PROTOCOLS FOR FORENSIC PCR ANALYSIS

VERSION 4.5

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

Initials: RCS Date: 2/6/98

Initials: RCJ Date: 2/6/98

Initials: RCS Date: 2/6/98

Initials: RCJ Date: 2/6/98

General PCR Guidelines

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

2. Duplicate analyses must be performed on all evidence samples as follows:
   A. Concordant reverse dot blot, RFLP or STR match results from at least one locus in a different technique is considered a duplicate analysis.
   B. Identical typing among at least three samples including exemplars in a case is considered internal duplication.
   C. If after the first PCR analysis, there is no evidence of a mixture then the duplicate analysis may begin at the extraction stage (preferable) or the amplification stage. If there are additional alleles or unbalanced intensity of dots then either C or D (see below) apply.
   D. If after the first PCR analysis, there are additional alleles less than 50 or the "C" or "S" dots or the alleles are slightly unbalanced in intensity, then the duplicate analysis may begin at the extraction stage (preferable) or the amplification stage
   E. If after the first PCR analysis, there are three or more alleles greater than 50 or the "C" or "S" dots or the alleles are very unbalanced in intensity, then the duplicate analysis must begin at the amplification stage.

   Note: Duplicate analyses do not have to be performed on exemplars.

3. Duplicate analyses are performed to verify the typing results. At least one run (or the single run if there is only one) must have ***no*** visible dots or peaks in the extraction reagent control, amplification negative control and no extraneous dots or peaks in the positive control. The other run which is only used for verification, can have extraneous dots or peaks less than 50 or the "C"or "S" dots in the extraction reagent, amplification negative and positive controls.

4. The substrate control can have visible dots or peaks. These dots or peaks do *not* invalidate the results of the accompanying stains. The presence of dots or peaks 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 DNA extraction, for PCR set-up, and for handling amplified DNA 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 in designated racks should move between areas.

Initials: RCJ Date: 2/6/98

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.

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 pipetting 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. 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.

Initials: RG Date: 2/6/98

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 of DNA.

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, amplified DNA's are discarded. **The remainder of the DNA extract's must be retained.**

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

29. A positive and interpretable, STR, PM, HLA DQA1 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.

Initials: RCJ Date: 2/6/98

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 138) for suggestions on how to modify procedures.

1. Use Kimwipes to open sample tubes.

2. Only one tube should be uncapped at a time.

3. 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.

4. 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.

5. For pipetting Chelex, the pipette tip used must have a relatively large bore--1 mL pipet man tips are adequate.

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

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

8. 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.

9. 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.

10. If a sample is found to contain <0.15 ng/20 µL of DNA by QuantiBlot analysis the sample should not be amplified and it should be reported as containing insufficient DNA. If a sample is found to contain 0.15 ng/20 µL of DNA by QuantiBlot analysis, it should not be amplified in the PM + HLA DQA1 system. It can either be re-extracted, reported as containing insufficient DNA or concentrated using a Microcon-100 (see Troubleshooting section). The choice is at the discretion of the interpreting analyst. Other DNA concentrations (especially 0.31 and 0.62 ng/20µL) may also be concentrated and purified using a Microcon-100 if the DNA is suspected of being degraded or containing an inhibitor of PCR.

11. 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: RCJ Date: 2/6/98

Protein and DNA Extraction of Stains and Swabs

The following procedure is used for samples in which Biochemical, Hematological, and PCR analyses may be performed on the same sample.

1. Suspend the sample in approximately 75-200 µL of sterile dH_2O (or 0.05 M DTT if no semen is present) in a 1.5 mL microcentrifuge tube.

2. Centrifuge the tube in the microcentrifuge for 2 minute at 10,000 to 15,0000 x g.

3. Carefully pipet off all but 50 µL of the supernatant containing the enzyme fraction to a fresh microcentrifuge tube for serological analysis. Lyophilize or store the enzyme fraction frozen if it is not going to be analyzed immediately.

4. Proceed with the appropriate DNA extraction on the following pages adding the deionized water directly to the tube containing the cell debris pellet and the fabric or swab substrate.

Initials: RCJ Date: 2/6/98

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

1. Remove the extraction rack from the refrigerator. Extract either evidence or exemplars. Do not extract both together.

2. Pipette 1 mL of sterile deionized water into each of the tubes in the extraction rack.

3. Mix the tubes by inversion or vortexing.

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

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

6. 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).

7. Add 175 µL of 5% Chelex.

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

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

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

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

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

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

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

Initials: RCJ Date: 2/6/98

Chelex DNA Extraction From Liquid Semen

1. Remove the extraction rack from the refrigerator.

2. Pipette 200 µL of 5% Chelex into each tube in the extraction rack.

3. Add 1 µL of 20 mg/mL Proteinase K and 7 µL of 1 M DTT to each of the tubes. Mix gently.

4. Mix gently.

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

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

7. Follow the protocol for Whole Blood/Blood Stains (page 11) beginning with Step 10.

Initials: RC Date: 2/6/98

Chelex DNA Extraction from Semen Stains or Swabs

1. Remove the extraction rack from the refrigerator.

2. Pipette 1 mL of PBS into each tube in the extraction rack.

3. Mix by inversion or vortexing

4. Incubate at room temperature for 30 minutes.

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

6. Remove the swab or other substrate from the sample tube, one tube at a time, using sterile tweezers (or with a hot needle punch a hole in the bottom of the tube and collect the supernatant in a piggy backed bottom tube) and close tubes. Sterilize tweezers with ethanol before the removal of each sample. Store swab or substrate in a sterile tube for the substrate remains fraction.

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

8. 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).

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

10. 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 swab remain fraction may either be combined with the resuspended sample or both fractions processed separately. If no epithelial cells are observed, the differential extraction procedure may be omitted and the samples may be processed beginning with step 21.

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

Initials: RC Date: 2/6/98

12. Add 1 µL of 20 mg/mL Proteinase K. Vortex briefly to resuspend the pellet.

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

14. 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

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

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

17. 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.

18. 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.

19. 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.

20. To the approximately 50 µL resuspended sperm fraction and to the tubes containing the substrate remains and the sperm fraction extraction negative, add 150 µL of 5% Chelex, 1 µL of 20 mg/mL Proteinase K, and 7 µL of 1M DTT. Mix gently.

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

Initials: RCl Date: 2/6/98

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

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

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

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

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

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

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

Initials: RCS Date: 2/6/98

Chelex DNA Extraction From Hair

Microscopic examination of hair should be completed before attempts at DNA typing.

1. Fill out the extraction worksheet.

2. Handling hair with clean forceps, examine the hair under a dissecting microscope for the presence of sheath material. The hair may be placed on a clean piece of white paper. Note possible presence of body fluid on hair.

3. Wash the hair containing sheath material to reduce surface dirt and contaminants by immersing the hair in sterile, deionized water in a clean 50 mL beaker.

4. Return the hair to the dissecting microscope. Use a clean scalpel to cut a 1 cm portion from the root end of the hair. Because hair may contain cellular material on the surface which may or may not originate from the hair donor, it is advisable to cut off a 1 cm section of the shaft adjacent to the root portion for separate analysis as a control.

5. Add the root portion of the hair to 200 µL of 5% Chelex in a 1.5 mL microcentrifuge tube.

6. Incubate at 56°C (at least 6 to 8 hours) or overnight.

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

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

   Note: Check that the hair is completely immersed in the Chelex solution before boiling.

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

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

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

12. Store the remainder of the supernatant at either 2 to 8°C or frozen. To re-use, repeat Steps 8 through 11.

Initials: RCJ Date: 2/6/98

Chelex Extraction From Non-Organically and Organically Extracted Samples

The amount of DNA in non-organically and organically extracted samples is usually quantitated by yield gel. See the RFLP manual for organic extraction, non-organic extraction and yield gel analysis. The target amount of DNA to add to each tube for Chelex Extraction is 50 ng in 50 µL. Table I lists the volumes of (non-)organic extract to add to the Chelex tube for the various yield gel concentrations.

When a differential extraction has been performed on semen samples, both semen and epithelial cell fraction reagent blanks are generated. Both reagent blanks must be Chelex extracted and amplified if their corresponding fraction contains DNA.

Table I: Non-Organic or Organic Extract for Amplification

yield gel conc (ng/µL)	volume dH_2O (µL)	volume (non-)organic extract (µL)	volume 5% Chelex (µL)
100	49.5	0.5	150
50	49	1.0	150
40	48.7	1.3	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
2.0	25	25	150
≤1.0	0	50	150

1. Fill out the extraction worksheet.

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

Initials: RCJ Date: 2/6/98

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. The target amount of DNA to add to each Chelex Extraction Tube is 50 ng.

4. Add 50 µL dH_2O and 150 µL 5% Chelex to an empty tube marked as a Chelex Extraction Negative.

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

6. Vortex.

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

8. Vortex.

9. Microfuge for 2-3 minutes.

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

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

Initials: RCI Date: 2/6/98

Estimation of DNA Quantity from QuantiBlot Analysis

See the RFLP manual for the Estimation of DNA Quantity from a Yield Gel

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-DQA1 - 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^{-4} 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 ½ 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 DQA1 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: 2/6/98

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: RCJ Date: 2/6/98

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 Kimwipe, 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 DQA1 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.

Hybridization

12. Add 30 mL of pre-warmed HLA DQA1 Hybridization Solution to the tray. Tilt the tray to one side and add 20 µL of QuantiBlot D17Z1 Probe to the HLA DQA1 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.

Initials: RCJ Date: 2/6/98

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).

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.

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.

Initials: RCJ Date: 2/6/98

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 ½ 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.

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: JCJ Date: 2/6/98

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^{-4} Buffer. Any $MgCl_2$ can be removed from samples by microdialysis using Centricon 100 spin units (follow manufacture's directions).

Initials: RCJ Date: 2/6/98

	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 sig-n-ificantly 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 mem-b-rane, 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: RCJ Date: 2/6/98

	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^{-4} 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.

Initials: JC Date: 2/6/98

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: KCJ Date: 2/6/98

PM+DQA1 Analysis

Amplification of PM+DQA1

A positive control-external, an amplification negative control, an extraction reagent control and substrate controls, if applicable, should be included with each batch of samples being amplified to demonstrate procedural integrity. The positive control-external is a control whose alleles are known to the analyst.

1. For each sample to be amplified, label a new tube. Do not amplify samples in which no DNA was detected by QuantiBlot.

2. To each labeled tube, add TE^{-4} and DNA as specified in Table II.

   NOTE: Steps 1 and 2 may be done in the DNA extraction or the DNA set-up areas of the laboratory. The following steps should be done in the DNA set-up area.

3. Add the following amounts of TE^{-4} and DNA to new labeled tubes:

Sample	TE^{-4}	DNA
amplification positive control-external	---	20 µL
extraction reagent control	---	20 µL
amplification negative control	20 µL	---

Initials: RCJ Date: 2/6/98

. Table II- Amount of DNA to be Amplified for the PM+DQA1 Kit

DNA Concentration* (ng/20μL)	Target Volume (μL) to be amplified	TE^{-4} (μL) for Target Volume	Range of Volumes (μL) which can be amplified
≥50	Dilute 1:10 and use the dilution for amplification		
25	1.6	18.4	0.5-8**
12.5	3.2	16.8	0.9-16**
6.2	6.5	13.5	1.6-20**
5	8	12	2-20**
2.5	16	4	4-20**
1.25	20	0	8-20**
0.62	20	0	-
0.31	20	0	-
<0.31	Do not amplify	-	-

* If the QuantiBlot result is ≥10 ng/20 μL, dilute the sample 1:10 with TE^{-4} and re-quantitate. Multiply the diluted concentration by 10 to obtain the original sample concentration. Repeat the procedure if the diluted sample is still ≥10 ng/20 μL.

Note: When a dilution is made to determine DNA concentration, it is preferable to calculate the DNA concentration in the undiluted DNA extract and to amplify the undiluted DNA extract and not amplify the dilution.

** Add TE^{-4} to a final volume of 20 μL.

Initials: JC Date: 2/6/98

5. Turn on the Perkin Elmer Thermal Cycler. (See manufacturer's instructions). Step File #14 on all 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 all machines for an additional 7 minutes incubation at 72°C.

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

6. Determine the number of samples to be amplified, including controls and label a PCR reaction mix tube for each sample.

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

8. 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.

9. Pipet 40 µL PM Primer Set into each reaction mix tube 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.

   Note: It is important to begin the cycling processing within 20 minutes after addition of the PM Primer Set to the PCR Reaction Mix.

10. 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.**

11. Close all of the tubes.

12. **Note: Use a new sterile filter pipet tip for each sample addition. Open only one tube at a time for sample addition.** For PM+HLA DQA1 the final volume is 100 µL. Add the contents (20 µL) of the sample DNAs and sterile H_2O tubes which were prepared above 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.**

Initials: RC Date: 2/6/98

13. 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.

14. Start the Thermal Cycler amplification program (File #14). 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.

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

16. In the hybridization room, open the tubes one at a time and add 5 µL of 200 mM EDTA. Use a new pipet tip for each addition. Insert the pipet through the mineral oil layer. Discard the pipet tip and re-cap the tube before proceeding to the next tube. It may be convenient to remove a 5 µL aliquot for gel electrophoresis before recapping the tube.

17. 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 fourteen days, or at -20° for at least 6 months in the appropriate refrigerator or freezer.

Initials: RCJ Date: 2/6/98

Optional Step to Verify Amplification

Agarose Gel Preparation

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

2. Add agarose to the appropriate amount of 1X TBE in a flask. Volume required will vary depending on size of the minigel apparatus. Prepare enough agarose to pour a 0.45cm 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 0.5 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 TBE to fill the buffer tanks and cover the gel to a depth of about 2 mm.

9. Carefully remove slot forms. Avoid touching wells.

Gel Loading and Electrophoresis

1. Add 2 µL Gel Loading Buffer to a 0.5 mL microcentrifuge tube for each sample or to a well of a 96 well microplate for each sample.. Add 5 µL of the DNA sample to the loading buffer and mix by tapping gently.

2. Dilute the Gibco BRL 123 bp ladder to 40 ng/µL with sterile water. Add 5 µL of the diluted ladder to 2 µL of Gel Loading Buffer.

3. Do not use the outside lanes of the gel. In the first and last useable lanes of the gel, pipet 123 bp ladder.

Initials: RCJ Date: 2/6/98

4. Carefully pipette samples into the remaining wells.

5. Connect leads so that the DNA migrates toward the positive (+) electrode. Run the minigel at 100 volts, (or 7.5 v/cm) at room temperature for about two hours or until the bromophenol blue (faster-migrating) dye is 7.5 cm from the wells of the gel.

6. 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.

Interpretation of Gel Electrophoresis Patterns

The HLA DQA1 amplification products will appear as 242/239 bp bands. The PM products will appear as 214 bp (LDLR), 190 bp (GYPA), 172 bp (HBGG), 151 bp (D7S8), and 138 bp (GC). Primer dimer bands and unincorporated primers may appear as broad bands near the bottom of the gel in the region of lower molecular weight. Occasionally,, non-specific bands or smearing can be observed above and/or below the six specific bands, but they do not compromise the typing results.

For PM+DQA1 six bands should be present. Otherwise do not proceed.

Initials: RCJ Date: 2/6/98

PM+DQA1 Hybridization

1. Heat a shaking water bath to 55°C. The water level should be 1/4 - ½ inch above the shaking platform. The water level should not be higher than ½ 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 37-55°C. Warm the HLA DQA1 Hybridization Solution and the HLA DQA1 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. **Make sure the tube is at room temperature before removing the strips.** 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.

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

5. Add one drop of oil to each well that will be used. 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. Tilt the Typing Tray towards the labeled end of the strips. Add 3 mL of DQA1 Hybridization Solution at the labeled end of each strip.

Initials: RCJ Date: 2/6/98

7. **Note: Perform the following steps for each tube of amplified DNA. When pipetting 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 20 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 20 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.
      I. If using only PM strip or only DQA1 strips for each amplified sample, set the tube aside.
      ii. If using both PM **and** DQA1 strips for the same amplified sample, return the tube to the 95°C heat block for 3-10 minutes after probe addition to the first strip. Add the remaining DNA samples to the first strip of each PM and DQA1 set. After the required time has passed, add the probe to the second strip of each set. Set the tube aside after addition to the second strip.
   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 2-8°C for 2 months, at -20°C for 6 months or discarded when the analysis is complete.

8. Mix the tray by carefully rocking and place the clear plastic lid on the tray. Ensure that each strip is completely wet. 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. **Once the hybridization has begun, strips should remain wet through the conclusion of the Color Development and Photography step.**

9. Hybridize the amplified DNA samples to the Probe Strips by incubating at 55°C for 15±2 minutes at 50-70 rpm. Adjust the water level and check the tray position so that water does not splash into the wells of the tray.

10. Approximately 5 minutes before the end of the hybridization step, prepare the Enzyme Conjugate Solution in a glass flask either according to Table III or the following formula:

    # Strips x 3.3mL pre-warmed DQA1 Hybridization Solution
    # strips x 27 µL Enzyme Conjugate: HRP:SA

    Mix by swirling.

Initials: RG Date: 2/6/98

Table III- DQA1 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

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. Do not use a paper towel. Replace the water bath cover and keep the water bath rotating between incubation steps to maintain the temperature at 55°C±1°C

12. **Note: DQA1 Wash Solution solids must be in solution before use.**

    Dispense 5 mL of DQA1 Wash Solution into each well. Rinse by rocking for several seconds, then pour the solution from each well. Wipe the tray lid with a Kimwipe.

13. Dispense 3 mL of the enzyme Conjugate Solution prepared in Section 10 into each well. Cover tray with lid and weight. Place into the 55°C in the shaking water bath for 5±1 minutes at 50-70 RPM.

Initials: LCJ Date: 2/6/98

14. After incubation, remove the tray from the water bath and pour out the contents of each well. Wipe the tray lid with a Kimwipe. Do not use a paper towel. Replace the water bath cover and keep the water bath rotating between incubation steps to maintain the temperature at 55°C±1°C

15. Dispense 5 mL of DQA1 Wash Solution into each well. Rinse by rocking for several seconds, then pour the solution from each well. Wipe the tray lid with a Kimwipe.

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

    Stringent Wash:
    Dispense 5 mL of DQA1 Wash Solution into each well. Cover tray with lid and weight. Place into the 55°C shaking water bath for 12 ±1 minutes, at about 50-70 rpm.

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

18. Dispense 5 mL of DQA1 Wash Solution into each well. Rinse by rocking for several seconds, then pour the solution from each well. Wipe the tray lid with a Kimwipe.

Initials: RG Date: 2/6/98

PM and PM+DQA1 Color Development

1. Dispense 5 mL of citrate buffer into each well. Wipe the tray lid with a Kimwipe . 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. A tube of hydrogen peroxide can be used for one week. Discard the remaining hydrogen peroxide after one week. 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 IV or the following formula. Add the reagents in order:

strips x 5 mL HLA DQA1 Citrate Buffer
strips x 0.5 µL 30% Hydrogen Peroxide or 5µL 3% Hydrogen Peroxide
strips x 0.25 mL Chromogen Solution

Mix by swirling 2-3 times. Do Not Vortex. Protect from light.

Table IV- Development Solution

number of strips	citrate buffer	hydrogen peroxide 30% (3%)	chromogen
3	15 mL	1.5 (15) µL	0.75 mL
4	20 mL	2.0 (20) µL	1.0 0 mL
5	25 mL	2.5 (25) µL	1.25 mL
6	30 mL	3.0 (30) µL	1.50 mL
7	35 mL	3.5 (35) µL	1.75 mL
8	40 mL	4.0 (40) µL	2.00 mL
9	45 mL	4.5 (45) µL	2.25 mL
10	50 mL	5.0 (50) µL	2.50 mL
11	55 mL	5.5 (55) µL	2.75 mL
12	60 mL	6.0 (60) µL	3.00 mL
13	65 mL	6.5 (65) µL	3.25 mL
14	70 mL	7.0 (70) µL	3.50 mL
15	75 mL	7.5 (75) µL	3.75 mL
16	80 mL	8.0 (80) µL	4.00 mL

Initials: RH Date: 2/6/98

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 DQA1 Citrate Buffer. Add 5 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 5 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.

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 PM and/or HLA DQA1 type for each sample from the photograph. (See Results Interpretation section.)

Initials: RG Date: 2/6/98

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 ½ second and f16 for type 665 film.

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

6. 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.

7. 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.

