2 - 10 ng DNA; repeat test.
	AmpliType® PM Primer Set not added to AmpliType PM PCR Reaction Mix.	Add AmpliType PM Primer Set; repeat test.
	GeneAmp® PCR Instrument System failure or wrong program.	See GeneAmp PCR Instrument System Manual and check instrument calibration.
	Tubes not seated tightly in the DNA Thermal Cycler 480 block during amplification.	Push tubes firmly into contact with block after first cycle; repeat test.
	MicroAmp® Base used with tray and tubes in GeneAmp PCR System 9600.	Remove MicroAmp Base; repeat test.
1.2 Amplified product visible on gel but no signal or faint signal on AmpliType DNA Probe Strips.	Hybridization and/or Stringent Wash temperature too high.	Check that the rotating water bath temperature is at 55°C (± 1°C) with an immersible thermometer; repeat test.
	Hybridization and/or Wash Solution salt concentration too low.	Prepare new solutions; repeat test.
	Stringent Wash time too long.	Repeat test, washing for 12 minutes (±1minute) only.

Observation	Possible Cause	Recommended Action
1.2 (continued) Amplified product visible on gel but no signal or faint signal on AmpliType® DNA Probe Strips.	Inadequate agitation of the DNA probe strips during hybridization.	Check speed of rotating water bath (50 to 70 rpm); verify that hybridization solution is washing over the strips; repeat test.
	Amplified DNA was not added to DNA probe strips.	Repeat test, adding amplified DNA to DNA probe strips.
	Amplified DNA was not denatured.	Check GeneAmp® PCR Instrument System block temperature is 95°C; leave sample in block >3 minutes using the heated block cover; repeat test following instructions in **Section 8.6**.
	Enzyme Conjugate: HRP-SA was not added to the Enzyme Conjugate Solution.	Prepare new diluted Enzyme Conjugate: HRP-SA solution; repeat test.
	Hydrogen peroxide was not added or too much was added to the Color Development Solution.	Make new Color Development Solution with correct amount of hydrogen peroxide; repeat test.
	Hydrogen peroxide inactive.	Make new Color Development Solution using new bottle or dilution of hydrogen peroxide; repeat test.
	Chromogen: TMB was not added to the Color Development Solution.	Make new Color Development Solution adding Chromogen: TMB; repeat test.
	The original AmpliType HLA DQα instead of AmpliType PM and PM+DQA1 typing protocol was followed.	Repeat test following the typing protocol in **Section 8.0.**
2. Positive signal from Control DNA 1, but no signal from DNA test sample.	Quantity of DNA test sample is below the assay sensitivity.	Quantitate DNA and add 2 - 10 ng DNA; repeat test.

Observation	Possible Cause	Recommended Action
2. (continued) Positive signal from Control DNA 1, but no signal from DNA test sample.	Test sample contains PCR inhibitor (e.g., heme compounds, certain dyes).	Any or all of the following actions may be taken: 1. Quantitate DNA and add minimum necessary volume; repeat test. 2. Wash the sample in Centricon® 100 (see **Reference 11**); repeat test. 3. Add BSA to reaction and see **References 15 and 31**; repeat test.
	Test sample DNA is degraded.	If possible, evaluate the quality of the DNA sample by running an agarose gel (see **Reference 18**). If the DNA is degraded, reamplify with an increased amount of DNA.
3. High DNA probe strip background color.	Low or lack of SDS in Hybridization and/or Wash Solution.	Prepare new Hybridization and/or Wash Solution with correct amount of SDS; repeat test.
	Inadequate agitation of the DNA probe strips during hybridization and washing steps.	Check speed of rotating water bath (50 to 70 rpm); verify that solutions are washing over strips; repeat test.
	Tray lid not wiped adequately.	Wipe lid (**Section 8.19**); repeat test.
	Excess amounts of Enzyme Conjugate: HRP-SA added to Enzyme Conjugate Solution.	Prepare new Enzyme Conjugate Solution with correct amount of Enzyme Conjugate: HRP-SA; repeat test.
	Exposure to light during Color Development.	Cover tray lid with foil during Color Development steps; repeat test.

Observation	Possible Cause	Recommended Action
3. (continued) High DNA probe strip background color.	Use of water other than deionized or glass distilled water for water rinses.	Repeat test using deionized or glass distilled water for water rinses.
4. High DNA probe strip background color upon storage.	Exposure to strong light and oxidizing agents.	Store strips in the dark away from oxidizing agents.
	Insufficient water washes after Color Development.	Increase number of water washes in future assays.
5. Presence of unexpected or additional dots in the amplified Control DNA 1 sample.	Cross-hybridization caused by Hybridization and/or Stringent Wash temperature being too low.	Check that the rotating water bath temperature is at 55°C (± 1°C) with a total immersion thermometer; repeat test.
	Cross-hybridization caused by Hybridization and/or Wash Solution salt concentration being too high.	Prepare new solutions; repeat test.
	Cross-hybridization caused by Stringent Wash time being too short.	Repeat test, washing for 12 minutes (± 1 minute).
	Contamination by amplified product or samples.	See **References 18** and **28**.
6. Signals weaker than "S" or "C" dot on the same strip (also see Observations 12 and 13).	Hybridization and/or Stringent Wash temperature too high or too low.	Check that the rotating water bath temperature is at 55°C (± 1°C) with an immersible thermometer; repeat test.
	Hybridization and/or Wash Solution salt concentration too high or too low.	Prepare new solutions; repeat test.
	Stringent wash time too long or too short.	Repeat test, washing for 12 minutes (± 1 minute).
	Mixed sample or contamination.	See **References 14, 15, 17, 18,** and **28**.

