PROTOCOLS FOR FORENSIC PCR ANALYSIS

Version 2.0

Office of the Chief Medical Examiner
Forensic Biology Department
520 First Ave
New York, N.Y. 10016

Initials: RCS Date: 4/7/94

Initials: RCS Date: 4/7/94

Initials: RY Date: 6/21/95

General PCR Guidelines

1. The general laboratory policy is to identify physiological fluids (see the Forensic Biochemistry and Hematology Laboratory Manual) before individualization is attempted. However, circumstances will exist when this is not be possible.

2. Duplicate analyses must be performed. Preferably, this should begin at the DNA extraction stage. However, if an additional extraction would consume >75% of the sample (or a complex mixture needs to be duplicated) then the duplicate analysis can begin at the amplification stage.

3. Duplicate analyses are performed to verify the typing results. At least one run must have *no* visible dots in the extraction reagent control, amplifcation negative control and no extraneous dots in the positive control. The other run which is only used for verification, can have extraneous dots less than the "C" dot in the extraction reagent, amplification negative and positive controls.

4. The substrate control can have visible dots. These dots do *not* invalidate the results of the accompanying stains. The presence of dots in the substrate control should be noted in the report.

5. To minimize the potential for carry-over contamination, the laboratory is organized so that the areas for handling amplified DNA, for DNA extraction and for PCR set-up are physically isolated from each other. Each of the three areas is in a separate room. Dedicated equipment such as pipettors should not leave their designated areas. Only the samples should move between areas.

6. Samples that have not yet been amplified should never come in contact with equipment in the amplified DNA work area. Samples that have been amplified should never come in contact with equipment in the unamplified work area.

7. Handle all samples aseptically to prevent contamination by extraneous DNA.

8. The DNA extraction and PCR setup of evidence samples should be performed at a separate time from the DNA extraction and PCR setup of exemplars. This precaution will help to prevent potential cross-contamination between evidence samples and exemplars.

9. Change gloves frequently to avoid sample-to-sample contamination. Change them whenever they might have been contaminated with DNA and whenever exiting a work area.

Initials: RC Date: 4/7/94

General PCR Guidelines

1. The general laboratory policy is to identify physiological fluids (see the Forensic Biochemistry and Hematology Laboratory Manual) before individualization is attempted. However, circumstances will exist when this is not be possible.

2. Duplicate analyses must be performed. Preferably, this should begin at the DNA extraction stage. However, if an additional extraction would consume >75% of the sample (or a complex mixture needs to be duplicated) then the duplicate analysis can begin at the amplification stage.

3. Duplicate analyses are performed to verify the typing results. At least one run must have *no* visible dots in the extraction reagent control and no extraneous dots in the positive control. The other run which is only used for verification, can have extraneous dots less than the "C" dot in the extraction reagent and positive controls.

4. The substrate control can have visible dots. These dots do *not* invalidate the results of the accompanying stains. The presence of dots in the substrate control should be noted in the report.

5. To minimize the potential for carry-over contamination, the laboratory is organized so that the areas for handling amplified DNA, for DNA extraction and for PCR set-up are physically isolated from each other. Each of the three areas is in a separate room. Dedicated equipment such as pipettors should not leave their designated areas. Only the samples should move between areas.

6. Samples that have not yet been amplified should never come in contact with equipment in the amplified DNA work area. Samples that have been amplified should never come in contact with equipment in the unamplified work area.

7. Handle all samples aseptically to prevent contamination by extraneous DNA.

8. The DNA extraction and PCR setup of evidence samples should be performed at a separate time from the DNA extraction and PCR setup of exemplars. This precaution will help to prevent potential cross-contamination between evidence samples and exemplars.

9. Change gloves frequently to avoid sample-to-sample contamination. Change them whenever they might have been contaminated with DNA and whenever exiting a work area.

Initials: RU Date: 4/7/94

10. Always change pipette tips between handling each sample.

11. Never "blow out" the last bit of sample from a pipettor. Blowing out increases the potential for aerosols, which may contaminate a sample with DNA from other samples. The accuracy of liquid volume delivered is not critical enough to justify blowing out.

12. Use filter pipet tips for pipeting all DNA and use whenever possible for other reagents. Use the appropriate filter tips for the different sized pipetmen. The tip of the pipetman should never touch the filter.

13. Avoid splashes. Centrifuge all liquid to the bottom of the closed tube before opening it.

14. Avoid touching the inside surface of the tube caps.

15. Use disposable bench paper to prevent the accumulation of human DNA on permanent work surfaces. Bleach should be used periodically to decontaminate exposed work surfaces.

16. Limit the quantity of samples handled in a single run to a manageable number (approximately 16). This precaution will reduce the risk of sample mix-up and the potential for sample-to sample contamination.

17. Store the DNA Amplification Reagents together in the box provided which will serve as a barrier to possible contamination by exogenous DNA. The box should be stored in PCR set-up refrigerator.

18. Store evidence and unamplified DNA in a separate refrigerator or freezer from the amplified DNA.

19. Each sample handling area should have its own microfuge racks. The racks should only leave their designated area to transport samples to the next area. Immediately after transporting samples, the racks should be returned to their designated area.

20. Avoid exposing mineral oil to UV light. Exposure to UV light causes the mineral oil to inhibit PCR.

21. Use the Thermal Cycler only for amplification and denaturation of amplified DNA for typing.

Initials: RJ Date: 4/7/94

22. Keep bleach away from the color development area. Small quantities of bleach can inhibit dot color development.

23. Make sure lab coat sleeves do not touch the caps of open tubes.

24. Discard pipette tips if they accidentally touch the bench paper or any other surface.

25. Wipe the outside of the pipetman with 10% bleach solution if the barrel goes inside a tube.

26. During analysis, all evidence, unamplified DNA, and amplified DNA should be stored refrigerated or frozen. Freezing is generally better for long term storage.

27. After the report is issued, DNA extracts and amplified DNA's are discarded. **However if a sample is consumed during analysis, the remainder of that sample's unamplified DNA (i.e. DNA extracts) must be retained.**

28. Make sure worksheets and logbooks are completely filled out.

Initials: RC Date: 4/7/94

DNA Extraction

DNA Extraction Guidelines

Slightly different extraction procedures may be required for each type of specimen. Due to the varied nature of evidence samples, the user may need to modify procedures. See the trouble shooting guide (page 52) for suggestions on how to modify procedures.

1. Use a clean cutting surface for each sample such as a Kimwipe.

2. Clean scissors thoroughly with 70% ethanol or use fresh razor blades for cutting each evidence sample.

3. Swabs should be cut into two or three pieces of equal size. Sections which are not to be analyzed immediately should be stored frozen.

4. Use Kimwipes to open sample tubes and blood tubes.

5. Only one tube should be uncapped at a time. When a sample is added to a tube, the tube should be re-capped and the scissors and work area cleaned before the next tube is uncapped.

6. Try not to consume more than 75% of the sample, when possible.

7. When pouring or pipetting Chelex solutions, the resin beads must be distributed evenly in solution. This can be achieved by shaking or vortexing the tubes containing the Chelex stock solution before aliquoting.

8. Pour an aliquot of the Chelex solution from the stock tube into a sterile disposable working tube before adding to samples. The stock tube can be used multiple times. The working tube is discarded after each batch of extractions.

9. For pipeting Chelex, the pipette tip used must have a relatively large bore--1 mL pipetman tips are adequate.

10. Keep the Chelex extraction reagents and equipment separated from the rest of the laboratory equipment.

11. Be aware of small particles of fabric which may cling to the outside of tubes.

Initials: RC   Date: 4/7/97

12. Include an extraction reagent control with each batch of extractions to demonstrate extraction integrity. The extraction reagent control contains water in place of biological fluids or stains. If DNA is found in the extraction reagent control by QuantiBlot analysis, the extraction of all the samples in the batch should be repeated and the samples should not be amplified. However, if no DNA is found then the extraction reagent controls are treated as normal samples and extracted, amplified and typed along with the test samples.

13. Obtain substrate controls from unstained fabric or substrate as close to each stain as feasible. It is not always possible to find an unstained substrate control for each evidence sample. If no DNA is found in a substrate control by QuantiBlot analysis, the substrate control is discarded without amplification because no DNA is present. However if DNA is found in the substrate control either a new substrate control should be extracted and quantitated or the substrate control should be amplified and typed along with the test samples.

14. If a sample is found to contain < 0.15 ng of DNA by QuantiBlot analysis, the sample should not be amplified and it should be reported as containing no detectable DNA.

15. After extraction, the tubes containing the unamplified DNA should be transferred to a box and stored in the appropriate refrigerator or freezer. The microtube rack used to hold the DNA extraction tubes should be washed with 10% bleach. The tubes should *not* be stored in the extraction racks

Initials: RG Date: 4/7/94

Chelex DNA Extraction From Whole Blood, Bloodstains, or Blood Scraped or Swabbed Off a Surface

1. Fill out the extraction worksheet.

2. Pipette 1 mL of sterile deionized water into a 1.5 mL microcentrifuge tube for each sample. Cap all of the tubes.

3. A. Open one tube at a time with a Kimwipe and add one of the following:
    a) 3 μL whole blood
    b) portion of bloodstain or swab about 3 mm square
    c) enough scrapings to give a light straw colored extract

   B. Cap the tube before adding sample to the next tube.

4. Mix the tubes by inversion or vortexing.

5. Incubate at room temperature for 15 to 30 minutes. Mix occasionally by inversion or vortexing.

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

7. Carefully remove supernatant (all but 20 to 30 μL). If the sample is a bloodstain or swab, leave the substrate in the tube with pellet. (The supernatant can be frozen and retained for analysis according to the Forensic Biochemistry and Hematology Manual or it can be discarded if it is not needed).

8. Add 175 μL of 5% Chelex.

9. Incubate at 56°C for 15 to 30 minutes.

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

11. Incubate at 100°C for 8 minutes using a screw down rack.

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

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

Initials: KC Date: 4/7/94

14. Pipet 20 μL into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration.

15. Store the remainder of the supernatant at 2 to 8°C or frozen.

16. To re-use a sample, thaw and repeat steps 12-13.

Initials: RCI Date: 4/12/94

Chelex DNA Extraction From Liquid Semen

1. Fill out the extraction worksheet.

2. Add 200 μL of 5% Chelex into a 1.5 mL microcentrifuge tube for each sample.

3. Add 2 μL of 10 mg/mL Proteinase K and 7 μL of 1 M DTT to each of the tubes. Cap all of the tubes and mix gently.

4. Open one tube at a time with a Kimwipe and add 3 μL of whole semen. Cap the tube before adding sample to the next tube.