8 Place strips between sheets of blotting paper **(do not use paper towels)** and allow to dry; minimize exposure to light

9. When strips are dry, 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: RCJ Date: 1/6/98

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: CY Date: 2/6/98

Results Interpretation

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

1. Reading and Interpreting the AmpliType PM DNA Probe Strips

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

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

When an "S" dot is visible on the AmpliType PM DNA Probe Strip, the intensities of the dots at the remaining twelve positions are compared to the intensity of the "S" dot. Those dots that appear either darker than or equivalent to the "S" dot are considered positive. Each positive dot indicates the presence of the corresponding allele. Dots with signals less than the "S" dot should be noted and interpreted with caution.[9,11,28 14,15]

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

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

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

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

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

Initials: RCJ Date: 2/6/98

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

A sample from a single individual should produce balanced dot intensities within each locus for which the individual is heterozygous.

2. Reading and Interpreting the AmpliType HLA DQA1 DNA Probe Strips

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

To read the developed HLA DQA1 DNA Probe Strips, the "C" dot is examined first and then the remaining dots are examined. The control probe "C" on the HLA DQA1 DNA Probe Strips detects all of the HLA DQA1 alleles. The "C" dot is designed to be the lightest typing dot on the strip and it indicates that adequate amplification and typing of the HLA DQA1 alleles in the sample have occurred. If the "C" dot is absent, an accurate determination of the type cannot be made. HLA DQA1 DNA Probe Strips with no visible "C" dot should not be typed.

The "C" probe serves two functions.

A. To indicate adequate amplification and typing of the HLA DQA1 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 HLA DQA2 pseudogene, HLA DQA1 type 1.3,4, or sub-types of the HLA DQA1 4 allele (See page 50, 121).

The accurate interpretation of the HLA DQA1 results depends on the presence and intensity of the "C" dot. The intensities of the dots at the remaining ten positions are compared to the intensity of the "C" dot. Those dots that appear either darker than or equivalent to the "C" dot are considered positive. Each positive dot indicates the presence of the corresponding HLA

Initials: RC Date: 2/6/98

DQA1 allele. Dots with signals less than the "C" dot should be noted and interpreted with caution.[9,11,28]

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

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

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

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

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

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

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

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

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

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

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

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

The "All but 1.3" dot is positive in the presence of all HLA DQA1 alleles EXCEPT 1.3. This probe is necessary to differentiate the 1.2, 1.3 genotype from the 1.3, 1.3 genotype.

Note: The "All but 1.3" dot can be equal to or lighter than the "C" dot when the genotype has a HLA DQA1 1.3 allele paired with a HLA DQA1 4.1, 4.2, or 4.3 allele because the HLA DQA1 4.1, 4.2, and 4.3 alleles have a single partially destabilizing mismatch to the "All but 1.3" probe.[18] The partially

destabilizing mismatch allows these three alleles to bind to this probe weakly relative to the HLA DQA1 1.1, 1.2, 2, and 3 alleles. The "All but 1.3" may also sometimes be weaker than the "C" dot for genotypes with two of the 4 alleles.

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

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

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

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.

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

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

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

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

3. Reporting Procedures

All alleles which meet reporting criteria are listed in the laboratory report regardless of intensity differences. Genotypes are not reported and should not be inferred. i.e. if only a "3" allele is found, it should be reported as 3. The reporting criteria are as follows:

Initials: RCJ Date: 2-16-98

A. If an allele has an intensity greater than or equal to "C" or "S" in two runs (or one run if only one run is required and performed) then the allele is listed in the report. (i.e. 2, 3 or 2, 3, 4.1)

B. If an allele has an intensity greater than or equal to "C" or "S" in one run and an intensity of less than "C" or "S" in at least one additional run then the allele is listed in the report in brackets. (i.e. [2]). At the bottom of the table, the brackets are defined as "[ ]= The presence of the allele above a minimum threshold could not be duplicated."

C. If an allele has an intensity of less than "C" or "S" in at least one run and does not fit into categories A and B above than the allele is reported as **. The ** is defined at the bottom of the table as " ** = additional alleles were detected which did not meet laboratory criteria for allele identification; therefore, these additional alleles are not reported."

D. If an allele has an intensity greater than or equal to "C" or "S" in one run and its presence can not be duplicated (greater or less than the "C" or "S") when duplication is required, then the allele is reported as **.

E. If due to the nature of the case, the presence of the 1.2 allele is suspected of being masked (see the Interpretation of Complex HLA DQA1 Results section), the possible presence of the 1.2 allele is reported as (1.2). The (1.2) is defined at the bottom of the table as "Indicates that this sample may contain a 1.2 allele; however, due to the number of alleles detected in the sample it is not possible to make a definitive determination.."

F. If no alleles are detected in a sample then the sample is reported as "Neg= no alleles detected."

G. If DNA below the minimum threshold is found on QuantiBlot Analysis then the sample is reported as "INS =Insufficient human DNA was detected; therefore, this sample was neither amplified nor typed."

H. If there is a large intensity difference between alleles from a locus, the intensity difference should be noted on the report with ***. The *** is defined at the bottom of the table as "***= Large intensity difference between alleles suggests a mixture of DNA."

I. Other symbols or reporting procedures will be used if necessary depending on the details of the case.

Initials: RCJ Date: 2 6 98

Comparison of Samples and Interpretation of Results in Report

A. Determine whether it is likely that a sample contains a mixture of DNA (i.e. more than two alleles for a locus, intensity differences between alleles within a locus, reproducible pattern of alleles less than "C" or "S", or the facts of the case suggest a potential mixture.) State in the report whether a sample contains a mixture or possible mixture of DNA, determine the minimum number of individuals who could have contributed to a mixture and the likely source of each component of the mixture

B. Compare all possible evidence and exemplar pairs and all possible evidence pairs to determine inclusions and exclusions. For inclusions look at the examination table in the report and compare all alleles reported regardless of whether they are in parentheses or brackets. For exclusions in addition to the preceding, consider all clear dots which in each of two runs have an intensity less than the "C" or "S" dots.

C. Assuming a single physiological fluid donor, two samples could derive from a common biological source (inclusion) if all the alleles in the evidence sample could be accounted for by the alleles in the exemplar sample. If however a mixture is possible in the evidence sample, there may be alleles in the evidence sample that are not accounted for by the exemplar sample. If an inclusion requires the presence of more than one physiological fluid donor, this should be stated in the report. (i.e.. (S) 4.1, 4.1; vaginal swab sperm fract. 1.1, 4.1 (4.1>1.1); Assuming a single semen donor, the suspect can be eliminated as the semen donor. However if there is more than one semen donor, the suspect can not be eliminated as a possible semen donor.)

D. Statistics are calculated for probative inclusions only where: (1) The sample is apparently unmixed. (2) The sample appears to be a mixture of two components and the source of one component is known. (i.e. when vaginal epithelial cells are present in the sperm fraction from a vaginal swab.) See below for the calculation of statistics.

E. Statistics are not calculated for expected inclusions such as vaginal epithelial cells from a vaginal swab consistent with the victim

Initials: RCJ Date: 2/6/98

4. Extraction Reagent, Amplification Negative and Substrate Controls

The extraction reagent control and amplification negative control are a check for the possible contamination of the reagents in the PM and/or HLA DQA1 test by other human DNA or by amplified PM and/or HLA DQA1 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. 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 control, amplification negative control and substrate control 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 PM and/or HLA DQA1 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" or "S" dots not visible, and the test samples are easily typeable with clearly visible "C" or "S" dots, the contamination problem is not serious. If the "C" or "S" dot is visible on the typing strip of the extraction reagent, amplification negative or substrate controls, the contamination problem is more serious. See Table VI for interpretation guidelines.

Initials: CS Date: 2/6/98

The appearance of signals in extraction reagent, amplification 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 VI for interpretation guidelines.

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

Control	"C" / "S" 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.

5. Amplification Positive Control- External

Control DNA 1 which is provided in the PM+DQA1 kits is a positive control- external which is used with each batch of samples typed to demonstrate that the kit is performing properly. Control DNA 1 has an HLA DQA1 type of **1.1, 4.1** and a PM type of LDLR **BB**, GYPA **AB**, HBGG **AA**, D7S8 **AB**, GC **BB**.

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: RCJ Date: 2/6/90

Interpretation of Complex PM+HLA DQA1 Results

Occasionally typing results may appear markedly different from the standard 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, HLA DQA2.

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 DQA1 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 DQA1 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 DQA1 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 DQA1 genotypes in which a three allele mixture could contain an undetected 1.2 allele. These five patterns are listed in Table VII. These five patterns correspond to 25 of the 166 possible mixtures of two genotypes containing three alleles in total.

Initials: RCJ Date: 4/6/98

Table VII: HLA DQA1 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 DQA1 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 HLA DQA2 pseudogene.

2. Subtypes of the HLA DQA1 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 DQA1 4 allele. 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.

3. Weak Amplification of the Related HLA DQA2 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, HLA DQA2. **HLA DQA2 can not be used for comparison of samples.**

HLA DQA2 is a non-expressed pseudogene that has some sequence similarity to HLA DQA1. Sequence information shows that one HLA DQA1 primer has two base-pair mismatches with the second exon of HLA DQA2 and the other HLA DQA1 primer has a one base-pair mismatch. Under the primer annealing conditions of this kit, the efficiency of HLA DQA2 amplification is always very low compare to HLA DQA1; however signals at the "1.1" dot which can be attributed to weak HLA DQA2 amplification are observed sporadically. The amplified segment of HLA DQA2 will hybridized to the HLA DQA1 "1.1" dot but not to the "1" dot. This hybridization pattern - "1" dot negative, "1.1" dot weak - is an indication of HLA DQA2 amplification. This signal should not confuse the typing result since 1.1 signal due solely to HLA DQA2 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 HLA DQA2 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 HLA DQA2 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: RCJ Date: 2/2/98

Troubleshooting

Observation	Possible Cause	Recommended Action
1. No signal or faint signal from both the positive control and the DNA test samples at all loci.	No PCR amplification or insufficient PCR amplification of all markers.	Check amplified DNA on agarose gel (see **Observation 1.1**).
	Improper hybridization or assay condition.	Repeat test from Hybridization step (see **Observation 1.2**).
1.1 No amplified product visible on gel.	No DNA added or insufficient DNA added to PCR Reaction Mix.	Quantitate DNA and add 0.31-10 ng DNA; repeat test.
	AmpliType PM Primer Set not added to AmpliType PCR Reaction Mix.	Add AmpliType PM Primer Set; repeat test.
	GeneAmp PCR Instrument System failure or wrong program.	See GeneAmp PCR Instrument System Manual and check instrument calibration.
	Tubes not seated tightly in the DNA Thermal Cycler 480 block during amplification.	Push tubes firmly into contact with block after first cycle; repeat test.
	MicroAmp Base used with tray and tubes in GeneAmp PCR System 9600.	Removed MicroAmp Base; repeat test.
1.2 Amplified product visible on gel but no signal or faint signal on AmpliType DNA Probe Strips.	Hybridization and/or Stringent Wash temperature too high.	Check that the rotating water bath temperature is at 55°C (±1°C) with an immersible thermometer; repeat test.
	DQA1 Hybridization and/or DQA1 Wash Solution salt concentration too low.	Prepare new solutions; repeat test.

Initials: RCJ Date: 2/6/98

Observation	Possible Cause	Recommended Action
	Stringent Wash time too long.	Repeat test, washing for 12 minutes (±1 minute) only.
	Inadequate agitation of the DNA probe strips during hybridization.	Check speed of rotating water bath (50 to 70 rpm); verify that hybridization solution is washing over the strips; repeat test.
	Amplified DNA was not added to DNA probe strips.	Repeat test, adding amplified DNA to DNA probe strips.
	Amplified DNA was not denatured.	Check GeneAmp+ PCR Instrument System block temperature is 95°C; leave sample in block >3 minutes. Repeat the test.
	Enzyme Conjugate: HRP-SA was not added to the appropriate solution.	Prepared new diluted Enzyme Conjugate: HRP-SA solution; repeat test.
	Hydrogen peroxide was not added or too much was added to the Color Development Solution.	Make new Color Development Solution with correct amount of hydrogen peroxide; repeat test.
	Hydrogen peroxide inactive.	Make new Color Development Solution using new bottle or dilution of hydrogen peroxide; repeat test.
	Chromogen: TMB was not added to the Color Development Solution.	Make new Color Development Solution adding Chromogen: TMB; repeat test.
	For HLA DQA1 kits, the original HLA DQA1 instead of PM or PM + DQA1 typing protocol was followed.	Repeat test following the PM + DQA1 typing protocol.

Initials: RCJ Date: 2/6/98

Observation	Possible Cause	Recommended Action
2. Positive signal from positive control, but no signal from DNA test sample.	Quantity of DNA test sample is below the assay sensitivity.	Quantitate DNA and add 0.31-10 ng DNA; repeat test.
	Test sample contains PCR inhibitor (e.g. heme com pounds, certain dyes).	Any or all of the following actions may be taken: 1. Quantitate DNA and add minimum necessary volume; repeat test. 2. Wash the sample in Centricon+ 100 (see **Reference 11**); repeat test. 3. Add BSA to reaction and see **Reference 15 and 31**; repeat test.
	Test sample DNA is degraded	If possible evaluate the quality of the DNA sample by running an agarose gel (see **Reference 18**). If the DNA is degraded, reamplify with an increased amount of DNA.
3. High DNA probe strip background color.	Low or lack of SDS in DQA1 Hybridization and/or DQA1 Wash Solution.	Prepare new DQA1 Hybridization and/or DQA1 Wash Solution with correct amount of SDS; repeat test
	Inadequate agitation of the DNA probe strips during hybridization and steps.	Check speed of rotating water bath (50 to 70 rpm); verify that solutions are washing over strips; repeat test
	Tray lid not wiped ade-quately.	Wipe lid; repeat test.
	Excess amounts of Enzyme Conjugate; HRP-SA added Solution.	Prepared new solution with correct amount of Enzyme Conjugate: HRP-SA; repeat test.

Initials: AC/ Date: 2/6/98

Observation	Possible Cause	Recommended Action
	Expose to light during Color Development.	Cover tray lid with foil during Color Development steps; repeat test.
	Use of water other than deionized or glass distilled water for water rinses.	Repeat test using deionized or glass distilled water for water rinses.
4. High DNA probe strip background color upon storage.	Exposure to strong light and oxidizing agents.	Store strips in the dark away from oxidizing agents.
	Insufficient water washes after Color Development.	Increase number of water washes in future assays.
5. Presence of unexpected or additional dots in the amplified positive control sample.	Cross-hybridization caused by Hybridization and/or Stringent Wash temperature being too low.	Check that the rotating water bath temperature is at 55°C (±1°C) with a total immersion thermometer; repeat test.
	Cross-hybridization caused by DQA1 Hybridization and/or DQA1 Wash Solution salt concentration being too high.	Prepare new solutions; repeat test.
	Cross-hybridization caused by Stringent Wash time being too short.	Repeat test, washing for 12 minutes (±1 minute).
	Contamination by amplified product or samples.	See **Reference 18** and **28**.

Initials: ACJ Date: 2/6/98

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

Initials: ACJ Date: 2/6/98

Observation	Possible Cause	Recommended Action
	Amplification of HLA DQA2 pseudogene (faint 1.1 dot).	See **Reference 18** and **30**.
8. Some, but not all, loci visible on gel of AmpliType PM PCR products.	Test sample DNA is degraded.	If possible, evaluate the quality of the DNA sample by running an agarose gel (see **Reference 18**). If the DNA is degraded, reamplify with an increased amount of DNA.
	Test sample contains PCR inhibitor (e.g. heme com pounds, certain dyes).	Any or all of the following actions may be taken: 1. Quantitate DNA and add minimum necessary volume; repeat test. 2. Wash the sample in Centricon+ 100 (see **Reference 11**); repeat test. 3. Add BSA to reaction and see **Reference 15 and 31**; repeat test.
	Input DNA and/or PCR product was not denatured sufficiently during amplification.	Check calibration of the GeneAmp+ PCR Instrument System using the appropriate Temperature Verification System.
9. Some, but not all, loci visible on AmpliType PM DNA Probe Strip.	Not all loci amplified.	Verify the presence of amplified loci on an agarose gel (see **Observation 8**).
	Amplified DNA was not denatured.	Check GeneAmp PCR Instrument System block temperature is 95°C; leave sample in block >3 minutes. Repeat the test

Initials: RCJ Date: 2/6/98

Observation	Possible Cause	Recommended Action
10. Imbalanced dot intensities within a locus on the AmpliType PM DNA Probe Strip (this kit is designed to produce balance dot intensities when heterozygous samples are typed, except as described in interpretation section)	DQA1 Hybridization and/or DQA1 Stringent Wash temperature too high or too low.	Check that the rotating water bath temperature is at 55°C (±1°C) with a total immersion thermometer; repeat test.
	DQA1 Hybridization and/or DQA1 Wash Solution salt concentration too high or too low.	Prepare new solutions; repeat test.
	Stringent Wash time too long or too short.	Repeat test, washing for 12 minutes (±1 minute).
	Mixed sample or contamination.	See **Reference 14, 15, 17, 18,** and **28.**
	EDTA not added to the reaction prior to the heat denaturation step of the DNA hybridization protocol.	Add EDTA to amplified sample; repeat test.
11. Weak or absent "4.1" dot on the AmpliType HLA DQA1 or HLA DQA1 DNA Probe Strips in the amplified positive control sample.	EDTA not added to the reaction prior to the heat denaturation step of the DNA hybridization protocol.	Add EDTA to amplified sample; repeat test.
12. "1.2, 1.3,4" dot weaker than "C" dot on AmpliType HLA DQA1 DNA Probe Strips.	Genotype of sample has a HLA DQA1 4.2 or 4.3 allele paired with a HLA DQA1 1.1, 2, 3, 4.2, or 4.3 allele.	See **Reference 18**.
13. "1.1" dot weaker than "C" dot but on AmpliType HLA DQA1 DNA Probe Strip.	Amplification of HLA DQA2 pseudogene (faint 1.1 dot).	See **Reference 18** and **30**.
14. "All but 1.3" signal weaker than "C" dot on AmpliType HLA DQA1 DNA Probe Strip.	Genotype of sample has a HLA DQA1 1.3 allele paired with a HLA DQA1 4.1, 4.2, or 4.3 allele.	See **Reference 18**.

Initials: RCJ Date: 2/6/98

Initials: RCJ Date: 2/6/98

References

1. von Beroldingen, C.H., Blake, E.T., Higuchi, R., Sensabaugh, G.F., and Erlich H.A. 1989. Applications of PCR to the analysis of biological evidence. In: PCR Technology, Principles and Applications for DNA Amplification, ed. Erlich, H.A., Stockton Press, Inc. New York, NY, p. 209-223.

2. Reynolds, R., Sensabaugh G., and Blake, E. 1991, Analysis of genetic markers in forensic DNA samples using the polymerase chain reaction. Analytical Chemistry **63**: 2-15.

3. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**; 487-491.

4. Higuchi, R., von Beroldingen, C.H., Sensabaugh, G.F., and Erlich, H.A. 1988. DNA typing from single hairs. Nature **332**; 543-546.

5. Paabo, S., Gifford, J.A., and Wilson, A.C. 1988. Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Research **16**: 9775-9787.

6. Hochmeister, M.N., Budowle, B., Jung J., Borer, U.V., Comey, C.T., and Dirnhofer, R. 1991. PCR-based typing of DNA extracted from cigarette butts. International Journal of Legal Medicine **104**: 229-233.

7. Hochmeister, M.N., Budowle, B., Borer, U.V., Eggman, U., Corney, C.T., and Dirnhofer, R. 1991. Typing of deoxyribonucleic and (DNA) extracted from compact bone from human remains. Journal of Forensic Sciences **36**: 1649-1661.

8. Sajantila, A., Strom M., Budowle, B., Karhunen, P.J., and Peltonen, L. 1991. The polymerase chain reaction and post-mortem forensic identity testing: application of amplified D1S80 and HLA-DQ alpha loci to the identification of fire victims. Forensic Science International **51**: 23-34.

9. Blake, E., Mihalovich, J., Higuchi, R., Walsh, P.S., and Erlich, H. 1992. Polymerase Chain Reaction (PCR) amplification and human leukocyte casework experience. Journal of Forensic Sciences **37**: 700-726.

10. Corney, C.T. and Budowle, B. 1991. Validation studies on the analysis of the HLA DQA1 locus using the polymerase chain reaction. Journal of Forensic Sciences **36**: 1633-1648.

Initials: RCJ Date: 2/6/98

11. Corney, C.T., Budowle, B., Adams, D.E., Baumstark, A.L., Lindsey, J.A., and Presley, L.A. 1993. PCR amplification and typing of the HLA DQA1 gene in forensic samples. Journal of Forensic Sciences **38**: 239-249.

12. Sullivan, K., et. al. 1992. Characterization of HLA DQA1 for forensic purposes. Allele and genotype frequencies in British Caucasian, Afro-Caribbean and Asian populations. International Journal of Legal Medicine **105**: 17-20.

13. Saiki, R.K., Walsh, P.S., Levenson, C.H., and Erlich, H.A. 1989. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes Proceedings of the National Academy of Sciences, USA **86**: 6230-6234.