Observation	Possible Cause	Recommended Action
6. (continued) Signals weaker than "S" or "C" dot on the same strip (also see Observations 12 and 13).	Amplification of HLA DQA2 pseudogene (faint "1.1" dot).	See **References 18** and **30**.
	EDTA not added to the reaction prior to the heat denaturation step of the DNA hybridization protocol.	Add EDTA to amplified sample (**Section 4.0**); repeat test.
7. More than two alleles present on the AmpliType HLA DQA1 DNA Probe Strip or at the HBGG and/or GC marker on the AmpliType PM DNA Probe Strip.	Cross-hybridization caused by Hybridization and/or Stringent Wash temperature too low.	Check that the rotating water bath temperature is at 55°C (± 1°C) with a total immersion thermometer; repeat test.
	Cross-hybridization caused by Hybridization and/or Wash Solution salt concentration too high.	Prepare new solutions; repeat test.
	Stringent Wash time too short.	Repeat test washing for 12 minutes (± 1 minute).
	Mixed sample or contamination.	See **References 14, 15, 17, 18,** and **28**.
8. Some, but not all, loci visible on gel of AmpliType PM PCR products.	Amplification of HLA DQA2 pseudogene (faint 1.1 dot).	See **References 18** and **30**.
	Test sample DNA is degraded.	If possible, evaluate the quality of the DNA sample by running an agarose gel (see **Reference 18**). If the DNA is degraded, reamplify with an increased amount of DNA.

Observation	Possible Cause	Recommended Action
8. (continued) Some, but not all, loci visible on gel of AmpliType PM PCR products.	Test sample contains PCR inhibitor (e.g., heme compounds, certain dyes).	Any or all of the following actions may be taken: 1. Quantitate DNA and add minimum necessary volume; repeat test. 2. Wash the sample in Centricon® 100 (see **Reference 11**); repeat test. 3. Add BSA to reaction and see **References 15 and 31**; repeat test.
	Input DNA and/or PCR product was not denatured sufficiently during amplification.	Check calibration of the GeneAmp® PCR Instrument System using the appropriate Temperature Verification System.
9. Some, but not all, loci visible on AmpliType PM DNA Probe Strip.	Not all loci amplified.	Verify the presence of amplified loci on an agarose gel (see **Observation 8**).
	Amplified DNA was not denatured.	Check GeneAmp PCR Instrument System block temperature is 95°C; leave sample in block > 3 minutes using the heated block cover; repeat test following instructions in **Section 8.6.**
10. Imbalanced dot intensities within a locus on the AmpliType PM DNA Probe Strip (this kit is designed to produce balanced dot intensities when heterozygous samples are typed, except as described in **Section 12.1**).	Hybridization and/or Stringent Wash temperature too high or too low.	Check that the rotating water bath temperature is at 55°C (± 1°C) with a total immersion thermometer; repeat test.
	Hybridization and/or Wash Solution salt concentration too high or too low.	Prepare new solutions; repeat test.
	Stringent Wash time too long or too short.	Repeat test, washing for 12 minutes (± 1 minute).

Observation	Possible Cause	Recommended Action
10. (continued) Imbalanced dot intensities within a locus on the AmpliType PM DNA Probe Strip (this kit is designed to produce balanced dot intensities when heterozygous samples are typed, except as described in **Section 12.1**).	Mixed sample or contamination.	See **References 14, 15, 17, 18,** and **28.**
	EDTA not added to the reaction prior to the heat denaturation step of the DNA hybridization protocol.	Add EDTA to amplified sample (**Section 4.0**); repeat test.
11. Weak or absent "4.1" dot on the AmpliType HLA DQA1 DNA Probe Strip in the amplified Control DNA 1 sample.	EDTA not added to the reaction prior to the heat denaturation step of the DNA hybridization protocol.	Add EDTA to amplified sample (**Section 4.0**); repeat test.
12. "1.2, 1.3, 4" dot weaker than "C" dot on AmpliType HLA DQA1 DNA Probe Strip.	Genotype of sample has a HLA DQA1 4.2 or 4.3 allele paired with a HLA DQA1 1.1, 2, 3, 4.2, or 4.3 allele.	See **Reference 18.**
13. "1.1" dot weaker than "C" dot but no signal for "1" dot on AmpliType HLA DQA1 DNA Probe Strip.	Amplification of HLA DQA2 pseudogene (faint 1.1 dot).	See **References 18** and **30.**
14. "All but 1.3" signal weaker than "C" dot on AmpliType HLA DQA1 DNA Probe Strip.	Genotype of sample has a HLA DQA1 1.3 allele paired with a HLA DQA1 4.1, 4.2, or 4.3 allele.	See **Reference 18.**

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QuantiBlot™

PERKIN ELMER

Human DNA Quantitation Kit

FOR FORENSIC AND RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

Part No. N808-0114

READ ENTIRE PROCEDURE PRIOR TO USE OF KIT

TABLE OF CONTENTS

LIST OF KIT COMPONENTS

IMPORTANT: Do NOT freeze any of the kit components.

Store the QuantiBlot™ Human DNA Quantitation Kit components at 2° to 8°C. Under these conditions, components of the kit are stable through the control date printed on the label.

Note: The QuantiBlot™ Human DNA Quantitation Kit contains reagents for at least 10 hybridization reactions. Each hybridization reaction requires the DNA Standards and DNA Calibrators with space for testing up to 38 samples.

Reagent	Volume	Description
QuantiBlot™ D17Z1 Probe	220 µL	1 vial containing 1 pmole/µL biotinylated DNA oligonucleotide probe of the following sequence: 5'-biotin-TAGAAGCATTCTCAGAA ACTACTTTGTGATGATTGCATTC-3'.
Enzyme Conjugate: HRP-SA .	2.0 mL	1 vial containing Horseradish Peroxidase-Streptavidin (HRP-SA) conjugate supplied in buffer.
Bromothymol Blue Solution	200 µL	1 vial containing 0.04% Bromothymol Blue in water.
QuantiBlot™ DNA Standard A	250 µL	1 vial containing 2 ng/µL human genomic DNA in TE buffer.
QuantiBlot™ DNA Calibrator 1	100 µL	1 vial containing 0.7 ng/µL human genomic DNA in TE buffer.
QuantiBlot™ DNA Calibrator 2	100 µL	1 vial containing 0.1 ng/µL human genomic DNA in TE buffer.
Package Insert		QuantiBlot™ Human DNA Quantitation Kit protocol.

