PROTOCOLS FOR FORENSIC STR ANALYSIS

VERSION 7

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

Initials: RG Date: 5/16/00

TABLE OF CONTENTS

Initials: BG Date: 5/16/00

Initials: RC Date: 5/16/00

Initials: RCQ Date: 5/16/00

General PCR Guidelines

Body fluid identification

1. The general laboratory policy is to identify the stain type, i.e., usually blood, semen, or saliva (see the Biochemistry Manual) before individualization is attempted. However, circumstances will exist when this may not be possible.

2. A positive and interpretable QuantiBlot and/or STR result can be considered primate positive. Identification of the specific physiological fluid may be accomplished using the procedures described in the Biochemistry Manual.

Laboratory organization

3. 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.

4. 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.

5. Dedicated equipment such as pipettors should not leave their designated areas. Only the samples in designated racks should move between areas.

Microfuge tube and pipet handling

6. Avoid splashes and aerosols. Centrifuge all liquid to the bottom of a closed microfuge tube before opening it.

7. Avoid touching the inside surface of the tube caps with pipets, gloves, or lab coat sleeves.

8. Use the correct pipet for the volume to be pipetted. Generally, the range of a pipet begins at 10% of its maximum volume (i.e., a 100 uL pipet can be used for volumes of 10 - 100 uL).

9. Use filter pipet tips for pipetting all DNA and use whenever possible for other reagents. Use the appropriate filter tips for the different sized pipets; the tip of the pipet should never touch the filter.

10. Always change pipet tips between handling each sample.

Initials: RCS Date: 5/16/00

11. Never "blow out" the last bit of sample from a pipet. 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. Discard pipet tips if they accidentally touch the bench paper or any other surface.

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

Sample handling

14. 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.

15. 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.

16. Use disposable bench paper to prevent the accumulation of human DNA on permanent work surfaces. 10% bleach should be used periodically to decontaminate exposed work surfaces.

17. 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.

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

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

Controls for PCR analysis

20. The following controls have to be processed along side the sample analysis:
    a. A positive control is a DNA sample where the STR alleles for the relevant STR loci are known. The positive control tests the success and the specificity of the amplification, and during the detection and analysis stage the electrophoretical separation and the correct allele calling.

       A positive control has to be included with every batch of amplified samples, every gel run and with every set of samples run on a CE that will be analyzed in one genotyper file. If the positive control has been shown to give the correct type, this confirms the integrity of the amplification. If individual samples from this amplification set need to

be re-analyzed on a second gel or CE run the positive control does not have to be repeated, if another positive control is included in the run.

If the failure of a positive control is based on an amplification problem, all samples in that set are inconclusive and have to be re-amplified. In case of a failure due to an electrophoresis problem, e.g. the alleles are present but called incorrectly, all samples of that run have to be re-run.

b. An extraction negative control consists of all reagents used in the extraction process, and is necessary to detect DNA contamination of these reagents. A clean result - the absence of detectable DNA - on an extraction negative in one of the PCR multiplexes is sufficient to show the absence of contamination.

If peaks attributed to DNA are detected in an extraction negative control, the first step is to repeat the amplification to test if the extract is truly contaminated or if something occurred during a later stage. If the peaks could be caused by spill over from an adjacent lane, it may be possible to just re-run the extraction negative in order to show that it was actually clean. Only if DNA peaks are still present following the second amplification, all samples have to be re-extracted.

c. An amplification negative control consists of only amplification reagents without the addition of DNA, and is used to detect DNA contamination of the amplification reagents. If peaks attributed to DNA are detected in an amplification negative control, all samples in that amplification are inconclusive and have to be repeated. The only exception is, if the peaks might be have been caused by spill over from an adjacent lane and the amplification negative can be shown to be clean by repeating the electrophoresis step.

Concordant analyses and the triplicate rule

21. The general laboratory policy is to confirm DNA results by having concordant DNA results within a case - confirmation of a match or exclusion being the most common situation. **Consistent DNA typing results from at least one locus in a different amplification (same DNA system or a different DNA system) is considered a concordant analysis.** Concordant analysis is also used to detect sample mix-up and confirm the presence of DNA mixtures. Depending on the case and sample, the policy can be satisfied by:

Two separate Quad amps and typing
Quad amp and typing followed by Cofiler, ProfilerPlus, or Y M1 amp and typing
Two separate Y M1 amps and typing

Initials: RCJ Date: 5/16/00

In many cases, the "triplicate rule" supplies the concordant results: identical DNA profiles among at least three items (three evidence samples or two evidence samples plus an exemplar) within a case is considered internally concordant results.

A. For evidence samples, the following guidelines apply:

1. An epithelial cell fraction or swab remains fraction of a stain or a swab that matches the victim in the case does not require further analysis.

2. Identical DNA profiles among at least three items (three evidence samples or two evidence samples plus an exemplar) within a case is considered internally concordant results.

3. If after the first DNA analysis there is no evidence of a mixture, then further analysis (if needed) may begin at the extraction stage or the amplification stage. If there are additional alleles then either 4 or 5 (see below) apply.

4. If after the first DNA analysis there are additional alleles less than 50 (gel based systems: Quad, YM1, Profiler Plus) or less than 100 (capillary based systems: Cofiler), then further analysis (if needed) may begin at the extraction stage or the amplification stage.

5. If after the first DNA analysis, there are three or more alleles greater than 50 (gel based systems) or greater than 100 (capillary based systems), a mixture of DNA is present. To confirm the presence of a mixture the sample must have further analysis. The further analysis must begin at the amplification stage, in either the same DNA system or a different DNA system.

B. For exemplar samples, the following guidelines apply:

1. If the DNA profile of an exemplar does not match any of the DNA profiles of evidence samples in the case, including mixtures, the exemplar has to be duplicated to eliminate the possibility of an exemplar mix-up. *This is because it is highly likely that an exemplar mix-up would generate a false exclusion.*

   The exemplar must be duplicated starting with a second independent extraction, with the exemplar cut and submitted for extraction by a different analyst. If there is no additional exemplar material available, the duplication may begin at the amplification stage.

Initials: RCJ Date: 5/16/04

Since duplicate exemplar analyses are performed to confirm the exclusion, a partial DNA profile (at least two complete loci) that demonstrates an exclusion is sufficient.

2. If the DNA profile of an exemplar matches any of the DNA profiles of evidence in the case, or is present in an mixture, the exemplar does not have to be duplicated. *This is because it is highly unlikely that a sample mix-up would generate a false inclusion.*

C. For evidence samples or exemplar samples analyzed in DNA systems containing overlapping loci, the DNA results for the overlapping loci must be consistent.

DNA storage

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

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

24. Amplified DNA is discarded after the Genotyper analysis is completed.

25. DNA extracts are retained refrigerated for a period of time, then frozen for long-term storage.

Miscellaneous

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

27. Use the Thermal Cycler only for amplification of DNA.

Initials: RCJ Date: 5/16/00

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.

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 negatives 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 negative control with each batch of extractions to demonstrate extraction integrity. The extraction negative control contains water in place of biological fluids or stains. If DNA is found in the extraction negative 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 negative controls are treated as normal samples and extracted, amplified and typed along with the test samples.

9. If a sample is found to contain <0.15 ng/20 µL of DNA by QuantiBlot analysis the sample should not be amplified. 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.

Initials: RCJ Date: 5/16/00

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

Initials: RG Date: 5/16/00

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: 5/16/00

Chelex DNA Extraction From Blood, Tissue, or Saliva

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 neat and also a 1/10 dilution 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: RCS Date: 5/16/00

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 13) beginning with Step 10.

Initials: RCJ Date: 5/16/00

Non-differential Chelex DNA Extraction from Semen Stains or Swabs

1. Remove the extraction rack from the refrigerator.
2. Pipette 1 mL of autoclaved deionized water 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 shortly and spin in a microcentrifuge for 5 minutes at 10,000 to 15,000 x g.
6. Without squeezing the substrate, remove most of the supernatant.
7. Add 175 µL of 5% Chelex, 1 µL of 20 mg/mL Proteinase K, and 7 µL of 1M DTT. to all tubes. Use the pipette tip when adding the DTT to thoroughly mix the contents of the tubes.
8. Incubate at 56°C for approximately 2 hours.
9. Vortex at high speed for 10 to 30 seconds.
10. Incubate in at 100°C for 8 minutes using a screw down rack.
11. Vortex at high speed for 10 to 30 seconds.
12. Spin in a microcentrifuge for 2 to 3 minutes at 10,000 to 15,000 x g.
13. Store the extract at 2 to 8°C or frozen.
14. Do not submit these extracts for QuantiBlot.

Initials: RCJ Date: 5/16/05

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 overnight or for a minimum of 1 hour minutes using a shaking platform.

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 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 11. 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).

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

Initials: RCS Date: 5/16/00

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 negative 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 21.

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.

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

Initials: RCS Date: 5/16/00

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 neat and also a 1/10 dilution 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: RG Date: 5/16/00

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 neat and also a 1/10 dilution 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: RS Date: 5/16/00

Organic Extraction Procedure

A Proteinase K Incubation for Blood and Bone Marrow

1. Process an extraction negative with every batch of extractions.

2. Add 0.7 mL of well mixed blood or bone marrow if liquid, otherwise a 1.5 x 1.5 cm scoop of bone marrow, to a microcentrifuge tube.

3. Add 1.0 mL CLB. Vortex.

4. Centrifuge samples for 1 minute.

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

6. Prepare a master mix for N+2 samples:

	1 Sample	5 Samples	25 Samples	50 Samples
0.2M Na Acetate	375 µL	1.9 mL	9.5 mL	19.0 mL
10% SDS	25 µL	125 µL	625 µL	1.3 mL
Proteinase K (20 mg/mL)	5 µL	25 µL	125 µL	625 µL

7. Add 400 µL of the master mix to each pellet. Vortex briefly.

8. Place tubes in a 56°C heat block and incubate for 1 hour.

Go to section D for the Phenol Chloroform Extraction and Ethanol Precipitation

Initials: RG Date: 5/16/00

B Proteinase K Incubation for Bloodstains and Tissue

1. Process a extraction negative control extractions for every batch of samples.

2. Stains and tissues up to 1.5 x 1.5 cm can be extracted. Trim excess unstained fabric from stains.

   If a stain is very diffuse or is on a bulky substrate, the extraction can be scaled up appropriately.

   For swabs remove cotton swab from applicator stick with a razor blade.

   For scrapings, use an amount to approximate 10 - 50 µL.

3. Cut stained fabric into small pieces (3 x 3 mm). Mince tissues (<1 mm^2) with a new razor blade into small pieces. The mincing should be done in a weigh boat in an ice bath.

4. Place fabric, swab, tissue, or scrapings into a microcentrifuge tube.

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

6. Prepare a master mix of Stain Extraction Buffer and Proteinase K for N+2 samples.

	1 Sample	5 Samples	25 Samples	50 Samples
Stain Extraction Buffer	400 µL	2.0 mL	10.0 mL	20.0 mL
Proteinase K (20 mg/mL)	10 µL	50 µL	250 µL	500 µL

7. Add 410 µL of master mix to fabric, tissue or cotton swab. Make certain the fabric, tissue, or swab is totally submerged.

8. Place tubes in a 56°C heat block and incubate for 14-18 hours. Occasionally, vortex at high speed for 30 seconds to insure the fabric or tissue is resuspended or use a shaking 56°C heat block..