14. Herrin, G., Jr., Fides, N., and Reynolds, R. 1994. Evaluation of the AmpliType+ PM DNA test system on forensic case sample. Journal of Forensic Sciences **39**: 1247-1253.

15. Budowle, B., Lindsey, J.A., DeCou. J.A., Kooms, B.W., Giusti, A.M., and Comey, C.T. 1995. Validation and population studies of the loci LDLR, GYPA, HBGG, D7S8, and GC (PM loci), and HLA DQA1 using a multiplex amplification and typing procedure. Journal of Forensic Sciences **40**: 45-50.

16. Roy, R. and Reynolds, R. 1995. AmpliType+PM and HLA DQA1 typing from pap smear, semen smear and post-coital slides. Journal of Forensic Sciences **40**: 266-269.

17. Fildes, N. and Reynolds, R. 1995. Consistency and reproducibility of AmpliType+PM results between seven laboratories: Field trial results. Journal of Forensic Sciences **40**: 279-286.

18. Perkin-Elmer. 1990. AmpliType+ Users Guide, Version 2.

19. Gyllensten, U.B. and Erlich, H.A. 1988. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA DQA1 locus. Proceedings of the National Academy of Sciences, USA **85**: 7652-7656.

20. Yamamoto, T., Davis, C.G., Brown, M.S., Schneider, W.J., Casey, M.L., Goldstein, J.L., and Russell, D.W. 1984. The Human LDL Receptor: A cysteine rich protein with multiple Alu sequences in its mRNA. Cell **39**: 27-38.

21. Kudo, Shinichi and Fukuda, Minoru. 1989. Structural organization of glycophorin A and B genes: Glycophorin B gene evolved homologous recombination at Alu repeat sequences. Proceedings of the National Academy of Sciences, USA **86**: 4619-4623.

Initials: RCJ Date: 2/6/98

22. Slightom, J.L., Blechi, A.E., and Smithies, O. 1980. Human fetal $^{G}\Upsilon$- and $^{A}\Upsilon$- globin genes: complete nucleotide sequences suggest that DNA can be exchanged between these duplicated genes. Cell **21**: 627-638.

23. Horn, G.T., Richards, B., Merrill, J.J., and Klinger, K.W. 1980. Characterization and rapid diagnostic analysis of DNA polymorphisms closely linked to the cystic fibrosis locus. Clinical Chemistry **36**: 1614-1619.

24. Yang, F., Brune, J.L., Naylor, S.L., Cupples, R.L., Naberhaus, K.H., and Bowman, B.H. 1985. Human group-specific component (Gc) is a member of the albumin family. Proceedings of the National Academy of Sciences, USA **82**: 7994-7998.

25. Reynolds, R.L. and Sensabaugh, G.F. 1990. Use of the Polymerase Chain Reaction for typing GC variants. In: Advances in Forensic Haemogenetics 3, ed. Polesky, H.F. Mayr, W.R., Springer-Verlag Berlin Heidelberg, p 158-161.

26. Erlich, H.A. and Bugawan, T.L. 1989. HLA Class II Gene Polymorphism: DNA typing, evolution, and relationship to disease susceptibility. In: PCR Technology, Principles and Applications for DNA Amplification, ed. Erlich, H.A., Stockton Press, Inc. New York, NY, p 193-208.

27. Fildes, N., Reynolds, R., and Erlich, H.A. In preparation, Roche Molecular Systems.

28. Higuchi, R. and Kwok, S. 1989. Avoiding false positives with PCR. Nature **339**; 237-238.

29. Watson, R. 1989. The formation of primer artifacts in polymerase chain reactions. Amplifications - A Forum for PCR Users (Perkin-Elmer Newsletter) **2**: 5-6.

30. Crouse, C.A., Vincek, V., and Caraballo, B.K. 1994. Analysis and interpretation of the HLA DQA1 "1.1 weak-signal" observed during the PCR-based typing method. Journal of Forensic Sciences **39**: 41-51.

31. Comey, C.T., Koons, B.W., Presley, K.W., Smerick, J.B., Sobieralski, C.A., Stanley, D.M., and Baechtel, F.S. 1994. DNA Extraction Strategies for Amplified Fragment Length Polymorphism Analysis. Journal of Forensic Sciences **39**: 1254-1269.

32. Helmuth, R., Fildes, N., Blake, E., Luce, M.C., Chimera, J., Madej, R., Gorodezky, C., Stoneking, M., Schmill, N., Klitz, W., Higuchi, R., and Erlich, H.A. 1990. HLA-DQA1 allele and genotype frequencies in various human populations, determined by using enzymatic amplification and oligonucleotide probes. Am J. Hum Genet. **47**: 515-523.

Initials: ACJ Date: 2/6/98

STR Analysis

Amplification of the Quad Multiplex System (VWA, THO1, F13A, FES/FPS)

A positive control-external, an amplification negative control, an extraction reagent control and substrate controls, if applicable, should be included with each batch of samples being amplified to demonstrate procedural integrity. The positive control-external is a control whose alleles are known to the analyst. The STR PCR reaction mix already contains BSA. **Do not add additional BSA**

1. For each sample to be amplified, label a new tube. Do not amplify samples in which no DNA was detected by QuantiBlot.

2. To each labeled tube, add TE^{-4} and DNA as specified in Table VIII.

   NOTE: Steps 1 and 2 may be done in the DNA extraction or the DNA set-up areas of the laboratory. The following steps should be done in the DNA set-up area.

3. Add the following amounts of TE^{-4} and DNA to new labeled tubes:

Sample	TE^{-4}	DNA
amplification positive control-external	7 μL	20 µL
extraction reagent control	7 μL	20 µL
amplification negative control	27 μL	---

Initials: RCJ Date: 2/6/98

Table VIII- Amount of DNA to be amplified for the Quad multiplex

QuantiBlot DNA Concentration* (ng/20μL)	Target Volume (μL) to be amplified	TE^{-4} (μL) for Target Volume	Range of Volumes (μL) which can be amplified (corresponds to approx. 0.15 - 5 ng of DNA)
≥25	Dilute 1:10 and use the dilution for amplification		
12.5	1.6	25.4	0.3 - 8**
6.2	3.3	23.7	0.5 - 16**
5	4.0	23.0	0.6 - 20**
2.5	8.0	19.0	1.2 - 27**
1.25	16.0	11.0	2.4 - 27**
0.62	27.0	0	4.8 - 27**
0.31	27.0	0	9.6 - 27**
0.15	27.0	0	19.2 - 27**
< 0.15	Do not amplify	-	-

* If the QuantiBlot result is ≥ 10 ng/20 μL, dilute the sample 1:10 with TE^{-4} and re-quantitate. Multiply the diluted concentration by 10 to obtain the original sample concentration. Repeat the procedure if the diluted sample is still ≥ 10 ng/20 μL.

Note: When a dilution is made to determine DNA concentration, it is preferable to calculate the DNA concentration in the undiluted DNA extract. Then amplify the undiluted DNA extract, not the dilution.

** Add TE^{-4} to a final volume of 27 μL.

Initials: JCJ Date: 2 6 98

4. Turn on the Perkin Elmer Thermal Cycler. (See manufacturer's instructions).

   Step Cycle File #11 on all machines should include the following:

   Presoak at 80°C for 10 seconds; presoak at 80°C for 5 minutes

   Link to Thermo-Cycle File #15 on all machines:

   Denature at 94°C for 1 second; denature at 94°C for 45 seconds
   Anneal at 54°C for 2 minutes; anneal at 54°C for 1 minute
   Extend at 72°C for 1 second; extend at 72°C for 1 minute

   Repeat the above for 28 cycles

   Link to Time Delay File #16 on all machines for an additional 10 minutes incubation at 72°C.

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

   If Files #11, 12, 15, or 16 are not correct, bring this to the attention of the QC/QA coordinator and a supervisor.

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

6. Determine the number of samples to be amplified, including controls and label a PCR reaction mix tube for each sample.

7. 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.**

8. Pipet 3μL of 25mM $MgCl_2$ into each reaction mix tube with a sterile pipet tip. Pipet carefully in the solution at the bottom of the tube. Use a fresh pipette tip for each tube.

   Note: The samples should not sit for an extended period after adding the $MgCl_2$ solution to the PCR Reaction Mix. Proceed with the set up and begin the cycling processing as soon as possible.

9. Carefully add 1 drop of sterile Mineral Oil to all reaction mixture tubes including the controls. **Do not touch tube.**

Initials: RCJ Date: 2/6/98

10. Close all of the tubes.

11. **Note: Use a new sterile filter pipet tip for each sample addition. Open only one tube at a time for sample addition.** The final aqueous volume in the PCR reaction mix tubes will be 50 µL. Add the content (27 µL) of the sample DNAs and sterile H_2O tubes which were prepared above 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.**

12. 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.

13. Start the Thermal Cycler amplification program (File #11). 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.

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

15. After the amplification process, the samples are ready to be loaded on the Genescan gels. They may be stored in the appropriate refrigerator at 2-8°C for a period of up to 6 months, if they need to be stored further they should be transferred to a -20° freezer.

Initials: RC) Date: 2/6/98

Analysis of the Quad Multiplex System on the ABI 373 DNA Sequencer

Gel Casting for ABI 373 Sequencer

The STR alleles are separated on 6% denaturing polyacrylamide gels. Two different lengths of glass plates are being used on the two 373 sequencing machines: for the 373A 16.75 inch long glass plates have to be used, which from the bottom of the well to the laser read area result in a 24 cm separation distance (24 WTR). For the 373 Stretch 14.4 inch glass plates have to be used, which also result in a 24 cm separation distance from the bottom of the well to the laser read area (24 WTR). For the first time use of new plates, see the Quality Control manual for additional plate preparation. During set up, care has to be taken that the correct sides are facing the inside (see step 3.).

1. Pour 25 g of pre-weighed urea in 100 mL Erlenmeyer flask.
   Add 10 mL of deionized water
   5 mL of 10x TBE buffer
   7.5 mL of 40% polyacrylamide/bisacrylamide solution 19:1

   Add stir bar and place on stirrer with low heat.

2. Clean appropriate set (one notched, one plain) of glass plates (see above) as follows: Clean both sides with soap and paper towel, rinse with water, rinse with deionized water. Remove from sink and dry with low lint Kimwipes. Repeat the deionized water wash step until plates appear clear. Rinse with isopropanol or methanol and dry with low lint Kimwipes. Use a Kimwipe moistened with isopropanol or methanol to remove all remaining lint from plates.

   NOTE: Use only high quality isopropanol or methanol to wipe the plates. Do not use ethanol!

3. Prepare lab bench with bench paper and square box. Place plain plate on box such that the etched letters "L" and "R" for left and right are readable and are on the correct sides. Place white spacers on each side of plate. Put notched plate on top of plain plate such that the etched letters "L" and "R" for left and right are readable and are on the correct sides. Place three clamps on each side of the sandwiched plates.

4. The urea in the gel solution should be completely dissolved before proceeding. Pour the gel solution into a 50 mL sterile tube and adjust the volume to 50 mL with deionized water. Filter the solution using disposable 0.2 μm pore size filter units. Pour the filtered solution back into the 50 mL sterile tube. Let the solution cool down to room temperature.

Initials: RCJ Date: 1/6/98

5. Before proceeding, have a 20 mL glass pipette and 24 wells gel comb ready to use. Add 250μL of 10% ammonium persulfate (APS) and 25 μL of TEMED to the polyacrylamide -urea solution.

NOTE: The APS cannot be used if older than a week. Check date on tube. If necessary make up new APS solution by adding 5 mL of deionized water to pre-weighed 0.5g of APS, and note date on tube.

Mix gently and immediately pour the gel using a 20 mL glass pipette to apply solution to the notched area while continuously tapping the plates to prevent air bubble formation. Make sure that there is always enough gel solution in the notched area, so that the gel does not run dry. After the gel solution reaches the bottom of the sandwiched plates, insert comb slowly and carefully to prevent splatter and formation of air bubbles

Allow to polymerize for 1.5-2.0 hours.

Gels can be stored over night at room temperature. They do not have to be wrapped in clear plastic wrap. However if they are wrapped, wrap only the very ends.

Troubleshooting

The occurrence of air bubbles doesn't mean the whole gel has to be discarded. Just avoid this area of the gel while loading. Sometimes air bubbles in the comb area can be removed by pulling the comb out again, adding more gel solution, and inserting the comb again. Depending on the room temperature in the laboratory sometimes the urea in the gel crystallizes out. This process will be reversed if the gel is allowed to warm up.

Initials: RCJ Date: 2/6/98

Gel Electrophoresis on ABI 373 Sequencer

Setting the Run Parameters

1. Switch on the sequencer.

2. While waiting for the Sequencer to warm up, check the analysis parameters on the computer.

   On the computer screen double click on the **GS ANALYSIS** icon. Under heading **ANALYSIS** select **Preprocess parameters.**

   The settings should be:
   Multi component matrix should be checked
   Matrix File: Matrix-6% D.PAGE
   Gel Scaling
   Estimated peak heights: 3500
   Process Amount
   Start at Scan #: 1100
   Stop at Scan #: 2600
   Process Functions:
   Auto Lane Track should be checked
   Baseline Data should be checked

   Change settings if necessary, click **OK**.
   Quit **GS ANALYSIS** program.

3. On the computer screen double click on **GS COLLECTION** icon. Under heading **Edit** select **Settings**.

   The time setting should be: Collection length (hours) 5 (minutes) 30
   Name the gel adhering to this format: 98-S001 up to 98-S999

   The old name will be from the most recent gel. The next sequential number should be used. If the preceding gel was a research gel, which are named differently, consult the gel logbook for the appropriate gel number. Click **OK**.

   Enter the gel number in log book.

Initials: RCJ Date: 2/6/98

4. Check the electrophoresis settings on the Sequencer. Under Main Menu press **Set up run.** It should read: scan delay 0:00 timer 5:30, Volt 2500, mAmp 40, Watt 30.

   The only parameter that might have to be changed is the run time. To change place cursor by using the arrow buttons, type in the new time. Press **Main Menu**.

   Place the gel in the Sequencer.

Preparing the Plates

5. Open the Sequencer door. Place lower buffer chamber in the bottom of the electrophoresis unit with the electrode to the front. Pull the beam-stop bar towards you until it stops.

6. Remove clamps from gel. Use a razor blade to remove gel crusts on the outside of the plate. Clean gel plates with low lint Kimwipes and deionized water, followed by isopropanol or methanol. **The laser read area must be lint free.** Use Kimwipe moistened with isopropanol or methanol for last wipe, and let dry.

7. Place gel in the electrophoresis unit with the notched plate facing **away** from you. The bottom edge of the plates should rest on the notched supports in the bottom of the lower buffer chamber. Press gel against the back of the chamber and push the beam-stop bar against the gel. Center the plates with relation to the beam-stop bar and then latch the bar in place. Also close clamps connecting gel with metal back plate.

   Close door

8. Press the **Main Menu** button of the sequencer. Select **Start/Prerun** and then **Plate Check**. Select **Full Scan**.

9. On the computer screen, click on **Scan** button on the **GS Collection Controller Panel**. The scan window should show a relatively flat line across the screen in each of the four colors. Peaks appearing here indicate dirt on the glass or in the gel. If that is the case, reclean the plates as described below (step10), and then scan the plates again.

10. On the Sequencer press **Main Menu** and **Abort Run**. Open the instrument door, unlatch the beam-stop bar, and slowly pull the bar towards you. Don't remove plate, first try to clean the laser read region with a Kimwipe moistened with isopropanol. Then perform another plate check. If the plate check still shows peaks, the gel must be removed and both sides of the read area cleaned. If peaks persist, it must be a gel problem. The lanes in this region of the gel cannot be used.

Initials: ACS Date: 2/6/98

11. When the plates are clean according to the Scan, check the Y-axis values of the scan lines. The lowest line, usually the blue line, should be between 600 and 900. The highest line, usually the red line, should be between 1100 and 1400. If the values differ from this, the PMT voltage has to be adjusted. If the values are o.k. proceed with step 12.

12. To change the PMT setting, press **Main Menu** - press **Calibration** - press **Configure** - press **More** until " PMT voltage is --- volts." appears on the screen. Using the number keyboard to the right, raise or lower the value by steps of 10 and press **Change**. Observe the effect of these changes on the scan window. If desired height is reached press **Main Menu**.

Pre-Running the Gel

13. Open door and place upper buffer chamber in electrophoresis unit at the top of the gel. Place the clear plastic bar in front of the plate, and tighten the screws equally to maintain even pressure on the plates. Turn the screw just until the rubber gasket seal starts to flatten - **do not over tighten**.

14. Fill the upper buffer chamber with approximately 800 mL 1xTBE buffer. If the buffer chamber is leaking, tighten the screws some more. Gently remove the comb from the gel by pulling it straight up. If sample wells appear to be distorted, they can be put back into shape with a flat gel loading pipette tip. To remove excess polyacrylamide in the loading area clean the rim of the notched plate with gloved finger. Flush sample wells with the running buffer using a syringe or a pipette.

15. Fill the lower buffer chamber with approximately 600 mL 1xTBE buffer. Avoid splashing the buffer against the back of the chamber.

16. Attach the top and bottom electrode leads located on the left side of the electrophoresis chamber and gently close the door.

17. Press the **Main Menu** button of the Sequencer. Select **Start/Prerun** and then **Pre-Run Gel.**

The Pre-Run is programmed for one hour, but 15 minutes is sufficient to preheat the gel. Generally the samples should be prepared for loading during the Pre-Run, and electrophoresis can be started immediately after the samples are ready.

Initials: RCJ Date: 2/6/98

Sample Preparation

Determine which samples are to be run and fill out the gel sheet. Adhere to the following rules: **exemplars and controls can be loaded in adjacent lanes, evidence samples have to have an empty lane on both sides. After a 15 minute run (see below), the empty lanes can then be loaded with additional evidence samples. One positive control has to be run on each gel!**

18. After filling out the gel sheet, label the appropriate number of GeneAmp Thin-walled Reaction Tubes with the numbers of the lanes that will be loaded.

19. For N+2 samples mix 3 μL of deionized formamide with 2 μL of GS500 standard in loading buffer. Consult the following table:

sample no.+ 2	Formamide	GS 500
12	36 μL	24 μL
16	48 μL	32 μL
20	60 μL	40 μL
24	72 μL	48 μL

20. Aliquot 5 μL of the formamide/standard mix into each labeled tube. Add 2 μL of PCR product per tube according to the gel loading sheet.

21. Heat denature samples in the 95°C heat block for 2-3 minutes. Immediately afterwards place samples on ice, wait for 1 minute, then briefly spin down the samples using the microcentrifuge in the hybridization room.

    Put samples back on ice.

22. Stop Pre-Run by pressing **Abort Run** on **Main Menu**. Open door and repeat flushing the sample wells.

23. Carefully load 7 μL of each sample per well.

24. On **Main Menu** select **Choose Run**. Then select **Genescan Run.**

Initials: RCJ Date: 2/6/98

25. If there are additional evidence samples to be loaded, allow the gel to run for 15 minutes and then click **Pause** to stop the run. Add 7 µL of each sample to the wells that were originally skipped. Select **Resume** to continue the run.

26. On the screen click on the **Collect** button on the **GS Collection Controller Panel**. Data collection will begin.

    Close the scan window. The data collection can be viewed at any time during the run by selecting either the **Scan**, **Map** or **Gel** buttons. However the windows should not be left open during the run.

27. After the run is completed switch the Sequencer off. To clean the gel plates, open the door, unlatch the beam-stop bar and remove the gel plates together with the upper buffer chamber from the electrophoresis unit. Empty the upper buffer chamber, separate it from the gel and rinse it with water. Pry the gel plates apart, roll down and discard the polyacrylamide gel. Clean the plates and spacers with soap and water. Empty the lower buffer chamber and rinse with water. Remove most of the buffer from lower buffer chamber before taking it out.

    Clean TBE buffer crusts from the sequencer electrophoresis unit.

Initials: RCJ Date: 2/6/98

STR Gel Analysis Of Gels on Macintosh Terminals Connected to the ABI 373 Sequencer

The gel may either be analyzed on the Apple Macintosh terminals connected to the ABI 373 Sequencer or analyzed on the separate Apple Macintosh terminals number **1** and **2**. The Genescan analysis software running on the computer connected to the 373 is version 1.2, while the version on the Apple terminals is the newer 2.1 software. The difference between both versions consists mainly in the structure of the results file. Version 2.1 creates an independent results file for each sample, and combines all samples in a "project". When reanalysing a sample the previous sample file with the previous analysis result gets overwritten. Working with version 1.2 each gel processing creates a dated and consecutively numbered results file containing all samples that were analyzed. When using version 2.1 samples and their controls cannot be recombined in separate projects; they have to be analyzed together. The Apple/Macintosh terminals number **1** and **2** contain copies of all relevant matrix files for the different sequencing instruments. It is very important to choose the correct matrix for each gel run.