5. Mix gently.

6. Incubate at 56°C for 30 to 60 minutes. Vortex at high speed 5 to 10 seconds.

7. Spin in a microcentrifuge for 10 to 20 seconds at 10,000 - 15,000 x g.

8. Follow the protocol for Whole Blood/Blood Stains (page 6) beginning with Step 10.

Initials: RLS Date: 4/7/94

Chelex DNA Extraction from Semen Stains or Swabs

1. Fill out the extraction worksheet.

2. Pipette 1 mL of PBS into a 1.5 mL microcentrifuge tube for each sample. Cap all of the tubes.

3. A. Open one tube at a time with a Kimwipe and add one of the following:
    a) portion of semen stain about 3 mm square
    b) one third of a swab
    c) scrapings of a stain

   B. Cap the tube before adding sample to the next tube.

4. Mix by inversion or vortexing

5. Incubate at room temperature for 30 minutes.

6. Vortex or sonicate the substrate or swab for at least 2 minutes to agitate the cells off of the substrate or swab.

7. Remove the swab or other substrate from the sample tube, one tube at a time, using sterile tweezers and close tubes. Sterilize tweezers with ethanol before the removal of each sample.

   It is advisable not to discard the substrate until microscopic analysis (Step 11) shows that the sample contains sperm. Store swab or substrate in a sterile tube.

8. Spin in a microcentrifuge for 5 minutes at 10,000 to 15,000 x g.

9. Without disturbing the pellet, remove all but 50 μL of the supernatant. (The supernatant may be frozen or lyophilized and retained for analysis according to the Biochemistry and Hematology Laboratory Manual or discarded if not needed).

10. Resuspend the pellet in the remaining 50 μL by stirring with a sterile pipette tip.

Initials: RCJ Date: 4/7/94

11. Remove about 3 μL of the resuspended sample for a Christmas Tree Stain (see Biochemistry and Hematology Laboratory Manual). After staining, the slide should be labeled and saved as evidence. If sperm are not visible microscopically, the substrate may be put back into the PBS and vortexed more vigorously (step 6) to try to dislodge additional sperm.

    Note: If epithelial cells are detected, proceed with the differential extraction procedure beginning with step 12. If no epithelial cells are observed, the differential extraction procedure may be omitted and the sample may be processed beginning with step 21.

12. To the approximately 50 μL of resuspended cell debris pellet, add 150 μL sterile deionized water (final volume of 200 μL).

13. Add 2 μL of 10 mg/mL Proteinase K. Vortex briefly to resuspend the pellet.

14. Incubate at 56°C for about 60 minutes to lyse epithelial cells, but for no more than 75 minutes, to minimize sperm lysis.

15. During the incubation step do the following:

    a. Label a new tube for each sample, including the extraction reagent control. Mark each tube as an epithelial cell fraction
    b. Add 50 μL of 20% Chelex to each epithelial cell fraction tube
    c. Close tubes

16. Spin the extract in a microcentrifuge at 10,000 to 15,000 x g for 5 minutes.

17. Add 150 μL of the supernatant from each sample to its respective epithelial cell fraction sample tube. Store at 4°C or on ice until step 22.

18. Wash the sperm pellet with Digest Buffer as follows:
    a. Resuspend the pellet in 0.5 mL Digest Buffer.
    b. Vortex briefly to resuspend pellet.
    c. Spin in a microcentrifuge at 10,000 to 15,000 x g for 5 minutes.
    d. Remove all but 50 μL of the supernatant and discard the supernatant.
    e. Repeat steps a-d for a total of 5 times.

Initials: RCJ Date: 4/7/94

19. Wash the sperm pellet once with sterile dH_2O as follows:
    a. Resuspend the pellet in 1 mL sterile dH_2O.
    b. Vortex briefly to resuspend pellet.
    c. Spin in a microcentrifuge at 10,000 to 15,000 x g for 5 minutes.
    d. Remove all but 50 μL of the supernatant and discard the supernatant.

20. Resuspend the pellet by stirring with a sterile pipette tip. Remove about 3 μL of the resuspended sample and spot on a glass microscope slide for examination. Perform Christmas Tree stain (see Biochemistry and Hematology Laboratory Protocol). After staining the slide should be labeled and saved as evidence.

21. To the approximately 50 μL resuspended sperm fraction, add 150 μL of 5% Chelex, 2 μL of 10 mg/mL Proteinase K, and 7 μL of 1M DTT. Mix gently.

22. Vortex both the epithelial cell and sperm fractions. The following steps apply to both fractions.

23. Incubate at 56°C for approximately 60 minutes.

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

25. Incubate in at 100°C for 8 minutes using a screw down rack.

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

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

28. Pipet 20 μL into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration.

31. Store the remainder of the supernatant at 2 to 8°C or frozen.

32. To re-use a sample, thaw and repeat steps 26-27.

Initials: RC Date: 4/7/94

Non-Organic Extraction of DNA From Blood

1. Process a Cell Pellet Control and an Extraction Reagent Control (0.5 mL) with every batch of extractions.

2. Add 0.5 mL or of well mixed blood to a microcentrifuge tube.

3. Add 1.0 mL ice cold Cell Lysis Buffer (CLB). Vortex at high speed for one minute.

4. Centrifuge samples 5 minutes, 2700 x G at 4°C.

5. Decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue. Store tubes on ice.

6. Repeat steps 3-5 two (2) more times with CLB.

7. Repeat steps 3-5 one more time substituting ice cold Protein Lysis Buffer (PLB) for the CLB. Keep tubes on ice.

8. Thaw a tube of Proteinase K and mix thoroughly by gentle inversion of the tube.

9. Prepare a master mix of PLB and Proteinase K for N+2 samples.

   Master mix for 1 sample.
   225 μL PLB
   25 μL Proteinase K (10 mg/mL)

10. Process one sample at a time:
    Add 250 μL of master mix. Pipet up and down to resuspend pellet. Mix well. Place tube in 65°C heat block.

11. Incubate each tube for 2-2.5 hour. Vortex at high speed for $\approx$ 30 seconds every 15-20 minutes to insure nuclear pellet is resuspended.

12. Vortex at high speed for $\approx$ 30 seconds following complete incubation.

13. Centrifuge samples 5 minutes in a microcentrifuge at room temperature to remove particles. Transfer supernatant to a new tube.

Initials: RCJ Date: 4/7/94

14. Run a yield gel to determine the DNA concentration of each sample.

15. Store DNA at 4°C. It is stable for several months.

Initials: RCJ Date: 4/7/94

Chelex Extraction From Non-Organically Extracted Samples

The amount of DNA in non-organically extracted samples is usually quantitated by yield gel. The target amount of DNA to add to each amplification tube is 5 ng in 20 μL. The amount of DNA is normalized by adding different volumes of non-organic extract to each Chelex tube, depending upon the yield of the sample. Table I lists the volumes of non-organic extract to add to the Chelex tube for the various yield gel concentrations.

Table I: Non-Organic Extract for Amplification

yield gel conc (ng/μl)	volume dH_2O (μL)	volume non-organic extract (μL)	volume Chelex (μL)
100	49.5	0.5	150
50	49	1.0	150
25	48	2.0	150
20	47.5	2.5	150
12.5	46	4.0	150
10	45	5.0	150
5.0	40	10	150
2.5	30	20	150
≤1.0	25	25	150

1. Fill out the extraction worksheet.

2. Vortex and briefly microfuge the tubes containing the non-organically extracted DNA samples.

3. Add in order, the appropriate amount of dH_2O, non-organic extract, and 5% Chelex solution to each sample tube (Table I) for a final volume of 200 μL.

4. Incubate at 56°C for 15-30 minutes.

5. Vortex.

Initials: RCS Date: 4/7/94

6. Incubate in at 100°C for 8 minutes using a screw down rack.

7. Vortex.

8. Microfuge for 2-3 minutes.

9. Pipet 20 μL into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration. If QuantiBlot analysis is not performed, 20 μL of the supernatant may be added to the PCR reaction tube.

10. Store the remaining sample at 2-8°C or frozen.

Initials: RCI Date: 4/7/94

Estimation of DNA Quantity

Estimation of DNA Quantity from QuantiBlot

Sample Blotting

1. Vortex samples including, if applicable, Cell Pellet Control(s), Extraction Reagent Control(s), Substrate Control(s), DNA Standards, and DNA Calibrators 1 and 2. Centrifuge briefly to bring the contents to the bottom of the tube. If Chelex extracts are being used, centrifuge for 2 minutes.

2. While wearing gloves, label enough microfuge tubes for all samples and standards.

   Pipet samples and standards into the microfuge tubes, using the following amounts of each:

   A. DNA Standards and Calibrators - 5 μL

   B. Chelex extracts intended for HLA-DQα - 20 μL

   C. Non-organic extracts - using the yield gel concentration, estimate the volume needed to apply 1-5 ng DNA; prepare a dilution with 1X TE if necessary.

   The samples can be aliquoted ahead of time and stored at 4°C.

3. Heat a shaking water bath to 50°C. The water level should be 1/4 to 1/2 inch above the shaking platform. The temperature should not go below 49°C or above 51°C. **It is essential to check the temperature with a calibrated thermistor probe before the hybridization is performed. Also remember to record the temperature.**

   Heat a stationary water bath to between 37°C and 50°C. Warm the HLA DQα Hybridization Solution and the QuantiBlot Wash Solution in the water bath. All solids must be in solution before use.

4. Once you begin the rest of the QuantiBlot assay, you must finish. Allow approximately 3 hours.

   Add 150 μL of Spotting Solution to each tube. Vortex and centrifuge briefly to bring the contents to the bottom of the tube.

Initials: RCJ Date: 4/7/94

5. While wearing gloves, cut a piece of Biodyne B membrane to 11.5 x 7.9 cm. Cut a small notch in the upper left corner to mark its orientation. Place the membrane in a container containing 50 mL of Pre-Wetting Solution and incubate at room temperature for 1-30 minutes.

6. Using forceps, remove the membrane from the Pre-Wetting solution. Place the membrane on the gasket of the slot blotter, then place the top plate of the slot blotter on top of the membrane. Turn on the vacuum source (house vacuum or vacuum pump) to a vacuum of approximately 8 to 10 inches Hg. Turn off the sample vacuum and turn on the clamp vacuum on the slot blot apparatus. Push down to ensure a tight seal.

7. Load the membrane as follows:

   Using a new pipet tip for each sample, apply all of each sample into a separate well of the slot blotter. For best results, slowly dispense each sample directly into the center of the wells, with the pipet tip approximately 5 mm above the membrane.

Slot	Sample
1A	10 ng standard
1B	5 ng standard
1C	2.5 ng standard
1D	1.25 ng standard
1E	0.625 ng standard
1F	0.3125 ng standard
1G	0.15625 ng standard
1H	extraction reagent control (negative)
2A	3.5 ng Calibration 1 Std.
2B	0.5 ng Calibration 2 Std.
2C	0.15625 ng standard
2D-6H	samples and controls

Initials: RC) Date: 4/7/94

8. After all the samples have been applied, slowly turn on the sample vacuum. Leave the sample vacuum on until all samples have been drawn through the membrane. Inspect each slot that contains a sample for a uniform blue band. If a uniform blue band is not visible, make a note of it.