INTRODUCTION

The QuantiBlot™ Human DNA Quantitation Kit provides reagents and protocols necessary for the rapid quantitation of human DNA. The procedure is based on the hybridization of a biotinylated oligonucleotide probe to DNA samples immobilized on a nylon membrane.[1] The probe included in this kit is complementary to a primate-specific alpha satellite DNA sequence at the locus D17Z1.[2] Subsequent binding of Enzyme Conjugate:HRP-SA (horseradish peroxidase-streptavidin) to the biotin moiety of the probe allows for either colorimetric or chemiluminescent detection (protocols are provided for both detection schemes). In the case of colorimetric detection, the oxidation of 3,3',5,5'-tetramethylbenzidine (Chromogen:TMB) catalyzed by the horseradish peroxidase results in the formation of a blue-colored precipitate directly on the membrane. Alternatively, for chemiluminescent detection the oxidation of a luminol based reagent catalyzed by the horseradish peroxidase results in the emission of photons that are detected on standard autoradiography film.[3] This process is called Enhanced Chemiluminescence (ECL™). In both cases, the quantity of sample DNA is determined by comparison of the signal intensity to human DNA standards.

Using the protocols provided, 0.15 to 10.0 nanograms of human DNA can be quantitated. The entire protocol can be performed in less than 2 hours. DNA prepared by a variety of extraction methods, including phenol-chloroform, Chelex®, salting out[4], or binding to silica particles, can be quantitated.

The QuantiBlot Human DNA Quantitation Kit provides protocols for two alternative detection schemes: colorimetric and chemiluminescent. The advantages of the colorimetric detection scheme are as follows: 1) A darkroom equipped for X-ray film development is not necessary; and 2) The Chromogen:TMB reagent is available directly from Perkin-Elmer (**Part No. N808-0092**). The advantages of the chemiluminescent (ECL) detection scheme are as follows: 1) If desired, the sensitivity can be increased below 0.15 ng by performing long exposures to the X-ray film (see **Section 5.2.5**); and 2) The X-ray film result is a permanent record (no photography is required).

MATERIALS REQUIRED BUT NOT SUPPLIED

The items listed are those required for quantitation procedures. This list does not include reagents or equipment required for DNA extraction (for a list of DNA extraction materials see the AmpliType™ User Guide, Version 2, Section 3). Many of the items listed are available from major laboratory suppliers (MLS) such as Baxter Scientific Products, Fisher Scientific, or VWR unless otherwise noted. Equivalent sources may be acceptable where noted.

Materials	Source
AmpliType™ User Guide, Version 2	**Perkin-Elmer, Norwalk, CT (Part No. N808-0111).**
Reagents	
Use reagent grade unless otherwise noted.	
Crystalline citric acid, monohydrate	JT Baker, Phillipsburg, NJ (Cat. No. 0110-05).
Deionized or distilled water	
Disodium ethylenediaminetetraacetic acid, dihydrate	GIBCO BRL, Gaithersburg, MD (Cat. No. 5575UA), or Sigma St. Louis, MO (Cat. No. E5513).
Hydrochloric acid, concentrated	JT Baker (Cat. No. 9535-01).
Hydrogen peroxide, 30%	Sigma Chemical Company, (Cat. No.H1009).
Sodium chloride	MLS
Sodium dodecyl sulfate, ultra pure electrophoresis grade	GIBCO BRL (Cat. No. 5525UAA).
5N Sodium hydroxide solution	VWR, San Francisco, CA (Cat. No. JT5671-2).
Sodium hydroxide pellets	MLS
Sodium phosphate, monobasic, monohydrate	Sigma (Cat. No. S9638).
Tris base (Trizma base [FW 121.1])	GIBCO BRL (Cat. No. 5504UA), or Sigma (Cat. No. 8404).
Trisodium citrate, dihydrate	GIBCO BRL (Cat. No. 5584UA).
Detection Reagents	
For colorimetric detection, the following reagents are needed:	
Chromogen:TMB	**Perkin-Elmer (Part No. N808-0092).**
100% ethanol (in a glass container)	Quantum Chemical Company, Cincinnati, OH.
For chemiluminescent (ECL™) detection, the following reagents are needed:	
ECL™ Detection Reagents	Amersham, Arlington Heights, IL (Cat. No. RPN.2109).
Film processing GBX Fixer	Sigma (Cat. No. P-7167).
Film processing GBX Developer	Sigma (Cat. No. P-7042).
General Equipment	
Autoclave	MLS
Hybridization Tray	**Perkin-Elmer (Part No. N808-0136).**
Hybridization Tray Retainer	**Perkin-Elmer (Part No. N808-0137).**
Balance, accurate to 1.0 mg	MLS
Bellco Hot Shaker Plus™ rotating water bath, adjustable to 65°C	Bellco, Vineland, NJ [Model No. 7746-22110 (110V), or Model No. 7746-22220 (220V)].

General Equipment (continued)	Source (continued)
Complete immersion thermometer (N.B.S. or equivalent, graduated to 0.2°C, range bracketing 37° to 55°C)	Cole Parmer, Niles, IL (Cat No. G-08001-34).
Lead weights, (e.g., 1 kg lead ring)	VWR Bridgeport, NJ (Cat. No. 29700-048), or Cole Parmer Chicago, IL (Cat. No. G-06137-06).
Labware and glassware	MLS
Magnetic stirrer and stir bars	MLS
Polaroid™ Camera and Type 55 or 667 (black and white) film or Type 59 or 559 (color) film.	MLS
pH meter	MLS
Pipettors -adjustable to deliver 1-20 µL, 20 - 200 µL and 200-1000 µL	Rainin, Woburn, MA (P20, P200, and P1000), or equivalent.
Refrigerator (2° to 8°C)	MLS
Shaker, variable speed, orbital platform (capable of 100-125 rpm)	Bellco, Lab-Line (Model No. 3520 for RPM X100).
Slot blot apparatus, The Convertible™	GIBCO BRL (Cat. No. 1055AA and Cat. No. 1055AE).
Timer, 60 minute (±1 minute)	MLS
Vortex	MLS
Water bath, stationary, adjustable to 37°C or incubator	MLS
Additional equipment needed for chemiluminescent detection:	
Automatic film processor (optional)	Konica, Japan (Model QX-130 A plus Code No. 1521), or equivalent.
Film cassette	Amersham (Cat. No. RPN.1642).
Dark room	MLS
Supplies	
Biodyne® B nylon membrane, 0.45 µm	GIBCO BRL (Cat. No. 4811SA).
Gloves, disposable	MLS
Lab wipes or paper towels	MLS
Permanent ink marker (for marking tubes)	MLS
Pipet tips for adjustable pipettors with plugged tips (sterilized by irradiation or autoclaving)	GIBCO BRL (Cat. No. 4867SA, 4862SP, and 4869SA).
GeneAmp™ PCR Reaction Tubes (0.5 mL polypropylene tubes)	**Perkin-Elmer (Part No. N801-0180).**
Additional supplies needed for chemiluminescent detection:	
Saran Wrap®	MLS
Whatman Benchkote® (polythene backed)	VWR (Cat. No. 52855-001).
X-ray film (Hyperfilm ECL or Kodak XAR5 film)	Amersham (Cat. No. RPN.2103), Kodak, or equivalent.