The procedures for analysis on the ABI 373 Sequencer are below. See the next section for the procedures for analysis on the separate terminals.

After the gel run is complete, the **GS Collection** software automatically launches the **GS Analysis** program so that the preprocessed gel should be displayed on the computer screen and the top of the screen menu bar should display the **GS Analysis** commands. If the latter is not the case, the menu bar can be activated by clicking on the **GS Analysis** icon under **Finder.** A gel can also be opened and analyzed at a later time (see step10).

The **Gel Window** shows the DNA fragments in all lanes as colored bands. The lane number and a square **lane indicator box** appear at the top of each lane. By placing the cursor on the **lane indicator box** and clicking on the mouse a lane can be selected. A white line will be seen tracing this lane from the top to the bottom of the gel. To the left of the DNA fragment bands, the same information is shown as peaks for the selected lane, where each peak corresponds to the fragment bands.

Initials: RCJ Date: 2/6/98

Also displayed are the scan numbers for the analyzed segment. According to the preset **Pre-process Parameters** the scan numbers have to range from 1100 to 2600. Each lane has to contain 6 red standard fragments from 100 - 250 bp, which are distributed as follows:

top of gel —

—

—

—

—

bottom of gel —

If the preprocessed gel contains less than these 6 fragments; it has to be preprocessed again (see step 1 and 2). If all fragments are present proceed with step 3.

1. View the gel in order to decide if the shorter or longer size standard fragments are missing, in what direction, and how much to extend the processed gel region. Click on left hand corner of the gel window to close the gel. Under **Analysis** select **Preprocess Parameters**. Under **Process Amount** change the Start at Scan, End at Scan parameters according to the above assessment. Click **OK**.

2. Under **Analysis** select **Preprocess Collection File**. Select the folder which contains the gel file (can be desktop, hard drive, or 672 software folder) by moving the cursor on the folder name and clicking open until correct gel file is highlighted. The gel files can be distinguished from the results file by two things: the results files have a date assigned to them and a horizontal page as an icon, while the gel files have no date and a vertical page as icon. Click **open**. The collected raw data will be preprocessed again; this may take a couple of minutes. The preprocessing is finished when the gel picture appears on the screen. Continue with step 3.

Initials: RCS Date: 2/6/98

3. The gel picture displayed on the screen should have a black background and distinct blue, green, and red signals. If the background is blue or green, the colors have to be baselined. The electropherogram displayed on the left side of the gel also shows if all four colors share the same baseline.

   To baseline, under **Gel** choose **Base Line Gel**. Select the colors where you see the background problem and click **OK**.

   If all signals or only certain colors appear to be very weak, the screen contrast can be adjusted. This does not change the measured peak height for the PCR products, but creates a more intensive image on the screen, which improves the lane tracking. Select a lane which contains the color that has to be adjusted. Under **Gel** choose **Adjust Gel Contrast**. The **Adjust Gel Contrast** window shows the electropherogram peaks of the selected lane. Click the appropriate boxes to deselect the colors you do not want to enhance. (An X indicates that the color is selected.) With the mouse cursor, drag the upper triangle down if you want to raise the peak, or pull the lower triangle up, if you want to lower the peak. Click **Preview** to examine the effect of the changes. If you don't like then click **Restore** to undo the changes. After you found the contrast you like, click **OK**.

4. The lane tracking for the gel is done automatically by the **GS Analysis** software. The result has to be checked manually for each gel. In order to do that click on the **lane indicator box** for each lane that contains samples. The white line has to be drawn through the middle of each DNA fragment. All 6 red standard fragments have to be visible in the left peak display. Make sure that the lane assignments correspond to the gel sheet.

   To correct the lane tracking, move the cursor on the image of a **lock** at the bottom of the tracking line. Move the line to the left or to the right in order to meet the middle of the fragment or achieve optimal peak height on the peak display. After the tracking is satisfactory, click the image of a **lock** on the **Controller Panel** to save the lane adjustment.

Initials: RCJ Date: 2/6/98

5. After the tracking for all lanes is satisfactory, the analysis can proceed. Under **Analysis** select **Analyze Lanes**. The **Analysis Control** window will be displayed, which shows a diagram of the lane numbers versus the dye colors. Each color for each lane is represented by a square panel. On the left side of the dye colors the **STD** arrow allows the selection of different dyes for the size standard. This arrow has to be placed next to **R** for red.

   Before the analysis the size standard has to be defined. In the **Analysis Control** dialog box click on **Define New.** Enter a lane that contains the standard in the box asking for **Lane Number**. The **Dye Color** setting should be "red" and doesn't have to be changed. After entering the lane number click **OK**. The system displays an electropherogram of the designated sample. Each peak included in the electropherogram has to have a size assigned to it. Either click on the peak or in the table to highlight a number.

Required Sizes for the Peaks	
peak 1	100
peak 2	139
peak 3	150
peak 4	160
peak 5	200
peak 6	250

   After entering the number 250, click **return**. The high light should be back on peak 1. Click **Done**, click **o.k.** to "save user defined standard as untitled standard", click **replace**.

6. Analyze all lanes that contain samples by clicking on the white number of these lanes and afterwards deselecting the color yellow by clicking on the yellow fields. This way the blue, green and red labels get analyzed. Don't analyze empty lanes. Click on **Analyze**.

7. Close the **Analysis Control** window. In order to look at the results after the analysis is complete, the **Results Control** window has to be opened. Under **Results** go to **Electropherograms Results**. The **Results Control** window shows the title of the gel run and the same lane number versus color display as the **Analysis Control** window. The analyzed colors per lane are shown in dark grey. The white squares mean that this color has not been analyzed.

   Three icons represent the different possibilities to show the electropherogram results: each color per lane separately or "tiled"; as many as four colors per lane together or "stacked"; and a table with the sizing results. Maximum selection of colors per display is four. The table of sizing results can be combined with either tiled or stacked data.

Initials: RCJ Date: 2/6/98

8. To ensure that all the sizing results are correct, the labeling of the size standard peaks has to be checked for each lane. Click on tiled display, select four red lanes to look at, and click on **Show**. Check by clicking on each peak or by scanning the size table for intermediate sizes. Continue until every lane with samples has been checked.

   If an assignment is wrong the size standard has to be redefined for this lane only and the lane has to be reanalyzed with the newly defined standard (see step 5).

9. After the size standards have been checked it is optional to look at the allele peaks for each lane.

   Before proceeding with the **Genotyper** analysis, close the **Results Control** window, close the **Gel Window**, and click on **Save** for "save changes before closing". Quit **GS Analysis** under **File**.

10. In order to reopen a gel at a later date, under **File** select **open gel file** for gels that have not been analyzed yet or **open results file** for already analyzed gels. To analyze the gel start with step 1. To look at the **Genescan** electropherogram of a particular sample under **Results** select **Electropherogram results**. Then click on the colors and lanes you want to look at in either the stacked or overlapping format.

Initials: QC Date: 2/6 98

STR Gel Analysis and Genotyper for 373 Gels on Separate Apple Macintosh Terminals

If gels are analyzed on the separate terminals, they MUST use the Genotyper software on the separate terminals. They can not use the Genotyper on the terminals attached to the 373 Sequencer.

1. Copy the gel from the 373 to a JAZ transport disk. Then copy the gel from the transport disk to the Apple terminal hard disk, and delete the gel from the transport disk.

2. Open the **GS Analysis** software by clicking on the **373 / 377 analysis** icon.

3. Under **File** go to **Open.** From the listed possibilities click on existing **Collection gel**. Open the hard disk, go to the appropriate folders, and highlight the desired gel file. Gels are represented by vertical icons as opposed to folders.
Click **open.**

4. To the computer message "This is an old gel format. Please verify that the number of lanes is 24" click **OK**

   The next message will be "----- does not contain a gel image matrix. Do you want to cancel, continue without multi-componenting, or attach a gel image matrix?" Click **Attach a matrix**. The software automatically opens the GS Matrix folder for possible matrices. Open the correct instrument folder, and chose the correct Quad matrix.

   The computer will generate a gel image. For a description of this gel window see the **377 STR Gel Analysis and Allele Identification** section B "Collection Gel Processing", step 2.

5. Continue with step 3 of **377 STR Gel Analysis and Allele Identification** section B "Collection Gel Processing". Follow all of the steps until the end of the **Quad Genotyper Ver 2.0 Analysis on Separate Macintosh Terminal** section.

6. **Since a 373 gel instead of a 377 Gel is being processed, note the following deviations from the listed procedures:**

   A. Since the gel has been processed with a matrix, it is not necessary to install the matrix again for the sample files - skip **step 1** under **D "Project file analysis"**.

   B. Due to sizing differences between both platforms, the Genotyper categories differ slightly between the 373 and the 377 runs. Use the **Quad 373 Genotyper** icon when performing **G "QUAD Genotyper" step 1.**

Initials: RCS Date: 2/6/98

7. After analysis, the gel, the results, and the Genotyper file have to be archived in duplicate and removed from the hard disk. To do this follow the steps outlined in the **377 STR Gel Analysis and Allele Identification** section A "Gel file copying and archiving procedure".

Initials: QCJ Date: 2/6/98

Quad Genotyper Analysis of 373 Gels on Macintosh Terminals Connected to the ABI 373 Sequencer

If the gel was analyzed on the Macintosh terminal connected to the ABI 373 Sequencer, it may be Genotyped on either the Apple Macintosh terminals connected to the ABI 373 Sequencer or Genotyped on the separate Apple Macintosh terminals number **1** and **2**. The Genotyper software running on the computer connected to the 373 is version 1.1, while the version on the separate Apple terminals is the newer 2.0 software.

The procedures for Genotyping on the ABI 373 Sequencer are below. See the ABI 377 section for the procedures for Genotyping on the separate terminals.

After the gel analysis is completed the file has to be further processed using the Genotyper software. This program will assign allele names to the detected peaks and create the electropherogram output that has to go in the case file. The following steps have to be performed exactly as listed. **Do not change the order!** If the order has been changed, or unexpected events occur, see section 12 for trouble shooting.

1. Start **Genotyper** by double clicking on the **Quad Typer** icon.

   If there are data visible in the two upper windows, go to **Analysis** and select **Clear Dye Lane List** and **Clear Table**. Otherwise proceed with step 2.

2. Under **File** select **Import Results**. The **GS Analysis** results files are on the **Hard drive** in Folder **672Software**. Choose correct results file by double clicking on the appropriate folders until the results file name and the correct version is highlighted. Then click **open** and then click **import**.

3. In order to preserve the template file, the current file has to be saved under a new name!!! Therefore, immediately after importing the results, change the name of the Genotyper template to your initials and the gel file. Under **File** select **Save As**. Enter your initials and the name of the gel you are processing. Click **Save**.

Initials: QCI Date: 2/6/98

4. The next step is to type in the sample information for each analyzed lane. Under **View** select **Show Dye Lane Window**. Highlight the first lane, place the cursor in the field **Sample Information** either by hitting **Tab** or by using the mouse, and put the sample information in.

Note: It is important that the sample information for the blue and green labels in the same lane are completely identical; this can easily be achieved by using the **Copy** and **Paste** functions. If different lanes of the same gel have been loaded with duplicate samples, the sample names have to be different in order to be listed separately in the table (e.g. extr. neg. A, extr. neg. B). After all lanes have been filled in, close the **Dye Lane Window** by clicking on the left upper corner.

5. Run the **Quad Macro 1** by pressing the Apple key and the number 1. **Quad Macro 1** selects peaks that meet allele calling criteria (see results interpretation) by automatically performing the following steps: select blue lanes + select green lanes, clear labels, label category peaks with the size in bp, remove labels from peaks whose height is less than 10% of the highest peak in the category's range; then remove labels from peaks less than approximately 20% of a following higher, labeled peak within 0.00 to 5.00bp.

Under **View** select **Show Plot Window.** Check every lane and manually delete labels for extra peaks by placing the cursor on the peak above the baseline and clicking.

Extra peaks are defined as peaks that meet one of the following criteria:

1 small constant bands of the following size and color
Blue 180-181 bp, 230-232 bp, and 246-248 bp
Green 128-131 bp and 211-220 bp

2 pull-ups of green peaks caused by very high blue peaks in the same lane

3 shoulder peaks approx. 1-3 bp bigger than main allele

4 peaks caused by overflow of sample in the adjacent lane

5 -4 and +4 bp stutter if there is no indication for the presence of a mixture. (See interpretation section).

Fill out the Genotyper Editing sheet for each lane and note the reason for removal of a peak using the number code above.

Initials: RCJ Date: 2/6/98

If a locus displays only one peak and a distinct same color peak is visible but not labeled because its size is outside of the defined allele range, this peak might be a new, previously unreported allele. This possibility has to be considered especially, if the amplification for the other loci shows a clean profile. The presence of a possible new rare allele has to be pointed out to a DNA supervisor to start the steps necessary for confirmation (see Interpretation of complex Quad STR results). The "new" allele will not be automatically reported in the Genotyper table, but will be visible on the electropherograms.

Close **Plot Window**.

6. Run the **Allele Designation Macro** by pressing the Apple key and the number 2. This macro automatically assigns allele names to the labeled peaks based on the size categories for the different fragments and writes a results table. The following steps are carried out: clear table, change labels to the categories name, add rows with one sample per row, containing sample info in column1, up to 4 labels in columns 3-6, the text "check" in column 2 if number of labels >2 (titled "mixture."); put column titles in first row.

   Under **View** select **Show Table**. Under **File** select **Print**. Print Table. Close **Table Window**.

7. Under **View** select **Show Plot Window**. Under **Analysis** select **Change Labels**. In the Change Labels Dialog box check both "with size in bp" and " the categories name". Click **OK**.

8. The next step is to print the plot window. To achieve a more uniform format, the base pair range which should be printed has to be selected. Under **View** select **Zoom to**, type in 110 to 260. Click **OK**. The display on the screen will focus in on that range.

   Under **File** select **Print**. Print plot window or as it is called here "Graphical area". Close **Plot Window**.

9. Close template by clicking on upper left corner. Click on **Save**. The edited results are saved as a **Genotyper** file, placed in the **GS Analysis** results file for this gel and can be opened and re-edited.

10. Under **File** quit **Genotyper**.

Initials: RCJ Date: 2/6/98

11. All analyzed and raw data for each gel are being stored on optical disks. Casework gels have to be stored in duplicate on two different optical disks. After a gel has been copied to an optical disk it has to be deleted from the hard drive in order to keep the hard drive memory available.

    Switch optical drive on and insert appropriate disk. Open disk by clicking on it. Under **File** select **New Folder**. Name folder with gel name. Open **672 Software Folder** on Hard Drive. Drag gel file and the relevant (most recent results file which also should contain Genotyper analysis results) in the new folder on the optical disk . After copying process is complete, repeat above steps for case work gels.

    Drag gel file and all results files in the trash can. Under **Special** select **empty trash**. Click **Yes**.

Troubleshooting

If the wrong peak has been removed accidentally, it can be re-labeled by clicking on it again.

If the Apple Two Macro has been run before all extra peaks had been unlabeled, this extra allele will be listed in the table. To redo the table go back to the plot window, remove the label, and run the **Apple Two Macro** again. This can be repeated for all instances where the table needs to be rewritten (e.g. changing the lane information).

In order to place lanes next to each other for comparison purposes, lanes can be marked by double clicking on them. A black bullet appears in front of the lane number. If this happens accidentally a lane can be unmarked by either double clicking on it again or under **Edit** selecting **unmark**.

The Apple One Macro selects all blue and green results for further analysis. If these lanes have accidentally been deselected rerunning the Apple One Macro would re-label all extra peaks. To get back to a display of all green and blue lanes do the following: under **Edit** choose **select blue** and while pressing **shift** open **Edit** again and select + **select green**.

Initials: RCJ Date: 1/6/98

Analysis of the Quad Multiplex System on the ABI 377 DNA Sequencer

Gel Casting for the ABI 377 Sequencer

The STR alleles are separated on 5% denaturing Long Ranger gels. The plates that are being used result in a 36cm separation distance from the wells to the laser read area. The bottom plates have two side indentations that fit against a metal notch in the gel mounting cassette. The top plate has a cut out area at the top to allow buffer-gel contact. For the first time use of new plates, see the Quality Control manual (QC033) for additional plate preparation. During set up, insure that the correct sides are facing the inside (see step 3.).

1. When making **one** plate:
   Pour 10.8 g of pre-weighed urea in 100 mL Erlenmeyer flask.
   Add 10 mL of deionized water
   3 mL of 10x TBE buffer
   3 mL of 50% Long Ranger

   For **two** plates:
   Pour 18 g of pre-weighed urea in 100 mL Erlenmeyer flask.
   Add 20 mL of deionized water
   5 mL of 10x TBE buffer
   5 mL of 50% Long Ranger

   Add stir bar and place on stirrer with low heat. Do not boil!!!

2. Clean appropriate set of bottom and top glass plates as follows: Clean both sides with liquid detergent and paper towels, rinse with water, rinse with deionized water. Remove from sink and let dry or dry with low lint Kimwipes.

   NOTE: Do use solvents such as isopropanol sparingly on these plates. Do not use ethanol.

3. Prepare lab bench with bench paper and square box. Place bottom plate on box such that the etched letters "L" and "R" for left and right are readable and are on the correct sides. Place 0.2mm spacers on each side of plate with the jagged side facing the top of the plate. Put notched top plate on top of bottom plate such that the etched letters "L" and "R" for left and right are readable and are on the correct sides. Place three clamps on each side of the sandwiched plates.

Initials: RCJ Date: 2/6/98

4. The urea in the gel solution must be completely dissolved before proceeding. Pour the gel solution into a 50 mL tube:
   - for **one** plate adjust the volume to **30mL,**
   - for **two** plates adjust the volume to **50 mL** with deionized water.

   Filter the solution using disposable 100mL 0.2 μm pore size filter units and a vacuum pump. Degas the solution by leaving the filter attached to the running vacuum pump for **5 minutes**. Pour the filtered solution back into the 50 mL tube. Let the solution cool to room temperature.

5. Before proceeding, have a 20 mL glass pipette and a 0.2mm 24 wells gel comb ready to use.

   - For **one** plate (**30mL** volume) add **150μL** of 10% ammonium persulfate (APS) and **21μL** of TEMED to the Long Ranger/Urea solution.
   - For **two** plates (**50 mL** volume) add **250μL** of 10% ammonium persulfate (APS) and **35 μL** of TEMED to the Long Ranger/Urea solution.

   NOTE: APS older than FIVE days cannot be used. Check date on tube. If necessary make a new APS solution by adding 5 mL of deionized water to pre-weighed 0.5g of APS. Do not forget to date the tube.

   Mix gently and immediately pour the gels using a 20 mL glass pipette to apply solution to the notched area while continuously tapping the plates to prevent air bubble formation. Insure that there is always enough gel solution in the notched area, so that the gel does not run dry. After the gel solution reaches the bottom of the sandwiched plates, insert comb slowly and carefully to prevent splatter and formation of air bubbles. Place a clamp on the comb.

   Allow to polymerize for 1.0-1.5 hours.

   NOTE: since the gels are very thin, they dry out rapidly. A gel should be used immediately after the 1.5h polymerization.

   If it is not used immediately, wrap the gel after 1.5h. Remove the gel clips, **do not pull the comb!!** Place paper towels moistened with1xTBE or deionized water on the bottom and the top ends of the gel, cover the moist paper towels with clear plastic wrap, fold the plastic wrap over and hold in place using gel clips.

   Wrapped this way the gels can be stored over night at room temperature.

Initials: RCJ Date: 2/6/98

Troubleshooting

The occurrence of air bubbles does not mean the whole gel must be discarded. Just avoid this area of the gel while loading. Sometimes air bubbles in the comb area can be removed by pulling the comb out again, adding more gel solution, and inserting the comb again. Depending on the room temperature in the laboratory sometimes the urea in the gel crystallizes out. This process will be reversed if the gel is allowed to warm up.

Initials: RO Date: 2/6/98

Gel Electrophoresis on ABI 377 Sequencer

In order to run a gel on the 377, the run has to be set up both on the software and the instrument. Additionally, proper documentation of the gel run has to be prepared. The documentation consists of filling out a gel sheet that lists the samples to be loaded, the lot numbers of the reagents that were used, and the analyst's and the witnesses' initials. The run must be logged in the 377 instrument log book.

The program used to operate the run and collect the data is the **ABI Prism 377 Collection Software**. This program has to be started. Then the software preparations consist of creating a run folder (section B), and naming the gel file.

On the instrument, the gel has to be mounted into the electrophoresis unit (section A), checked for artefactual fluorescence (section C), and preheated before the run can be started. The instrument does not have manual control switches, and is operated from the computer terminal, utilizing the 377 collection software.