   Turn off the sample vacuum, the clamp vacuum, then the vacuum source.

10. Disassemble the slot blotter and remove the membrane. Proceed immediately to pre-hybridization. Do not allow the membrane to dry out.

    Clean the apparatus by soaking in enough 0.1% SDS to cover for 5-15 minutes. Using a kim wipe, clean the gasket and the side of the top plate that contacts the membrane. Rinse the slot blotter in H_2O and allow to dry at room temperature. **Never use bleach.**

11. Transfer the membrane to 100 mL of pre-warmed HLA DQα Hybridization Solution in the hybridization tray. Add 5 mL of 30% H_2O_2. Place the lid on the tray. Put the tray into the 50°C shaking water bath. Place a weight (e.g. lead ring) on the covered tray to prevent the tray from sliding or floating.

    Shake at 50°C for 15 minutes at 50-60 rpm. Pour off the solution.

Initials: RCS Date: 4/7/94

Hybridization

12. Add 30 mL of pre-warmed HLA DQα Hybridization Solution to the tray. Tilt the tray to one side and add 20 μL of QuantiBlot D17Z1 Probe to the HLA DQα Hybridization Solution. Cover tray with lid and weight.

    Shake at 50°C for 20 minutes at 50-60 rpm. Pour off the solution.

13. Add 100 mL of pre-warmed QuantiBlot Wash Solution to the tray. Rinse by rocking for several seconds, then pour off the solution.

14. Add 30 mL of pre-warmed QuantiBlot Wash Solution to the tray. Tilt the tray to one side and add 180 μL of Enzyme Conjugate. Cover tray with lid and weight.

    Shake at 50°C for 10 minutes at 50-60 rpm. Pour off the solution.

15. Add 100 mL of QuantiBlot Wash Solution to the tray. Rinse by rocking for 1 minute, then pour off the solution.

    Repeat for a total two washes.

16. Add 100 mL of QuantiBlot Wash Solution to the tray. Cover tray with lid and weight.

    Shake at room temperature for 15 minutes at 100-125 rpm. Pour off the solution. During this time, prepare the Color Development Solution (see below).

Initials: *QCJ* Date: *5/9/95*

Color Development

17. In a glass flask, prepare the Color Development Solution. Add the reagents in order:

    60 mL of Citrate Buffer
    3 mL Chromogen
    60 μL 3% H_2O_2.

    Mix thoroughly by swirling (do not vortex).

    Note: Do not prepare the Color Development Solution more than 10 minutes before use. Use a new tube of hydrogen peroxide for each batch of Color Development Solution. Discard the remaining hydrogen peroxide after use. Wrap the Chromogen bottle in Parafilm after each use to prevent oxidation.

18. Add 100 mL of Citrate Buffer to the tray. Rinse by rocking for several seconds, then pour off the solution.

19. Add the Color Development Solution to the tray. Cover tray with lid.

    Develop the membrane by shaking at room temperature for 20-30 minutes at 50-60 rpm. Pour off the solution.

20. Stop the color development by washing in 100 mL deionized H_2O. Cover tray with lid and shake at room temperature for 5-10 minutes at 50-60 rpm.

    Repeat for a total of 3 washes.

Initials: RCS Date: 4/7/94

Color Development

17. In a glass flask, prepare the Color Development Solution. Add the reagents in order:

    30 mL of Citrate Buffer
    1.5 mL Chromogen
    30 μL 3% H_2O_2.

    Mix thoroughly by swirling (do not vortex).

    Note: Do not prepare the Color Development Solution more than 10 minutes before use. Use a new tube of hydrogen peroxide for each batch of Color Development Solution. Discard the remaining hydrogen peroxide after use. Wrap the Chromogen bottle in Parafilm after each use to prevent oxidation.

18. Add 100 mL of Citrate Buffer to the tray. Rinse by rocking for several seconds, then pour off the solution.

19. Add the Color Development Solution to the tray. Cover tray with lid.

    Develop the membrane by shaking at room temperature for 20-30 minutes at 50-60 rpm. Pour off the solution.

20. Stop the color development by washing in 100 mL deionized H_2O. Cover tray with lid and shake at room temperature for 5-10 minutes at 50-60 rpm.

    Repeat for a total of 3 washes.

Initials: RCJ Date: 4/7/94

Photography

21. Photograph the membrane while wet. Place the membrane on a dark, flat, non-absorbent surface.

22. Use a Polaroid MP4 camera system with type 667 (preferred) or 665 film and a Wratten 23A or 22 (orange) filter.

23. Turn on the flood lights. Adjust the height of the camera and focus so that the membrane fills the entire viewing frame.

24. Photograph at 1/125 seconds and f22 for type 667 film. Photograph at 1/2 second and f16 for type 665 film.

25. Develop the film for 30-60 seconds. If the photograph is out of focus, not exposed properly, or does not accurately record the bands on the membrane, vary the exposure conditions and re-photograph.

    Discard the membrane once a good photograph is obtained.

26. Attach the photograph to the QuantiBlot worksheet. Once the photograph is reviewed, file in the appropriate binder or folder. For casework, the original and photocopies are retained in the case files.

Initials: RC Date: 4/7/94

Interpretation

27. At least one of the 0.15 ng standards must be visible. If not, the samples on the membrane with an amount of < 0.31 ng must be repeated.

28. Compare the intensity of Calibrator 1 (3.5 ng/5 μL) and Calibrator 2 (0.5 ng/5 μL) to the DNA Standards. The intensity of Calibrator 1 should be between 2.5 ng and 5 ng; the intensity of Calibrator 2 should be between 0.31 ng and 0.62 ng. If not, the membrane must be repeated.

29. Estimate the quantity (ng) and concentration (ng/μL) of DNA loaded for each sample by comparing the band intensity of the unknowns with those of the DNA Standards. If the band intensity is between two DNA Standards **match the sample to the DNA Standard with the lower amount of DNA.**

    The concentration (ng/μL) is the quantity (ng) divided by the amount of sample used (μL).

    For diluted samples (1/10), multiply DNA concentration by 10.

    If sample band intensity is > 10 ng, it must be diluted 1/10 and quantitated again.

30. Show the photograph to another Analyst or Scientist to review the DNA quantity and concentration. Disagreements should be settled by another Scientist, the Assistant Director and/or the Director.

Initials: RCS Date: 4/7/94

Troubleshooting of QuantiBlot

Observation	Possible Cause	Recommended Action
1. No signal or low sensitivity.	Use of a membrane other than Biodyne B.	Use Biodyne B nylon membrane. Do not use membranes that have neutral charge.
	Incorrect NaOH or EDTA concentrations in Spotting Solution.	Prepare Spotting Solution correctly.
	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.
	Hydrogen peroxide was inactive.	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).

Initials: RCS Date: 4/7/94

	Observation	Possible Cause	Recommended Action
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.
3.	Non-uniform signal intensity within a slot.	Bubbles(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.

Initials: RCl Date: 4/7/94

Observation	Possible Cause	Recommended Action
	Membrane was not pre-wetted 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.
	Lack of thorough rinsing.	Thoroughly rinse twice, for 1 minute each, using 100 mL of pre-warmed Wash Solution. 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.
5. The DNA Calibrators do not quantitate correctly with respect to the DNA Standards.	DNA Standard serial dilutions prepared incorrectly.	Prepare two-fold serial dilutions of DNA Standard in TE Buffer as described. Add 5 μL of Spotting Solution for slot blotting.
6. Signal 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 primates 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,* dog, cat, mouse, rat, pig, cow, chicken, fish and turkey.

Initials: RCS Date: 4/7/94

References for QuantiBlot

1. Walsh, P.S., Valaro, J., and Reynolds, R., 1992. A rapid chemiluminescent method for quantitation of human DNA. Nucleic Acids Research **20:** 5061-5065.

2. Wayne, J.S. and Willard, H.F., 1986. Structure, organization, and sequence of alpha satellite DNA from human chromosome 17: evidence for evolution by unequal Crossing-Over and an ancestral pentamer repeat shared with the human X chromosome. Molecular and Cellular Biology **6:** 3156-3165.

3. Whitehead, T.P., Thorpe, G.H.G., Carter, T.J.N., Groucutt, C., and Kricka, L.J., 1983. Enhanced luminescence procedure for sensitive determination of peroxidase-labeled conjugates in immunoassay. Nature **305**: 158-159.

4. Miller, S.A., Dykes, D.D., and Polesky, H.F., 1988. A simple salting out procedure for extraction DNA from human nucleated cells. Nucleic Acids Research **16:** 1215.

Initials: RCL Date: 4/7/94

Estimation of DNA Quantity and Quality From a Yield Gel

1. Preparation of 20 x 25 cm yield gel (BRL Apparatus).

   BRL gels are poured with a 30 lane, 2 mm comb. The gel may have 2 or more origins if many samples are analyzed. The BRL gels are cast in the electrophoresis tank using the combs and dams in the tank.

   a. For each gel, add 20 mL 10X TAE, 180 mL dH_2O and 1.8 g electrophoresis grade or DNA typing grade agarose to a flask of at least twice the liquid volume.

   b. Bring the flask to a boil in the microwave (4 minutes on high) to dissolve the agarose. Make sure the agarose is completely dissolved.

   c. Add 20 μL of ethidium bromide

   d. Allow to cool to approximately 56°C

   e. Pour agarose into gel form (be sure comb(s) are in place).

2. Pour approximately 1800 mL 1x TAE into the tank; enough buffer should be present to cover the gel. Remove comb(s).

3. Vortex samples including Cell Pellet Control, Negative Control, Yield Calibrators, Calibration Control and Lambda Marker tubes for 15 seconds.

   Microcentrifuge briefly to bring contents to the bottom of the tube.

4. Place the tubes in a 65°C heatblock and incubate for 5 minutes.

5. Microcentrifuge briefly to bring contents to the bottom of the tube.

Initials: RCS Date: 4/7/94

6. Samples can be prepared in microcentrifuge tubes or in wells of a microtiter plate. Store the remainder of the DNA extracts at 4°C.

   a. Stains, Tissue, Post-Mortem Blood, and Swabs - Mix 10 μL of DNA sample and 2 μL of Yield Gel Loading Buffer.

   b. Whole Blood, Bone Marrow, and Fresh Sperm- Mix 2 μL of DNA sample, 8 μL of dH_2O and 2 μL of Yield Gel Loading Buffer.

   c. Cell Pellet Control - Mix 5 μL of DNA sample, 5 μL of dH_2O, and 2 μL of Yield Gel Loading Buffer.