9. If the tissue is not dissolved or the stain not removed from the fabric, add 10 µL Proteinase K (20 mg/mL). Place the tubes in a 65°C heat block and incubate for 2-2.5 hours. During

Initials: RCS Date: 5/16/00

this period, vortex at high speed for 30 seconds every 15-20 minutes to insure tissue is resuspended.

10 Make sure the caps are on tight. Using a clean, sterile needle, punch a hole in the bottom of each tube (heating the needle makes punching the hole much easier).

11 Piggyback the tube containing the sample inside a new labeled tube. Collect the lysate by centrifuging at 2700 x G in a swinging bucket rotor for 2 minutes. Save the tube containing the fabric or swab until after looking at the DNA on a yield gel.

Go to section D for the Phenol Chloroform Extraction and Ethanol Precipitation

Initials: RO Date: 5/16/00

C Proteinase K Incubation for semen stains and swabs

1. Make sure that you process an extraction negative control for both cell fractions.

2. Stains up to 1.5 x 1.5 cm can be extracted. Trim excess fabric from stain.

   If a stain is very diffuse or is on a bulky substrate, the extraction can be scaled up appropriately.

   For swabs remove cotton swab from applicator stick with a razor blade.

3. Cut stained fabric or swab into small pieces (3x3 mm).

4. Place fabric or swab into a 1.5 mL microcentrifuge tube.

5. Prepare a master mix for N+2 samples as follows:

	1 Sample	5 Samples	25 Samples	50 Samples
1X TNE	400 µL	2.0 mL	10.0 mL	20.0 mL
20% Sarkosyl	25 µL	125 µL	625 µL	1.3 mL
Sterile H_2O	75 µL	375 µL	1.9 mL	3.8 mL
Proteinase K (20 mg/mL)	5 µL	25 µL	125 µL	250 µL

6. Add 500 µL of master mix to each sample.

7. Place the tubes in a 37°C heat block and incubate for 2 hours.

8. Make sure the caps are on tight. Using a clean, sterile needle, punch a hole in the bottom of each tube (heating the needle makes punching the hole much easier).

9. Piggyback the tube containing the sample inside a new labeled tube. Collect the lysate by centrifuging at 2700 x G in a swinging bucket rotor for 2 minutes. Save the tube containing the fabric or swab until after looking at the DNA on a yield gel. Optional- If the substrate remains are to be re-extracted for additional bound sperm, transfer the fabric or swab to a new marked tube.

Initials: RCS Date: 5/16/00

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

11. Without disturbing the pellet, pipet 450 µL of the supernatant in into a new labeled tube. This supernatant is called the epithelial cell fraction and is enriched for non-sperm DNA. Place the epithelial cell fraction and cell pellet control on ice until step 17. Discard the sperm fraction of the cell pellet control. In this step, the negative control is treated like a sample even though it should not have a pellet. The sperm negative is derived from the remaining lysate in the negative control and the epithelial negative is derived from the 450µL of supernatant which was pipetted off.

12. Wash the sperm pellet with TNE as follows:
    a. Resuspend the pellet in 0.5 mL TNE 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 3 times.

13. Resuspend the pellet in the remaining liquid. 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.

14. Prepare a master mix for N+2 samples as follows:

	1 Sample	5 Samples	25 Samples	50 Samples
1X TNE	150 µL	750 µL	3.8 mL	7.5 mL
20% Sarkosyl	50 µL	250 µL	1.3 mL	2.5 mL
0.39 M DTT	40 µL	200 µL	1.0 mL	2.0 mL
Sterile H_2O	150 µL	750 µL	3.8 mL	7.5 mL
Proteinase K (20 mg/mL)	10 µL	50 µL	250 µL	1.3 mL

15. Add 400 µL of the master mix to each pellet and optionally to the substrate remains.

16. Place the tubes in a 37°C heat block and incubate for 2 hours.

Go to section Organic Extraction Section D for the Phenol Chloroform Extraction and Ethanol Precipitation

Initials: RG Date: 5/16/00

D Phenol Chloroform Extraction

1. Add 400 µL Phenol. This step must be done in the fume hood. Shake the tube vigorously by hand or vortex to achieve a milky emulsion in the tube.

2. Centrifuge samples 2 minutes in a microcentrifuge at room temperature.

3. Carefully transfer the aqueous phase (top layer) to a new labeled tube. Do not disturb the layer of denatured protein that collects at the interface. Discard the organic layer into a waste bottle in the hood.

4. Repeat steps 10-12 with Phenol/Chloroform/Isoamyl Alcohol and then Chloroform/Isoamyl Alcohol.

5. If the sample is very discolored or dirty, repeat steps 10-13.

6. Add 1.0 mL, absolute EtOH. Mix by inversion of the tube. Place the tube at room temperature for at least 30 minutes. **The tubes can be stored indefinitely at this stage.**

7. Centrifuge for 15 minutes in a microcentrifuge at room temperature.

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

9. To the pellet, add approximately 1.0 mL room temperature 70% EtOH. Centrifuge samples 5 minutes in a microcentrifuge at room temperature.

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

11. Dry the pellet in the Speed-Vac centrifuge for 10 minutes to remove remaining EtOH.

12. Add 250 µL TE, mix, and resolubilize by placing tubes in a 56°C heat block and incubating for minimum of 2 hours.

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

14. Continue with the protocol on the next page, Organic Extraction Section E.

Initials: RG Date: 5/16/00

E Estimation of DNA Quantity And Quality From Yield Gel

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

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

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

   b. Bring the flask to a boil in the microwave (2.5-3 minutes on high) to dissolve the agarose. Make sure the agarose is completely dissolved.

   c. Add 20 µL of ethidium bromide.

   d. Allow to cool to approximately 56°C.

   e. Pour agarose into the tank (be sure comb(s) are in place).

   f. Let the gel cool for approximately 30-60 minutes.

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

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

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

5. Place the tubes in a 65°C heat block and incubate for 5 minutes.

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

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

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

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

Initials: RCS Date: 5/15/00

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

9. Load each row of each gel as follows:

Lane	Volume	Material	Description
1	10 µL	Lambda Marker	Hind III digested DNA
2	10 µL	Yield Calibrator A	30 ng/µL DNA (300 ng total)
3	10 µL	Yield Calibrator B	20 ng/µL DNA (200 ng total)
4	10 µL	Yield Calibrator C	10 ng/µL DNA (100 ng total)
5	10 µL	Yield Calibrator D	5 ng/µL DNA (50 ng total)
6	10 µL	Yield Calibrator E	2.5 ng/µL DNA (25 ng total)
7	10 µL	Yield Calibrator F	1 ng/µL DNA (10 ng total)
8	10 µL	Calibration Control	75 ng high M.W. human DNA
9-30	12 µL	Samples	Unknown samples

Include the Extraction Negative Control as samples.

9. Set the voltage at 100 volts on the dial. When the Bromophenol blue tracking dye has moved 4-5 cm (approx. 1.5-2 hours) from the origin, the run can be stopped.

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

Take a photograph of the gel using Polaroid film. For type 667 film the settings are *f*5.6 for ½-1 second. DO NOT EXPOSE YOURSELF TO THE UV LIGHT FOR AN EXCESSIVE AMOUNT OF TIME. ALWAYS WEAR U.V. GOGGLES WHEN WORKING WITH THE TRANSILLUMINATOR.

11. Interpretation:

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

Initials: RCS Date: 5/16/00

b. Compare the intensity of the Calibration Control (lane 8) to that of the Yield Calibrators. If the intensity is between Yield Calibrators C and D (lanes 4-5), the result is valid. Otherwise the gel must be repeated.

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

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

d. Show the yield gel to another analyst to independently determine DNA quality, concentrations, and yields. Disagreements should be settled by a supervisor.

	DNA CONCENTRATION[2]		
Sample Intensity[1]	2 µL Sample Loaded On Yield Gel	5 µL Sample Loaded On Yield Gel	10 µL Sample Loaded On Yield Gel
≥A	see note 3 below	see note 3 below	Repeat yield gel loading 2 µL of sample
B	100 ng/µL	40 ng/µL	20 ng/µL
C	50 ng/µL	20 ng/µL	10 ng/µL
D	25 ng/µL	10 ng/µL	5 ng/µL
E	12.5 ng/µL	5 ng/µL	2.5 ng/µL
≤F	Repeat yield gel loading 10 µL of sample	Repeat yield gel loading 10 µL of sample	≤1 ng/µL

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

Initials: RG Date: 5/16/00

Chelex Extraction From Organically Extracted Samples

The amount of DNA in organically and organically extracted samples is usually quantitated by yield gel. 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 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: 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 organically extracted DNA samples.

Initials: RG Date: 5/16/00

3. Add in order, the appropriate amount of dH_2O, 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 neat and also a 1/10 dilution into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration.

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

Initials: AG Date: 5/16/00

MICROCON DNA concentration and purification

Microcon 100 filter membranes have a pore size that retains all DNA fragments >100bp. This filtration device can be used to concentrate a sample (e.g. when the quantification reads <0.15ng/20µL) by simply removing excess liquid, or to purify a sample from low molecular weight contaminants (e.g. if the QuantiBlot is inconclusive or an amplification inhibitor is suspected). After the liquid reduction the volume might be as small as 2-5µL, it is therefore necessary to bring the volume back up by adding TE^{-4} . In order to allow duplicate amplifications the final volume shouldn't be smaller than 50µL. ***Do not store the DNA in the Microcon vials!*** The lids are not tight enough to prevent evaporation.

1. Label a sufficient number of blue Microcon sample reservoirs and insert them into the vials.

2. Fill out Microcon worksheet. Process 40µL of the appropriate extraction negative as an Microcon negative control.

3. 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!***

4. 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.***

   -Spin at 500 x g (2500 RPM, Eppendorf) for 15 minutes at room temperature.

5. For **purification** of the DNA sample add 200 µL of TE^{-4} solution and repeat step 4). Do this as often as necessary and when finished add enough TE^{-4} to reconstitute the starting volume, for **concentration** only proceed to step 6).

6. 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.

7. 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!

8. Remove from centrifuge and separate sample reservoir. Measure resulting volume using an adjustable Micropipette, adjust volume to 50µL using TE^{-4}. Transfer the DNA extracts and the microcon negative control to new labeled 1.5mL tubes and store extract for later use.

9. Calculate resulting concentration or submit 10µL for QuantiBlot.

Initials: RCJ Date: 5/16/00

Estimation of DNA Quantity from QuantiBlot Analysis

Sample Blotting

1. Vortex all samples including DNA Standards and 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. All other samples - 20 µL and 1/10 dilutions (with a final volume of 20 µL). When necessary, 1/100 and 1/1000 dilutions may also be run. Prepare all necessary dilutions in TE^{-4}. The samples can be aliquoted ahead of time and stored at 4°C.

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

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. Note: Either side of the Biodyne B membrane can be used as the side onto which samples are pipetted. **Always wear gloves when handling the membrane.**

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 vacuum pump to a vacuum pressure of approximately 200 to 250 mm

Initials: RCS Date: 5/16/00

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. **Note: Do not allow the pipet tip to touch the membrane since this may compromise the membrane at that spot.**

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 negative control (negative)
2A	3.5 ng Calibration 1 Std.
2B	0.5 ng Calibration 2 Std.
2C	0.15625 ng standard
2D-6F	samples and controls
6G	3.5 ng Calibration 1 Std.
6H	0.5 ng Calibration 2 Std.

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.