Before starting a run, the computer should be restarted. This removes hard drive memory fragmentation and prevents the software from crashing. Also to monitor instrument usage and help trouble shooting, a QC log sheet must be filled out for each electrophoresis run.

Initial Preparation

- RESTART THE COMPUTER BY UNDER **SPECIAL** SELECTING **RESTART.**
- OPEN THE 377 COLLECTION SOFTWARE BY DOUBLE CLICKING ON THE **ABI PRISM 377 COLLECTION** ICON.
- SWITCH THE INSTRUMENT ON.

A Mounting the Gel Cassette in the Electrophoresis Chamber

1. Remove the gel clips, and pull the well forming comb.
2. Clean the outside of the plates. Buffer crusts and gel spill-over must be removed. It is possible to rinse the plates under running tap water. Afterwards rinse with plenty of dH_2O. Also rinse the well area with dH_2O. Dry the plates with Kimwipes.

Initials: JCJ Date: 2/6/98

3. Place the plates back in the gel cassette with the bottom plates down, push the plates towards the bottom of the cassette until the rear plate indentations rest firmly against the metal stops.

   Turn cassette clamps #2, #3, #4 and #5 on both sides to lock the plates in position.

4. Check the laser read region for lint or other residue. Clean the laser read region with a moist Kimwipe and deionized water, then move the beam stopper into the down position, and turn both clamps #6 to hold it.

   * You are ready to place the cassette gel assembly into the instrument.

5. Open the front door of the ABI Prism 377 and place the lower buffer chamber on the bottom shelf of the electrophoresis unit.

6. Check the amber colored positioning pins, and the back plate for buffer crusts and dirt. Remove any residue with deionized water.

   ATTENTION: It is extremely important that the positioning pins are clean. They align the gel plate parallel to the laser scan plane.

7. With the beam stop bar and the upper notch facing towards you, place the gel cassette against the rear heat-transfer plate and turn the outer top and bottom clamps to hold the cassette. Close the door and proceed to section "B".

Initials: Date:

B Create a Run File

1. Choose **New** from the **File** menu and click the **GeneScan™ Run** icon. This automatically creates a new Run file, and opens the Run window.
   The default setting for the run parameters displayed in the run window are:
   Plate check module - **Plate check A**
   Prerun module - **GSPR36A-2400**
   Run module - **GS Run36A-2400**
   Collect time - **2.5h**
   Sample sheet - <none>
   Well to read distance - **36**
   Gel's Matrix file - **appropriate Genescan matrix**
   Lanes - **24**

   These settings should not be changed.

   If the settings appear to have been changed, click on the black arrow in the white field next to the parameter, and select the correct setting from the appearing list of options.

2. Import the sample sheet named "empty" by clicking on the black arrow in the white field next to sample sheet, and selecting the file name "empty".

3. The **run folder** is automatically named after the instrument, and with the date and time of the creation of the run file. This name appears on top of the GS Run window.

C Checking the Plates

1. Click on **Plate Check.**

2. Observe the Scan window that appears.
   The scan window should show a relatively flat line across the screen in each of the four colors. If the scan lines are flat, the plates are clean.

   Proceed to Pre-running the Gel.

Initials: RCJ Date: 2/6/98

3. Otherwise clean the plates in the laser read region by following the steps below:
A. Click **Pause** to pause the plate check.
B. Open the door, un-clamp clamps #6, and wipe the laser read region with a Kimwipe moistened with dH2O. Close clamps #6.
C. Close the door and click **Resume**.
D. Repeat until scan lines are flat. If it is necessary to clean the backside of the plates the gel/cassette assembly can be removed for that purpose.

If it is not possible to remove all spikes in the scan lines, a part of the gel still can be used. The horizontal position of the spikes corresponds to the horizontal position of the wells that have to be avoided. Load samples only in the areas with clean scan lines.

D Pre-Running the Gel

1. Click **Cancel** and then **Terminate** to end the plate check, then open the instrument door.

2. Un-clamp clamps #3,#4, and #5 on both sides. Place the front heat-transfer plate on top of the plate, re-clamp the clamps, and connect the electrical wire on the left side and the two water tubes on the right side.

NOTE: Make sure that you don't un-clamp all clamps at the same time. That way the gel plates are always secured.

3. Un-clamp both clamps #2. Place the upper buffer chamber on the top rim of the gel, press it down and re-clamp the clamps in a swift motion.

4. Fill the upper buffer chamber with 600 mL of **1X TBE Buffer** and the lower buffer chamber with 700 mL of **1X TBE Buffer**. Connect the top and bottom electrodes.

5. Flush out the wells using a flat 0.2mm pipette tip connected to a syringe.

6. In order to enhance the visibility of the wells, blue loading buffer is added to the wells before pre-running the gel. 50μL of the loading buffer are applied by using a 100μL pipette tip and dragging the pipette across the gel while expelling the blue buffer.

7. Place the lid on the upper buffer chamber, and close the instrument door.

Initials: RCJ Date: 2 16 98

8. Choose **Status** from the **Window** menu. In order to do the prerun the status should be :
Instrument state : idle
Laser power : stand by
Electrophoresis : off
Door : closed
Time set : 1:00 remaining appr. 1:00

The electrophoresis indicators should all be near zero.
The gray gel temperature indicator shows the temperature goal, the green indicator the actual temperature.

9. Click **Pre-Run.** Observe the status window to see if the electrophoresis parameters start. The actual temperature should start rising: it must reach 48 °C before the samples can be loaded.

NOTE THE PRERUN PARAMETERS IN THE QC CHECK BOOK.

Proceed with naming the gel and sample preparation.

E. Gel Naming and Sample Preparation

Determine which samples are to be run and fill out the gel sheet. Adhere to the following rules: **exemplars and controls can be loaded in adjacent lanes, evidence samples have to have an empty lane on both sides. After a 15 minute run (see below), the empty lanes can then be loaded with additional evidence samples. One positive control has to be run on each gel!**

1. Fill out a 377 gel worksheet for the appropriate set of samples based on the amplification worksheet.

The name for casework gels has to be in the following format:
98-Men001, 98-Mul001 and 98-Jef001.

This reflects the consecutive order of gel runs for that year, and the instrument that was used. Always check the instrument logbook for the next consecutive casework gel number. Chose descriptive names for non casework gels, such as QC urea, or Asian DB 1-30. The gel name will automatically be used as a project name during gel analysis.

Do not forget to note the **Runfolder date and time** on the gel sheet (see step 3 under B create a run file). The runfolder date and time are found on top of the GS run window.

Initials: RCI Date: 2/6/98

2. Make a mastermix of 5μl blue formamide and 0.55μl of Genescan 500 standard per sample for n+2 samples referring to the following table:

sample no.+ 2	Blue Formamide	GS 500
12	60 μL	6.6 μL
16	80 μL	8.8 μL
20	100 μL	11 μL
26	130 μL	14.3 μL
36	180 μL	19.8 μL

3. Label a sufficient number of 0.5mL sample tubes. Have another analyst witness your tube set up. Add 5μl of the mastermix to each tube, then add 4μl of amplified product. The amplified product must be pipetted directly into the formamide, otherwise the sample will not be denatured correctly.

4. Heat the samples at 95°C for two minutes and put on ice immediately. The samples can stay chilled for a maximum of 30 minutes. If they have not been loaded in that time span, the samples must be reheated.

F. Sample Loading and Starting the Run

1. Remember: the run cannot be started before the gel temperature is at least 48°C. Check the status window for the temperature readings.

2. Interrupt the prerun by clicking **pause.**

   NOTE: Do not cancel and terminate the run because this will cause the temperature to drop!!!

3. Open the instrument door, remove the lid from the upper buffer chamber, and flush out the wells using a 10mL syringe equipped with a flat 0.2mm pipette tip.

4. Referring to the gel work sheet, load 4μl of the amplified product mix in the appropriate lanes.

5. Place the lid on the upper buffer chamber. Close the instrument door.

Initials: RC Date: 2/6/98

6. End the prerun mode by clicking **Cancel** and then **Terminate**. Now click on **Run**.

   When prompted **Save as** "gel file" enter the correct gel name as noted on the gel sheet (see section E Name gel and prepare samples). Click **Save**.

7. In order to see if everything is functioning properly and in order to monitor the run, open the status window.
   The settings should be:
   Instrument state : running
   Laser power : on
   Electrophoresis : on
   Door : closed
   Time set total: 2:30

	Grey indicator **goal** Or **upper limit**	green indicator **actual**
Electrophoresis voltage (kV):	3.00	around 3.00
Current (mA)	60.0	35 - 45
Power (W)	200	95 - 160
Gel Temperature (°C)	51	>48
Laser Power (mW)	40	40

   NOTE THE ELECTROPHORESIS PARAMETERS IN THE QC CHECK BOOK.

8. Compare the **run folder date and time** noted on the gel sheet with the final run folder date and time, as listed on top of the GS Run window. There will be a discrepancy if the run was aborted during set up and a new run folder was created.

   Correct the gel worksheet if necessary. This is important in order to be able to locate the gel file for analysis.

9. If there are additional evidence samples to be loaded, allow the gel to run for 15 minutes and then repeat steps 2-5 for the additional samples. In place of step 6, click on **RESUME** to continue the run. Verify that the machine is running properly as described in step 7.

During the run all windows can be left open. The progress of the samples can be observed by clicking on **Window** and from the scroll down menu selecting **Gel image.** This window displays the scans, and it is possible to scroll up to look at earlier scans.

Initials: RCJ Date: 2/6/98

It is also possible to use the GSAnalysis software, and other Macintosh functions (e.g. copying of files) during the data collection. This should generally be avoided. It must be done with extreme care. Do not touch the Collection software.
After the gel run is finished the Collection Software quits automatically.

G. Removing the Used Gel and Clean-Up

1. Open the door, remove the lid from the upper buffer chamber, and remove the buffer from both buffer chambers before proceeding.

2. Disconnect all electrodes, the electrical wire for the heat plate and the tubings.

3. Un-clamp clamps #3,# 4,#5 on each side to release the heat plate. Remove the heat plate. Put clamps back to hold the gel plates.

   IMPORTANT: Do not remove the gel cassette with the front heat plate still being attached to it. The weight of the front heat plate could damage the cassette during that step.

   Occasionally, especially after a buffer spill, the heat transfer plate is sticking to the glass plate, and cannot be removed. In that situation, remove the upper buffer chamber first and then slide the heat plate upwards until it can be removed.

4. Un-clamp upper buffer chamber (clamps #2), remove it, and put clamps back to hold the gel plates.

5. Push the gel cassette towards the back plate, release the four outer clamps that hold the cassette, and remove the cassette/gel assembly from the instrument.

6. Place cassette/gel assembly on a bench top. Remove the gel plates from the cassette, pry open and clean the plates. Rinse the black gel cassette under running tap water to remove any electrophoresis buffer. Pay special attention to the beam stop bar and the clamps - these should not be allowed to be encrusted with dried buffer.

7. Go back to the instrument, and remove any TBE buffer from the amber positioning pins, the laser lens, the back plate, and the heat plate. Rinse the upper and lower buffer chambers with tap water.

Initials: RCS Date: 2/6/98

F. Troubleshooting Electrophoresis

1. **Communication problems** between the instrument and the Macintosh terminals are caused by corrupted memory files, and can cause several problems, e.g. the status window displays the message "door open" and will not let you start a run. In reality the instrument door is closed. To clear the memory the instrument must be reset:
   - press the reset button on the back of the instrument using a pencil
   - watch the back panel LEDs and wait for them to blink in a four on, four off sequence
   - while the four on, four off sequence is occurring press the rest button a second time

   The next time you start the data collection program a new firmware image will be copied to the instrument. (See also pages 7-6 to 7-9 in the ABI PRISM 377 DNA sequencer User's Manual).

   After resetting the instrument, the CCD pixel position has to be reentered. Under the collection software menu go to **Window**, select **Manual control**. Click on the arrow next to **Fxn name** and from the option list select **CCD pixel position**. The correct value varies from instrument to instrument, and was determined during the installation. The value is listed inside the door of each instrument. Enter the number, click execute, and close the manual control window.

2. **Inconsistent mobilities from gel to gel**, and poor resolution are most likely caused by low quality, too old, or simply the wrong gel casting reagents. Check the age of e.g. the APS, remake the 1xTBE buffer, make a fresh gel, and try again. When making the gel check if the right urea aliquots, the right spacers, and the right comb were used.

3. **Inconsistent mobilities within a gel** are most likely caused by incomplete denaturation of single samples. There is no strong effect if the denaturation temperature is up to 10°C lower than 95°C, but the mobility changes if the sample was mistakenly not denatured at all, or if the sample was allowed to sit more than 30 minutes after the denaturation. Samples will not be properly denatured if the amplified product was not pipetted into the formamide, but streaked along the tube wall.

   Another reason for inconsistent mobility and **smearing** up and down of bands ("black holes") are electrostatic charges caused by "over wiping" the glass plates with Kimwipes. Make a fresh gel and rerun the samples.

4. **Double peaks and shoulders** are also signs of incomplete denaturation. Another possible reason for these Z shaped bands is an uneven well bottom, or a piece of gel blocking the well. Clean wells carefully before loading to avoid loading misshaped wells.

Initials: RCJ Date: 2/6/98

5. **Fuzzy bands and hazy gels** can be caused by improper alignment of the gel plates with the laser plane. Reasons can be buffer crusts on the amber alignment pins (see section A step 6), a mistakenly un-clamped lower gel cassette holding clamp, or a worn out gel cassette holding clamp. Always apply pressure on the gel cassette when releasing the outer clamps in order to avoid a loosening of the clamps (see section G step 5).

6. **Horizontal streaks** on a gel are a sign of the laser being on it's way out. It must be replaced as soon as possible.

7. **Vertical streaks** are less disruptive since they do not cause artificial peaks in an electropherogram. Vertical streaks are caused by scratches or lint on the glass plates in the laser read region.

8. "Red rain" is a phenomenon where **red vertical streaks** appear late into the run. These red streaks are caused by bubbles between the glass plate and the gel matrix. The problem occurs when a gel dried out after sitting without being wrapped. Another reason is the appearance of hydrophobic patches on the glass surface. This can be solved by repeating the treatment of the plates with sodium hydroxide (QC033).

Initials: QCJ Date: 2/6/98

STR Gel Analysis of Gels Run on ABI 377

The gel analysis and allele identification steps will be performed using the ABD/PE **GS Analysis** and **Genotyper** software. Besides the 377 instruments, the Apple/Macintosh terminals number **1** and **2** are set up for 377 gel analysis; these terminals have copies of the **GS Analysis** and **Genotyper** programs, of all relevant matrix files, the size standards, and all Genotyper templates. It is preferable to analyze all gels at the separate terminals, so that the instrument computer is available for data collection. If necessary gels can be analyzed and printed at the 377 terminals, but in order to be archived on 4.2GB optical disks they then must be copied on a JAZ disk. To avoid the accumulation of empty runfolders, and gel files in various states of processing, and in order to ensure the correct archiving of the final version of the analyzed gel, please adhere strictly to the following copying and archiving rules.

The collection software places all run folders in the folder "**Current runs**", which is located on the hard drive.

The way to delete a file on an Apple Macintosh is to drag the file icon into the trash bin. Then under **Special** select **Empty trash**. To rename a file, place the cursor on the old file name, click once so that the name is highlighted, and then type in the new name. To close an application window, click on the upper left hand corner.

A Gel File Copying and Archiving Procedure

1. Before copying or analyzing a gel, identify the **Run folder** containing the gel file based on the date and time noted on the gel sheet. Rename this run folder with the gel name according to the following format:

   Raw data Gelname (e.g. Raw data 97-Men001)

   Occasionally, additional run folders with the same date and similar times are created during the electrophoresis set-up. These run folders do not contain the gel file with the raw data, are smaller in size, and should be deleted immediately.

2. Put a designated JAZ transport disk in the JAZ drive. The disk icon will be visible on the desk top. Drag the icon of the renamed run folder onto the JAZ disk icon. After copying is complete, drag the JAZ disk icon onto the trash icon to expel the disk.

   ATTENTION: Always remove external disks by dragging the disk icon into the trash icon. Do not press the expel button on the JAZ disk drive!!!

Initials: RCJ Date: 2/6/98

3. Insert the transport disk in the JAZ disk drive at terminal #1 or #2, double click on the disk icon to open the disk window, open the hard drive window the same way and copy the gel onto the hard drive. **Do not copy the gel onto the desktop!**

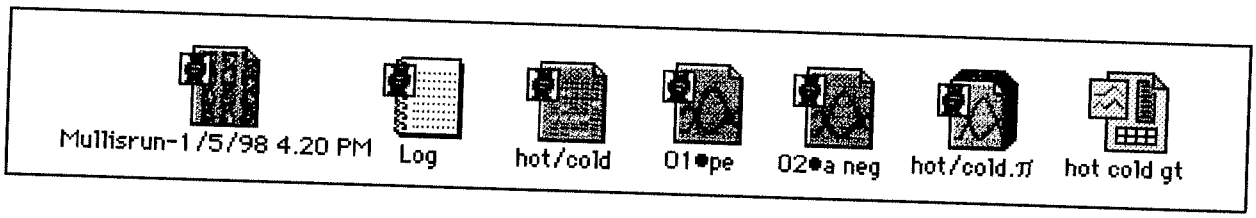
   After the copy process is complete, delete the raw data folder from the JAZ transport disk. Eject the JAZ disk by dragging it onto the trash can icon.

4. After the gel has been analyzed and run through the Genotyper the run folder should contain the following items:

   - a run file (65K ABI Prism 377 Collection document)
   - the run log (65K ABI Prism 377 Collection document)
   - the gel file (ca. 10 MB Genescan document)
   - sample files for all samples (33K Genescan documents)
   - a project file (65K Genescan document)
   - the genotyper file (ca. 400K Genotyper document)

   See picture below for appearance of file icons.

   It also should have been renamed (see Genotyper instructions), according to the following format: **GelName Files (e.g. 97-Men001 Files)**



5. Copy the analyzed gel in duplicate on 4.2GB optical disks. Make two copies and delete the **"File"**folder from the terminal. Go back to the instrument and delete the "**raw data**" folder.

B Collection Gel Processing

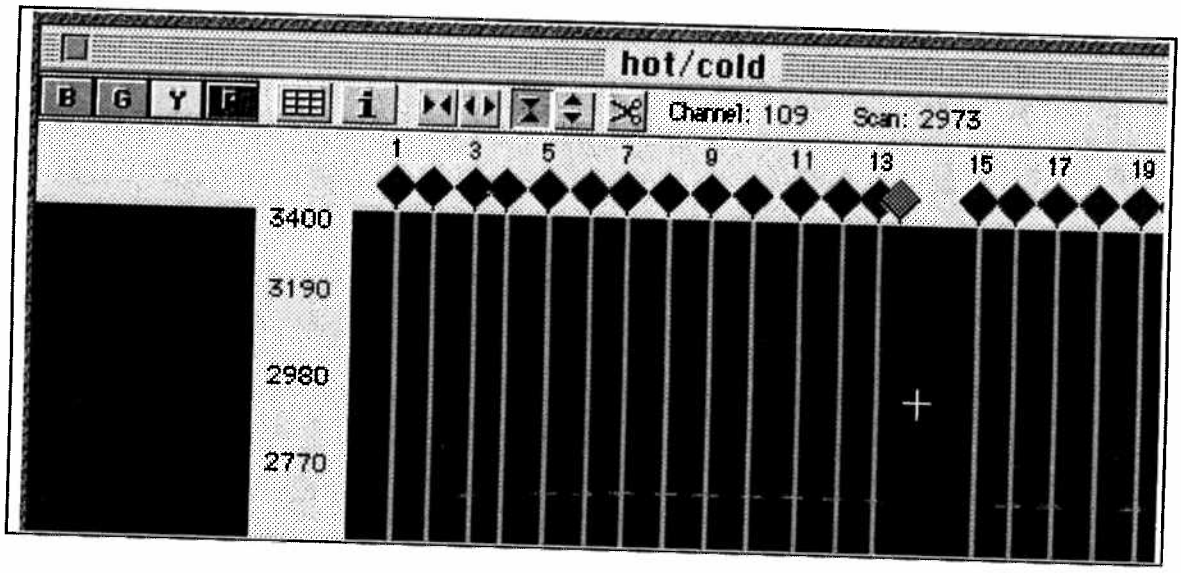
1. Open the **GS Analysis** software by clicking on the **373 / 377 analysis** icon.

2. Under **File** go to **Open.** From the listed possibilities click on existing **Collection gel**. Open windows until the desired gel file (in the raw data folder) is displayed and highlighted. Gels are represented by vertical icons as opposed to folders.
   Click open.

Initials: RD Date: 2/6/98

WINDOW OUTLINE

The analysis software will open the gel file and a gel image will be generated. The **Gel Window** shows the DNA fragments in all lanes as colored bands. The colors can be deselected by clicking on the color boxes in the upper left hand corner. A standard setting is the displays all four colors.