7. If using a microcentrifuge tube, microcentrifuge briefly to bring contents to the bottom of the tube.

8. Load each row of each gel as follows

Lane	Volume	Material	Description
1	10 μL	Lambda Marker	Hind III digested λ DNA
2	10 μL	Yield Calibrator A	30 ng/μL λ DNA (300 ng total)
3	10 μL	Yield Calibrator B	20 ng/μL λ DNA (200 ng total)
4	10 μL	Yield Calibrator C	10 ng/μL λ DNA (100 ng total)
5	10 μL	Yield Calibrator D	5 ng/μL λ DNA (50 ng total)
6	10 μL	Yield Calibrator E	2.5 ng/μL λ DNA (25 ng total)
7	10 μL	Yield Calibrator F	1 ng/μL λ DNA (10 ng total)
8	10 μL	Calibration Control	50 ng high M.W. human DNA
9-30	12 μL	Samples	Unknown samples

Include the Cell Pellet Control and Negative Control as samples.

9. Set the voltage at 100 volts on the dial. When the bromophenol blue tracking dye has moved 1-2 cm (approx. 45-60 min) from the origin, the run can be stopped.

Initials: RCS Date: 4/7/94

10. Switch off the power supply and remove the gel from the tank. Examine the gel on the ultraviolet light transilluminator.

    Take a photograph of the gel using Polaroid film. For type 667 film the settings are f5.6 for 1/2-1 second. DO NOT EXPOSE YOURSELF TO THE UV LIGHT FOR AN EXCESSIVE AMOUNT OF TIME. ALWAYS WEAR U.V. GOGGLES WHEN WORKING WITH THE TRANSILLUMINATOR.

11. Interpretation:

    A. Quality - Intact DNA will move as a band not far from the origin. A smear from the origin to, or past, the dye front indicates that the DNA has been fragmented.

    B. Compare the intensity of the Calibration Control (lane 8) to that of the Yield Calibrators. If the intensity between Yield Calibrators D and E (lanes 5-6), the result is valid. Otherwise the gel must be repeated.

    C. Quantity - Estimate the quantity (ng) and concentration (ng/μL) of high molecular weight DNA loaded for each sample by comparing the band intensity of the high molecular weight band of the unknowns with those of the Yield Calibrators. High molecular weight DNA should appear as a tight band slightly above the uppermost band of the Lambda Marker.

       If the sample shows "trailing", use only the region above the upper band of the Lambda Marker for quantifying high molecular weight DNA. If the band size is between two yield calibrators match the sample to the yield calibrator with the lowest amount of DNA. See table II below for interpretation.

    D. Show the yield gel to another Analyst or Scientist to independently determine DNA quality, concentrations, and yields. Disagreements should be settled by another Scientist and/or the Assistant Laboratory Director.

Initials: RC Date: 4/7/94

Table II- Interpretation of Yield Gels

	DNA CONCENTRATION[b]		
Sample Intensity[a]	2 μL Sample Loaded On Yield Gel	5 μL Sample Loaded On Yield Gel	10 μL Sample Loaded On Yield Gel
≥ A	see note c below	see note c below	Repeat yield gel loading 2 μL of sample
B	100 ng/μL	40 ng/mL	20 ng/μL
C	50 ng/μL	20 ng/mL	10 ng/μL
D	25 ng/μL	10 ng/mL	5 ng/μL
E	12.5 ng/μL	5 ng/mL	2.5 ng/μL
≤ F	Repeat yield gel loading 10 μL of sample	Repeat yield gel loading 10 μL of sample	≤ 1 ng/μL

a. Yield Calibrator of closest intensity
b. For diluted samples, multiply DNA concentration by 10.
c. Dilute 5 μL of sample with 45 μL of TE and mix. Run 2 μL on a new yield gel. Save the dilution at 4°C.

Initials: RCS Date: 4/7/94

PCR HLA DQα Test

Amplification

A positive control, an extraction reagent control and substrate controls, if applicable, should be included with each batch of samples being amplified to demonstrate procedural integrity.

1. Turn on the Perkin Elmer Thermal Cycler. (See manufacturer's instructions). File #14 on both machines should include the following:

   Denature at 94°C for 1 minute
   Anneal at 60°C for 30 seconds
   Extend at 72°C for 30 seconds

   Repeat the above for 32 cycles

   Link to Time Delay File #13 on both machines for an additional 7 minutes incubation at 72°C.

   Link to Soak File #12 on both machines for a 4°C soak.

   If Files #12, 13, or 14 are not correct, bring this to the attention of the QC/QA coordinator and a supervisor. Re-program the incorrect file(s) and note that the file(s) was changed in the log book for that run.

2. Determine the number of samples to be amplified, including controls.

3. Fill out the amplification worksheet and record the appropriate lot numbers.

4. Ensure that the solution is at the bottom of each PCR reaction mix tube by tapping the tube down onto a clean work surface or by centrifuging briefly. Label the caps of the PCR Reaction Mix tubes. Open caps using the microcentrifuge tube de-capping tool or a new Kimwipe. **Avoid touching the inside surface of the tube caps.**

Initials: ZY Date: 6/21/95

5. **Note: It is important to begin the cycling processing within 30 minutes after addition of the $MgCl_2$ Solution to the PCR Reaction Mix.**

   Pipet 50 μL of the $MgCl_2$ Solution provided into each tube including controls, with a sterile pipet tip. Pipet carefully at a slight angle to minimize mixing and to avoid splashing of solution. Use either a filter tip or a combi tip on the pipettor.

6. Carefully add 2 drops of the Mineral Oil from the dropper bottle provided in the kit to all tubes including the controls. **Do not actually touch tube.**

7. Close all of the tubes.

8. **Note: Use a new sterile filter pipet tip for each sample addition. Open only one tube at a time for sample addition.**

   Test Sample Tubes:
   See Table III for the amount of DNA to add to each tube. It is recommended that the target volume of DNA be amplified in the first attempt. If the target volume is exceeded and extra dots less than "c" are detected, then the target volume or less should be amplfied in subsequent attempts. Do not amplify samples in which no DNA was detected by QuantiBlot. Add the sample DNA to each labeled tube by inserting the pipet tip through the mineral oil layer. After the addition of the DNA, cap each sample before proceeding to the next tube. **Do not vortex or mix.**

   Positive Control Tubes:
   Add 20 μL of the 100 ng/mL Genomic Control DNA to the designated PCR Reaction Mix tube.

   Extraction Reagent Controls:
   Add 20 μL of the extraction reagent control to the designated PCR Reaction Mix tube.

   Amplification Negative Control Tubes:
   Do not add DNA

   The final aqueous volume in the PCR reaction mx tubes will vary between 101 μL and 140 μL, depending on the volume of DNA added.

Initials: RG Date: 10/5/97

5. **Note: It is important to begin the cycling processing within 30 minutes after addition of the $MgCl_2$ Solution to the PCR Reaction Mix.**

   Pipet 50 μL of the $MgCl_2$ Solution provided into each tube including controls, with a sterile pipet tip. Pipet carefully at a slight angle to minimize mixing and to avoid splashing of solution. Use either a filter tip or a combi tip on the pipettor.

6. Carefully add 2 drops of the Mineral Oil from the dropper bottle provided in the kit to all tubes including the controls. **Do not actually touch tube.**

7. Close all of the tubes.

8. **Note: Use a new sterile filter pipet tip for each sample addition. Open only one tube at a time for sample addition.**

   Test Sample Tubes:
   See Table III for the amount of DNA to add to each tube. It is recommended that the target volume of DNA be amplified in the first attempt. If the target volume is exceeded and extra dots less than "c" are detected, then the target volume or less should be amplfied in subsequent attempts. Do not amplify samples in which no DNA was detected by QuantiBlot. Add the sample DNA to each labeled tube by inserting the pipet tip through the mineral oil layer. After the addition of the DNA, cap each sample before proceeding to the next tube. **Do not vortex or mix.**

   Positive Control Tubes:
   Add 20 μL of the 100 ng/mL Genomic Control DNA to the designated PCR Reaction Mix tube.

   Extraction Reagent Control:
   Add 20 μL of the extraction reagent control to the designated PCR Reaction Mix tube.

   The final aqueous volume in the PCR reaction mx tubes will vary between 101 μL and 140 μL, depending on the volume of DNA added.

Initials: RCS Date: 4/7/94

5. **Note:** **It is important to begin the cycling processing within 30 minutes after addition of the $MgCl_2$ Solution to the PCR Reaction Mix.**

   Pipet 50 μL of the $MgCl_2$ Solution provided into each tube including controls, with a sterile pipet tip. Pipet carefully at a slight angle to minimize mixing and to avoid splashing of solution. Use either a filter tip or a combi tip on the pipettor.

6. Carefully add 2 drops of the Mineral Oil from the dropper bottle provided in the kit to all tubes including the controls. **Do not actually touch tube.**

7. Close all of the tubes.

8. **Note: Use a new sterile filter pipet tip for each sample addition. Open only one tube at a time for sample addition.**

   Test Sample Tubes:
   See Table III for the amount of DNA to add to each tube. Do not amplify samples in which no DNA was detected by QuantiBlot. Add the sample DNA to each labeled tube by inserting the pipet tip through the mineral oil layer. After the addition of the DNA, cap each sample before proceeding to the next tube. **Do not vortex or mix.**

   Positive Control Tubes:
   Add 20 μL of the 100 ng/mL Genomic Control DNA to the designated PCR Reaction Mix tube.

   Extraction Reagent Control:
   Add 20 μL of the extraction reagent control to the designated PCR Reaction Mix tube.

   The final aqueous volume in the PCR reaction mx tubes will vary between 101 μL and 140 μL, depending on the volume of DNA added.

Initials: RC   Date: 10/5/94

Table III- Amount of DNA to be Amplified at the HLA DQα Locus

QuantiBlot DNA Concentration (ng/20μL)	Target Volume (μL) to be amplified	Range of Volumes (μL) which can be amplified
≥10	Dilute 1:10 and re-quantitate	
5	5	1-40
2.5	10	2-40
1.25	20	3-40
0.62	40	10-40
0.31	40	20-40
0.15	40	20-40
<0.15	Do not amplify	-

9. **Note: PCR Reaction Mixes should be amplified in rows A-D in the Thermal Cycler**

   For each well of the Thermal Cycler heat block which will be used to amplify samples, add one drop of mineral oil to the well. Place the PCR Reaction Mix tubes into the Thermal Cycler. Push the tubes down completely into the heat block. Record the heat block position of each tube.

10. Start the Thermal Cycler amplification program. Verify the cycling parameters by monitoring the first cycle. The tubes should be checked after the first cycle and pressed further into the heat block so that they fit tightly.

11. Return the microtube rack used to set-up the samples for PCR to the PCR Set-Up Area.

12. After the amplification process, the samples are ready for DNA Hybridization and Color Development or they may be stored at 2-8°C for at least seven days, or at -20° for extended periods in the appropriate refrigerator or freezer.

Initials: RC Date: 4/7/94

Table III- Amount of DNA to be Amplified at the HLA DQα Locus

QuantiBlot DNA Concentration (ng/20μL)	Target Volume (μL) to be amplified	Range of Volumes (μL) which can be amplified
>10	Dilute 1:10 and re-quantitate	-
10	2.5	1-20
5	5	1-40
2.5	10	2-40
1.25	20	3-40
0.62	20	10-40
0.31	20	20-40
0.15	40	20-40
<0.15	Do not amplify	-

9. **Note: PCR Reaction Mixes should be amplified in rows A-D in the Thermal Cycler**
   For each well of the Thermal Cycler heat block which will be used to amplify samples, add one drop of mineral oil to the well. Place the PCR Reaction Mix tubes into the Thermal Cycler. Push the tubes down completely into the heat block. Record the heat block position of each tube.