1.0 REAGENT PREPARATION

1.1 Preparation of reagents not supplied

Use reagent grade chemicals unless otherwise noted. Prepare all solutions using deionized or glass distilled water (identified below as DI H_2O). Wear gloves and follow safety recommendations provided by manufacturer for handling chemicals. Comply with any and all laws, regulations, or orders with respect to the disposal of any hazardous or toxic chemical, material, substance or waste. Store all reagents at room temperature (15° to 30°C) unless otherwise noted. Reagents are prepared as follows:

1.1.1 0.5 M EDTA (1 L). Add 186.1 g of disodium ethylenediaminetetraacetic acid dihydrate ($Na_2EDTA•2H_2O$) to 800 mL of DI H_2O. Stir vigorously on a magnetic stirrer. To dissolve the EDTA powder, adjust the pH to 8.0 (± 0.2) by adding approximately 20 g of NaOH pellets. Check the pH and add 5N NaOH solution if needed (for small pH adjustments, pellet addition cannot be accurately controlled). Adjust the final volume to 1 liter with DI H_2O and mix thoroughly. The solution should be autoclaved or filtered through a 0.2 µm Nalgene™ filter.

1.1.2 20% (w/v) SDS (1 L). **CAUTION!!! Wear protective mask when weighing SDS.** Slowly dissolve 200 g electrophoresis-grade (ultra pure) sodium dodecyl sulfate (SDS) in 800 mL DI H_2O. Warming (e.g., in a 37°C water bath) may be required to dissolve solids completely. Adjust the final volume to 1 liter with DI H_2O and mix thoroughly.

1.1.3 1 M Tris-HCl, pH 8.0 (1 L). Dissolve 121.1 g Tris base in 800 mL DI H_2O. Adjust to pH 8.0 (± 0.2) at room temperature by adding approximately 45 mL of concentrated HCl. Adjust the final volume to 1 liter with DI H_2O and mix thoroughly. Sterilize by autoclaving.

1.1.4 TE Buffer [10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 (1 L)]. Add 10 mL of 1 M Tris-HCL, pH 8.0 (**Section 1.1.3**) and 0.2 mL of 0.5 M EDTA (**Section 1.1.1**) to 990 mL DI H_2O and mix thoroughly. Dispense 100 mL aliquots and sterilize by autoclaving.

1.1.5 Citrate Buffer [0.1 M Sodium Citrate, pH 5.0 (1 L)]. Dissolve 18.4 g trisodium citrate, dihydrate ($Na_3C_6H_5O_7•2H_2O$) in 800 mL DI H_2O. Adjust the pH to 5.0 (± 0.2) by addition of approximately 6 g of citric acid monohydrate ($C_6H_8O_7•H_2O$). Adjust the final volume to 1 liter with DI H_2O and mix thoroughly.

1.1.6 20X SSPE Buffer [3.6 M NaCl, 200 mM ($NaH_2PO_4•H_2O$), 20 mM EDTA, pH 7.4 (1 L)]. Dissolve 7.4 g of disodium ethylenediaminetetraacetic acid dihydrate ($Na_2EDTA•2H_2O$) in 800 mL DI H_2O. Adjust the pH to 8.0 (± 0.2) with 10 N NaOH solution. Add 210 g Sodium Chloride (NaCl) and 27.6 g Sodium Phosphate, monobasic, monohydrate ($NaH_2PO_4•H_2O$). Adjust the pH to 7.4 (±0.2) with 10 N NaOH (about 10 mL). Adjust the final volume to 1 liter with DI H_2O and mix thoroughly.

1.1.7 Hybridization Solution [5X SSPE, 0.5% w/v SDS (1 L)]. Add 250 mL 20X SSPE (**Section 1.1.6**) and 25 mL 20% w/v SDS (**Section 1.1.2**) to 725 mL DI H_2O and mix thoroughly. Hybridization Solution solids must be in solution before use; warming (e.g., in a 37°C water bath) may be required to dissolve solids completely. Preparation in a clear glass container is recommended to facilitate visual inspection for solids during warming.

1.1.8 Wash Solution [1.5X SSPE, 0.5% w/v SDS (2 L)]. Add 150 mL of 20 X SSPE (**Section 1.1.6**), and 50 mL of 20% w/v SDS (**Section 1.1.2**) to 1,800 mL of DI H_2O and mix thoroughly. Wash Solution solids must be in solution before use; warming (e.g., in a 37°C water bath) may be required to dissolve solids completely. Preparation in a glass container is recommended to facilitate visual inspection for solids during warming.

1.1.9 Spotting Solution [0.4N NaOH, 25 mM EDTA, 0.00008% Bromothymol Blue (75 mL)]. Add 6 mL of 5N NaOH, 3.75 mL of 0.5 M EDTA (**Section 1.1.1**) and 150 µL 0.04 % of Bromothymol Blue (provided in Kit) to 65 mL of DI H_2O and mix thoroughly. Spotting Solution is stable for at least three months at room temperature.