Other functions displayed on top of the gel image are:
- grid icon for opening the sample sheet
- **i** icon to read run information
- different < > icons for the zoom options
- ✂ icon to activate a lane tracking tool

The mouse cursor is shown as an arrow outside of the gel image and as a cross on the gel. It can be used to determine channel and scan coordinates for every point of the gel. To do this, the cursor is placed on the desired spot and the channel and scan numbers are read on the right side of the top display.

The lane number and a diamond shaped **lane indicator box** appear at the top of each lane. By placing the cursor on the **lane indicator box** and clicking on the mouse, a lane can be selected. Only after filling out the sample sheet and marking lane as used, white line will be seen tracing this lane from the top to the bottom of the gel. To the left of the DNA fragment bands, the same information is shown as peaks for the selected lane. Each peak corresponds to the fragment bands.

Initials: RCJ Date: 2/6/98

3. Proceed by filling out a sample sheet based on the 377 gel worksheet.

   Click on the **Grid icon** next to the color boxes. The empty sample sheet that appears on the screen is named after the gel. It shows the lane numbers and has spaces to be filled in for **file name**, the **sample name, sample info**, and **comments**. The four color options (blue, green, yellow, red) are displayed in the "Dye" column, with the diamond in the "STD" column specifying **red** as the fluorescence dye for the sizing standard. Which fluorescent dyes have been analyzed already and/or are present as labels for the PCR may be checked in the "A" and "P" columns.

4. For each used lane check the "used" box and enter the sample information.
   Fill out **only** the sample name and the sample info column, base the input on the amplification and the gel worksheets, the lane assignments must be identical with the gel sheet:
   - enter the tube labels as the sample name (do not use long sample names!!)
   - enter the full sample information including the case number as sample info for blue and green.

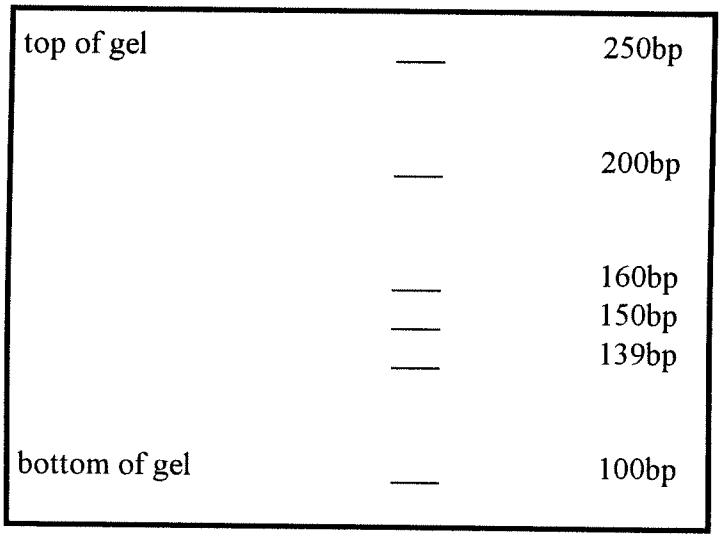
   The **edit copy, paste,** and **fill down** commands are used to fill out the sheet. The text for the blue and green sample info must be identical for the same sample!! For different samples, it is important not to use the same description as sample info, e.g. use "amplification negative 1" and "amplification negative 2" to distinguish between two different amplification negatives.

5. After entering all relevant information, close the sample sheet window. Choose **Save.**
   The gel image will display tracking lines for all used lanes.

Initials: RCJ Date: 2/6/98

6. Next choose the appropriate scan area to analyse by looking at the red size standards:

Each lane must contain 6 red standard fragments from 100 - 250 bp. They are distributed as follows.



If the red signals are too faint to be assessed properly go to step 8 and increase the gel contrast.

If the gel image contains less than these 6 fragments the gel image must be regenerated - go to step 7. If all fragments are present and the gel picture is as described in step 8, proceed with step 9.

7. If the not all of the six red size standard fragments are present, determine which ones are missing and decide how much to extend or shrink the gel region. If the gel must be cut off, the scan number can be measured by placing the cross on the desired area. If additional areas are needed the scan number has to be guessed.

Under **Gel**, select **Regenerate gel image**. Change the **Stop at Scan**, **Start at Scan** parameters according to the above assessment. Click **OK**.

The gel image will be regenerated for the new scan range. Repeat this step if necessary. After determining the final correct scan range, note the scan numbers on the gel sheet. You will need this information to adjust the analysis range.

Initials: JCJ Date: 1/16/98

8. The gel picture displayed on the screen should have a black background and distinct blue, green, and red signals. If the background is blue or green or if any of the colors are too faint to be seen clearly, the color contrast must be adjusted. This does not change the measured peak height for the PCR products, but creates a more intensive image on the screen and improves the lane tracking.

   Place a tracking line on one of the samples.

   Under **Gel** choose **Adjust Gel contrast.** The **Adjust Gel Contrast** window shows an example of the electropherogram peaks. All four colors are represented by colored triangles at the top and at the bottom of the electropherogram. Each color can be adjusted individually. Using the mouse cursor, drag the upper triangle down if you want to raise the peak. Pull the lower triangle up, if you want to lower the peak or reduce the baseline for that color. Click **Apply.**

9. Under **Gel** select **Track lanes**. Click on **Autotrack lanes**. This will launch the automatic lane tracking. Afterwards the result has to be checked manually for each gel.

10. Click on the **lane indicator diamond** for each used lane. The white line should drawn through the middle of each DNA fragment. All 6 red standard fragments have to be visible in the left peak display. Make sure that the lane assignments correspond to the gel sheet.

    To correct the lane tracking, keep the mouse button down and move the lane indicator diamond. Move the line to the left or to the right in order to find the middle of the fragment and to achieve optimal peak height on the peak display. If only parts of the lane tracking need to be adjusted, click on the scissors from the top buttons. This will transform the mouse cursor into a pair of scissors. Place the scissors on the area to be moved and click once. The movable area will become blurry and can be moved independently from the rest of the lane tracking line.

    It is easier to adjust the tracking of a band by zooming in on the lane using the zoom options.

11. After the tracking is satisfactory, click on the next lane. The changes for the previous lane will be saved automatically.

12. After the tracking for all lanes is satisfactory, the changes for the whole gel should be saved. Under **File** select **save.**

Initials: RU Date: 2/6/98

13. The last step is to extract the lanes that are going to be analyzed.

Under **Gel** select **Extract lanes**.
From the **extract lanes window** choose **extract from all used lanes**!!

From the remaining settings on the extract lanes window all options should be checked as follows:

- Overwrite original sample files should be checked.
- Create a new project is o.k.
- **Autoanalyze** sample files should be **deselected**.
- Save gel after extraction should be checked.

Always select all used lanes, except when a reanalysis for certain lanes is intended (see trouble shooting section for allowed exceptions) or QC or research samples have been co-loaded on a casework gel.

The software will combine all sample lanes in a so called **project file** that will be named after the gel file with the suffix ".". Each sample will be accompanied with the sample name, sample info, run information and the gel name.

For casework gels it is NOT acceptable to combine samples from different gels into one project!

C Troubleshooting Collection Gel Processing

1. If the automatic lane tracking results in completely fragmented tracking lanes, it might be easier to manually adapt the straight tracking lanes. Under **Gel** go to **Track lanes**, select **Revert to straight tracking.** Then move the tracking lanes appropriately.

2. If too many tracking lines (more lanes than samples loaded) are present, there must be a mistake on the sample sheet, e.g. an empty lane is marked as used. Click on the **sample sheet icon**, correct the sheet, close, and then click **save**.

3. For a regular 2.5h run it should always be possible to select a gel image range which includes the 250bp fragment. The maximum number of scans after 2.5h is 6000. If the upper scan number can not be increased through the regeneration of the gel image, the data collection must have been stopped at some point before the 2.5h was over. If the scan range is present but no fragments are visible for the upper gel area, the electrophoresis was terminated, while the laser was still collecting. If the maximum scan range shows

DNA fragments but does not include the 250bp fragment, the electrophoresis run was too slow.

For all of the above scenarios, the run must be repeated. Do not analyze the gel further.

D. Project File Analysis

After all sample lanes have been extracted, the **Analysis Control** window will be displayed automatically. If a project is being analyzed at a later date it can be opened under **File,** going to open, and then selecting **Project**.

WINDOW OUTLINE
This window shows in separate columns the lane numbers, boxes to select each color to be analyzed for each lane, the sample file names, size standard options, and analysis parameters to choose for each lane. The boxes for red should be marked with diamonds to indicate that red is the color for the size standard.

To make changes for one sample only, click on the sample until it is highlighted. To make changes for all samples click on top of the column so that all samples are highlighted.

1. Highlight all samples. Under **Samples** choose **Install new matrix**. To choose the correct matrix the fluorescent labels and the instrument on which the gel was run must be known. Open folders until the correct matrix file appears. Click **Open**.

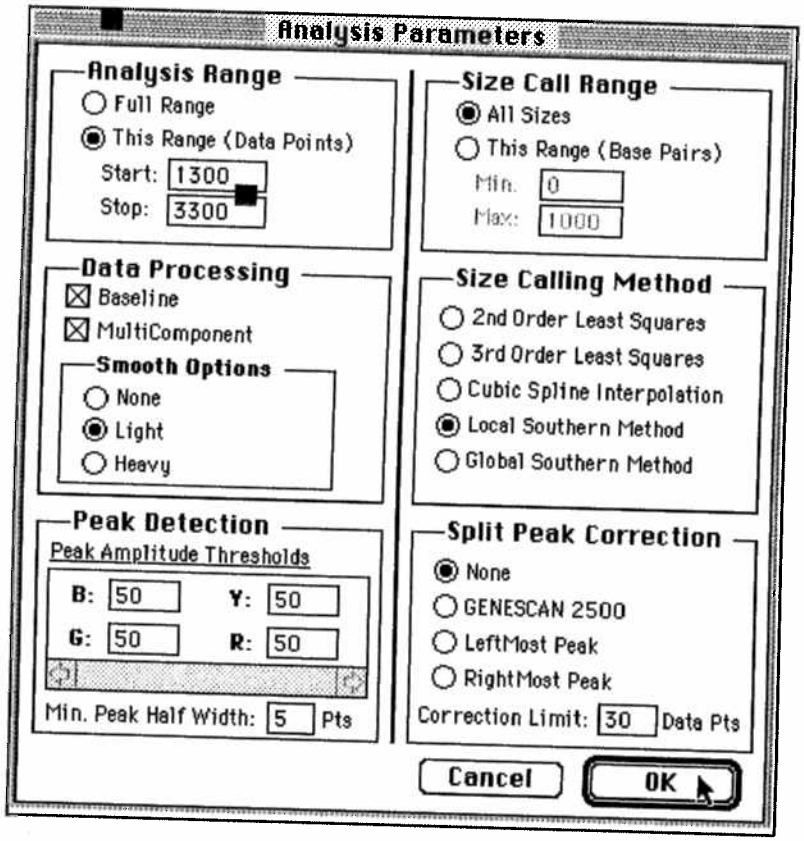
   After the matrix selection the software should displays a window stating that the matrix file was successfully installed within the selected sample files.

2. Click on the arrow of the top size standard box so that all lanes are highlighted, hold the mouse button down, and select **GS500 ROX**.

3. Under **Settings** choose **Analysis Parameters.** It is important that the **Analysis range** is consistent with the relevant scan range (see gel sheet) and contains all desired standard peaks. The settings for the remaining options are predefined and should not be changed.

   The default settings are shown in the following figure:

Initials: RCJ Date: 2/6/98



After the scan range is corrected, click **O.K.**

4. Click on the top blue, green, and red boxes to select these colors for the analysis for all lanes.

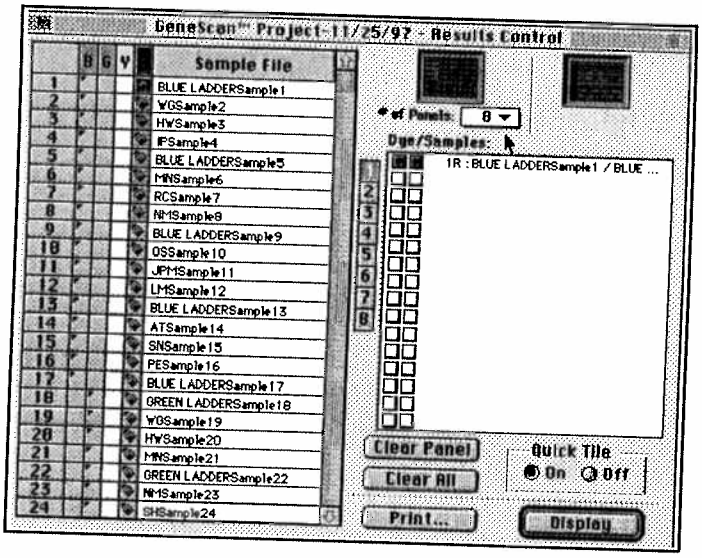
   Click on the **Analyze** button in the upper left corner. All selected samples will be analyzed. After the analysis a black triangle appears in the color boxes to indicate that this sample has been analyzed.

   An analysis log window will appear that states for each sample if the size calling was correct and whether and which size standards were skipped. Regardless of this message, the correct assignments of the size standard peaks must be checked manually.

5. Under **Window** go to **Results Control**.

   The results control window is displayed below.

Initials: RCJ Date: 2/6/98



It shows the title of the project, and the same lane numbers and color display as the **Analysis Control** window. The analyzed colors per lane are shown in dark grey. The white squares mean that this color has not been analyzed.

The electropherogram results can be displayed as a table with the sizing results only (deselect the left electropherogram icon), as electropherogram only (deselect the right table icon) and as a combination of both (standard setting). Up to 8 display panels can be seen in parallel. Each color per lane can be separately assigned to a panel by clicking on it and choosing the next panel for the next color. All colors can also be viewed as "stacked" electropherograms by assigning all colors to the same panel. It is optional to look at the allele peaks at this point.

6. Overlay up to16 size standards by clicking on the top of the red data column. The red standards for 16 lanes should be highlighted.

Click **display**.

Initials: QCJ Date: 2/6/98

7. All 16 size standards will be displayed on top of each other. They should align properly. Scroll down the sizing table and scan it for intermediate sizes. Note any lanes that show deviations. Close the electropherogram window by clicking on the upper left corner.

Required Sizes for the Peaks are:

peak 1	100
peak 2	139
peak 3	150
peak 4	160
peak 5	200
peak 6	250

8. Repeat step 6 for the remaining size standards.

9. If an assignment is wrong the size standard must be redefined for this lane only. The lane must be reanalyzed with the newly defined standard.

Under **Window** select **Analysis control.** Click on the arrow in the size standard column for the lane to be reanalyzed. Choose **define new**. The system displays an electropherogram of the size standard fragments in this lane. Highlight the first peak by clicking on it. Enter the number, 100, and press return. Continue to enter the above values for the size standard peaks.

After entering the number 250, click **return**. The highlight should be back on peak 1. Click **Save**, to "save changes to "untitled," before closing, click **Save**, to "save this document as "untitled," click **replace**.

Reanalyze the corrected lane.

10. Before proceeding with the **Genotyper** analysis, close the **Results Control** window. Under **File**, select **Quit**, and click on **Save** for "save changes before closing".

E. Troubleshooting Project File Analysis

1. If it is not possible to open a project because no project file can be highlighted in the desired run folder, means that the lanes have not been extracted. Start with section B collection gel processing.

Initials: JCL Date: 2/6/98

2. The assignment of the wrong sample information to a specific lane must be corrected on the sample sheet. Do not exit the project. Under **File,** go to **open** and select **Collection gel**. The collection gel belonging to the open project will highlight automatically. Correct the sample sheet as described in step 4 of section B. Close the sample sheet and re-extract all used lanes. Make sure that "overwrite original sample files" is checked and that the message "add to open project" is displayed. After re-extracting the lanes, all samples must be reanalyzed.

3. If an empty lane is extracted mistakenly, the sample file for the empty lane can be deleted by highlighting it. Click on **Project** and select **remove sample files**. Click **Remove** when asked " -- sample files are selected, are you sure you want to remove them."

   Do not delete sample files outside of a project. (e.g. by dragging them onto the trash icon.) A project is a compilation of all sample files and will still contain a "ghost" of the deleted sample file. These "ghosts" will cause problems when importing a project into the Genotyper template.

4. If all 6 peaks of the size standard are not present in the electropherogram, this could be due to a discrepancy in the selected scan range for lane tracking, and the scan range entered for the analysis parameters. Check the collection gel for the necessary scan range, correct the scan range entry as described in step 3, and reanalyze the samples.

Initials: EO Date: 2/6/98

QUAD Genotyper 2.0 Analysis of all ABI 377 Gels and those ABI 373 Gels Processed on Separate Apple Macintosh Terminals

After the gel analysis is completed, the file must be processed further using the Genotyper software. This program will assign allele names to the detected peaks and create the electropherogram output that goes into the case file. The following steps must be performed exactly as listed. **Do not change the order!** If the order is changed or an unexpected event occurs, see section 12 for trouble shooting.

The Genotyper version 2.0 has a short cut option for opening the different windows. On the right side of the main window, icons represent the dye lane window, the plot window, the table window and so on. Open these by clicking on the icons.

1. Start **Genotyper** by double clicking on the **Quad 377 Genotyper** icon.

   If data are visible in the two upper windows, go to **Analysis** and select **Clear Dye Lane List** and then **Clear Table**. Otherwise proceed with step 2.

2. Under **File,** select **Import Genescan files**. The analysis result files are in the run folder. Choose the correct run folder by double clicking on the appropriate folders until the sample file names appear. Go to the project file (gel name.π), and highlight it.

   IMPORTANT: Always deselect the option to import the raw data!!! This makes the Genotyper files much to large.

   Click **import**.

   Always import the complete project, unless a reanalysis for specific lanes is intended (see trouble shooting section for allowed exceptions), or QC or research samples have been co-loaded on a casework gel.

   For casework gels it is not acceptable to combine samples from different gels in one Genotyper file.

3. After importing the results, change the name of the Genotyper template to your initials and the gel file name. Under **File** select **Save As**. Enter your initials and the name of the gel you are processing. Click **Save**.

Initials: RCJ Date: 2/6/98

4. Run the **Quad Macro 1** by pressing the Apple key and the number 1, or by highlighting the macro name in the lower left hand corner and, under **Macro,** selecting **Run Macro**.

   Quad Macro 1 selects peaks that meet allele calling criteria (see results interpretation) by automatically performing the following steps: select blue lanes + select green lanes, clear labels, label category peaks with the size in bp, remove labels from peaks whose height is less than 10% of the highest peak in the category's range; then remove labels from peaks less than 20% of a following higher, labeled peak within 0.00 to 5.00bp.

5. Under **View** select **Show Plot Window.** Check all lanes. Manually delete labels for extra peaks by placing the cursor on the peak above the baseline and clicking.

   Extra peaks are defined as peaks that meet one of the following criteria:

   1 small constant bands of the following size and color
   Blue 180-181 bp, 230-232 bp, and 246-248 bp
   Green 127-131 bp and 211-228 bp

   2 pull-ups of green peaks caused by very high blue peaks in the same lane

   3 shoulder peaks approx. 1-3 bp bigger than main allele

   4 peaks caused by overflow of sample in the adjacent lane

   5 -4 and +4 bp stutter if there is no indication for the presence of a mixture. (See interpretation section)

   6 unknown.

   Fill out the Genotyper Editing sheet for each lane and note the reason for removal of a peak using the number code above.

   If a locus displays only one peak label and a distinct same color peak is visible but is not labeled because its size is outside of the defined allele range, this peak might be a new, previously unreported allele. This possibility must be considered especially if the other loci show a proper profile. The presence of a possible new rare allele must be pointed out to a DNA supervisor for confirmation (see Interpretation of complex Quad STR results). The "new" allele will not be automatically reported in the Genotyper table, but will be visible on the electropherograms. Click on the unlabeled peak in order to label it with the size in bp, which is necessary for allele identification.

Initials: RCJ Date: 2/6/98

7. Run the **Allele Designation Macro** by pressing the Apple key and the number 2, or by highlighting the macro name in the lower left hand corner and, under **Macro,** selecting **Run Macro**.

   This macro automatically assigns allele names to the labeled peaks based on the size categories for the different fragments and writes a results table. The following steps are carried out: clear table, change labels to the categories name, add rows with one sample per row, containing sample info in column1, up to 4 labels in columns 3-6, the text "check" in column 2 if number of labels >2 (titled "mixture."); put column titles in first row.

   Scroll through the plot window again to check for artefacts such as shoulders or pull-ups. Remove these peaks. To determine the size in bp for the editing documentation, click on the peak to remove the allele label, click again to re-label with size in bp, and click again to finally remove the label.