Initials: RCS Date: 5/16/00

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

9. 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. Following soaking in SDS, rinse the gasket and the side of the top plate that contacts the membrane **thoroughly** with H_2O (include a final rinse with deionized H_2O). Make sure that all the wells of the top plate are rinsed and clean. Rinse the bottom unit of the slot blotter with deionized H_2O and allow to dry at room temperature. **Never use bleach.**

10. Transfer the membrane to 100 mL of pre-warmed QuantiBlot 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 (eg. 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

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

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

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

13. 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.

14. 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.

15. 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

16. 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.

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

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

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

19. Stop the color development by washing in approximately 100 mL deionized H_2O. Repeat several times. After the last wash, store membrane in deionized H_2O. Cover tray with lid and proceed with photography.

Photography

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

21. Use a Polaroid MP4 camera system with type 667 or 664 film and a Wratten 23A or 22 (orange) filter.

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

Initials: RG Date: 5/16/00

23. Photograph at 1/125 seconds and f8 for type 667 film. Photograph at 1/2 second and f5.6 for type 664 film.

24. 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.

    Indicate the appropriate columns and rows on front of the photograph. Also, initial and date the photograph.

25. Attach the photograph to the QuantiBlot worksheet. Once the QuantiBlot passes review (see below), make photocopies and distribute to the appropriate analysts. File the original QuantiBlot worksheet along with photograph in the appropriate binder. Discard the membrane.
    Note: If an entire QuantiBlot or a portion of it does not pass review (see below), it is the responsibility of the analyst on the QuantiBlot rotation to resubmit those samples that are in question.

Quality Control

1. **The DNA standards should yield band intensities that decrease approximately two-fold for each subsequent dilution (see below for exceptions).**

2. Compare the intensities of calibrator 1 (3.5 ng) and calibrator 2 (0.5 ng) to those of the DNA standards. The intensity of calibrator 1 should be between 2.5 and 5 ng; the intensity of calibrator 2 should be between 0.31 and 0.62 ng. Calibrators 1 and 2 are loaded in duplicate on the membrane. At least three of these four calibrator samples must be consistent with the DNA ladder and each other. **If more than one of the calibrator samples are incorrect, the membrane must be repeated**.

3. Estimate the quantity (ng) loaded for each sample by comparing band intensities of the unknown samples 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.**

    It is recommended to run the neat and 1/10 dilution for each sample. As a general rule in determining the DNA quantity of an unknown sample, the band intensity must be less than the largest DNA standard (10 ng). If the sample band intensity is ≥ 10 ng, multiply the reading of the 1/10 dilution by 10 to determine the DNA quantity for that sample. If the sample band intensity of the 1/10 dilution is ≥ 10 ng, then submit 1/100 and 1/1000 (if necessary) dilutions for analysis. To determine the DNA quantity for these samples, multiply the 1/100 and 1/1000 dilution readings by 100 and 1000, respectively.

Initials: RCJ Date: 5/16/00

4. **Membranes that have compromised DNA standards may pass review, however, care must be taken in the interpretation of the results.** Examples of compromised DNA standards include bands that are not visible, nonuniform signal intensity within a slot, and samples that yield a band intensity that is not consistent with that of the other DNA standards.

The following guidelines must be followed:

a. At least one of the 0.15 ng standards must be visible. If not, samples that show band intensities of <0.31 ng and samples with no apparent DNA must be repeated, including the extraction negative control(s).

b. If one of the DNA standards other than 0.15 ng produces a band intensity that is not consistent with the other DNA standards and the DNA calibrators, then sample readings that fall at or near the amount of the compromised standard, and are between the adjacent two standards (one on either side of the compromised standard) are not valid for that membrane.

For example, if the 2.5 ng DNA standard is compromised, the sample readings that are <5 ng but >1.25 ng are not valid for that membrane. If the 10 ng DNA standard is compromised, sample readings >5 ng are not valid for that membrane.

c. If more than one of the DNA standards are compromised, the membrane can pass review if four consecutive DNA standards are correct as determined by comparing band intensities to those of the DNA calibrators. In such a case, the membrane will be valid for the determination of DNA sample amounts that yield values at or within those four DNA standards.

For example, if the 0.625, 0.31, and 0.15 ng DNA standards are compromised, the membrane in question is still valid for readings ≥1.25 ng given that the 10, 5, 2.5, and 1.25 ng DNA standards are consistent with the concentrations of DNA calibrators 1 and 2.

5. If the membrane fails review for the determination of DNA amounts, it can still be used as a guide in the resubmission of neat samples and/or dilutions. Also, any bands that are produced on the membrane are indicative of the presence of human DNA*, provided that the corresponding plate negative and extraction negative controls do not produce a signal.

6. Submit a photograph and QuantiBlot Worksheet for review by the QuantiBlot Station supervisor.

* Non-human primate DNA may give comparable results to that of human DNA using this procedure (Perkin Elmer Corp. 1996).

Initials: RCJ Date: 5/16/00

Quantiblot interpretation

1. The Quantiblot procedure, done properly, gives reproducible and fairly accurate determinations of the total amount of human DNA. The neat sample and 1/10 dilution should give quantitation results that make sense - the neat and 1/10 samples should correlate with one another.

neat	1/10 dilution	1/10 dilution
	results OK	**review photo to determine best concentration**
≥ 10	5, 2.5, 1.25	0.62
5	0.62	1.25, 0.31
2.5	0.31	0.62, 0.15
1.25	0.15	0.31, blank
0.62	< 0.15	0.15
0.31	blank	
0.15	blank	

2. If you have any pairs of results other than those above, you need to take steps to correct the problem **before** amplification. A review of the photograph may be all that is needed to determine the discrepancy of the results (eg. incorrectly called results, nonuniform signal intensity, thick or thin bands) and obtain a reasonable estimate of the DNA concentration.

1. If neat and 1/10 dilution are both ≥ 10 ng, submit additional dilutions for Quantiblot.
2. If neat and 1/10 dilution are of equal intensity, resubmit neat and 1/10 dilution for Quantiblot.
3. If neat and 1/10 dilution are too far apart in intensity (eg. 5 and 0.15 ng, 2.5 ng and blank), resubmit neat and 1/10 dilution for Quantiblot.
4. If neat and 1/10 dilutions are both "*" due to colored impurities, then the sample may need cleaning up using a Microcon spin filter followed by Quantiblot.

Initials: RCJ Date: 5/16/00

Troubleshooting of QuantiBlot
(taken from the Perkin Elmer QuantiBlot package insert, see QuantiBlot references)

	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: RCS Date: 5/16/00

	Observation	Possible Cause	Recommended Action
2.	Areas of low sensitivity across the membrane.	Membrane slipped up onto the side of the Hybridization Tray during Hybridization or Stringent Wash steps.	Reduce the rotation rate of the water bath to 50-60 rpm. Check that the membrane is fully submerged in the bottom of the Hybridization Tray before shaking.
		Membrane dried-out significantly at some point in the protocol.	Do not allow the membrane to dry at any point in the protocol.
3.	Non-uniform signal intensity within a slot.	Bubbles(s) in slot blot wells when sample was pipetted into well, or when vacuum was applied.	Slowly pipet the Spotting Solution directly over the center of the wells of the slot blot apparatus, with the pipet tip raised approximately 5 mm above the membrane. Turn on the sample vacuum slowly, not all at once. After being drawn through the membrane, the sample should appear as a uniform blue band on the membrane. If the entire sample is not drawn through the membrane, turn off the sample vacuum. Pipet the sample back into the pipet tip; then pipet the sample back into the well of the slot blot apparatus. Turn on the sample vacuum to draw the sample through the membrane.
4.	Filter background	No or low SDS in the Hybridization Solution or in the Wash Solution	Prepare solutions with proper concentrations of SDS.

Initials: RCS Date: 5/16/00

	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 each dilution to 150 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: RCS Date: 5/16/00

PCR amplification

A positive control, an amplification negative control, an extraction negative control, and a female negative control, if applicable, should be included with each batch of samples being amplified to demonstrate procedural integrity. The positive control is a control whose alleles are known to the analyst. Samples that were extracted together should all be amplified together, so that every sample is run parallel to the extraction negative control.

Do not forget to sign the DNA Extracts Chain of Custody sheet when removing a sample from its storage location.

The STR PCR reaction mix already contains BSA. **Do not add additional BSA.**

Follow the general PCR guidelines for handling the tubes and cleaning of the work surface. The following steps have to be performed in the appropriate dedicated areas. Evidence samples and exemplars should not be handled at the same time.

A Preparing DNA aliquots for the amplification

1.) Do not amplify samples in which insufficient DNA was detected by QuantiBlot (0 or <0.15ng/20µL).

2.) The target amounts to be amplified are 1 ng of DNA for the QUAD, Profiler Plus and Cofiler multiplexes, and 2ng for the Y-STR systems. Due to the varying volumes of reaction mix and magnesium chloride for the different multiplex reactions, the DNA aliquots have to be adjusted accordingly to achieve a final volume of 50µL. Follow the appropriate tables when setting up the aliquots.

3.) For each sample to be amplified, label a new tube. Add DNA and TE^{-4} and as specified in Tables II, IV, VI, and VII.

4.) Remember the following general rules:

If the neat and 1/10 QuantiBlot results are ≥10 ng/20 µL, dilute the sample 1/100 with TE^{-4} and re-quantitate. Multiply the diluted concentration by 100 to obtain the original sample concentration. Repeat the procedure if the diluted sample is still ≥10 ng/20 µL.

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. *If possible always amplify the optimal target amount of 1ng (2ng for YM1).*

5.) Tables III, V, and VII list the preparation of the controls for each system.

Initials: AS Date: 5/16/00

Quad multiplex

Table II - 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	0.2 - 8**
6.2	3.3	23.7	0.5 - 16**
5	4.0	23.0	0.6 - 20**
3.1	6.5	20.5	1.0 - 27**
2.5	8.0	19.0	1.2 - 27**
1.5	13.3	13.7	2.0 - 27**
1.25	16.0	11.0	2.4 - 27**
0.62	27.0	0	4.8 - 27**
0.31	27.0	0	9.7 - 27**
0.15	27.0	0	20 - 27**
< 0.15	Do not amplify	-	-

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

Table III - Control samples for the Quad multiplex

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

Initials: RS Date: 5/16/00

AmpFlSTR Cofiler and Profiler Plus Kits

Table IV - Amount of DNA to be Amplified for the AmpFlSTR Cofiler and Profiler Plus Kits

DNA Concentration (ng/20µL)	Target Volume (µL) to be amplified	TE^{-4} (µL) for Target Volume	Range of Volumes (µL) which can be amplified
≥25	Dilute 1/10 and use the dilution for amplification		
12.5	1.6	18.4	0.5-8**
6.2	3.2	16.8	0.5-16**
5	4	16	0.6-20**
3.1	6.5	13.5	1.0 - 20**
2.5	8	12	1.2-20**
1.5	13.3	6.7	2.0 - 20**
1.25	16	4	2.4-20**
0.62	20	0	4.8-20**
0.31	20	0	9.6 -20**
0.15	20	0	19.2-20**
< 0.15	Do not amplify	-	-

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

Table V - Control samples Cofiler and Profiler Plus Kits

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

Initials: RCJ Date: 5/16/00

Y STR multiplex YMI

The amplification of exemplars, sperm cell fractions of samples extracted by differential lysis and semen stains, where no epithelial cells were seen during the differential lysis, is based on the QuantiBlot results (see Table VI). Semen positive swabs taken from female individuals that were extracted using the non-differential semen extraction and the swab remains fractions of differential lysis samples are amplified using the amounts specified in Table VII.