1.1.10 Pre-Wetting Solution [0.4N NaOH, 25 mM EDTA (500 mL)]. Add 40 mL of 5N NaOH, 25 mL of 0.5 M EDTA (**Section 1.1.1**) to 435 mL of DI H_2O and mix thoroughly.

1.1.11 3% Hydrogen Peroxide (1 mL) **[For Colorimetric Detection Only]**. Add 100 µL of 30% H_2O_2 to 900 µL of DI H_2O and vortex to mix. Protect from light. Store at 2° to 8°C. 3% Hydrogen Peroxide has a shelf life of approximately 4 weeks when stored at 2° to 8°C.

1.1.12 Chromogen:TMB Solution. Bring the Chromogen:TMB (TMB) to room temperature (15° to 30°C). Before opening the bottle, tap it on the lab bench to shake the TMB to the bottom of the bottle. Remove the stopper carefully to prevent loss of the powder. Slowly add 30 mL of room temperature reagent grade 100% ethanol to the bottle. **Do NOT use ethanol that has been stored in a metal container. Do NOT use 95% ethanol or other alcohols.** Recap the bottle. Seal the stopper with Parafilm. Shake in an upright position on an orbital shaker for 30 minutes or until completely dissolved. Store in bottle at 2° to 8°C and protect from rust. Under these conditions the Chromogen Solution is stable for six months after preparation.

1.2 Preparation of reagents supplied

1.2.1 Human DNA Standards. Prepare a two-fold serial dilution of the DNA Standard A (provided in Kit) in TE Buffer (**Section 1.1.4**) as follows:

1. Label seven 0.5 mL autoclaved GeneAmp™ PCR Reaction Tubes A through G.
2. Vortex the DNA Standard A to mix it thoroughly.
3. Transfer 120 µL of DNA Standard A into the tube labeled A.
4. Aliquot 60 µL of TE Buffer (**Section 1.1.4**) into each of the six remaining tubes labeled B through G.
5. Add 60 µL of DNA Standard A (tube A) to the 60 µL of TE Buffer in tube B. **Vortex to mix thoroughly.**
6. Add 60 µL of diluted DNA Standard B (tube B) to the 60 µL of TE Buffer in tube C. **Vortex to mix thoroughly.**
7. Add 60 µL of diluted DNA Standard C (tube C) to the 60 µL of TE Buffer in tube D. **Vortex to mix thoroughly.**
8. Continue the serial dilution through tube G.

9. If the dilution steps are performed as described in **Section 1.2.1** above, the seven DNA Standard tubes (tubes A through G) will have the concentrations of human DNA listed in **Table 1.**

Table 1: DNA Standards and Concentrations

DNA Standard	Concentration (ng/ µL)	Quantity DNA per 5 µL (ng)
A	2	10
B	1	5
C	0.5	2.5
D	0.25	1.25
E	0.125	0.625
F	0.0625	0.3125
G	0.03125	0.15625

Note: Store the diluted DNA Standards at 2° to 8°C. The DNA Standards A through G are stable for at least three months at 2° to 8°C.

2.0 INFORMATION REGARDING PROTOCOLS

This entire section should be read before slot blotting/immobilization of DNA.

The QuantiBlot™ Human DNA Quantitation Kit contains reagents for at least 10 hybridization reactions. Each hybridization reaction must include the following ten control samples: seven DNA Standards, the two DNA Calibrators and one blank [Spotting Solution (**Section 1.1.9**) only]. An additional 38 samples can be spotted on the membrane, for a total of up to 48 samples per hybridization reaction. DNA Calibrators are provided as an internal control for DNA Standard performance.

3.0 SLOT BLOTTING/IMMOBILIZATION OF DNA

Note: Wear clean disposable laboratory gloves while preparing samples. Follow safety recommendations provided by manufacturer for handling chemicals. Comply with any and all laws, regulations or orders with respect to the disposal of any hazardous or toxic chemical, material, substance or waste.

Before beginning this section, assemble the required reagents, supplies and equipment as follows:

DNA Standards (A through G)
DNA Calibrator 1 (provided in Kit)
DNA Calibrator 2 (provided in Kit)
Slot Blot Apparatus
Spotting Solution (**Section 1.1.9**)
Pre-Wetting Solution (**Section 1.1.10**)
Biodyne® B nylon membrane

3.1 Determine the number of samples to be analyzed including the seven Human DNA Standards (A through G), the DNA Calibrators 1 and 2 (provided in Kit), and the one blank (Spotting Solution only). Aliquot 150 µL of Spotting Solution into a new 0.5 mL GeneAmp PCR Reaction Tube for each sample.

3.2 Label seven of the tubes containing 150 µL Spotting Solution as follows: A, B, C, D, E, F, and G and label two of the tubes containing 150 µL of Spotting Solution as follows: DNA Calibrator 1 and DNA Calibrator 2.

3.3 Vortex the seven DNA standards and the two DNA Calibrators. Add 5 µL of each solution to the corresponding labeled tube containing 150 µL of Spotting Solution.

Note: Sample DNA should be $MgCl_2$-free. See Troubleshooting Section.

3.4 Add 1 to 5 µL of each test sample DNA to the remaining tubes containing 150 µL of Spotting Solution.

3.5 While wearing clean gloves, cut a piece of Biodyne® B membrane to 11.0 cm x 7.9 cm. Cut a small notch in the upper right corner of the membrane to mark orientation. Place the membrane in the Hybridization Tray (**Part No. N808- 0136**) containing 50 mL of Pre-Wetting Solution. Incubate at room temperature for 1 to 30 minutes.

Note: The following protocol is for use with GIBCO BRL The Convertible™ slot blot apparatus. Refer to GIBCO BRL instructions for additional details. The vacuum source must have a pressure of at least 8 to 10 inches Hg.

3.6 Using forceps, remove the membrane from the Pre-Wetting Solution. Place the membrane on the gasket of the slot blot apparatus, then place the top plate of the slot blot apparatus on top of the membrane. **Turn on the vacuum source** (i.e., turn on house vacuum line or vacuum pump). **Turn off the sample vacuum** and **turn on the clamp vacuum** on the slot blot apparatus. Push down on the top plate to ensure the formation of a tight seal. Pour off the Pre-Wetting solution and rinse the Hybridization Tray thoroughly with DI H_2O.