10. Start the Thermal Cycler amplification program. Verify the cycling parameters by monitoring the first cycle. The tubes should be checked after the first cycle and pressed further into the heat block so that they fit tightly.

11. Return the microtube rack used to set-up the samples for PCR to the PCR Set-Up Area.

12. After the amplification process, the samples are ready for DNA Hybridization and Color Development or they may be stored at 2-8°C for at least seven days, or at -20° for extended periods in the appropriate refrigerator or freezer.

Initials: RCL Date: 4/7/94

Optional Step to Verify Amplification

The presence of amplified HLA DQα sequences can be verified by gel electrophoresis. The Amplitype HLA DQα gene amplification product will be either 239 or 242 base pairs long, depending on the particular allele.

Agarose Gel Preparation

1. Weigh out agarose for a 3% Nusieve™ plus 1% Seakem™ GTG gel

2. Add agarose to the appropriate amount of 1X TAE in a flask. Volume required will vary depending on size of the minigel apparatus. Prepare enough agarose to pour a 3 mm gel.

3. To dissolve agarose, heat in a microwave oven for 1 to 2 minutes. Swirl flask to aid in dissolving agarose.

4. Cool the solution to 55°C in a water bath.

5. Add a volume of 10 mg/mL Ethidium Bromide to the molten agarose to achieve a final concentration of 1 μg/mL Ethidium Bromide.

6. To form the gel, pour liquified agarose/ethidium bromide solution in minigel apparatus for a 3 mm thick gel. Immediately insert slot forms and adjust to assure that the apparatus is level.

7. Allow the gel to set for 20 minutes at room temperature, or until completely solidified.

8. When gel is ready, add a sufficient volume of 1X TAE to fill the buffer tanks and cover the gel to a depth of about 1 mm.

9. Carefully remove slot forms. Avoid touching wells.

Initials: RCS Date: 4/7/94

Gel Loading and Electrophoresis

1. Add 5 μL DNA sample to a 0.5 mL microcentrifuge tube. Add 1 μL Analytical Gel Loading Buffer and mix by tapping gently.

2. Do not use the outside lanes of the gel. In the first and last useable lanes of the gel, pipet 6 μL of the Phi X Marker.

3. Carefully pipette samples into the remaining wells.

4. Connect leads so that the DNA migrates toward the positive (+) electrode. Run the minigel at 50 to 100 volts, (or 5 to 15 v/cm) at room temperature for about one to two hours or until the bromophenol blue (faster-migrating) dye is one to two cm from the end of the gel.

5. Check for the extent of DNA migration by examining visually on a 300 nm UV transilluminator. **WEAR PROTECTIVE EYEWEAR AND HANDLE THE GEL WITH GLOVES.**

   To photograph gel, place on a UV transilluminator box under a stationary camera with a Kodak 23A Wratten orange filter. Photograph in the dark under UV illumination.

   Note: Sample evaluation gels and PCR-product gels are both run in the DNA Amplification Work Area. However, the pre-amplification samples must never be brought into that area. Only the aliquot of sample prepared for gel loading should be removed from the DNA Extraction Work Area.

Interpretation of Gel Electrophoresis Patterns

The HLA DQα amplification products will appear between the 281 bp/271 bp doublet (sixth band from the origin) and the 234 bp fragment (seventh band from the origin) of the Phi X Marker. A "primer dimer" band will be frequently observed at slightly lower molecular weight than the 72 bp marker fragment. Unincorporated primers will appear as a broad band at still lower molecular weight.

Occasionally the HLA DQα amplification product will appear as a doublet band.

Initials: RG Date: 4/7/94

DNA Hybridization

1. Heat a shaking water bath to 55°C. The water level should be 1/4 - 1/2 inch above the shaking platform. The water level should not be higher than 1/2 inch, as higher levels may result in water splashing into wells. **The temperature should not go below 54°C or above 56°C. It is essential to check the temperature with a calibrated thermistor probe before the hybridization is performed.**

2. Heat a stationary water bath to 37-65°C. Warm the HLA DQα Hybridization Solution and the HLA DQα Wash Solution in the water bath. All solids must be in solution before use.

3. Fill out the hybridization worksheet.

4. Using filter forceps, remove the required number of DNA Probe Strips from the glass tube. Place one Probe Strip in each clean well of the Amplitype DNA Typing Tray. With a waterproof marking pen, label each strip in the space provided.

5. Place the tubes in a 95°C heat block. Press the tubes down tightly in the heat block. Denature the amplified DNA by incubation at 95°C for 3-10 minutes. Keep each tube at 95°C until use.

6. **Note: Do not prepare the HLA DQα Hybridization/Enzyme conjugate Solution more than 15 minutes before use.**

   Prepare the hybridization solution in a glass flask either according to Table IV or the following formula:

   # Strips x 3.3mL pre-warmed HLA DQα Hybridization Solution
   # strips x 27 μL Enzyme Conjugate

   Mix by swirling.

Initials: RCI Date: 4/7/94

Table IV- HLA DQα Hybridization Solution

number of strips	hyb solution	enzyme conjugate
3	9.9 ml	81 μl
4	13.2 ml	108 μl
5	16.5 ml	135 μl
6	19.8 ml	162 μl
7	23.1 ml	189 μl
8	26.4 ml	216 μl
9	29.7 ml	243 μl
10	33.0 ml	270 μl
11	36.3 ml	297 μl
12	39.6 ml	324 μl
13	42.9 ml	351 μl
14	46.2 ml	378 μl
15	49.5 ml	405 μl
16	52.8 ml	432 μl

7. Tilt the Typing Tray towards the labelled end of the strips. Add 3 mL of the freshly prepared HLA DQα Hybridization/Enzyme Conjugate Solution at the labelled end of each strip.

Initials: RCJ Date: 4/7/94

8. **Note:** **Perform the following steps for each tube of amplified DNA. When pipeting amplified extract, wait for the bead of mineral oil to expel from the tip before drawing up. For each tube, perform steps a-c within 30 seconds.**

   a. Remove the tube from the 95°C heat block.
   b. Carefully open the tube. (Use the microcentrifuge tube decapping device or a new Kimwipe).
   c. Withdraw 35 μL amplified DNA from the aqueous (bottom) layer and immediately add to the contents of the well (pipet below the surface of the hybridization solution) at the labeled end of the corresponding Probe Strip.
   d. Cap the tube and set aside.
   e. Repeat until each amplified DNA sample has been added to the corresponding well. Use a new pipet tip for each addition.

   The remaining amplified DNA samples can be stored at -20°C or discarded when the analysis is complete.

9. Mix the tray by carefully rocking and place the clear plastic lid on the tray. Put the tray into the 55°C shaking water bath. Place a weight (e.g., lead ring) on the covered tray to prevent the tray from sliding or floating.

   Note: **Do not shake the tray after the samples have been added to the strips. It is important that the strips are completely submerged in the hybridization solution before placing the tray in the water bath, but cross contamination can occur at this step if the solution is shaken out of the wells. Gentle rocking of the tray is sufficient to moisten the strips.**

10. Hybridize the amplified DNA samples to the Probe Strips by incubating at 55°C for 20 minutes at 50-90 rpm. Adjust the water level and check the tray position so that water does not splash into the wells of the tray.

11. After hybridization, remove the tray from the water bath and pour out the contents of each well. Wipe the tray lid with a Kimwipe or paper towel.

12. Note: **HLA DQα Wash Solution solids must be in solution before use.**

    Dispense 10 mL of HLA DQα Wash Solution into each well. Rinse by rocking for several seconds, then pour the solution from each well.

Initials: RCS Date: 4/7/94

13. **Note: The temperature and timing of the Stringent Wash are CRITICAL.**

    Stringent Wash:
    Dispense 10 mL of HLA DQα Wash Solution into each well. Cover tray with lid and weight. Place into the 55°C shaking water bath for 12 minutes, ±2 minutes, at about 50 rpm.

14. Remove the tray from the water bath, take off the lid and pour the solution from each well.

15. Dispense 10 mL of HLA DQα Wash Solution into each well. Cover and place on an orbital shaker at room temperature for 5 minutes at about 50 rpm.

16. Remove the tray from the orbital shaker, take off the lid and pour the solution from each well.

Initials: RG Date: 4/7/94

Color Development

1. Dispense 10 ml of citrate buffer into each well. Wipe the tray lid with a Kimwipe or paper towel. Cover tray and place on an orbital shaker for 5 minutes at about 50 rpm.

2. Note: **Do not prepare the Color Development Solution more than 10 minutes before use. Use a new tube of hydrogen peroxide for each batch of Color Development Solution. Discard the remaining hydrogen peroxide after use. Cover the chromogen bottle with parafilm after each use to prevent oxidation.**

During this wash step, prepare the Color Development Solution in an Erlenmeyer flask either according to Table V or the following formula. Add the reagents in order:

strips x 10 mL HLA DQα Citrate Buffer
strips x 10 μL 3% Hydrogen Peroxide
strips x 0.5 mL Chromogen Solution

Mix by swirling 2-3 times. Do Not Vortex

Initials: RCS Date: 4/7/94

Table V- Development Solution

number of strips	citrate buffer	hydrogen peroxide	chromogen
3	30 ml	30 μl	1.5 ml
4	40 ml	40 μl	2.0 ml
5	50 ml	50 μl	2.5 ml
6	60 ml	60 μl	3.0 ml
7	70 ml	70 μl	3.5 ml
8	80 ml	80 μl	4.0 ml
9	90 ml	90 μl	4.5 ml
10	100 ml	100 μl	5.0 ml
11	110 ml	110 μl	5.5 ml
12	120 ml	120 μl	6.0 ml
13	130 ml	130 μl	6.5 ml
14	140 ml	140 μl	7.0 ml
15	150 ml	150 μl	7.5 ml
16	160 ml	160 μl	8.0 ml

3. **Note:** **Place lid on tray and cover lid with aluminum foil during steps, 3,5 and 6 to protect from strong light.**

   Remove the tray from the orbital shaker, remove the cover and slowly pour off the HLA DQα Citrate Buffer. Add 10 mL of the newly prepared Color Development Solution to each well. Develop the strips at room temperature by shaking on the orbital shaker at about 50 rpm for 20-30 minutes.

4. Remove tray from shaker and slowly pour off the contents from each well.

5. Stop the color development by washing the strips in deionized water. Dispense approximately 10 mL of water into each well. Place tray on orbital shaker at about 50 rpm for 5-10 minutes. Slowly pour off the contents of each well.

Initials: RC Date: 4/7/94

6. Repeat Step 4 and Step 5 twice for a minimum of three water washes. Additional 5-10 minutes washes will reduce the potential for developing background color.