Table VI - Amount of DNA to be amplified for the Y M1 based on QuantiBlot results.

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	3.2	22.8	0.3 - 8**
6.2	6.6	19.4	0.5 - 16**
5	8.0	18.0	0.6 - 20**
3.1	13.0	13.0	1.0 - 26**
2.5	16.0	10.0	1.2 - 26**
1.5	26.0	0	2.0 - 26**
1.25	26.0	0	2.4 - 26**
0.62	26.0	0	4.8 - 26**
0.31	26.0	0	9.7 - 26**
0.15	26.0	0	20 - 26**
< 0.15	Do not amplify	-	-

** Add TE^{-4} to a final volume of 26 µL.

Initials: RCJ Date: 5/16/00

Table VII - Amount of DNA extract from a non-differential semen extraction or from the swab/substrate remains fraction of a differential lysis sample to be amplified for YM1**.

P 30 result for the 2ng subtraction (body cavity swabs)	P 30 result for the 0.05A units subtraction (stains or penile swabs)	Target Volume (µL) to be amplified	TE^{-4} (µL) for Target Volume	Range of Volumes (µL) which can be amplified
ERR for the neat	ERR for the neat	5	21	1 - 26*
between 1.1 - 3.0	between 1.1 - 3.0	10	16	2 - 26*
> 0 - 1.0	> 0 - 1.0	26	0	5 - 26*

* Add TE^{-4} to a final volume of 26 µL.

** Use 10µL for samples where P30 was not determined because sperm cells were seen.

Table VIII - Control samples Y STR multiplex YMI

Sample	DNA	TE^{-4}
in house amplification positive control	20 µL	6 µL
extraction negative control	20 µL	6 µL
amplification negative control	---	26 µL

Initials: RCJ Date: 5/26/01

B Amplification set-up

1. For each system fill out the amplification worksheet and record the appropriate lot numbers.

2. Determine the number of samples to be amplified, including controls and label a PCR reaction mix tube for each sample. Be sure to use the correct tubes for the thermocycler you plan to use. If you are amplifying in a 480 thermocycler use the large tubes (0.5mL); for the 9600 and 9700 thermocyclers, use the small tubes (0.2mL).

3. 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.**

4. According to the multiplex that is being amplified the following reagents have to be added to each tube:

	System		
	Quad Multiplex	Y STR YM1	Cofiler, Profiler Plus
reagent	3µL of $MgCl_2$	4µL of $MgCl_2$	10µL of specific primer mix

5. Pipet carefully in the solution at the bottom of the tube. Use a fresh sterile pipette tip for each tube.

6. **Only** for QUAD which can be amplified in the Perkin Elmer **480** thermocyclers, carefully add 1 drop of sterile Mineral Oil to all reaction mixture tubes including the controls. **Do not touch tube.**

   This step can be skipped for PCR reactions carried out in the Perkin Elmer **9600** and **9700** thermocyclers.

7. Close all of the tubes. At this stage have another analyst witness the sample set-up.

8. **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 50µL. Transfer the DNA aliquot prepared earlier to the labeled PCR reaction mix tubes; insert the pipet tip through the mineral oil layer (if present). After the addition of the DNA, cap each sample before proceeding to the next tube. **Do not vortex or mix.**

9. After all samples have been added take the rack to the amplified DNA area.

Initials: KCS Date: 3/30/01

B Amplification set-up

1. For each system fill out the amplification worksheet and record the appropriate lot numbers.

2. Determine the number of samples to be amplified, including controls and label a PCR reaction mix tube for each sample. Be sure to use the correct tubes for the thermocycler you plan to use. If you are amplifying in a 480 thermocycler use the large tubes (0.5mL); for the 9600 and 9700 thermocyclers, use the small tubes (0.2mL).

3. 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.**

4. According to the multiplex that is being amplified the following reagents have to be added to each tube:

	System		
	Quad Multiplex	Y STR YM1	Cofiler, Profiler Plus
reagent	3µL of $MgCl_2$	4µL of $MgCl_2$	10µL of specific primer mix

5. Pipet carefully in the solution at the bottom of the tube. Use a fresh sterile pipette tip for each tube.

6. For QUAD and YM1 being amplified in the Perkin Elmer **480** thermocyclers **only**, carefully add 1 drop of sterile Mineral Oil to all reaction mixture tubes including the controls. **Do not touch tube.**

   This step can be skipped for PCR reactions carried out in the Perkin Elmer **9600** and **9700** thermocyclers .

7. Close all of the tubes. At this stage have another analyst witness the sample set-up.

8. **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 50µL. Transfer the DNA aliquot prepared earlier to the labeled PCR reaction mix tubes; insert the pipet tip through the mineral oil layer (if present). After the addition of the DNA, cap each sample before proceeding to the next tube. **Do not vortex or mix.**

9. After all samples have been added take the rack to the amplified DNA area.

Initials: PG Date: 5/24/05

C Thermocycling

Turn on the Perkin Elmer 480, 9600, or 9700 Thermal Cycler. (See manufacturer's instructions).

Choose the following files in order to amplify each system:

instrument	Quad	YM1	Cofiler	Profiler Plus
480	file: 11	n/a	n/a	n/a
9600	n/a	n/a	file: 38	file: 38
9700	user: casewk file: quad	user: casewk file: ym1	user: casewk file: profiler/cofiler	user: casewk file: profiler/cofiler

Tables IX - XI list the conditions that should be included in each file. If the files are not correct, bring this to the attention of the QA coordinator and a supervisor.

Table IX **PCR conditions for the PerkinElmer DNA ThermalCycler 480.**

480 **QUAD** file # **11**	Taq Gold activation soak at 95°C for 11 minutes and at 96°C for 1 minute. Link to Thermo-Cycle File #15: 29 cycles: ▸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 Link to Time Delay File #16 for an additional 10 minutes extension at 72°C. Link to Soak File #12 on all machines for a 4°C soak.

Initials: RCA Date: 3/20/01

C Thermocycling

Turn on the Perkin Elmer 480, 9600, or 9700 Thermal Cycler. (See manufacturer's instructions).

Choose the following files in order to amplify each system:

instrument	Quad	YM1	Cofiler	Profiler Plus
480	file: 11	file: 24	n/a	n/a
9600	n/a	n/a	file: 38	file: 38
9700	user: casewk file: quad	n/a n/a	user: casewk file: profiler/cofiler	user: casewk file: profiler/cofiler

Tables IX - XI list the conditions that should be included in each file. If the files are not correct, bring this to the attention of the QA coordinator and a supervisor.

Table IX **PCR conditions for the PerkinElmer DNA ThermalCycler 480.**

480 **QUAD** file # **11**	Taq Gold activation soak at 95°C for 11 minutes and at 96°C for 1 minute. Link to Thermo-Cycle File #15: 29 cycles: ▸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 Link to Time Delay File #16 for an additional 10 minutes extension at 72°C. Link to Soak File #12 on all machines for a 4°C soak.
480 **YM1** file # **24**	Taq Gold activation soak at 95°C for 11 minutes Link to Thermo-Cycle File #23: 30 cycles: ▸Denature at 94°C for 1 minute ▸Anneal at 56°C for 2 minutes ▸Extend at 72°C for 2 minutes Link to Time Delay File #22 for an additional 30 minutes incubation at 60°C. Link to Soak File #21 for the 25°C storage soak.

Initials: RCS Date: 5/21/01

Table X **PCR Conditions for the Perkin Elmer GeneAmp PCR System 9600**

9600 **Cofiler/** **Profiler** **Plus** file # 38	File # 38 is the program for the following file combination: # 1, then # 8, then # 10, then # 26, then # 4: The individual linked files contain the following parameters: File # 1: Soak at 95°C for 11 minutes File # 8: ▸Denature at 94°C for 1 minute 28 cycles: ▸Anneal at 59°C for 1 minute ▸Extend at 72°C for 1 minute File # 10: 45 minutes incubation at 60°C. File # 26: storage soak 10 hours at 25°C File # 4: storage soak indefinitely at 4°C

Table XI **PCR Conditions for the Perkin Elmer GeneAmp PCR System 9700**

9700 **QUAD** user: casewk file: quad	The Quad file is as follows: Soak at 95°C for 11 minutes Soak at 96°C for 1 minute ▸Denature at 94°C for 45 seconds 29 Cycles: ▸Ramp 2 minutes to 54°C ▸Anneal at 54°C for 1 minute ▸Extend at 72°C for 40 seconds 10 minutes incubation at 72°C. Storage soak indefinitely at 4°C
9700 **Cofiler/** **Profiler** **Plus** user: casewk file: cofiler/profiler	The Cofiler/Profiler Plus file is as follows: Soak at 95°C for 11 minutes ▸Denature at 94°C for 1 minute 28 Cycles: ▸Anneal at 59°C for 1 minute ▸Extend at 72°C for 1 minute 45 minutes incubation at 60°C. storage soak 90 minutes at 25°C storage soak indefinitely at 4°C
9700 **Y M1** user: casewk file: ym1	The YM1 file is as follows: Soak at 95°C for 10 minutes ▸Denature at 94°C for 45 seconds 30 Cycles: ▸Anneal at 58°C for 58 seconds ▸Extend at 72°C for 1 minute 15 seconds 30 minutes incubation at 60°C. storage soak indefinitely at 4°C

Initials: KC/ Date: 3/30/01

Table X **PCR Conditions for the Perkin Elmer GeneAmp PCR System 9600**

9600 **Cofiler/** **Profiler** **Plus** file # 38	File # 38 is the program for the following file combination: # 1, then # 8, then # 10, then # 26, then # 4: The individual linked files contain the following parameters: File # 1: Soak at 95°C for 11 minutes File # 8: ▸Denature at 94°C for 1 minute 28 cycles: ▸Anneal at 59°C for 1 minute ▸Extend at 72°C for 1 minute File # 10: 45 minutes incubation at 60°C. File # 26: storage soak 10 hours at 25°C File # 4: storage soak indefinitely at 4°C

Table XI **PCR Conditions for the Perkin Elmer GeneAmp PCR System 9700**

9700 **QUAD** user: casewk file: quad	The Quad file is as follows: Soak at 95°C for 11 minutes Soak at 96°C for 1 minute ▸Denature at 94°C for 45 seconds 29 Cycles: ▸Ramp 2 minutes to 54°C ▸Anneal at 54°C for 1 minute ▸Extend at 72°C for 40 seconds 10 minutes incubation at 72°C. Storage soak indefinitely at 4°C
9700 **Cofiler/** **Profiler** **Plus** user: casewk file: cofiler/profiler	The Cofiler/Profiler Plus file is as follows: Soak at 95°C for 11 minutes ▸Denature at 94°C for 1 minute 28 Cycles: ▸Anneal at 59°C for 1 minute ▸Extend at 72°C for 1 minute 45 minutes incubation at 60°C. storage soak 90 minutes at 25°C storage soak indefinitely at 4°C

Initials: RG Date: 5/21/01

480 INSTRUCTIONS

1. 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.

2. Start the Thermal Cycler amplification program by pressing **FILE**, then the file number and **ENTER**. The cycling parameters may be verified by monitoring the values for the first cycle after the initial denaturation. While the instrument is running, the screen displays the heat block's current temperature (Blk=), the target temperature (End=), the time (counting down), and the cycle number (counting down). For hold steps, the instrument displays cycle=0.

   The tubes should be checked after the first cycle and pressed further into the heat block so that they fit tightly.