3.7 Use a new pipette tip for each sample. Pipet each sample (approximately 155 µL) into a different well of the slot blot apparatus. Slowly dispense each sample directly into the center of each well of the slot blot apparatus ensuring that the pipet tip is approximately 5 mm above the membrane.

3.8 After all samples have been pipetted into the wells of the slot blot apparatus, **slowly turn on the sample vacuum.** Leave the sample vacuum on until all of the samples have been drawn through the membrane (approximately 30 seconds). Inspect each slot that contains a sample for a uniform blue band. (If a uniform blue band is not visible, refer to the **Troubleshooting Section.**) **Turn off the sample vacuum.**

3.9 Turn off the clamp vacuum. Turn off the vacuum source. Disassemble the slot blot apparatus and remove the membrane. **Proceed to Section 4 immediately. Do NOT allow the membrane to dry-out.**

Note: After each use, soak the slot blot apparatus in a large volume of 0.1% SDS solution (approximately 5 to 15 minutes). Using a disposable lab towel, clean the gasket and the side of the top plate that contacts the membrane. Then rinse the slot blot apparatus with an excess of water and allow to dry at room temperature. Never use bleach.

4.0 DNA HYBRIDIZATION

The following section involves the hybridization of biotinylated QuantiBlot™ D17Z1 Probe to DNA samples immobilized on the nylon membrane, the binding of Enzyme Conjugate:HRP-SA to the hybridized probe and a stringent wash to remove non-specifically bound probe.

Before starting the DNA Hybridization procedure, assemble the required reagents and equipment as follows:

QuantiBlot D17Z1 Probe (provided in Kit)
Enzyme Conjugate:HRP-SA (provided in Kit)
Hybridization Solution (**Section 1.1.7**)
Wash Solution (**Section 1.1.8**)
Citrate Buffer (**Section 1.1.5**)
30% Hydrogen Peroxide
Hybridization Tray and lid
Hybridization Tray Retainer

Do NOT allow the membrane to dry at any point in the protocol. Minimize the time the membrane is not submerged in solution. Use the Hybridization Tray with lid for all steps.

Warm the Hybridization Solution and the Wash Solution to between 37° and 50°C in either a water bath or an incubator. **All solids must be in solution before use.** Mix well.

Note: Clean, disposable gloves should be worn throughout the DNA Hybridization (Section 4.0) and Detection steps (Section 5.0).

4.1 Pre-hybridization: Transfer the membrane to 100 mL of pre-warmed Hybridization Solution in the Hybridization Tray. Add 5 mL of 30% H_2O_2. Place the lid on the tray. Use the Hybridization Tray Retainer or a lead weight to keep tray from floating in the water bath. Rotate in a 50°C (±1°C) water bath (50 to 60 rpm) for 15 minutes (±2 minutes). Pour off the solution.

4.2 Hybridization: Add 30 mL of Hybridization Solution to the Hybridization Tray containing the membrane. Tilt the tray to one side and add 20 µL of QuantiBlot D17Z1 Probe to the Hybridization Solution. Place the lid on the tray. Rotate in a 50°C (±1°C) water bath (50 to 60 rpm) for 20 minutes (±2 minutes). Pour off the solution.

4.3 Rinse the membrane briefly in 100 mL of pre-warmed Wash Solution by rocking the tray for several seconds. Pour off the solution.

Note: Step 4.4 (below) describes the addition of two alternative volumes of Enzyme Conjugate:HRP-SA. The volume of Enzyme Conjugate:HRP-SA used depends on the detection scheme chosen: colorimetric (TMB) or chemiluminescent (ECL™) detection. (See Introduction on page 2 for more general information on these two alternative detection schemes.)

4.4 Stringent Wash/Conjugation: Add 30 mL of the pre-warmed Wash Solution to the Hybridization Tray. Tilt the tray to one side and add the Enzyme Conjugate:HRP-SA to the 30 mL of Wash Solution as follows: for colorimetric (TMB) detection add 180 µL of Enzyme Conjugate:HRP-SA **OR** for chemiluminescent (ECL) detection add 90 µL of Enzyme Conjugate:HRP-SA. Place the lid on the tray. Rotate in a 50°C (±1°C) water bath (50 to 60 rpm) for 10 minutes (±1 minute). Pour off the solution.

4.5 Rinse the membrane thoroughly for 1 minute in 100 mL of pre-warmed Wash Solution by rocking the tray or rotating it on an orbital shaker (100 to 125 rpm) at room temperature. Pour off the solution. Rinse again for 1 minute. Pour off the solution.

4.6 Wash the membrane by adding 100 mL of pre-warmed Wash Solution to the tray. Place the lid on the tray. Rotate at room temperature on an orbital shaker (100 to 125 rpm) at room temperature for 15 minutes. Pour off the solution.

4.7 Rinse the membrane briefly in 100 mL of Citrate Buffer by rocking the tray. Pour off the solution.

5.0 DETECTION STEPS

If 180 µL of Enzyme Conjugate:HRP-SA was used in Section 4.4, use the colorimetric detection steps in Section 5.1; if 90 µL of Enzyme Conjugate:HRP-SA was used in Section 4.4, proceed to Section 5.2 for chemiluminescent detection.

5.1 Colorimetric Detection

5.1.1 Prepare the Color Development Solution **not more than 10 minutes before use.** Add the following reagents in the order listed to a glass flask and mix thoroughly by swirling. **Do NOT vortex.** To 30 mL of Citrate Buffer (**Section 1.1.5**), add 1.5 mL of Chromogen:TMB Solution (**Section 1.1.12**) and 30 µL of 3% H_2O_2 (**Section 1.1.11**).