7. Photographs must be taken for a permanent record (see the next section).

8. Determine and record the HLA DQα type for each sample from the photograph. (See Results Interpretation section.)

Initials: RCS Date: 4/7/94

Photography And Storage

1. Place wet strips on a flat surface as close together as possible. Keep strips wet throughout the photographic procedure. Minimize exposure to strong light.

   For casework photograph up to approximately 8 strips at a time. For QC and validation photograph up to 16 strips at a time.

2. Use a Polaroid MP4 camera system with type 667 or 665 film and a Wratten 23A or 22 orange filter. Type 667 is the preferred film.

3. Turn on the flood lights. Adjust the height of the camera and focus so that the strips fill the **entire** viewing frame.

4. Photograph at 1/125 second and f22 for type 667 film. Photograph at 1/2 second and f16 for type 665 film.

5. Develop at room temperature for ≈ 30-60 seconds.

6. Label the photograph using a permanent marking pen with the date, analyst's initials, and case number.

7. If the photograph is not exposed properly or does not accurately record the dots on the strips, vary the exposure conditions and re-photograph.

   Make sure there is at least one good photograph of each set of strips.

8. Attach the photographs to the hybridization worksheets and file in the appropriate binder or folder. For cases the worksheets are retained in the case file.

9. Following photography, store the strips in a plastic folder in the appropriate binder or folder. Protect from light and oxidizing agents. Color may fade somewhat on drying.

Initials: RCS Date: 4/7/94

Results Interpretation

Results are interpreted by reading the pattern of dots on the Polaroid photograph of the DNA Probe Strips to determine which HLA DQα alleles are present in the DNA sample.

1. The "C" Dot

The strip is read by first examining the "C" dot. **DNA Probe Strips with no visible "C" dot should not be HLA DQα typed.** Those dots equivalent to or stronger than the "C" dot are considered positive. Dots with signal less than the "C" dot should be noted but not used in comparisons.

The "C" probe serves two functions.

A. To indicate adequate amplification and typing of the HLA DQα alleles in the sample.

The "C" dot is usually the weakest on the strip. If the "C" dot is absent, an accurate determination of the type cannot be made since there is a possibility that other probe signals are also below the threshold of detection. The presence of a "C" dot provides assurance that the appropriate typing and sub-typing dots should be clearly visible.

B. To indicate a possible procedural error, mixed sample, or DNA contamination.

The presence of visible dots with a signal intensity less than the "C" dot may indicate any of the above or amplification of DXα, HLA DQα type 1.3,4, or sub-types of the HLA DQα 4 allele (See page 49).

2. Typing and Sub-Typing Probes

The typing and sub-typing probes are used to determine the HLA DQα genotype of the DNA sample analyzed. Each positive dot indicates the presence of the corresponding HLA DQα allele (see Table VI).

The "1" dot is positive in the presence of the HLA DQα 1.1, 1.2, and 1.3 alleles.
The "2" dot is positive only in the presence of the HLA DQα 2 allele.
The "3" dot is positive only in the presence of the HLA DQα 3 allele.
The "4" dot is positive only in the presence of the HLA DQα 4 allele (4.1, 4.2, and 4.3 alleles).

Initials: RCS Date: 4/7/94

The four HLA DQα sub-typing probes differentiate HLA DQα 1.1, 1.2, and 1.3.
The "1.1" dot is positive only in the presence of the HLA DQα 1.1 allele. (see discussion of DXα, page 51)
The "1.3" dot is positive only in the presence of the HLA DQα 1.3 allele.
There is no probe unique to HLA DQα 1.2. The "1.2, 1.3, 4" dot is positive in the presence of HLA DQα 1.2, 1.3, and 4 alleles.
The remaining sub-typing probe, "All but 1.3", is necessary to differentiate the 1.2, 1.3 genotype from the 1.3, 1.3 genotype. This dot is positive in the presence of all HLA DQα alleles EXCEPT 1.3.

NOTE: **The "All but 1.3" dot can be weaker than the "C" dot with the 1.3, 4 genotype. The "All but 1.3" probe has a base pair mismatch with both the HLA DQα 1.3 sequence and the HLA DQα 4 sequence. The mismatch with the HLA DQα 4 sequence pairs a G with a T. This mismatch is only partially destabilizing, and allows the 4 sequence to give a signal at this dot, albeit a weaker signal. The "All but 1.3" dot is used only to distinguish the 1.2, 1.3 from the 1.3, 1.3 genotype. The "All but 1.3" may also sometimes be weaker than the "C" dot for the 4,4 genotype.**

There is also variation among the typing dots on a single probe strip. For instance, for the genotype 1.1, 3 the "1" dot is often darker than the "3" dot for high, medium and low signal levels. The difference is most pronounced at the high level. These differences in intensity are quite consistent from typing to typing.

Amplified samples which have been stored for even a short time often show low intensity dots which were not visible during the first hybridization. These dots are less intense than the "C" dot and do not affect the typing results.

TABLE VI

PROBE	ALLELES DETECTED
1	HLA DQα 1.1, 1.2, 1.3
2	HLA DQα 2
3	HLA DQα 3
4	HLA DQα 4 (4.1, 4.2, 4.3)
C	HLA DQα 1.1, 1.2, 1.3, 2, 3, 4 (All HLA DQα Alleles)
1.1	HLA DQα 1.1
1.2, 1.3, 4	HLA DQα 1.2, 1.3, 4
1.3	HLA DQα 1.3
All but 1.3	HLA DQα 1.1, 1.2, 2, 3, 4

Initials: RU Date: 6/21/95

3. Extraction Reagent, Amplification Negative and Substrate Controls

The extraction reagent and amplification negative controls are a check for the possible contamination of the reagents in the HLA DQα test by other human DNA or by amplified HLA DQα DNA. The extraction reagent control is performed by carrying out the extraction in a tube containing no sample. The amplification negative control contains no added DNA and checks for contamination at the amplification step.

The substrate control is a check for the possible contamination of substrate by an undetected stain containing biological material (e.g. human DNA). This contamination could be pre-existing, it could be deposited on the substrate during the commission of the crime, or it could be deposited during the handling and processing of the evidence. The substrate control is also a check for the contamination of the reagents in the HLA DQα test by other human DNA or by amplified HLA DQα DNA. In addition, the substrate control extract can be used to verify that the substrate contains an extractable PCR inhibitor. The substrate control is performed by carrying out the DNA extraction on unstained substrates (e.g. piece of fabric) located as close to each stain as possible.

The extraction reagent, amplification negative and substrate controls are amplified and typed along with the test samples. The appearance of signals in the typing of these controls indicates any or all of the following:

a. The sample preparation reagents may have been contaminated.

b. Cross-contamination between samples may be occurring during preparation.

c. The substrate might have an undetected stain containing biological material.

d. Human DNA or amplified HLA DQα DNA may be getting into the samples from some other source.

Clearly, if the test sample does not show any probe signal in common with the controls, the test sample is not affected by the same source of contamination.

If the signals on the typing strips for the extraction reagent, amplification negative or substrate controls are very faint with the "C" dots not visible, and the test samples are easily typeable with clearly visible "C" dots, the contamination problem is not serious. If the "C" dot is visible on the typing strip of the extraction reagent or substrate controls, the contamination problem is more serious. **See Table VII for interpretation guidelines.**

Initials: RCS Date: 4/7/94

3. Extraction Reagent and Substrate Controls

The extraction reagent control is a check for the possible contamination of the reagents in the HLA DQα test by other human DNA or by amplified HLA DQα DNA. The extraction reagent control is performed by carrying out the extraction in a tube containing no sample.

The substrate control is a check for the possible contamination of substrate by an undetected stain containing biological material (e.g. human DNA). This contamination could be pre-existing, it could be deposited on the substrate during the commission of the crime, or it could be deposited during the handling and processing of the evidence. The substrate control is also a check for the contamination of the reagents in the HLA DQα test by other human DNA or by amplified HLA DQα DNA. In addition, the substrate control extract can be used to verify that the substrate contains an extractable PCR inhibitor. The substrate control is performed by carrying out the DNA extraction on unstained substrates (e.g. piece of fabric) located as close to each stain as possible.

The extraction reagent and substrate controls are amplified and typed along with the test samples. The appearance of signals in the typing of these controls indicates any or all of the following:

a. The sample preparation reagents may have been contaminated.

b. Cross-contamination between samples may be occurring during preparation.

c. The substrate might have an undetected stain containing biological material.

d. Human DNA or amplified HLA DQα DNA may be getting into the samples from some other source.

Clearly, if the test sample does not show any probe signal in common with the controls, the test sample is not affected by the same source of contamination.

If the signals on the typing strips for the extraction reagent or substrate controls are very faint with the "C" dots not visible, and the test samples are easily typeable with clearly visible "C" dots, the contamination problem is not serious. If the "C" dot is visible on the typing strip of the extraction reagent or substrate controls, the contamination problem is more serious. **See Table VII for interpretation guidelines.**

Initials: RJ Date: 6/21/95

The appearance of signals in extraction reagent, amplication negative or substrate controls does not necessarily mean that the types obtained for the test samples are incorrect because of the following:

a. The contamination might be due to a single event limited to the control.

b. The level of contamination might be inconsequential compared to the amount of DNA being amplified and typed in the test samples.

c. The contamination might be easily distinguished from the test samples because the contamination and test samples do not have any alleles in common.

However, further testing may be necessary to support these possibilities.

See Table VII for interpretation guidelines.

Table VII- Guideline to the Interpretation of Visible Dots in the Extraction Reagent and Substrate Controls

Control	"C" dot visible?	Interpretation of Test Sample
Extraction Reagent/ Amplification Neg.	yes	All test samples inconclusive
	no	Test samples are conclusive if there is a duplicate run with no visible dots.
Substrate	yes	Sample is conclusive and alleles matching the control are not attributed to the stain.
	no	Sample is conclusive. The presence of additional alleles is noted in the report.

4. Amplification Positive Control

The HLA DQα 1.1, 4 control DNA provided in the Amplitype™ HLA DQα kit is a positive control which is used with each batch of samples typed to demonstrate that the kit is performing properly.

If the positive control does not produce a correct and readable type, the amplification must be repeated and the test samples are considered inconclusive.

Initials: RCS Date: 4/7/94

The appearance of signals in extraction reagent or substrate controls does not necessarily mean that the types obtained for the test samples are incorrect because of the following:

a. The contamination might be due to a single event limited to the control.

b. The level of contamination might be inconsequential compared to the amount of DNA being amplified and typed in the test samples.

c. The contamination might be easily distinguished from the test samples because the contamination and test samples do not have any alleles in common.

However, further testing may be necessary to support these possibilities.

See Table VII for interpretation guidelines.