   Upon completion of the amplification, remove samples and press the STOP button repeatedly until the main menu is displayed. Wipe any condensation from the heat block with a Kimwipe and close the lid to prevent dust from collecting on the heat block. Turn the instrument off.

9600 INSTRUCTIONS

1. Place the tubes in the heat block **(do not add mineral oil)**, slide the heated lid over the tubes, and fasten the lid by turning the top screw. Do not make it too tight, the white triangles should line up.

2. Start the file by doing the following steps:

   The main menu options are RUN - CREATE - EDIT - UTIL

   If this is visible and the cursor is blinking underneath RUN, press ENTER. If this is not displayed press STOP as often as is necessary to get the main menu.

   You will be prompted to Enter program #. Enter the desired program number and press ENTER again. At the next screen verify that Select tube is set to MICRO, if it is not, press the OPTION button until MICRO appears. Verify that Reaction vol is set to 50µL, if it is not, set it to 50µL using the numeric key pad. Press ENTER.

Initials: RCJ Date: 3/30/09

480 INSTRUCTIONS

1. 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.

2. Start the Thermal Cycler amplification program by pressing **FILE**, then the file number and **ENTER**. The cycling parameters may be verified by monitoring the values for the first cycle after the initial denaturation. While the instrument is running, the screen displays the heat block's current temperature (Blk=), the target temperature (End=), the time (counting down), and the cycle number (counting down). For hold steps, the instrument displays cycle=0.

   The tubes should be checked after the first cycle and pressed further into the heat block so that they fit tightly.

   Upon completion of the amplification, remove samples and press the STOP button repeatedly until the main menu is displayed. Wipe any condensation from the heat block with a Kimwipe and close the lid to prevent dust from collecting on the heat block. Turn the instrument off.

9600 INSTRUCTIONS

1. Place the tubes in the heat block **(do not add mineral oil)**, slide the heated lid over the tubes, and fasten the lid by turning the top screw. Do not make it too tight, the white triangles should line up.

2. Start the file by doing the following steps:

   The main menu options are RUN - CREATE - EDIT - UTIL

   If this is visible and the cursor is blinking underneath RUN, press ENTER. If this is not displayed press STOP as often as is necessary to get the main menu.

   You will be prompted to Enter program #. Enter the desired program number and press ENTER again. At the next screen verify that Select tube is set to MICRO, if it is not, press the OPTION button until MICRO appears. Verify that Reaction vol is set to 50µL, if it is not, set it to 50µL using the numeric key pad. Press ENTER.

Initials: RCJ Date: 5/21/01

The run starts when the cover temperature is 100°C. The cycling parameters may be verified by monitoring the first cycle after the initial denaturation. While the instrument is running, the screen displays the target temperature (either 'Ramp to' or 'Hold at'), the current temperature, and the time, counting down. During hold steps, the linked program number is displayed; for cycle steps, the cycle number is displayed counting up.

Upon completion of the amplification, remove samples and press the STOP button repeatedly until the main menu is displayed. Wipe any condensation from the heat block with a Kimwipe and pull the lid closed to prevent dust from collecting on the heat block. Turn the instrument off.

9700 INSTRUCTIONS

1. Place the tubes in the tray in the heat block **(do not add mineral oil)**, slide the heated lid over the tubes, and fasten the lid by pulling the handle forward. Make sure you use a tray that has a 9700 label.

2. Start the run by performing the following steps:

The main menu options are RUN CREATE EDIT UTIL USER. To select an option, press the F key (F1...F5) directly under that menu option.

Verify that user is set to "casewk." If it is not, select the USER option (F5) to display the "Select User Name" screen.

Use the circular arrow pad to highlight "casewk." Select the ACCEPT option (F1).

Select the RUN option (F1).

Use the circular arrow pad to highlight the desired STR system. Select the START option (F1). The "Select Method Options" screen will appear.

Verify that the reaction volume is set to **50**µL and the ramp speed is set to **9600 (very important)**.

If all is correct, select the START option (F1).

The run will start when the heated cover reaches 103°C. The screen will then display a flow chart of the run conditions. A flashing line indicates the step being performed, hold time is counted down. Cycle number is indicated at the top of the screen, counting up.

Initials: KD Date: 3/30/01

The run starts when the cover temperature is 100°C. The cycling parameters may be verified by monitoring the first cycle after the initial denaturation. While the instrument is running, the screen displays the target temperature (either 'Ramp to' or 'Hold at'), the current temperature, and the time, counting down. During hold steps, the linked program number is displayed; for cycle steps, the cycle number is displayed counting up.

Upon completion of the amplification, remove samples and press the STOP button repeatedly until the main menu is displayed. Wipe any condensation from the heat block with a Kimwipe and pull the lid closed to prevent dust from collecting on the heat block. Turn the instrument off.

9700 INSTRUCTIONS

1. Place the tubes in the heat block **(do not add mineral oil)**, slide the heated lid over the tubes, and fasten the lid by pulling the handle forward.

2. Start the run by performing the following steps:

The main menu options are RUN CREATE EDIT UTIL USER. To select an option, press the F key (F1...F5) directly under that menu option.

Verify that user is set to "casewk." If it is not, select the USER option (F5) to display the "Select User Name" screen.

Use the circular arrow pad to highlight "casewk." Select the ACCEPT option (F1).

Select the RUN option (F1).

Use the circular arrow pad to highlight the desired STR system. Select the START option (F1). The "Select Method Options" screen will appear.

Verify that the reaction volume is set to **50**µL and the ramp speed is set to **9600 (very important)**.

If all is correct, select the START option (F1).

The run will start when the heated cover reaches 103°C. The screen will then display a flow chart of the run conditions. A flashing line indicates the step being performed, hold time is counted down. Cycle number is indicated at the top of the screen, counting up.

Initials: RC Date: 5/2014

Upon completion of the amplification, remove samples and press the STOP button repeatedly until the "End of Run" screen is displayed. Select the EXIT option (F5). Wipe any condensation from the heat block with a Kimwipe and pull the lid closed to prevent dust from collecting on the heat block. . Turn the instrument off.

For all systems return the microtube rack used to set-up the samples for PCR to the PCR Set-Up Area.

Turn instruments off **ONLY** when the Main Menu is displayed, otherwise there will be a Power Failure message the next time the instrument is turned on. It will prompt you to review the run history. Unless you have reason to believe that there was indeed a power failure, this is not necessary. Instead, press the STOP button repeatedly until the Main Menu appears.

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.

Initials: RCJ Date: 3/30/01

Upon completion of the amplification, remove samples and press the STOP button repeatedly until the "End of Run" screen is displayed. Select the EXIT option (F5). Wipe any condensation from the heat block with a Kimwipe and pull the lid closed to prevent dust from collecting on the heat block. Turn the instrument off.

For all systems return the microtube rack used to set-up the samples for PCR to the PCR Set-Up Area.

Turn instruments off **ONLY** when the Main Menu is displayed, otherwise there will be a Power Failure message the next time the instrument is turned on. It will prompt you to review the run history. Unless you have reason to believe that there was indeed a power failure, this is not necessary. Instead, press the STOP button repeatedly until the Main Menu appears.

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.

Initials: RCS Date: 5/16/00

Amplification Troubleshooting

If the Chelex extracted DNA samples fail to amplify this might be due to several factors:

1. A mistake during the amplification set-up, such as not adding one of the components or not starting the thermocycler should affect all samples including the positive control. In this case the amplification must be repeated.

2. A Taq polymerase inhibitor such as hemoglobin or an organic dye is present in the DNA extract. One of the following remedies might overcome this problem:

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

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

   (3) Re-extract the sample using a smaller area of the stain to dilute potential Taq Gold polymerase inhibitors.

   (4) Re-extract the samples using the organic extraction procedure followed by a Chelex extraction.

   (5) Purify the extracted DNA using a Microcon 100 ultrafiltration device as described in the Microcon section.

3. The amount of DNA was insufficient or the DNA is severely degraded. Do one of the following:

   (1) Amplify a larger aliquot of the DNA extract.

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

   (3) Concentrate the extracted DNA using a Microcon 100 ultrafiltration device as described in the Microcon section.

The decision which of the above approaches is the most promising should be made after consultation with a supervisor.

Initials: RCS Date: 5/16/00

General Guidelines for fluorescent STR Analysis

Batch processing

1. Exemplars and evidence samples have to be handled separately at all times. These samples should never be together on the same gel or in the same run.

2. Samples from one amplification sheet should be processed together, so that each sample is accompanied by the appropriate controls.

3. Use the correct worksheet for the specific sample type, and make sure the sample preparation set-up and the gel loading set-up are witnessed properly.

4. Samples that need to be rerun must be loaded on a gel with the appropriate sample type. It is not necessary to rerun all of the amplification controls for the repeated sample.

5. Attention: Each gel or capillary run that is performed has to have at least one positive control.

Sample handling

6. Prior to loading on the gel, the amplified samples are stored at +4°C in the amplified DNA area. The tubes containing the amplified product should never leave the amplified DNA area.

7. See the general PCR Guidelines for microfuge tube and pipet handling.

8. Samples that have been loaded on a gel or in a sample tray, should be stored until the electrophoresis results are known. After it has been determined that the amplification products do not require repeated testing, the samples should be discarded.

Instrument and computer maintenance

9. Be gentle with all glass plates, instrument parts and instruments. Keep everything clean.

10. The Macintosh computer should be restarted before each run. This prevents the hard drive from becoming too fragmented.

11. Delete data files and other non-essential files from the hard disk at least once a week.

12. Notify QC if any problems are noted.

13. Fill out all log books.

Initials: RG Date: 5/16/00

Data File Copying and Archiving Procedure for 377 gels

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 Q00-Men001, Y00-Mul001 etc)

   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.

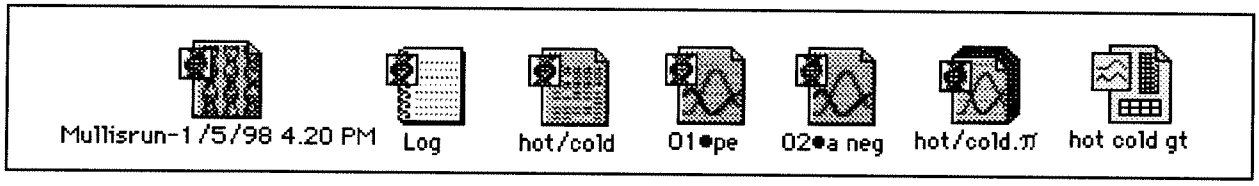
2. Put the appropriate JAZ archive disk in the JAZ drive. The disk icon will be visible on the desk top. Drag the icon of the renamed run folder onto the JAZ disk icon. After copying is complete, drag the JAZ disk icon onto the trash icon to expel the disk.

   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!!!

3. Insert the disk in the JAZ disk drive at terminal #1 or #2, double click on the disk icon to open the disk window. There is no need to copy the gel on the hard drive. You can analyze the gel while it stays on the JAZ disk.

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.



Initials: RG Date: 5/11/00

It also should have been renamed (see Genotyper instructions), according to the following format: **Gelname Files (e.g. Q00-Men001 Files, Y00-Mul001 Files etc)**

5. After you are satisfied with the analysis and the archiving of the gel, don't forget to go back to the instrument and delete the "**raw data**" folder.