   If extra peaks were removed after running Macro 2, you must run Macro 2 again in order to rewrite the table.

8. Under **View**, select **Show Table**. Under **File**, select **Print**. Print Table. Close **Table Window**.

9. Under **View,** select **Show Plot Window**. Under **Analysis,** select **Change Labels**. In the Change Labels Dialog box check both "with size in bp" and " the categories name." Click **OK**.

10. The next step is to print the plot window. To achieve an uniform format, the base pair range, which should be printed, must be selected. Under **View,** select **Zoom to.** Enter 110 to 260. Click **OK**. The display on the screen will focus on that range.

    Under **File,** select **Print**. Print plot window or as it is called here "Graphical area". Close **Plot Window**.

11. Close template by clicking on upper left corner. Click **Save**. The edited results are saved as a **Genotyper** file, placed in the **GS Analysis** results file for this gel. It can be opened and re-edited.

12. Under **File**, quit **Genotyper**.

Initials: RCJ Date: 2/6/98

13. As already listed in section A the run folder should now contain the following items:
- a run file (65K ABI Prism 377 Collection document)
- the run log (65K ABI Prism 377 Collection document)
- the gel file (ca. 10 MB Genescan document)
- sample files for all samples (33K Genescan documents)
- a project file (65K Genescan document)
- the genotyper file (ca. 400K Genotyper document)

If all these items are present rename the run folder as an indication that analysis is complete, according to the following format:

Gelname Files (e.g. 97-Men001 Files)

Refer to 377 STR Gel Analysis and Allele identification section A for archiving.

Troubleshooting QUAD Genotyper

1. The sample information can be corrected for the Genotyper file by opening the dye lane list, highlighting the lane, and retyping the sample information. The sample name cannot be changed here. It can only be changed on the sample sheet level (section B, step 4).

2. If an incorrect peak has been removed accidentally, it can be re-labeled by placing the cursor above the baseline and clicking on it again. Re-labeling always labels the peak with the size in base pairs. To re-label with the category name, run Macro Apple 2 again.

3. If the table is inconsistent with the information displayed in the plot window, the most likely reason is that something was changed (corrected sample info, removed extra peak) after the Apple 2 Macro had been run. To rewrite the table, simply run the **Apple 2 Macro** again. This has to be repeated for all instances where anything was changed.

4. If a lane has been placed out of order at the top of the plot window, it probably has been marked accidentally with a black bullet after double clicking on the dye lane. This feature is meant for placing lanes next to each other for comparison purposes. Remove black bullets by double clicking on it again or, under **Edit**, selecting **unmark**.

Initials: RCJ Date: 2/6/98

5. The Apple 1 Macro selects all blue and green results for further analysis. If these lanes have accidentally or purposefully (e.g. when highlighting a single dye lane for correcting the sample information) been deselected do the following. Under **Edit,** choose **select blue.** While pressing **shift,** open **Edit** again and select + **select green**. Shortcut for Genotyper 2.0: in the upper left hand corner, click on the first color, press **shift,** and click on the second color.

   Do not rerun Apple Macro 1 in order to re-select the blue and green lanes, this will re-label all edited peaks.

6. **Unlabeled peaks** that are clearly higher than 50 fluorescent units, can be caused by several reasons:

   A) The peaks have not been sized. This occurs especially for the FES locus when the 250bp size fragment was not present on the gel or has not been included in the analysis area (see Troubleshooting sections C and E). Quit Genotyper and correct any errors made during **Collection gel processing** or **Project file analysis**. Rerun the sample if the 250bp fragment data are not in the collection data.

   B) The peak is outside of the allele calling window because it is a new allele (see step 5, section F).

   C) The peak is outside of the allele calling window because of an aberrant electrophoretic mobility. Rerun the sample.

7. The Genotyper printout should have a standard format: green lanes, then blue lanes. The table should have 4 rows for each locus. The order of the loci in the table should be VWA, F13A1, THO1 and FES. If this is not the case the defaults have been changed.

   Default settings are: Dye lane sorting: 1. Lane number - ascending
   2. Dye color - descending
   Category sorting: 1. Size/scan - ascending

   The table set up is defined in the macros. See steps 4 and 7 of the QUAD Genotyper section for correct setting.

Initials: RCJ Date: 2/6/98

STR Results Interpretation

Allele Calling Criteria

Results are interpreted by observing the occurrence of electropherogram peaks for the four loci that are amplified simultaneously - HumVWA, HumTHO1, HumF13A1, and HumFES. The identification of a peak as an allele is determined by the labeling color of the locus specific primers and the length of the amplified fragment as follows:

Locus	Color	Size range
HumVWA	green	127±1.5bp to 172 ±1.5bp
HumTHO1	blue	150±1.5bp to 179 ±1.5bp
HumF13A1	green	181±0.75bp to 247 ±1.5bp
HumFES/FPS	blue	206±1.5bp to 238 ±1.5bp

For each locus an individual can be either homozygous and show one allele, or heterozygous and show two alleles. In order to eliminate possible background and stutter peaks, only peaks that display an intensity **>50** (arbitrary units), and have a **peak height >10% of the major peak** are called alleles by the instrument. Due to the systematic occurrence of stutter peaks smaller than 4bp of an allele peak, peaks at this position that are lower than the empirically determined stutter threshold of 20% are also disregarded.

The raw data collected by the Genescan Software undergo the following computer processing steps:

- recalculating fluorescence peaks using the instrument specific matrix in order to correct for the overlapping spectra of the four fluorescent dyes (automatically -GS collection software; ABD/PE).

- calculating the fragment length for the detected peaks using the known in-lane standard fragments (see the STR Gel Analysis section of this manual).

- labeling of all sized fragments that are >50 (arbitrary units) and >10% of the highest peak at that locus, and are >20% of a following 0-5bp bigger peak (see the Genotyper section of this manual).

Initials: RCJ Date: 2/16/98

Additional non-allelic peaks may occur under the following instances:

- peaks approx. 1-3 bp larger than main allele, caused by a flat decline of main peak fluorescence

- green peaks of exactly the same length as the blue peaks in the same lane ("pull-ups"), which are caused by insufficient filtering of the fluorescence of very high blue peaks

- constant peaks at the following sizes: blue 180-181bp, 230-232bp, and 246-248bp, green 128-131bp, and 211-220bp.

- overflow of sample that has been loaded in the adjacent lane

- -4 and +4 bp stutter if there is no indication for the presence of a mixture. (See interpretation section).

If these additional non-allelic peaks are labeled by the Genotyper program, the labels can be removed manually. The removal of a label must be recorded in the Genotyper editing sheet.

After the assigning of allele names to the remaining labeled peaks the Genotyper software prepares a result table where all peaks that meet the above listed criteria are listed as alleles. The allele nomenclature follows the recommendations of the International Society for Forensic Haemogenetics (ISFH), (DNA recommendations, 1994) and reflects the number of 4bp core repeat units for the different alleles.

Genotyper Results Table

The Genotyper results table is the basis for result interpretation and reporting of results. The results table reflects the number and allele assignments of the labeled peaks visible on the Genotyper Plot print out. During the interpretation the analyst has to refer to the plot print outs for peak height differences and should compare the sample plots to the table.

There are two instances where the data processed by the Genotyper software are not sufficient for proper interpretation and it is necessary to refer to the Genescan Analysis results for a sample.

1.) If a sample other than a control sample has no alleles listed for all four loci in the Genotyper results table and the Genotyper plot states "no size data" it is possible that the original Genescan Analysis electropherogram displays visible peaks <50. If only one peak is >50 and got called by the Genotyper software a plot will be displayed and can be used for further interpretation. If the plot only states "no size data" the analyst has to look at the original Genescan electropherogram in order to determine if peaks <50 were visible for this sample (see STR gel analysis 10).

Initials: RCJ Date: 2/6/98

2.) If a sample has only one allele at one of the loci but a distinct peak outside the previously reported size range is visible on the plot, this sample could have a "new" allele (see Interpretation of complex Quad STR results). In this case the analyst has to look at the original Genescan Analysis electropherogram in order to get the size in bp for this peak (see STR gel analysis 10).

Reporting Procedures

All alleles which meet reporting criteria and therefore are listed in the results table are listed in the laboratory report regardless of intensity differences. Genotypes are not reported and should not be inferred, i.e. if only a "7" allele is found, it should be reported as 7. The reporting criteria are as follows:

A. If an allele meets the above reporting criteria in two runs then the allele is listed in the report.

B. If an allele meets the above reporting criteria in one run and is visible on the electropherogram but does not meet criteria in at least one additional run then the allele is listed in the report in brackets. (i.e. [7]). At the bottom of the table, the brackets are defined as "[ ]= The presence of the allele above a minimum threshold could not be duplicated."

C. If an allele peak is visible but does not meets the above reporting criteria in at least one run and does not fit into categories A and B above then the allele is reported as **. The ** is defined at the bottom of the table as " ** = additional alleles were detected which did not meet laboratory criteria for allele identification; therefore, these additional alleles are not reported."

D. If an allele meets the above reporting criteria in one run and is not visible during the duplication, then the allele is reported as **.

E. If no alleles are detected in a sample then the sample is reported as "Neg= no alleles detected."

F. If DNA below the minimum threshold is found on QuantiBlot Analysis then the sample is reported as "INS =Insufficient human DNA was detected; therefore, this sample was neither amplified nor typed."

G. If there is a large intensity difference between alleles from a locus, the intensity difference should be noted on the report with ***. The *** is defined at the bottom of the table as "***= Large intensity difference between alleles suggests a mixture of DNA."

Initials: RCJ Date: 2/6/98

H. Other symbols or reporting procedures will be used if necessary depending on the details of the case.

Comparison of Samples and Interpretation of Results in Report

A. Determine whether it is likely that a sample contains a mixture of DNA (i.e. more than two alleles for a locus, intensity differences between alleles within a locus, reproducible pattern of visible but weak peaks, or the facts of the case suggest a potential mixture.) State in the report whether a sample contains a mixture or possible mixture of DNA, determine the minimum number of individuals who could have contributed to a mixture and the likely source of each component of the mixture

B. Compare all possible evidence and exemplar pairs and all possible evidence pairs to determine inclusions and exclusions. For inclusions look at the examination table in the report and compare all alleles reported regardless of whether they are in brackets.

C. Assuming a single physiological fluid donor, two samples could derive from a common biological source (inclusion) if all the alleles in the evidence sample are accounted for by the alleles in the exemplar sample. If however a mixture is possible in the evidence sample, there may be alleles that are not accounted for by the exemplar sample. If an inclusion requires the presence of more than one physiological fluid donor, this must be stated in the report. (i.e.. Hum THO1 (S) 7; (V) 6,9 vaginal swab sperm fract. 7, 9.3/10 (7>9.3/10); Assuming a single semen donor, the suspect can be eliminated as the semen donor. However if there is more than one semen donor, the suspect can not be eliminated as a possible semen donor.

D. Statistics are calculated for probative inclusions only where: (1) The sample is apparently unmixed. (2) The sample appears to be a mixture of two components and the source of one component is known. (i.e. when vaginal epithelial cells are present in the sperm fraction from a vaginal swab.) or if there is a large difference in peak heights between the major and minor components and then the genotype of the major component is easily inferred. The minor component genotype can only be determined if four alleles are present at a locur (or a smaller difference in peak heights) because other alleles may be masked by the major component alleles. See below for the calculation of statistics.

E. Statistics are not calculated for expected inclusions such as vaginal epithelial cells from a vaginal swab consistent with the victim.

Extraction Reagent, Amplification Negative and Substrate Controls

The extraction reagent control and amplification negative control are a check for the possible contamination of the reagents in the Quad STR test by other human DNA or by amplified STR

alleles. 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. In addition, the substrate control extract can be used to verify that the substrate contains an extractable PCR inhibitor. The substrate control, when taken, is performed by carrying out the DNA extraction on unstained substrates (e.g. piece of fabric) located as close to a stain or group of stains as possible.

The extraction reagent control, amplification negative control and substrate control (only if the presence of DNA has been shown by QuantiBlot) 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 QUAD STR DNA may be getting into the samples from some other source.

e. Sample overflow from the adjacent lane might have happened during gel loading.

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

If the extraction reagent, amplification negative or substrate controls show very weak peaks below 50 (arbitrary units) and the test samples show distinct peaks that meet the reporting criteria, the contamination problem is not serious. If the extraction reagent, amplification negative or substrate controls show peaks above 50, the contamination problem is more serious. **See Table IX for interpretation guidelines.**

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

Initials: RCS Date: 2/6/98

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 IX for interpretation guidelines.

Table IX- Guideline to the Interpretation of Visible Peaks in the Extraction Reagent and Substrate Controls

Control	above 50	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 peaks.
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.

Amplification Positive Control- External

The positive control- external DNA is DNA from an individual heterozygous for all Quad loci which is used with each batch of samples typed to demonstrate that the kit is performing properly. Positive control-external DNA A has the following types:

VWA	17, 19
THO1	6, 9.3/10
F13A1	5, 7
FES/FPS	11, 12

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

Initials: RCJ Date: 2/6/98

Interpretation of Complex QUAD STR Results

Occasionally typing results may appear markedly different from the standard patterns. Such results could be due to a procedural error, mixtures of DNA's (multiple contributors to the sample), or DNA degradation. It can be advisable to repeat the test with increased or decreased amounts of DNA, in order to help with the interpretation. This option should be discussed with a supervisor.

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 STR 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, e.g. VWA 15,17, is a mixture of approximately equal contributions from a homozygous 15,15 individual and a homozygous 17,17 individual. Such mixtures are not detected from typing results alone, as they reveal only two alleles which could have similar peak heights.

Other possible combinations that would result in a two allele pattern are mixtures of individuals with e.g. VWA alleles 15,15 + 15,17 or 17,17 + 15, 17. In these cases, the electropherogram should reveal unequal peak heights caused by the triplicate presence of one of the alleles. The peak heights for the Quad STR may vary among the four loci, but the maximum difference for a two peak pattern of an heterozygous individual has been shown to be 25%. Therefore a difference in peak heights of more than 25% for a two allele pattern is either an indication for a mixture or degradation (see Gill et al. 1995).

B. Mixtures with different level of starting DNA

Another scenario that could lead to unequal peak heights is the presence of unequal amounts of heterologous DNA in a sample (Lygo et al. 1994, Gill et al. 1995) . It is not possible to compare peak heights among the different loci.

A VWA typing profile 18>16>14 can be caused by unequal amounts of 14,16 and 18,18 but also by a mixture of two individuals with 14, 18 and 16, 18. Only in cases where it is known that two individuals contributed to the mixture and four alleles are present at a locus with two each matching in height, a genotype can be

inferred. If there is a large difference in peak heights, then the genotype of the major component can be inferred without four alleles present at the locus.

C. Possible mixture components masked by -4bp stutter

Due to enzyme slippage when replicating repetitive DNA stretches, an additional peak of a length exactly -4bp shorter than the main allele peak is a frequent occurrence for STR polymorphisms (Lygo et al. 1994, Gill et al. 1995). Depending on the locus, 25% to 61% of all peaks show such a stutter peak. The average size of a stutter peak for the four loci ranges from 4.1% to 9.5%, with maxima from 11.6% - 24.1%. Therefore peaks in a -4bp position from a main peak and less than 20% of this peak height are not reported as true alleles (see STR Results Interpretation Section). In a mixture the -4bp stutter could mask a real mixture component. Therefore individuals cannot be excluded from being a minor contributor to a mixture if their alleles are in the -4bp position of an allele from another individual.

2. Partial Profiles: not all four loci display allele peaks

A. Degradation

DNA degradation is the process of the very long (>40,000 bp) DNA double strand being broken down into smaller pieces. With increasing degradation the DNA fragments get very short, until the target sequences for the PCR reaction which at least have to contain both primer annealing sites are also broken down. The four QUAD STRs have different allele size ranges, with FES/FPS being the longest (210-238) and VWA being the shortest (127-172) locus.

The longer alleles are more likely not to be present in partially degraded DNA (Lygo et al. 1994, Gill et al. 1995). A QUAD result that displays only VWA and THO1 but no F13A1 and FES/FPS alleles, can be explained as being caused by DNA degradation. A profile with no VWA result but callable FES/FPS alleles cannot be caused by degradation but must have other reasons (e.g. see the following paragraph B).

Due to the allele size differences within a locus, degradation can also cause partial profiles for heterozygous DNA types, e.g. for the F13A1 type 3, 17, allele 3 (128bp) can be present while allele 17 (236bp) drops out. Parallel to the disappearing of the larger size allele, an imbalanced peak height with the larger allele peak being smaller, can be explained by DNA degradation.

Initials: RCJ Date: 2/6/98

B. Detection limit

Due to the different detection sensitivity of the green and blue dye, the green peaks are generally lower than the blue peaks. If the DNA sample is at the lower limit of the testing sensitivity it is therefore possible to get a partial profile of only the blue peaks, or the blue and one of the green systems.

C. Reporting partial profiles

Duplicated alleles at single loci can be used for comparison purposes even if not all loci could be typed for this sample.

If only one allele meets the reporting criteria at a locus and the second allele is visible but too weak to be called, the evidence DNA extract should be re-amplified using a higher amount of DNA (see Troubleshooting 8). If it is not possible to improve the result for the weaker peak, the callable allele can be used for comparison purposes. Then the presence of the weaker allele in the exemplar does not exclude this individual.

3. Detection Of Previously Unreported Rare Alleles

A. Definition

A distinct peak of the same labeling color outside the allelic range could be a rare new allele for this locus. This possibility should be considered if

- the overall amplification for the other loci displays distinct peaks >50 and does not show artefacts,
- the same color locus closest to the new size peak does not have more than one allele peak, and
- the new size peak is also detected in the duplicate run.

The presence of a previously unreported rare alleles has be confirmed by an additional third amplification and co-electrophoresis with a sample containing the closest known allele.

B. Reporting previously unreported rare alleles

Initials: RCS Date: 2/6/98

A match based on the presence of a new size allele in both the exemplar and the evidence DNA can be reported. Statistics can be calculated using the minimum frequency. The new size allele should be reported using the size in bp and a footnote stating the fact that this allele has not been observed for this locus (see reporting procedures H.).

An exclusion only based on the presence of a new size allele, where there is a match for all other tested polymorphisms, has to be reported as inconclusive.

4. Samples with High Background Levels

A sample which has more than two allele peaks per locus and a high background with multiple extra peaks of unknown origin outside of the allelic range has to be interpreted extra carefully and can be typed as inconclusive.