5.1.2 Add Color Development Solution to the membrane in the tray. **Cover the tray with the lid to protect the membrane from strong light.**

5.1.3 Shake at room temperature on an orbital shaker (50 to 60 rpm) for 20 to 30 minutes.

5.1.4 Remove tray from shaker, pour off liquid.

5.1.5 Stop the color development by washing in deionized H_2O (100 mL). Shake for 5 to 10 minutes (50 to 60 rpm) with the lid on the tray. Repeat for a total of three washes.

5.1.6 Photograph the membrane when it is wet. Saran Wrap® may be placed over the membrane during photography to prevent it from drying out.

1. Place the wet membrane on a flat non-absorbent surface. Keep the membrane wet throughout the photographic procedure. Minimize exposure to strong light.
2. Use a Polaroid camera with Type 55 or 667 (black and white) film or Type 59 or 559 (color) film.
3. Follow Polaroid film exposure and development instructions.

   Note: For black and white photography, an orange filter (Wratten 22 or 23A) will enhance contrast.
4. Following photography, the membrane may be air-dried on any hard non-absorbent surface. Protect from light and oxidizing agents (e.g., acid treated paper, bleach and nitric acid). The blue color on the membrane will fade upon drying.

5.2 Chemiluminescent Detection

Before beginning this section, assemble the required reagents, supplies and equipment as follows:

ECL™ Detection Reagents
Whatman® Benchkote
Saran Wrap®
X-ray Film
Film Cassette

Note: ECL Reagents 1 and 2 should be stored separately (at 2° to 8°C) and not allowed to cross-contaminate each other.

5.2.1 To 5 mL of ECL Reagent 2, add 5 mL of ECL Reagent 1. **Do NOT prepare this mixture more than 5 minutes before use.** Add the 10 mL of ECL reagent mixture to the membrane in the Hybridization Tray and shake for exactly 1 minute at room temperature. Pour off the solution.

Note: For maximum sensitivity, expose the membrane to X-ray film within 10 minutes of incubation in ECL reagents.

5.2.2 Cut a piece of Benchkote to approximately 12 x 16 cm. Place the damp membrane DNA-side-up on the plastic-coated side of the Benchkote. Cover the membrane with a piece of Saran Wrap that is a few centimeters larger than the BenchKote. Use a paper towel to smooth out any wrinkles or air bubbles in the Saran Wrap. Fold the Saran Wrap behind the BenchKote on the top and bottom sides. Again, use a paper towel to flatten the Saran Wrap and remove any air bubbles. Fold the Saran Wrap behind the Benchkote on the two remaining sides.

5.2.3 In a darkroom, place a piece of Hyperfilm ECL or Kodak XAR5 film in the film cassette. Carefully place the covered membrane on top of the film such that the DNA side is in contact with the film. **Do NOT move the membrane once it is placed on top of the film; movement may cause blurring of the resulting image or a "double image".** Close the film cassette. **It is very important that the film is in tight, uniform contact with the covered membrane.**

5.2.4 Expose the film for 15 minutes at room temperature.

5.2.5 Process the film with an automatic film processor. If an automatic film processor is not available, use Kodak GBX Developer and GBX Fixer as follows:

1. Dilute the Developer and Fixer solutions as instructed on the reagent bottles.
2. In the darkroom, incubate the film in the Developer for 90 seconds before rinsing it in DI H_2O for a few seconds. Then incubate it in the Fixer for 90 seconds.
3. Rinse the film in a continuous flow of fresh DI H_2O for 30 minutes. Hang to dry.

Note: If desired, additional X-ray film exposures can be obtained by repeating steps 5.2.3 through 5.2.5 above. Exposures longer than 15 minutes (up to several hours or overnight) will result in increased sensitivity. Preparation of additional further-diluted DNA Standards may be necessary to quantitate samples containing less than 0.15 nanograms of DNA.

6.0 RESULTS INTERPRETATION

Results are interpreted by comparing the signal intensity of the DNA test sample to the signal intensity obtained for the DNA Standards. The signal intensity for a sample reflects the total amount of DNA spotted on the membrane. The seven DNA Standards represent the following quantities of DNA spotted on the membrane: 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 ng (**See Table 1**).

The DNA Calibrators are used to provide DNA of a known concentration to verify that the DNA Standards were correctly diluted and are providing correct results for the test samples. For example, the DNA Calibrator 1 has a stock concentration of 0.7 ng/µL. Five µL of this control was added to 150 µL of Spotting Solution and the entire 155 µL was spotted on the membrane. Thus, 3.5 ng of this sample was spotted on the membrane (0.7 ng/µL • 5 µL = 3.5 ng). The signal obtained for this control sample should have an intensity that is between the 2.5 and 5 ng DNA Standards. Likewise, the DNA Calibrator 2 should have an intensity that is between the 0.3125 and 0.625 ng DNA Standards. If not, see the **Troubleshooting Section**.

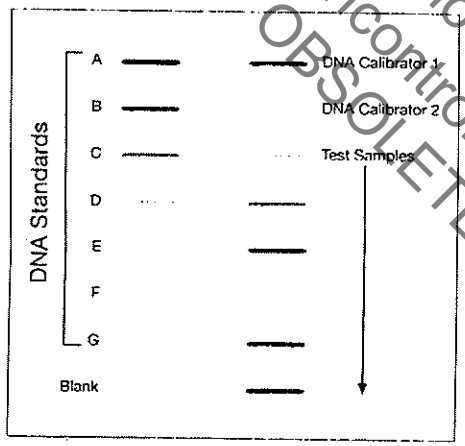

Figure 1: Example of QuantiBlot™ results (using colorimetric (TMB) detection method). The seven DNA Standards represent the following quantities of DNA spotted on the membrane: A = 10 ng; B = 5 ng; C = 2.5 ng; D = 1.25 ng; E = 0.625 ng; F = 0.3125 ng and G = 0.15625 ng. DNA Calibrator 1 should have an intensity that is between DNA Standards B and C. DNA Calibrator 2 should have an intensity that is between DNA Standards E and F. Quantities for the test samples are determined by comparison of signal intensities to the DNA Standards.

The concentration of a DNA test sample is determined as follows:

1. Determine the quantity of DNA test sample spotted on the membrane by comparing its signal intensity to the intensity of the DNA Standards.
2. Divide this quantity by the volume of DNA test sample added to the Spotting Solution (typically 5 µL of DNA test sample is added to 150 µL of Spotting Solution).