Data File Copying and Archiving Procedure for 310 capillary runs

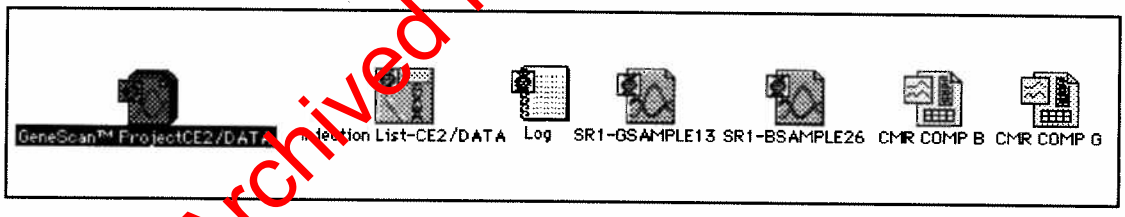
6. Before copying a run, identify the **Run folder** containing the project file based on the date and time noted on the sample sheet. Rename this run folder with the run name according to the following format:

CE1/00-001 files
CE2/00-001 files respectively,

After the project has been analyzed and typed, the run folder should contain the following items:

- a project file (39K ABI Prism 310 Collection document)
- the run log (65K ABI Prism 310 Collection document)
- an injection list (39K ABI Prism 310 Collection document)
- sample files for all samples (98K Genescan documents)
- the Genotyper files (ca. 400K Genotyper document)

See picture below for appearance of file icons.



7. Put a designated JAZ archiving 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.

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

8. After the copy process is complete, delete the run folder from the CE computer hard drive.

Initials: RCS Date: 5/16/00

STR Analysis on the ABI Prism 310 Capillary Electrophoresis (CE) Genetic Analyzer

Only the AmpFISTR Cofiler multiplex has been validated for analysis on the capillary and must be run on these instruments.

In order to run samples on the 310 CE, the run has to be set up on both the software and the instrument. The program that is used to operate the run and collect the data is the ABI PRISM 310 COLLECTION SOFTWARE. This program has to be started and the software preparations consist of filling out a sample sheet and generating an injection list (section E). On the instrument level, the capillary must be checked, installed or changed (section A), the buffer must be changed (section D) and, depending on whether or not the capillary has been changed, the autosampler must be calibrated (section B).

Initial Preparation

- RESTART THE COMPUTER BY UNDER **SPECIAL** SELECTING **RESTART.**
- OPEN THE 310 COLLECTION SOFTWARE BY DOUBLE CLICKING ON THE **ABI PRISM 310 COLLECTION** ICON.
- CHECK THE CAPILLARY COUNT!

To check the capillary count, select **changing the capillary** on the collection software window under the **INSTRUMENT** menu. The life-span of the capillary is limited to 150 injections. Determine if the samples you are about to run will exceed this number by counting the samples on the amplification sheets and adding the allelic ladder injections. Change the capillary when this limit is reached. The capillary count should be double checked in the instrument logbook. The capillary also must be changed if the electrode end of the capillary has remained outside of the buffer for an extended period of time.

IF THE CAPILLARY DOES NOT NEED CHANGING, CLICK **CANCEL**. **IMPORTANT**: DO NOT CLICK **OK**, BECAUSE THAT WILL RESET THE CAPILLARY COUNT TO ZERO.

Here is how to decide at which point of the manual to start:

1.) If the number of your samples will exceed 150 injections, proceed with section **A** of instrument preparation. You will have to change the capillary, and calibrate the autosampler.

2.) If the amount of POP4 in the installed syringe is low (<200µL) you will have to refill it. Start with section **C** of instrument preparation.

Initials: RCJ Date: 5/16/00

3.) If capillary and syringe are fine, start with section **D** of instrument preparation. You only have to change the buffer and the deionized water for the run.

IMPORTANT - CHECK THE SYRINGE FOR CRYSTALLIZED SOLUTION. THIS INDICATES A LEAK. THE CRYSTALS CAN CAUSE SPIKES AND THE LEAKING CAUSES INCOMPLETE FILLING OF THE CAPILLARY. CLEAN AND REFILL THE SYRINGE IN THAT CASE.

Initials: RCS Date: 5/16/00

Instrument Preparation

A. Changing and Installing the Capillary

When changing the capillary also rinse the pump block.

A1. Removing The Old Capillary

1. Unscrew the ferrule on the right side of the pump block and remove the capillary from the screw.

2. Open the heat block door and the laser detector door. Remove the capillary from the laser detector groove and from the electrode thumb screw. Discard the capillary.

A2. Removing, Rinsing, And Replacing The Pump Block

3. With the Genescan Collection program open, select **Manual Control** from the **Windows** menu.

4. Under the **Function** pop-up menu, select **Home Syringe.** Click **Execute** until the syringe drive is clear of the end of the plunger of the glass syringe. Swing the pump toggle to the left. Unscrew the syringe from the block grasping the silver part at the bottom of the syringe (NOTE: Do not unscrew the syringe while holding onto the glass). Set it aside.

5. To remove the buffer chamber make sure the buffer valve is open (on the **Manual Control Window** select **Buffer Valve Open**, click **Execute**), twist and pull the chamber gently until it is released. To remove the pump block from the CE pull the block firmly away from the machine.

6. Fill a plastic syringe with water and screw it into the top right side of the pump block (where the glass syringe goes). Wash different sections of the pump block by opening one valve at a time and flushing the open passage with water. One can also use the distilled water squeeze bottle. Thoroughly dry the pump block with Kimwipes.

   DO NOT USE ORGANIC SOLVENTS ON ANY PART OF THE PUMP BLOCK!

Initials: RC Date: 5/16/00

7. Make sure all the different plugs, valves, and ferrules of the pump block are removed and thoroughly cleaned with water (especially make sure the hole in the ferrule (through which the capillary runs) is clear of an Performance Optimized Polymer (POP 4)). Replace all pieces to the proper positions on the block.

8. Before replacing the pump block verify that the gold electrode socket on the back of the block is dry.

9. Align the steel shafts with the two large holes of the block, then push the block gently onto the shafts. Before pushing the block all the way to the back, align the buffer valve plunger with the activator arm. Slide the pump block until flush against the instrument.

A3. Installing The Capillary Onto an Empty Pump Block

The STR alleles are separated in a Performance Optimized Polymer (POP4). The DNA samples are electrokinetically injected through a 47cm x 50µm i.d. Capillary. Carefully handle the capillary to ensure that the glass window is clean and unbroken.

10. Choose the correct capillary. For STR separation use a 47cm x 50µm I.D. Capillary; this is distinguished from the sequencing capillary by a green mark on the window side of the capillary.

11. Carefully pull the capillary from the plastic storage tube.

12. CAREFULLY Inspect and clean the capillary detection window with a Kimwipe and isopropanol. **The window area is very fragile and breaks easily!**

13. To connect the capillary to the pump block make sure the door covering the heat plate and the laser detector are open, then partially unscrew the plastic ferrule on the right side of the pump block.

14. Insert the window end of the capillary through the center of the plastic screw and through the cone-shaped ferrule. The green dot should be above the laser window.

15. Screw the capillary and ferrule into the pump block. As the ferrule begins to seat, adjust the end of the capillary so that it is positioned a bit to the right of the opening to the glass syringe. Firmly tighten the ferrule into the pump block to secure the capillary.

Initials: RG Date: 5/16/00

16. Position the capillary in the vertical groove of the laser detector.

    The central portion of the capillary window should rest directly over the laser detector window without putting strain or tension on the capillary. As a positioning aid, align the green mark on the capillary with the top edge of the detector plate.

    GENTLY close the laser detector door over the capillary to secure its position. (Note: if the door slams closed, the capillary window will break. Check it).

17. Thread the other end of the capillary through the capillary hole in the electrode thumbscrew until it protrudes past the tip of the electrode by about 0.5 mm. The capillary and electrode should be as close to each other as possible WITHOUT TOUCHING. Tape the capillary into position at the lower right-hand corner of the heat plate.

18. **Gently** close the door to the heat plate.

19. Reset count by selecting under **Instrument** menu **Changing Capillary** and reset to 0 (zero) by clicking **OK**.

Note: The life of the capillary is 150 injections, or approximately three full 48 sample trays. When you begin a run the computer will alert you if the number of injections for your run in addition to the samples already injected with the same capillary exceeds 100. Ignore this message, you want to continue at this point. Only if your samples in addition to the previous count exceed 150, the capillary must be changed. If the sum of injections is less than 150, click **Cancel** and continue your run.

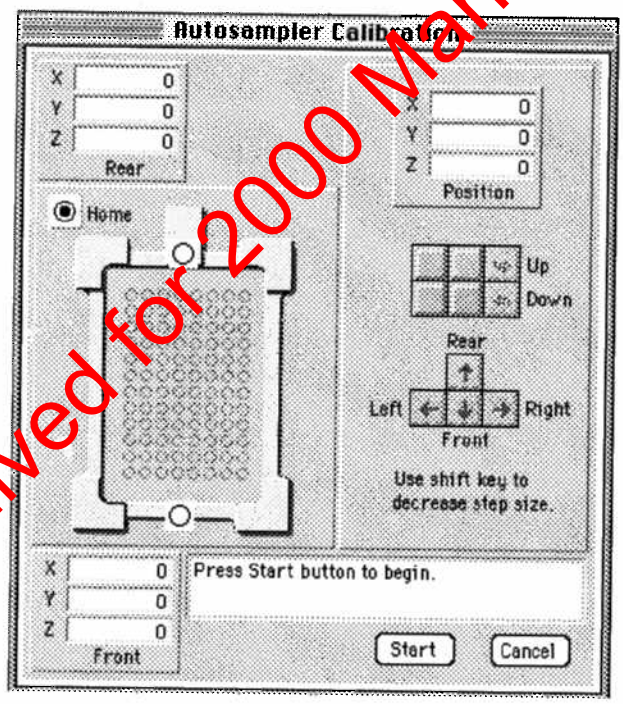
Initials: RG Date: 5/16/00

B. Calibrating the Autosampler

In order for the capillary to correctly inject each sample, its position must be carefully aligned each time a new electrode or capillary is installed or whenever an incident occurs, such as a collision between the capillary and the septa caps on the sample or buffer tubes. At the front and back of the autosampler platform is a black dot for calibrating tray movement along the x, y and z axes.

1. On the desktop double click on the PRISM 310 Collection icon if the collection program is not currently open.

2. On the top of the screen pull down the **Instrument** menu and select **Autosampler Calibration**. The autosampler calibration window will appear on the screen.



3. Click **Start**.

4. The machine will present the sample tray for removal. It is IMPERATIVE that the sample tray is removed. After removal click on **resume**, the autosampler platform will retreat.

Initials: AG Date: 5/16/00

5. Click on the directional buttons on the right side of the window to align the tip of the capillary (not the electrode) with the black dot on the front of the autosampler. Get the capillary as close as possible to the center of the black dot without the capillary touching the dot.(For very small directional steps hold down the SHIFT key while clicking on the directional arrows). When you're finished click **Set**.

6. Repeat step 5 for the rear calibration dot. When you're finished click **Set**. The autosampler will again present itself; replace the sample tray. Click **Done**.

C. Filling and Installing the Glass Syringe and the Pump Block

Check the amount of polymer left in the syringe. Each sample requires only 1µL of POP4, but there should be at least twice the required amount present. Don't refill the capillary if there is sufficient polymer for the number of samples in your run in the syringe. Do refill if you see urea crystals on top of the syringe. **Never add fresh polymer to old polymer.**

1. Under **Window** go to **Manual Control Window**.

   Under the **Function** pop-up menu in the **Manual Control Window** select **Syringe Home** and click **Execute** until the syringe drive is clear of the end of the plunger of the glass syringe. Swing the pump toggle to the left. Unscrew the syringe from the block grasping the silver part at the bottom of the syringe (NOTE: do not unscrew the syringe while holding onto the glass). RINSE THE GLASS SYRINGE THOROUGHLY BY FLUSHING HOT WATER THROUGH IT SEVERAL TIMES. CHECK THAT THERE IS NO OLD POP4 RESIDUE.