Initials: RCS Date: 2/6/98

QUAD Genotyper Categories Table for ABI 373

VWA

11	Highest peak at 127.00 ± 1.50 bp in green with height ≥50
12	Highest peak at 131.00 ±1.50 bp in green with height ≥50
13	Highest peak at 135.00 ±1.50 bp in green with height ≥50
14	Highest peak at 139.20 ±1.50 bp in green with height ≥50
15	Highest peak at 143.68 ±1.50 bp in green with height ≥50
16	Highest peak at 148.03 ±1.50 bp in green with height ≥50
17	Highest peak at 152.19 ±1.50 bp in green with height ≥50
18	Highest peak at 156.07 ±1.50 bp in green with height ≥50
19	Highest peak at 159.90 ±1.50 bp in green with height ≥50
20	Highest peak at 163.66 ±1.50 bp in green with height ≥50
21	Highest peak at 167.75 ±1.50 bp in green with height ≥50
22	Highest peak at 171.75 ±1.50 bp in green with height ≥50

THO1

4	Highest peak at 150.25 ±1.50 bp in blue with height ≥50
5	Highest peak at 154.25 ±1.50 bp in blue with height ≥50
6	Highest peak at 159.19 ±1.50 bp in blue with height ≥50
7	Highest peak at 163.10 ±1.50 bp in blue with height ≥50
8	Highest peak at 167.02 ±1.50 bp in blue with height ≥50
9	Highest peak at 170.96 ±1.50 bp in blue with height ≥50
9.3/10	Highest peak from 173.0 to 176.75 bp in blue with height ≥50
11	Highest peak at 179.25 ±1.50 bp in blue with height ≥50

Initials: RCS Date: 2/6/98

F13A1

3	Highest peak at 181.34 ±0.75 bp in green with height ≥50
4	Highest peak from 182.75 to 184.75 bp in green with height ≥50
5	Highest peak at 187.30 ±1.50 bp in green with height ≥50
6	Highest peak at 191.23 ±1.50 bp in green with height ≥50
7	Highest peak at 195.25 ±1.50 bp in green with height ≥50
8	Highest peak at 199.15 ±1.50 bp in green with height ≥50
9	Highest peak at 203.22 ±1.50 bp in green with height ≥50
10	Highest peak at 207.25 ±1.50 bp in green with height ≥50
11	Highest peak at 211.23 ±1.50 bp in green with height ≥50
12	Highest peak at 215.25 ±1.50 bp in green with height ≥50
13	Highest peak at 219.20 ±1.50 bp in green with height ≥50
14	Highest peak at 223.12 ±1.50 bp in green with height ≥50
15	Highest peak at 227.29 ±1.50 bp in green with height ≥50
16	Highest peak at 231.25 ±1.50 bp in green with height ≥50
17	Highest peak at 235.25 ±1.50 bp in green with height ≥50
18	Highest peak at 239.25 ±1.50 bp in green with height ≥50
19	Highest peak at 243.25 ±1.50 bp in green with height ≥ 50
20	Highest peak at 247.25 ±1.50 bp in green with height ≥50

FES

7	Highest peak at 206.25 ±1.50 bp in blue with height ≥50
8	Highest peak at 210.33 ±1.50 bp in blue with height ≥50
9	Highest peak at 214.27 ±1.50 bp in blue with height ≥50
10	Highest peak at 218.32 ±1.50 bp in blue with height ≥50
11	Highest peak at 222.32 ±1.50 bp in blue with height ≥50
12	Highest peak at 226.32 ±1.50 bp in blue with height ≥50
13	Highest peak at 230.34 ±1.50 bp in blue with height ≥50
14	Highest peak at 234.58 ±1.50 bp in blue with height ≥50
15	Highest peak at 238.39 ±1.50 bp in blue with height ≥50

Initials: RC Date: 2/6/98

QUAD Genotyper Categories Table for ABI 377

VWA	
11	Highest peak at 127.21 ± 1.50 bp in green with height ≥50
12	Highest peak at 131.74 ±1.50 bp in green with height ≥50
13	Highest peak at 135.74 ±1.50 bp in green with height ≥50
14	Highest peak at 139.70 ±1.50 bp in green with height ≥50
15	Highest peak at 143.70 ±1.50 bp in green with height ≥50
16	Highest peak at 147.70 ±1.50 bp in green with height ≥50
17	Highest peak at 151.70±1.50 bp in green with height ≥50
18	Highest peak at 156.55±1.50 bp in green with height ≥50
19	Highest peak at 160.55 ±1.50 bp in green with height ≥50
20	Highest peak at 164.50 ±1.50 bp in green with height ≥50
21	Highest peak at 168.50 ±1.50 bp in green with height ≥50
22	Highest peak at 172.45 ±1.50 bp in green with height ≥50
THO1	
4	Highest peak at 150.25 ±1.50 bp in blue with height ≥50
5	Highest peak at 154.25 ±1.50 bp in blue with height ≥50
6	Highest peak at 159.25 ±1.50 bp in blue with height ≥50
7	Highest peak at 163.25 ±1.50 bp in blue with height ≥50
8	Highest peak at 167.25 ±1.50 bp in blue with height ≥50
9	Highest peak at 171.25 ±1.50 bp in blue with height ≥50
9.3/10	Highest peak from 173.0 to 176.75 bp in blue with height ≥50
11	Highest peak at 179.25 ±1.50 bp in blue with height ≥50

Initials: RCJ Date: 2/6/98

F13A1

3	Highest peak at 181.75 ±0.75 bp in green with height ≥50
4	Highest peak from 182.75 to 184.75 bp in green with height ≥50
5	Highest peak at 187.75 ±1.50 bp in green with height ≥50
6	Highest peak at 191.75 ±1.50 bp in green with height ≥50
7	Highest peak at 195.75 ±1.50 bp in green with height ≥50
8	Highest peak at 199.75 ±1.50 bp in green with height ≥50
9	Highest peak at 203.75 ±1.50 bp in green with height ≥50
10	Highest peak at 207.75 ±1.50 bp in green with height ≥50
11	Highest peak at 211.75 ±1.50 bp in green with height ≥50
12	Highest peak at 215.75 ±1.50 bp in green with height ≥50
13	Highest peak at 219.75 ±1.50 bp in green with height ≥50
14	Highest peak at 223.75 ±1.50 bp in green with height ≥50
15	Highest peak at 227.75 ±1.50 bp in green with height ≥50
16	Highest peak at 231.75 ±1.50 bp in green with height ≥50
17	Highest peak at 235.75 ±1.50 bp in green with height ≥50
18	Highest peak at 239.75 ±1.50 bp in green with height ≥50
19	Highest peak at 243.75 ±1.50 bp in green with height ≥ 50
20	Highest peak at 247.75 ±1.50 bp in green with height ≥50

FES

7	Highest peak at 206.59 ±1.50 bp in blue with height ≥50
8	Highest peak at 210.59 ±1.50 bp in blue with height ≥50
9	Highest peak at 214.49 ±1.50 bp in blue with height ≥50
10	Highest peak at 218.60 ±1.50 bp in blue with height ≥50
11	Highest peak at 222.74 ±1.50 bp in blue with height ≥50
12	Highest peak at 226.12 ±1.50 bp in blue with height ≥50
13	Highest peak at 230.54 ±1.50 bp in blue with height ≥50
14	Highest peak at 234.56 ±1.50 bp in blue with height ≥50
15	Highest peak at 238.39 ±1.50 bp in blue with height ≥50

Initials: RCJ Date: 2/6/98

Troubleshooting

Observation	Possible Cause	Recommended Action
1. No peaks or only weak peaks from both the positive control and the DNA test samples at all loci, but the red ladder peaks are present.	Prepared sample tubes with loading buffer and forgot to add PCR product or added only oil supernatant.	Rerun samples on a new gel
	No PCR amplification or insufficient PCR amplification of all markers, which can be caused by:	
	No DNA added or insufficient DNA added to PCR Reaction Mix.	Quantitate DNA and add 1-10 ng DNA; repeat test.
	Mg Cl_2 or deionized water not added to QUAD PCR Reaction Mix.	Add Mg Cl_2or deionized water; repeat test.
	GeneAmp PCR Instrument System failure or wrong program.	See GeneAmp PCR Instrument System Manual and check instrument calibration.
	Tubes not seated tightly in the DNA Thermal Cycler 480 block during amplification.	Push tubes firmly into contact with block after first cycle; repeat test.
	MicroAmp Base used with tray and tubes in GeneAmp PCR System 9600.	Removed MicroAmp Base; repeat test.

Initials: RCJ Date: 2/6/98

Observation	Possible Cause	Recommended Action
2. No peaks, only weak peaks or peaks missing from the positive controls, the DNA test samples at all loci, and the red ladder.	Hardware electrophoresis or gel problems	Rerun samples (see **Observation 2.1**)
	Collection or processing settings were wrong	Rerun samples (see **Observation 2.2**)
2.1 The whole gel is black.	No data got collected due to laser or scanner motor failure.	Check laser and scan motor action and rerun samples.
	Wrong gel matrix was used.	Make new gel; rerun samples.
	Forgot to load samples.	Make new gel; rerun samples
2.2 Not all expected ladder fragments and/or only part of the allele peaks are visible.	Preprocessing range was too small.	Under Analysis parameters check Preprocess parameters, correct to appropriate range and Preprocess again.
	Collection time or electrophoresis time too short.	Check the instrument setting for run time and the GS Collection setting for collection time, correct, and rerun samples on new gel.
	Wrong preparation of loading buffer e.g. insufficient mixing.	Rerun samples.

Initials: RCJ Date: 2/6/97

Observation	Possible Cause	Recommended Action
3. Positive signal from the positive controls, but no or below 50 signal from DNA test sample.	Quantity of DNA test sample is below the assay sensitivity.	Quantitate DNA and add 1-10 ng DNA; repeat test.
		Extract larger area of the stain to achieve higher DNA yield; repeat test.
		Concentrate DNA sample by Centricon centrifugation (see page 63 of the PCR manual); repeat test.
	Test sample contains PCR inhibitor (e.g. heme compounds, certain dyes).	Any or all of the following actions may be taken: use more Taq enzyme, dilute extract, use BSA, purify using Centricon (see page 63 of the manual for detailed procedures and more options).
	Test sample DNA is de graded.	Reamplify with an increased amount of DNA.
	Sample loss during the loading of the test sample.	Rerun sample on new gel.

Initials: RCJ Date: 2/6/98

Observation	Possible Cause	Recommended Action
4. Allele peaks for the positive controls and the test samples are visible but not sized correctly.	Size standard was not defined correctly.	Under Analysis open define size standard, check peak assignment and entered sizes. Reanalyze gel with correct standard, check each lane for proper peak assignment.
	Gel composition was wrong.	Prepare new polyacrylamide/ bisacrylamide solution, pour new gel and rerun samples.
	Samples were not loaded with formamide or not properly heat denatured before loading.	Rerun samples.
5. Presence of unexpected or additional peaks in the amplified positive controls sample.	Spill over from an adjacent lane.	Rerun sample on new gel.
	Contamination by amplified product or samples.	See **Reference 18** and **28**.
6. More than two alleles present for the test samples at one or more of the four loci.	Presence of a mixture of DNA, mixed sample or contamination.	See interpretation of Complex Quad STR Results Section
7. Some, but not all, loci are visible for the test samples.	Quantity of DNA test sample is below the assay sensitivity.	(See Troubleshooting **3**.).
	Test sample DNA is de graded.	(See Troubleshooting **3**.)
	Test sample contains PCR inhibitor (e.g. heme compounds, certain dyes).	(See Troubleshooting **3**.).

Initials: RCJ Date: 2/6/98

Observation	Possible Cause	Recommended Action
	Input DNA and/or PCR product was not denatured sufficiently during amplification.	Check calibration of the GeneAmp+ PCR Instrument System using the appropriate Temperature Verification System.
8. Imbalanced peak intensities within a locus on the electropherogram (the multiplex is designed to produce balanced peak heights within a locus when heterozygous samples are typed, except as described in interpretation section)	Presence of a DNA mixture, DNA degradation (see Interpretation section).	(see Interpretation section)

Initials: RCJ Date: 2/6/98

References

1. Applied Biosystems, Genescan 672 Software User's manual. The Perkin Elmer Corporation 1993.

2. ABI PRISM™ Genescan Analysis 2.1 User's manual. The Perkin Elmer Corporation 1996.

3. Applied Biosystems, Genotyper DNA fragment Analysis Software User's Manual Set. The Perkin Elmer Corporation, 1994.

4. ABI PRISM™ Genotyper 2.0 User's Manual. The Perkin Elmer Corporation, 1996.

5. Applied Biosystems, 373 DNA Sequencing System User's Manual. The Perkin Elmer Corporation, 1994.

6. ABI PRISM™ 377 DNA Sequencer User's Manual. The Perkin Elmer Corporation, 1995.

7. Lygo, J.E.; Johnson, P.E.; Holdaway, D.J.; Woodroffe, S.; Whitaker, J.P.; Clayton, T.M.; Kimpton, C.P.; Gill, P. The Validation of Short Tandem Repeat (STR) Loci for Use in Forensic Casework. Int. J. Leg. Med. (1994) 107: 77-89.

8. Kimpton, Colin; Fisher, Deborah; Watson, Stephanie; Adams, Maia; Urquhart, Andy; Lygo, Joan; Gill, Peter. Evaluation of an automated DNA Profiling System Employing Multiplex Amplification of Four Tetrameric STR Loci. Int. J. Leg. Med. (1994) 106: 302-311.

9. DNA recommendations-1994 report concerning further recommendations of the DNA Commission of the ISFH regarding PCR-based polymorphisms in STR (short tandem repeat) systems. Int. J. Leg. Med. (1994) 107: 159-160.

10. Whitaker, J.P.; Clayton, T.M.; Urquhart, A.J.; Millican, E.S.; Downes, T.J.; Kimpton, C.P.; Gill, P. Short Tandem Repeat Typing of Bodies from a Mass Disaster: High Success Rate and Characteristic Amplification Patterns in Highly Degraded Samples. Biotechniques, Vol.18 No. 4 (1995) p.671-677.

11. van Oorschot, R.A.H.; Gutowski, S.J.; Robinson, S.L. HUMTHO1: Amplification, species specificity, population genetics and forensic applications. Int. J. Leg. Med. (1994) 107: 121-126.

12. Kimpton, C.; Gill, P.; D'Aloja, E.; Andersen, J.F.; Bar, W.; Holgersson, S.; Jacobsen, S.; Johnsson, V.; Kloosterman, A.D.; Lareu, M.V.; Nellemann, L.; Pfitsinger, H.; Phillips, C.P.; Rand, S.; Schmitter, H.; Schneider, P.M.; Sternersen, M.; Vide, M.C. Report on the second EDNAP collaborative STR exercise. Forensic Science International 71 (1995) 137-152.

Initials: RCJ Date: 2/6/98

13. Kimpton, C.P.; Gill, P.; Walton, A.; Urquhart, A.; Millican, E.S.; Adams, M. Automated DNA Profiling Employing Multiplex Amplification of Short Tandem Repeat Loci. PCR Methods and Application, Cold Spring Harbor Laboratory Press, 1993,3: 13-22

14. Gill, P.; Ivanov, P.L.; Kimpton, C.; Piercy, R.; Benson, N.; Tully, G.; Evett, I. Hagerlberg, E.; Sullivan, K. Identification of the remains of the Romanov Family by DNA analysis. Nature Genetics Vol. 6, February 1994, p.130-135.

15. Gill ,P.; Kimpton, C.P.; Urquhart, A.; Oldroyd, N.; Millican, E.S.; Watson, S.K.; Downes, T.J. Automated short tandem repeats (STR) analysis in forensic casework - a strategy for the future. Electrophoresis 1995. 16: 1543-1552.

16. Leclair, B.; Carmody, G.; Fourney, R.M. Automated fluorescent STR technology: An update on troubleshooting and analytical precision issues. Poster presented at the Eight International Symposium on Human Identification, Promega Corp., Scottsdale, 1997.

17, Frazier, R.R.E.; Millican, E.S.; Watson, S.K.; Oldroyd, N.J.; Sparkes, R.L.; Taylor, K.M.; Panchal, S.; Bark, L.; Kimpton, C.P.; Gill, P.D. Validation of the Applied Biosystems Prism ™ 377 automated sequencer for forensic short tandem repeat analysis. Electrophoresis (1996) 17: 1550-1552.

Initials: RCJ Date: 2/2/98

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 STR, HLA DQA1 or PM alleles of the relevant evidence sample is different from the alleles of the suspect's reference sample, then the subject is "excluded", and cannot be the donor of the biological evidence being tested. An exclusion is independent of the frequency of the alleles in the population.

If the subject and evidence samples have the same alleles, then the subject 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 the alleles in the relevant population. Population frequencies are calculated separately for the Black, Caucasian and Hispanic populations and each population's frequency is listed in the laboratory report regardless of the population group of subject(s) in the case. Additional population frequencies may be used for other population groups. If a source contains more than one frequency for a single population group, than the highest frequency is used for calculations. Allele frequencies are used for all calculations. Locus frequencies are calculated according to the National Research Council report entitled *The Evaluation of Forensic DNA Evidence* (National Academy Press 1996, pp. 4-36 to 4-37). In the standard scenario, for each group, homozygotes are calculated using the formula $p^2+p(1-p)\theta$ for values of $\theta= 0.01$ and 0.03 and heterozygotes are calculated using the formula $2p_ip_j$. The overall frequency for each group is calculated by multiplying the individual locus frequencies if the loci are unlinked. If the loci are linked then only the locus with the lowest locus frequency is used in the calculation. In addition, locus frequencies are calculated for "evidence and subject from the same subgroup" and for relatives using the formulas in the National Research Council Report and $\theta= 0.01$ and $\theta= 0.03$. Overall frequencies are calculated as described above

Unless there is reason to subject that the "evidence DNA and subject are from the same subgroup" or a relative of the subject left the biological sample (and the relative is unavailable for testing) , only the standard scenario, overall frequency for each group with $\theta=0.03$ is listed in the report. The other calculations and allele frequencies are retained in the casefile for referral at a later date if necessary.

The overall frequency for each racial group is reported as occurring in 1 out of x individuals (i.e. 1/overall frequency). The overall frequency is reported to two significant digits. For example, 1 out of 12,345 would be reported as 1 out of 12,000, 1 out of 1,234 would be reported as 1 out 1,200 and 1 out of 123 would be reported as 1 out of 120. A Quattro Pro Spreadsheet is used to automate the calculation of the racial specific loci and overall frequencies. The spreadsheet is located in the popstat subdirectory on the network and explanations for its use are included with the spreadsheet

Initials: RCJ Date: 2/6/98

The population frequencies are derived from the following sources:

Locus	**Reference**
HLA-DQA1	Helmuth et. al (1990) Am J. Hum Genet. **47**: 515-523
HLA-DQA1 4 subtypes	PM+HLA DQA1 Kit Package Insert
PM loci	Budowle et. al (1995) J. For. Sciences **40**: 45-50.
VWA, THO1, F13A1, FES/FPS	OCME Database

Initials: RCJ Date: 2/6/98

Amplification 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 or amplify a larger aliquot of the DNA extract because the sample is highly degraded.

(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 extraction procedure in the RFLP manual followed by a Chelex extraction.

(6) For PM + DQA1 only, add BSA to a final concentration of 160 µg/mL in the amplification reaction.

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

a) Assemble the filter unit according to the manufacturer's directions and label each unit.

b) Add 1.5 mL TE^{-4} Buffer to the upper Centricon-100 reservoir.

c) Add the entire extracted DNA sample to the top of the TE^{-4} 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^{-4} 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.

Initials: RCJ Date: 2/6/98

d) Add 2 mL of TE^{-4} 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. QuantiBlot 5 µL. 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.

(8) Purify the extracted DNA on a Microcon ultrafiltration device as follows:

I. Microcon Operation for DNA Concentration

a) Insert Microcon sample reservoir into vial.

b) Fill out Microcon worksheet. Process 20μL of the appropriate extraction negative as an Microcon negative control.

c) Pipette 100 μL of TE^{-4} solution into labeled sample reservoir. Add DNA sample (0.4 mL maximum volume) to buffer. Don't transfer any Chelex beads, or in case of an organic extraction sample any organic solvent! Seal with attached cap. ***Avoid touching the membrane with the pipette tip!***

d) Place the assembly into an variable speed microcentrifuge. Make sure all tubes are balanced! ***To prevent failure of device, do not exceed recommended g-forces.***

i) Microcon-100 (blue colored top)-Spin at 500 x g (2500 RPM, Eppendorf) for 15 minutes at room temperature.

ii) Microcon-50 (rose colored top)-Spin at 14,000 x g (13,000 RPM, Eppendorf) for 4 minutes at room temperature.

e) For additional purification of the DNA sample add 200μL TE^{-4} and repeat step d), for concentration proceed to step f).

f) Remove assembly from centrifuge. Open attached cap and add 20μL TE^{-4}. ***Avoid touching the membrane with the pipette tip!*** Separate vial from sample reservoir.

g) Place sample reservoir upside down in a new labeled vial, then spin 3 minutes at 1000 x g (3500 RPM Eppendorf) for 3 minutes. Make sure all tubes are balanced!

Initials: JC Date: 2/6/98

h) Remove from centrifuge and separate sample reservoir. Measure resulting volume using an adjustable Micropipette, adjust volume to 50μL using TE^{-4}. Calculate resulting concentration. Snap sealing cap shut to store product for later use.

II. Microcon Operation for DNA Concentration and Clean-up

a) Place a Micropure-EZ into a Microcon sample reservoir and insert both into a vial.

b) Pipette solution into sample reservoir (250 µL maximum volume). Seal with attached cap. ***Avoid touching the membrane with the pipette tip!***

c) Place the assembly into an variable speed microcentrifuge. Make sure all tubes are balanced! Spin at for 30 sec at room temperature at the following speeds. ***To prevent failure of device, do not exceed recommended g-forces.***

i) Microcon-100 (blue colored top)-Spin at 500 x g (2500 RPM, Eppendorf)

ii) Microcon-50 (rose colored top)-Spin at 14,000 x g (13,000 RPM, Eppendorf).

d) Add 100 µL TE^{-4} to the Micropure-EZ reservoir.

e) Microcon -100- Spin for 15 minutes at room temperature at the above speed.
Microcon -50- Spin for 4 minutes at room temperature at the above speed.

f) Remove and discard Micropure-EZ

g) Add 500 µL of sterile water or TE^{-4} to the Microcon and spin at the appropriate speed listed in section c and the appropriate time listed in section e.

f) Remove assembly from centrifuge. Open attached cap and add 20μL TE^{-4}. ***Avoid touching the membrane with the pipette tip!*** Separate vial from sample reservoir.

g) Place sample reservoir upside down in a new labeled vial, then spin 3 minutes at 1000 x g (3500 RPM Eppendorf) for 3 minutes. Make sure all tubes are balanced!

h) Remove from centrifuge and separate sample reservoir. Measure resulting volume using an adjustable Micropipette, adjust volume to 50μL using TE^{-4}. Calculate resulting concentration. Snap sealing cap shut to store product for later use.