Table VII- Guideline to the Interpretation of Visible Dots in the Extraction Reagent and Substrate Controls

Control	"C" dot visible?	Interpretation of Test Sample
Extraction Reagent	yes	All test samples inconclusive
	no	Test samples are conclusive if there is a duplicate run with no visible dots.
Substrate	yes	Sample is conclusive and alleles matching the control are not attributed to the stain.
	no	Sample is conclusive. The presence of additional alleles is noted in the report.

4. Amplification Positive Control

The HLA DQα 1.1, 4 control DNA provided in the Amplitype™ HLA DQα kit is a positive control which is used with each batch of samples typed to demonstrate that the kit is performing properly.

If the positive control does not produce a correct and readable type, the amplification must be repeated and the test samples are considered inconclusive.

Initials: RC Date: 4/7/94

5. Species Specificity

A positive and interpretable HLA DQα and/or QuantiBlot result can be considered primate positive. Identification of the specific physiological fluid may be accomplished using the procedures described in the Forensic Biochemistry and Hematology Laboratory Manual.

6. Population Frequencies

To interpret the significance of a match between genetically typed samples, it is necessary to know the population distribution of alleles at the locus in question. For example, if the HLA DQα genotype of the relevant evidence sample is different from the type of the suspect's reference sample, then the suspect is "excluded", and cannot be the donor of the biological evidence being tested. An exclusion is independent of the frequency of the two genotypes in the population.

If the suspect and evidence samples have the same genotype, then the suspect is "included", and could be the source of the evidence sample. The probability that another, unrelated, individual would also match the evidence sample is equal to the frequency of that genotype in the relevant population.

Initials: RCS Date: 4/7/94

Interpretation of Complex Results

Occasionally typing results may appear markedly different from the standard 21 patterns. Such results could be due to a procedural error, mixtures of DNA's (multiple contributors to the sample) the presence of the uncommon subtypes 4.2 or 4.3, or the presence of an allele of the related pseudogene, DXα.

1. Mixtures of DNA: more than one genotype present in the DNA sample.

   A. General Mixtures

   Evidence samples may contain DNA from more than one individual either because of the nature of the sample or from contamination. The possibility of multiple contributors should be considered when interpreting the HLA DQα typing results. For any typing system in which heterozygous genotypes are analyzed, the detection of more than two alleles indicates a mixed sample. Furthermore, there is a possibility that a phenotype read for example as HLA DQα 3,4 is a mixture of approximately equal contributions from a homozygous 3,3 individual and a homozygous 4,4 individual or from {3,3 + 3,4} or {4,4 + 3,4} mixtures. Such mixtures would not be detected from typing results alone, as they would reveal only two alleles.

   There are 210 possible mixtures of two different HLA DQα genotypes. Of these, 166 are mixtures that would contain three or more alleles and 44 are mixtures of the type that may not be easily determined to be mixtures as demonstrated by the example above.

   B. Mixtures containing the 1.2 allele.

   Since there is no probe uniquely specific for the 1.2 allele, it could be overlooked in some three-allele mixtures. There are five hybridization patterns corresponding to normal HLA DQα genotypes in which a three allele mixture could contain an undetected 1.2 allele. These five patterns are listed in Table VIII. These five patterns correspond to 25 of the 166 possible mixtures of two genotypes containing three alleles in total.

Table VIII: HLA DQα phenotypes that can contain hidden 1.2 alleles

three allele combinations in which 1.2 can be present but not specifically detected	apparent heterozygous type
1.1, 1.2, 4	1.1, 4
1.1, 1.2, 1.3	1.1, 1.3
1.2, 1.3, 2	1.3, 2
1.2, 1.3, 3	1.3, 3
1.2, 1.3, 4	1.3, 4

C. Mixtures with different level of starting DNA

Mixtures may be present in unequal amounts. For example, the typing result of a mixture of ten parts type HLA DQα 3,3 and one part of type 4,4 is usually recognizable as being different than a true heterozygous type 3,4. When the dot intensities from the mixture are compared to the dot intensities in a heterozygous individual, the dot or dots corresponding to 3 are darker than they should be relative to the other dots, and the dots corresponding to 4 may be fainter than the "C" dot. This type of a result is a flag for the possible presence of a mixture or contamination.

D. Apparent mixtures containing a weak "1.1" allele

The presence of a weak "1.1" allele may not be the result of a mixture or contamination but instead could be due to the amplification of the related DXα pseudogene (see page 51 for a discussion of this topic).

2. Subtypes of the HLA DQα 4 allele

Occasionally, the signal intensity of the "1.2, 1.3, 4" dot will be much weaker than the "C" dot, even though a strong "4" dot is present. This phenomena is due to the relatively rare subtypes of the HLA DQα 4 allele which are not discriminated by this kit. The 4.2 and 4.3 allele sequences contain a single mismatch to the "1.2, 1.3, 4" probe. This mismatch, close to one end of the probe sequence, is not completely destabilizing so that a reduced signal is obtained with these alleles.

Initials: RCS Date: 4/7/94

3. Weak Amplification of the Related DXα allele

Occasionally a very weak signal, much weaker than the "C" dot, is seen at the "1.1" dot of a probe strip on which other signals are strong, even when there is no dot present for the "1" probe. This may be due to the relatively low-level amplification of a related gene, DXα. **DXα can not be used for comparison of samples.**

DXα is a non-expressed pseudogene that has some sequence similarity to HLA DQα. Sequence information shows that one HLA DQα primer has two base-pair mismatches with the second exon of DXα and the other HLA DQα primer has a one base-pair mismatch. Under the primer annealing conditions of this kit, the efficiency of DXα amplification is always very low compare to HLA DQα; however signals at the "1.1" dot which can be attributed to weak DXα amplification are observed sporadically. The amplified segment of DXα will hybridized to the HLA DQα "1.1" dot but not to the "1" dot. This hybridization pattern - "1" dot negative, "1.1" dot weak - is an indication of DXα amplification. This signal should not confuse the typing result since 1.1 signal due solely to DXα will always be much weaker than the "C" probe and as such should be noted but not considered as part of the genotype.

Another possible indication of DXα amplification is the presence of a weak "1.1" and strong hybridization to the other dots in types {1.2, 1.3}, {1.2, 1.2}, {1.3, 1.3}, {1.2, 3}, {1.2, 4}, {1.3, 3}, or {1.3, 4}. The "1" dot is positive (darker than the "C" dot) in all of these types because they contain the 1 allele. However the "1.1" dot is weaker than the "C" dot. Therefore the presence of a weak "1.1" may indicate amplification of the DXα pseudogene and does not necessarily suggest that a sample is composed of a mixture or is contaminated.

4. Amplified Samples Which Have Been Stored in the Refrigerator Prior to Hybridization

Amplified samples which have been stored for even a short time at 4°C often show low intensity dots which were not visible if the samples had been hybridized immediately after amplification. These dots are less intense than the "C" dot and do not affect the typing results.

Initials: RC Date: 4/7/94

Troubleshooting

If the Chelex extracted DNA samples fail to amplify try one or several of the following: The choice of which procedure to use is up to the discretion of the Analyst in consultation with a Scientist or Assistant Laboratory Director.

(1) Amplify using an additional 10 units of Taq polymerase.

(2) Amplify a smaller aliquot of the DNA extract to dilute potential Taq polymerase inhibitors.

(3) Amplify up to 40 μL of the DNA extract to ensure sufficient high molecular weight DNA is present.

(3) Re-extract the sample using a smaller area of the stain or less biological fluid to prevent saturation of the Chelex and to dilute potential Taq polymerase inhibitors.

(4) Re-extract the sample using a larger area of the stain or more biological fluid to ensure sufficient high molecular DNA is present.

(5) Re-extract the samples following the appropriate Non-Organic DNA Extraction Procedure (page 12). Amplify 0.3-10 ng of material as calculated by the yield gel.

(6) Add BSA to a final concentration of 160 μg/mL in the amplification reaction.

(7) Add Ficoll to a final concentration of 0.5%, in the amplification reaction.

(8) Prior to amplification, heat treat the extracted DNA at 94°C for 30 minutes.

Initials: RCS Date: 4/7/94

(9) Purify the extracted DNA on a Centricon-100 ultrafiltration device as follows:

a) Assemble the Centricon-100 unit according to the manufacturer's directions and label each unit.

b) Add 1.5 mL TE Buffer to the upper Centricon-100 reservoir.

c) Add the entire extracted DNA sample to the top of the TE Buffer. Cover the tube with Parafilm™. Use a sterile needle to punch a pinhole in the Parafilm being careful not to touch the solution with the needle. Centrifuge in a fixed-angle rotor (see Centricon instructions) at 1000 x g at room temperature for 20 minutes. The DNA sample will remain concentrated in about 20 to 40 μL of TE Buffer in the bottom of the upper Centricon reservoir and molecules with molecular weights of less than about 100,00 daltons will pass through the filter. Discard the effluent in the lower reservoir.

Note: The Centricon units are sensitive to rotor force. Do not centrifuge above 1,000 x g. Centrifugation time can be increased if the volume does not reduce to 40 μL in the specified 20 minutes.

d) Add 2 mL of TE Buffer to the concentrated DNA solution in the upper Centricon reservoir.

e) Repeat the centrifugation and wash steps in c through d twice for a total of 3 washes. After the last wash cycle collect the approximately 40 μL 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 retentate cup. The sample is now ready for the PCR amplification process. Store the sample at 2 to 8°C or freeze at -20°C until ready to perform PCR.

Initials: RCS Date: 4/7/94

Troubleshooting of HLA DQα

Problem	Possible Cause	Remedy
1. No or faint signal from both the Genomic Control DNA and DNA test samples	No or insufficient amplification	Run Amplified DNA on agarose gel (see page 34)
		If no product on gel see below
No product on gel: Repeat test from Amplification Step	No or insufficient DNA added to PCR Reaction Mix	Add >2 ng DNA
	$MgCl_2$ not added to PCR Reaction Mix	Add $MgCl_2$ Solution
	Thermal Cycler failure or wrong program	See Thermal Cycler Manual
	Tubes popped up from Thermal Cycler heating block during amplification	Push tubes firmly into contact with Thermal Cycler heat block after first cycle.
		If 239-242 bp product visible on gel see other possible causes
Product visible on gel: Repeat test from hybridization Step using new Probe Strips	Hybridization or Stringent Wash temperature too high	Check water bath temperature, should be 55°C
	HLA DQα Hybridization or HLA DQα Wash Solution salt concentration too low	Check HLA DQα Hybridization and HLA DQα Wash Solutions; prepare new solutions
	Stringent Wash time too long	Wash for correct time
	Inadequate agitation of the DNA Probe Strips during hybridization	Check rotation speed of shaking water bath
	Amplified DNA was not added to Probe Strips	Add Amplified DNA