This calculation gives DNA concentration in ng/µL.

PERFORMANCE CHARACTERISTICS

When the recommended protocols are followed, using the colormetric detection method with Chromogen:TMB, the user of the QuantiBlot™ Human DNA Quantitation Kit will be able to detect and quantitate 0.15 to 10 ng of human DNA per test sample.

TROUBLESHOOTING

Observation	Possible Cause	Recommended Action
1. No signal or low sensitivity (0.15 ng DNA Standard not visible).	Use of a membrane other than Biodyne® B.	Use Biodyne B nylon membrane. Do not use membranes that have a neutral charge.
	Incorrect NaOH or EDTA concentrations in Spotting Solution.	Prepare Spotting Solution correctly (**Section 1.1.9**).
	Water bath temperature too high.	Water bath temperature should be 50°C (±1°C).
	DNA Probe was not added at hybridization step.	Add QuantiBlot™ D17Z1 Probe.
	Enzyme conjugate was not added.	Add Enzyme Conjugate: HRP-SA at indicated step in protocol. Use 180 µL of Enzyme Conjugate:HRP-SA for colorimetric (TMB) detection, and use 90 µL of HRP-SA for chemiluminescent (ECL™) detection.
	Hydrogen peroxide was inactive (colorimetric detection only).	Prepare a new Color Development Solution using a fresh bottle of hydrogen peroxide.
	Presence of $MgCl_2$ in the DNA sample.	Concentrations of $MgCl_2$ >0.3 mM can result in reduced sensitivity. Prepare all DNA dilutions in TE Buffer. Any $MgCl_2$ can be removed from samples by microdialysis using Centricon® 100 spin units (follow manufacture's directions).
	Cross-contamination of ECL Reagents (chemiluminescent detection method only).	ECL Reagents 1 and 2 should be stored separately and should not be mixed until 5 minutes prior to use.
	ECL Reagents stored at room temperature (chemiluminescent detection method only).	Order fresh ECL Reagents. Store ECL Reagents at 2° to 8°C.
2. Areas of low sensitivity across the membrane.	Membrane slipped up onto the side of the Hybridization Tray during Hybridization or Stringent Wash steps.	Reduce the rotation rate of the water bath to 50-60 rpm. Check that the membrane is fully submerged in the bottom of the Hybridization Tray before shaking.
	Membrane dried-out significantly at some point in the protocol.	Do not allow the membrane to dry at any point in the protocol.

Observation	Possible Cause	Recommended Action
2. Areas of low sensitivity across the membrane.	Poor contact between the film and the covered membrane during film exposure for chemiluminescent detection.	Smooth-out any wrinkles or bubbles in the Saran Wrap® covering the membrane and make sure that the film cassette provides tight, uniform contact between the covered membrane and the film.
3. Non-uniform signal intensity within a slot.	Bubble(s) in slot blot wells when sample was pipetted into well, or when vacuum was applied.	Slowly pipet the Spotting Solution directly over the center of the wells of the slot blot apparatus, with the pipet tip raised approximately 5 mm above the membrane. Turn on the sample vacuum slowly, not all at once. After being drawn through the membrane, the sample should appear as a uniform blue band on the membrane. If the entire sample is not drawn through the membrane, turn off the sample vacuum. Pipet the sample back into the pipet tip; then pipet the sample back into the well of the slot blot apparatus. Turn on the sample vacuum to draw the sample through the membrane.
4. Filter background	No or low SDS in the Hybridization Solution or in the Wash Solution.	Prepare solutions with proper concentrations of SDS.
	Membrane was not prewetted prior to slot blotting.	Pre-wet the membrane in Pre-Wetting Solution prior to slot blotting.
	Too much Enzyme Conjugate:HRP-SA was added.	Use 180 µL of Enzyme Conjugate:HRP-SA for colorimetric detection or 90 µL of Enzyme Conjugate:HRP-SA for chemiluminescent detection.
	Lack of thorough rinsing at **Section 4.5** of the DNA Hybridization protocol.	Thoroughly rinse twice, for 1 minute each, using 100 mL of pre-warmed Wash Solution at this step. These two rinse times can be extended beyond 1 minute if necessary.
	Slot blot apparatus not cleaned thoroughly after last use.	Immediately after each use, soak the slot blot apparatus in a large volume of 0.1% SDS solution. Never use bleach.

Observation	Possible Cause	Recommended Action
5. Blurry or mis-shapen slots (chemiluminescent detection only).	Bubbles or wrinkles in Saran Wrap covering membrane during film exposure.	Carefully smooth-out wrinkles or air pockets in the Saran Wrap that covers the membrane during film exposure.
6. Blurry slots and/or double image (chemiluminescent detection only).	Membrane was moved after it was placed in contact with the film.	Carefully place the membrane directly on top of the film. Do not move the membrane once it has been placed on top of the film.
7. The DNA Calibrators do not quantitate correctly with respect to the DNA Stan-dards.	DNA Standard serial dilutions prepared incorrectly.	Prepare two-fold serial dilutions of DNA Standard A in TE Buffer as described. Add 5 µL of each dilution to 150 µL of Spotting Solution for slot blotting.
8. Signals obtained for non-human DNA samples.*	Water bath temperature too low.	Water bath temperature should be 50°C (±1°C).
	SSPE concentration too high in Wash Solution.	Check that the 20X SSPE solution and the Wash Solution were prepared correctly.

* DNA from primate species may give signals similar to those obtained from equivalent amounts of human DNA. In Roche Molecular Systems (RMS) laboratories, 30 ng to 300 ng quantities of non-primate DNA samples result in either no signals or signals that are less than or equal to the signal obtained for 0.15 ng of human DNA. The following non-primate DNA samples have been tested in RMS laboratories: *E. coli*, yeast, dog, cat, mouse, rat, pig, cow, chicken, fish and turkey.

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PROTOCOL FOR EXTRACTION AND QUANTITATION OF CASE EVIDENCE

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