2. The last rinsing of the syringe should be deionized water. Insert the plunger. Avoid moving the plunger up and down in a dry syringe, since this wears out the plunger.

3. Let the POP4 warm up to room temperature before using it. Make sure there are no crystals or other particles in the POP4, the formation of crystal can be prevented by storing the vials upside down. Manually draw 600µl (for three full trays of samples) of POP4 into a 1mL glass syringe making sure to keep the syringe free of any and all air bubbles. Rinse exterior with deionized water and dry with a Kimwipe. (Each sample injection requires approximately 1µL of polymer, plus extra to fill the pump block and eradicate any air bubbles which may be present).

4. Remove any air bubbles in the syringe before installing! Turn the syringe upside down and gently tap on the side to dislodge the air bubbles. Expel the air bubbles with some

of the solution. This is **important**: the presence of air bubbles might cause false leak alarms. Clean the outside of the syringe with water and dry it with a Kimwipe.

5. Slide syringe through the guide and screw the glass syringe filled with POP4 into the right side of the pump block by holding the silver portion of the syringe.

6. Open the **Manual Control** menu and from the **Function** pop up menu select **Buffer Valve Close,** (click **Execute**). Manually open waste valve below syringe. Press down on syringe plunger to remove any air bubbles. Tighten waste valve.

7. In the **Manual Control** menu, select **Buffer Valve Open** (click **Execute**). Manually push plunger to fill polymer channel and remove any air bubbles.

   MAKE SURE THERE ARE NO AIR BUBBLES IN THE PUMP BLOCK. If you see an air bubble anywhere in the pump block passage, open the appropriate valve and plunge the syringe until the bubble is flushed through the open valve.

8. Tighten all valves. Under **Manual Control** choose **Buffer Valve Close**, click **Execute**.

9. Swing the pump toggle to the right and in the **Manual Control** menu, select **Syringe Down**. Click **Execute** as many times as necessary to move the syringe drive toggle down until bottom of drive almost touches the top of the syringe plunger. DO NOT MOVE THE SYRINGE PLUNGER.

10. Fill capillary by selecting under **Manual Control** under **Module** the command **Seq Fill Capillary**. Click **Start.** This step will take 10 min.

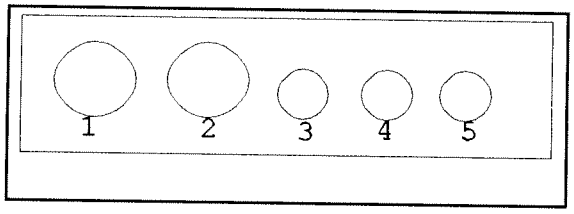
D. Changing the Buffer and Baseline Check

1. Empty, rinse and fill anode buffer chamber with 1X Genetic Analyzer Buffer with EDTA up to the fill line and fit securely to its position on the pump block. The 1X buffer is made by aliquoting 5mL of 10X buffer with EDTA into a plastic tube and adding 45mL of deionized water. (Check refrigerator #7 for 1X buffer before making more)! Label the tube with the lot number, the date of manufacture and your initials

2. Present the autosampler platform by pressing the TRAY button on the left of the inside of the instrument. Empty, rinse, and fill cathode buffer tube with 1X Genetic Analyzer EDTA buffer to the fill line, replace cap and septum, and place in position

Initials: RS Date: 5/16/00

1 on the autosampler platform (**Anode and Cathode Buffers should be changed before each run**). Fill the glass tube with deionized water, replace the cap, septum, and place in position 2 on the autosampler platform.



3. Fill a 1.5 mL Eppendorf tube (with cap clipped off) with deionized water (3/4 full) and place in position 3 on the autosampler platform.

In order to check the color detection baseline (based on laser performance and state of the capillary window) do the following:

ATTENTION: the tray must be in the autosampler for this step.

4. Under **File** go to **Open**, and open the Injection list stored in the current run folder named **Injection list - CCD4 color test**.

5. Click **Run**. Click **O.K.** to the instrument alert "EP current is Zero". The scan window should display all four color lines at about the same level; the level should be below 2048. If a problem is detected, open the heat plate door and the laser window door and then clean the capillary window with a moist Kimwipe. Redo the scan.

6. Close the scan window. Cancel the run, and click **terminate**. Close the injection list window, click **Don't save**.

E. Filling Out a Sample Sheet

Before starting a run you must fill out a run control sheet, record the sample information in the computer sample sheet, and generate an injection list. The sample sheet associates sample information (name and type of analysis) with the sample tube position, which becomes the sample identification.

1. Fill out a CE run Control Sheet for the appropriate set of samples based on the amplification worksheet and the tray set-up rules:

Initials: RCS Date: 5/16/00

A) All samples of one amplification must stay together

B) One amplification of 20 samples should have three evenly spaced allelic ladders.

C) A full tray consists of two amplification sheets and two run control sheets.

D) Evidence (semen and blood) have to be run separately from exemplar samples. They cannot be in the same tray together.

E. Two amplifications of the same sample type, either evidence or exemplars, can be in one tray.

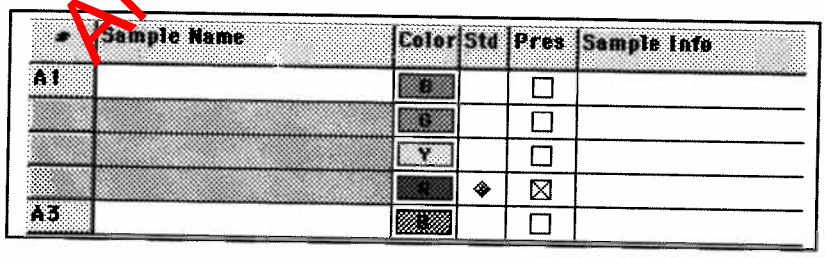
F) Do not skip a space in the tray. Place all samples consecutively.

G) Samples that need to be rerun (e.g. because of a separation problem or the presence of a partial profile) must be placed with the appropriate sample type and amplification system. In order to improve the signal strength for samples that showed a partial profile, it is possible to increase the injection time from 5 to 10 seconds.

2. Double click on the 310 PRISM COLLECTION icon, if the collection program is not already open.

3. Under **File** select New..., The Create New Dialogue box appears. Then click on **Genescan Sample Sheet, 48 tube** icon.

4. A Genescan sample sheet appears.



In the STD column make sure a diamond is present next to the red box, this denotes that the standard dye is RED (this is the default setting).

Initials: RS Date: 5/16/00

5. Fill in the your first sample name in the first space of the **Sample Name** column (Your first sample must be an allelic ladder). The number of the sample (A1, A3, A5..etc) corresponds to the position of the sample in the autosampler tray. Your second sample name should be entered in the A3 position, and so on. ENTER ONLY THE NAMES OF THE SAMPLES THAT ARE PRESENT IN THE RUN. DO NOT FORGET ANY SAMPLES. Use a short name, e.g. the tube labels noted on the amplification sheet, as the sample "name". If you are going to inject a sample more than once **do not** enter the information in the sample sheet twice. For a reinjection see section F, step 3.

6. In the **Sample Info** column enter the complete sample identification, including the FB number in the box(es) corresponding to the dye colors present in your sample; the checkbox in the column labeled "**pres**" (present) automatically becomes selected.

   The **copy, paste, and fill down** functions should be used to fill out the sample info column. Fill out the sample information accurately because, when the data is imported into Genotyper, the sample names will appear on the Genotyper dye/lane list. The sample info has to be filled out for all three labeling colors.

   Also copy and paste all the entries from the **sample info** column also into the **sample comment** column

7. Save the sample sheet by selecting **Save As...**from the **File** menu. Enter the name of the run, using the following format :

   CE1/00-001, CE2/00-001, CE3/00-001 respectively,

For QC and validation runs use descriptive names, click **Save.** The sample sheet is automatically saved to the sample sheet folder. Note the sample sheet name and the run folder date on the run control sheets.

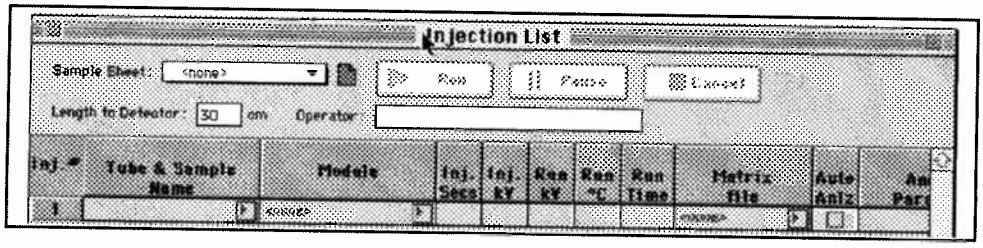
F. Preparing a Genescan Injection List and presetting the temperature

The injection list specifies the order in which each sample will run and the modules to be used for the run. Each row in the injection list corresponds to a single operation - an injection. The left column is the injection number and implies these operations are ordered (sequential).

1. Choose **New** from the **File** menu. The **Create New** Dialogue box appears.

2. Click the **Genescan Injection List** icon. A blank Injection list appears.

Initials: RCS Date: 5/16/00



3. Click the arrow in the **Sample Sheet** field to display a pop-up menu and select your sample sheet.

   The information recorded in the Sample Sheet will appear in the Sample Name Column of the Injection List. The Default module is "**GS STR POP4 1mL, F**" and should appear for each sample in the MODULE column. The run conditions are: Inj sec 5, Inj KV 15.0, Run KV 15.0, Run 60°C , Run time 24. Change the injection time to 2 seconds if necessary; do not touch the other settings. The matrix standard must be the most recent matrix made for the instrument. Size standard and Analysis Parameters are both preset analysis default settings.

Reinjecting a Sample

For each run it is advisable to inject the first ladder 2X. To accomplish this highlight, by clicking #1 on the injection list, injection #1, then from the **Edit** menu select **Insert**. A new row will appear at the top of the injection list. Click on the injection # of the sample you want to reinject (in this case the ladder), this will highlight the row. Under **Edit** select **Copy**. Then click on the blank row and under **Edit** select **Paste**.

4. Enter your initials in the OPERATOR box.

5. From the **Window** menu select **manual control**, choose **Temperature Set**.from the **Function** pop-up window

6. Set the temperature to 60°C, then click **Execute**. The instrument takes up to 30 minutes to reach the 60°C run temperature. Samples can be prepared while the instrument is heating.

7. Under **Windows** open the **Status** window to observe the temperature.

Initials: RCS Date: 5/16/00

Preparing and Running the DNA Samples

1. Label the sides of the Genescan tubes and place them in the autosampler tray according to the amplification and the run sheets.

2. For N+2 samples mix 24.5µL deionized formamide with 0.5µL CXR standard per sample.

Sample no. +2	Formamide	CXR
14	343 µL	7 µL
26	637 µL	13 µL
38	931 µL	19 µL
50	1225 µL	25 µL

Mix thoroughly by inversion.

3. Aliquot 25µl of the formamide/standard mixture into each labeled tube. Have someone witness tube set-up and sign the run sheet. Add 1µL of PCR product to each tube according to the injection list. When adding PCR product, make sure to pipet the solution into the formamide and flush the pipet tip a few times.