Initials: RCS Date: 4/7/94

Problem	Possible Cause	Remedy
	Amplified DNA was not denatured	Check heat block temperature (95°C) and complete DNA addition within 30 seconds
	Enzyme Conjugate was not added to the HLA DQα Hybridization/ Enzyme Conjugate Solution	Make new HLA DQα Hybridization/ Enzyme Conjugate Solution. Add Enzyme Conjugate
Product visible on gel: Repeat test from Color Development Steps using previously developed strips	Hydrogen peroxide was not added or too much was added to Color Development Solution	Use 3% hydrogen peroxide. Make new Color Development Solution
	Hydrogen peroxide inactive	Use new bottle of hydrogen peroxide. Make new Color Development Solution
	Chromogen was not added to the Color Development Solution	Make new Color Development Solution. Add Chromogen
2. Positive signal from Control DNA, no signal from DNA test sample	The quantity of DNA test sample is below the assay sensitivity	Repeat amplification increasing DNA volume, if possible
	The test sample contains PCR inhibitors e.g. heme compounds	Try adding additional Taq Polymerase or further purification procedures
3. High Probe Strip background color	Low or lack of SDS in HLA DQα Hybridization and/or HLA DQα Wash Solutions	Prepare new HLA DQα Hybridization and HLA DQα Wash Solutions with correct amount of SDS
	Inadequate agitation of the DNA Probe Strips during hybridization and washing steps	Check rotation speed of shaking water bath

Initials: RG Date: 4/7/94

Problem	Possible Cause	Remedy
	Excess amounts of Enzyme Conjugate added to HLA DQα Hybridization Solution	Make new HLA DQα Hybridization/ Enzyme Conjugate Solution with correct amount of Enzyme Conjugate
	Exposure to light during color development	Cover tray lid with foil
4. High 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
5. Presence of wrong alleles in Control DNA	Hybridization or Stringent Wash temperature too low	Check shaking water bath temperature, should be 55°C
	HLA DQα Hybridization or HLA DQα Wash Solution salt concentration too high	Check HLA DQα Hybridization and HLA DQα Wash Solutions; prepare new solutions
	Stringent Wash time too short	Wash for correct time
6. Signal weaker than "C"	Hybridization or Stringent Wash temperature too low or high	Check shaking water bath temperature, should be 55°C
	HLA DQα Hybridization or HLA DQα Wash Solution salt concentration too low or high	Check HLA DQα Hybridization and HLA DQα Wash Solutions; prepare new solutions
	Stringent Wash time too short or long	Wash for correct time
	Mixed sample or contamination	see page 49
	DXα	see page 49
	Subtypes of the HLA DQα4 allele	see page 49

Initials: RU Date: 4/7/94

Problem	Possible Cause	Remedy
"All but 1.3" signal weaker than "C" for a 1.3,4 or 4,4 genotype	Inherent property of type	see page 45
"1,2, 1.3, 4" signal weaker than "C" and "4" signal stronger than "C"	Subtype of the HLA DQα4 allele (Inherent property of subtype)	see page 49
Weak "1.1" signal with a strong signal for 1-2 additional alleles	May be DXα	see page 49
"1" dot darker than "3" dot	Inherent property of type	see page 45
More than two alleles present	Hybridization or Stringent Wash temperature too low	Check shaking water bath temperature, should be 55°C
	HLA DQα Hybridization or HLA DQα Wash Solution salt concentration too high	Check HLA DQα Hybridization and HLA DQα Wash Solutions; prepare new solutions
	Stringent Wash time too short	Wash for correct time
	Mixed sample or contamination	see page 49

Initials: ZCL Date: 4/7/94

References for PCR & HLA DQα

1. Higuchi, R., von Beroldingen, C.H., Sensabaugh, G.F., and Erlich, H.A. "DNA typing from single hair". Nature 332: 543-546, 1988.

2. Gyllensten, U.B., Erlich, H.A. "Generation of single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA DQα locus". Proc. Natl. Acad. Sci USA 85: 7652-7656 1988.

3. Saiki, R.K., Walsh, P.S., Levenson, C.H., and Erlich, H.A. "Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes". Pro. Natl. Acad. Sci USA 86: 6230-6234 1989.

4. Helmuth, R, et.al. "HLA DQα allele and genotype frequencies in various human population determined by using enzymatic amplification and oligonucleotide probes." Am. J. Hum. Genet. 47: 515-523.

5. von Beroldingen, C., Blake, E.T., Higuchi, R., Sensabaugh, G.F., Erlich, H. "Applications of PCR to the analysis of biological evidence" (Chapter 17, p 209), in PCR Technology: Principles and Applications for DNA Amplification, ed. Erlich, H., 1989.

6. Huguchi, R., Kwok, S. "Avoiding False Positive with PCR". Nature 339: 237-238, 1989.

7. Amplitype™ Users Guide, Version 2, Cetus Corporation. 1990.

8. HLA DQα Forensic DNA Amplification & Typing Kit, 50 Test Kit, Package Insert. Catalog No. N808-0056. 1991

9. Walsh, P.S., Metzger, D.A., and Higuchi, R. "Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10: 506-513. 1991.

Initials: RCS Date: 4/7/94

Appendix A

Storage and Stability of the Amplitype HLA DQα Kit

The AmpliType™ HLA DQα Forensic DNA Amplification and Typing Kit should be stored at 2-8°C.

The Kit should be isolated from any sources of contaminating DNA, especially amplified PCR product.

The DNA Probe Strips should be stored with the desiccant in the glass tube with the screw-cap securely tightened, protected from light, at 2-30°C.

Under these conditions, components of the kit are stable until the printed expiration date.

Initials: RCS Date: 4/7/94

Washing and Re-Use of the Hybridization Trays

1. To each well of the DNA typing tray, add approximately 10 mL of 95-100% ethanol.

2. Cover the DNA typing tray with the lid and carefully agitate for 15 to 30 seconds to dissolve any residual Chromogen.

3. Remove the lid and pour off the ethanol from each well. Visually inspect each well for the presence of Chromogen (faint blue color). If necessary repeat steps a and b to remove any residual Chromogen.

4. Rinse each tray well and the tray lid with dH_2O.

5. Allow trays and lids to air dry. The trays are now ready for re-use.

Initials: ZCS Date: 4/7/94

Appendix B- Equipment and Supplies for PCR Typing

General Equipment and Supplies

Autoclave
Balance
Deionizer column for water
Calibrated Thermocouples and Meter
Labware, glassware including beakers, graduated cylinders, Erlenmeyer flasks
Freezer, -80°C and -20°C
Refrigerator
Lab Coat
Microwave oven
pH meter with calomel (Tris) electrode
Reference buffers
Polaroid Camera with type 667 film
Stir plate, Stir bar
Thermometer (N.B.S or equivalent)
Timer, 60 minute (±1 minute)
Parafilm
U.V. Transilluminator
Absorbent bench paper
Absorbent tissues (e.g., Kimwipes)
Evidence bags or envelops (for storage)
Filter paper, Whatman 3M, Whatman #1
Lab marker, waterproof ink
Microscope slides and cover slips
Paper, white
Paper towels
Parafilm
Plastic disposable pasteur pipettes
Pipettes, plastic, sterile disposable to deliver 1 to 10 mL
Pipette bulbs or electric pipettors
Wash bottles

Initials: RCS Date: 4/7/94

DNA Extraction Work Area Dedicated Equipment and Supplies

This work area should be used only for evidence handling and for extraction and isolation of DNA. Microscopy, photography, and any other activities that involve the handling of evidence samples before amplification should also be performed in this work area. These items should never leave the DNA extraction work area.

1. Pipettors: Adjustable 2 to 20 μL, 20 to 200 μL and 200 to 100 μL. If possible, reserve one set of pipettor for reagent use only, and another set for the handling of samples containing DNA.
2. Microcentrifuge tube racks
3. Microcentrifuge tubes (1.5 mL)
4. Microcentrifuge
5. Refrigerated Centrifuge and Rotors
6. Scissors
7. Disposable razor blades
8. Pipette tips and Filter pipette tips
9. Pipette bulb
10. Refrigerator
11. Hot/Stir plate
12. Laboratory glassware
13. Disposable gloves
14. Forceps
15. Vortex mixer
16. Freezer to -20°C
17. Heat blocks and Heater
18. Laminar flow/biological/biosafety cabinet with U.V. source
19. Centricon-100 microconcentrators
20. Toothpicks
21. Boiling Water bath racks
22. 1 L Pyrex glass beakers
23. Ice
24. Ice bucket

Initials: RCS Date: 4/7/94

PCR Set Up Work Area Dedicated Equipment and Supplies

This work area is used only for adding $MgC1_2$ Solution, mineral oil, and sample DNA to the PCR Reaction Mix. These items should never leave the PCR Setup Work Area

1. Combitip™ repeat pipettor with combitips
2. Dedicated pipettor (adjustable 2 to 20 μL and 20 to 1000 μL for adding DNA samples to the PCR Reaction Mix.
3. Sterile filter pipette tips
4. Microtube de-capping devices, autoclavable
5. Microtube rack
6. Disposable gloves
7. Microcentrifuge
8. Heatblock and heater
9. Refrigerator
10. Vortex Mixer
11. Kimwipes

Initials: RCS Date: 4/7/94

Amplified DNA Work Area Dedicated Equipment and Supplies

This work area(s) is used only for those activities that involve the handling of amplified DNA. This includes DNA typing (Hybridization and Color Development), DNA yield determination, gel electrophoresis of amplified DNA, waste disposal of amplified DNA solutions, and storage of amplified DNA. Amplified DNA or equipment and supplies used to handle amplified DNA should not be taken out of Amplified DNA Work Area.

Samples that have not yet been amplified should never come in contact with this equipment.

1. PE Cetus DNA Thermal Cycler
2. 1 to 20 μL and 20 to 200 μL adjustable pipettors
3. Disposable gloves
4. Microtube de-capping device
5. Disposable serological pipettes
6. Pipettor for serological pipettes
7. Towel wipes
8. Kimwipes
9. Microtube racks
10. Filter Pipette tips
11. Laboratory glassware
12. Gel electrophoresis apparatus
13. Power supply for electrophoresis
14. Disposable gloves
15. Sink
16. Refrigerator
17. Aluminum foil
18. Seal-a-meal bags
19. Waterproof marking pen
20. Water bath, stationary
21. Weight, (approx. 1kg)
22. Shaker, variable speed, orbital platform
23. Shaker, water bath
24. Filter forceps
25. Microcentrifuge
26. Heat block and heater
27. Vortex Mixer
28. Microcentrifuge tubes, 0.5 mL & 1.5 mL
29. 2 to 10 mL multi-dispensers
30. Hybridization Tray
31. Heat block and heater

Initials: RCS Date: 4/7/94

QuantiBlot Equipment and Supplies

1. Bellco Hot Shaker Plus
2. Lead weights (approx. 1 kg)
3. Pipettors (P20, P200 and P1000)
4. Shaker, variable speed, orbital platform
5. Timer
6. Vortex
7. Water bath, stationary
8. Slot blot apparatus
9. Vacuum pump
10. Plastic Tubing
11. Vacuum trap
12. Biodyne B nylon membrane 0.45μM
13. Microcentrifuge tubes, 0.5 mL & 1.5 mL
14. Hybridization tray