4. Cap tubes with grey rubber septa. (Make sure the septum is flush with the top edge of the tube; this may take a little work).

Allelic ladders are prepared in the same way as the samples. The allelic ladder preparation can be reused for two weeks. The ladders should be labeled on top of the grey septa with the color and date. The allelic ladders have to be denatured before each run.

5. Heat denature samples in the 95°C heat block for 2-3 minutes. Place the autosampler tray on ice. Immediately after the denaturation replace tubes in the tray in the order according to the injection list. Leave on ice for at least 1 minute. Check the tubes for airbubbles.

6. Open the instrument doors and press the **Tray** button on the instrument (to the left of the pump block) to present the autosampler platform for tray replacement. Replace the tray so that position A1 sits at the rear right corner of the platform. The tray will fit only in this position. Press **Tray** button again to restore the platform to its home position.

7. Close both instrument doors.

8. Click **Run** on the injection list.

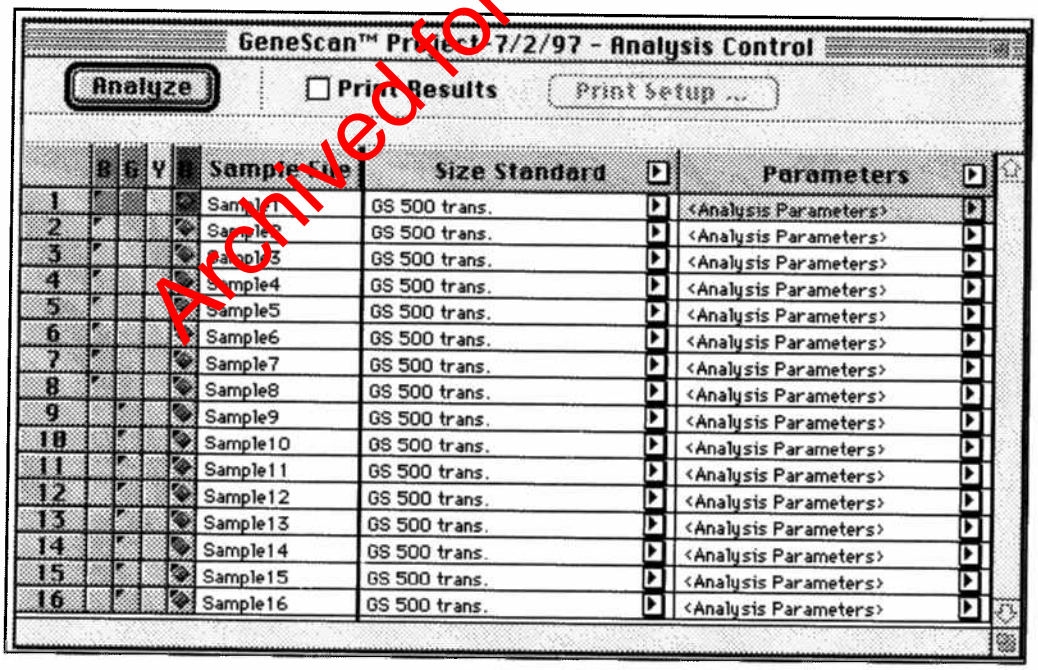
Initials: RCS Date: 5/16/00

To check the progress of the run, open the **Status** window under the **Windows** menu. The sample injections will begin automatically once the temperature has reached 60°C. When data is being collected, a band of 4 colors will show across the raw data window. These bands should be below the 2048 mark. If this is not the case refer to the Troubleshooting section.

STR Fragment Length Analysis for Cofiler

After the run is complete, the Genescan Collection software automatically launches the Genescan Analysis program so that the Analysis window will be displayed on the computer monitor. In order to access the Analysis window select **310 Genescan Analysis 2.1** from the pull-down computer menu on the upper right-hand corner of the screen. The Analysis Log will be displayed over the **Results Control** window. Follow the archiving procedure outlined in the General Guidelines for fluorescent STR analysis.

If the program is closed, access your project by double-clicking on the **310 Genescan Analysis** icon on the desktop. The Analysis program will launch. When it opens you will see a new menu at the top of the screen. Under File, select **open**. The **Open Existing** dialogue box will appear, click on the **project** icon. Your project will be housed in the **Current Runs** folder on the **desktop**. Click on the folder titled **runs**, a list of run folders will appear. Select your run by double clicking the run folder titled with the date you ran your samples. The **Analysis Control** window will appear.



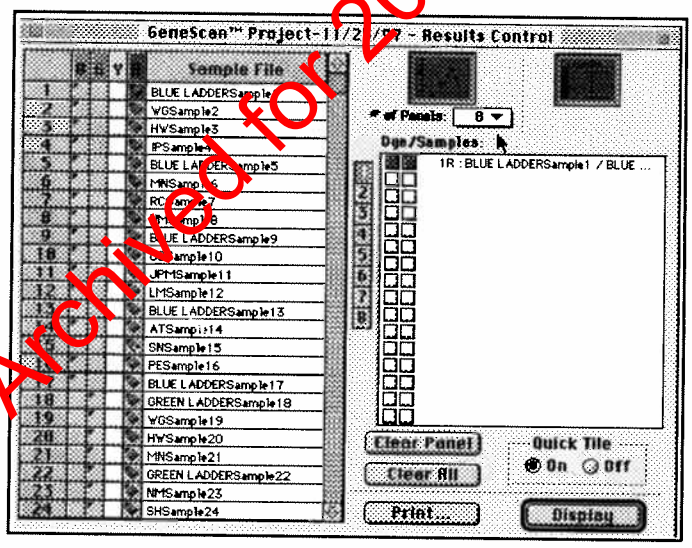
Initials: RCS Date: 5/16/00

Under the **windows** menu at the top of the screen, select **Analysis Log**. In the Analysis Log window there should be messages indicating that the standard peaks have been sized from 80-400 bp. for each sample. This tells you that each peak has been sized. If this is not the case or you have some warning message (e.g. "size standard skipped peak defined as 225bp") you will need to reanalyze the run making sure you have the correct size standard selected for analysis (the default setting is Promega CXR). To do this select the appropriate size standard from the Standard menu on the Results Control window.

The Analysis Control window shows the samples in order. The boxes corresponding to the dye colors are situated to the right of the sequential injections. A small black triangle in the dye box indicates that this sample has been analyzed. If this is not the case (if you've changed any analysis parameters, e.g. size standard, it won't be) select the samples for analysis by clicking on the red standard box and the corresponding color, then click **analyze**.

To ensure that all the sizing results are correct, check the labeling of the size standard peaks for each sample.

1. To view the analysis results select **Results Control** from the **Windows** menu at the top of the screen. The Results control window appears.



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.

Initials: Date:

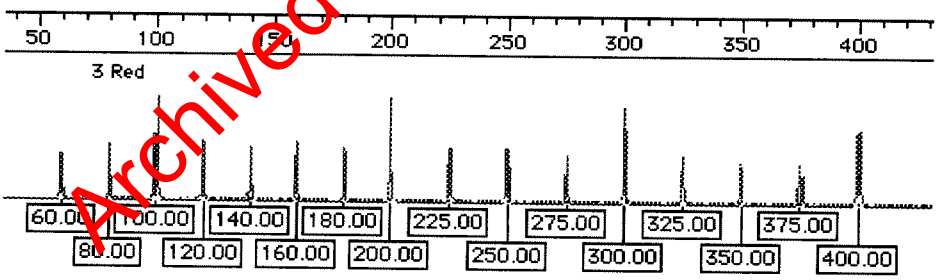
The electropherogram results can be displayed as a table with the sizing results only (deselect the left electropherogram icon), as electropherogram only (deselect the right table icon) and as a combination of both (standard setting). Up to 8 display panels can be seen in parallel. Each color per lane 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.

2. Check the size assignments to the red standard peaks. This can be done several ways: Option 1: Overlay up to16 size standards in one panel by clicking on the top of the red data column. The red standards for 16 lanes should be highlighted.

   Click **display**. 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. Repeat this step for the remaining lanes.

   Option 2: Select 8 panels and check Quick Tile. Click on panel 1, hold the mouse button down and drag the highlight down to sample 8. Click on **Display**. Each sample standard will be displayed in its own window; to view all 8 you must scroll through all the windows. Continue checking your size standards for the entire tray by going back to the **Results Control** window, clicking on **Clear All** and selecting the next 8 samples.

   The PROMEGA CXR standard peaks should be visible are as follows:



3. 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 80bp peak by clicking on it. Enter the number, 80, and press return. Continue to enter the above values for the size standard peaks.

Initials: RS Date: 5/16/00

After entering the number 400, click **return**. Click **Save**, to "save changes to "untitled," before closing, click **Save**, to "save this document as "untitled," click **replace**.

Reanalyze the corrected lane.

4. If not all peaks of the size standards are present, you might be looking at the wrong analysis range. Under **Settings** select **Analysis parameters.** The analysis range should be set to: Start: 3000 Stop: 6500

   The other settings are:
   Size call range: all sizes, Baseline checked, Multicomponent checked
   Smooth options: light, Size calling method: local Southern method
   Peak amplitude thresholds: 100 for all four colors, Minimum peak half width: 3
   Split peak correction: none

   If the analysis range has been changed, change it back and reanalyze the samples. If the range is correct but the standards fall out of this range, extend the range in the appropriate direction and reanalyze the samples.

5. Before proceeding with the Genotyper analysis under **File** select **Save Project**. The project will be named with the date of the run. Quit Genescan Analysis.

6. After quitting the Genescan Analysis and Collection programs, the raw data for each run is contained in a project file. A sample file is also created for each individual sample. Everything associated with your run is saved to the same run folder, unless otherwise directed.

FOR GENOTYPER ANALYSIS GO TO THE GENOTYPER SECTION AFTER GELANALYSIS.

Capillary Electrophoresis Troubleshooting

See below for a Capillary Instrument Electrophoresis and Analysis Troubleshooting Guide

Initials: QG Date: 5/16/00

Observation	Possible Cause	Recommended Action
1. Error Message: "leak detected"	There is a leak in the pump block.	Tighten all valves on the pump block including the ferrule in which the capillary is seated.
	There is a leak in the syringe.	Tighten, or remove and replace the syringe.
2. The 4 color bands are well above the 2048 mark in the raw data collection window.	There may be an air bubble in the pump block.	Flush the pump block of any air bubbles.
	There may be an air bubble or foreign particle in the capillary or on the capillary window.	Remove and replace the capillary. Clean capillary window with 95% ethanol and clean the capillary holder with deionized water.
3. Communication Problems	Corrupted memory files.	Turn off the instrument (power button is on the back of the instrument). Wait a few minutes then hold down the **Tray** button while turning the machine back on. Wait until the buzzing sound stops. Release the tray button. Restart the computer.
4. No Current	Too little or no buffer in anodic jar.	Replenish buffer jar.
	Too little or no buffer in position 1 of the autosampler.	Replenish buffer in position 1 of the autosampler.
	Electrode bent.	Replace or straighten electrode and recalibrate autosampler.

Initials: EGJ Date: 5/14/00

Observation	Possible Cause	Recommended Action
	Capillary bent away from electrode.	Tape capillary securely to heat plate to keep capillary from shifting position. Place the tape on the heat plate just above the electrode holder.
	Unfilled capillary, bubbles in capillary.	Replace capillary and rerun module.
	Pump blockage (pump is plugged with urea or crystallized buffer).	Remove and clean block.
	Loose valve fittings or syringe.	Tighten valve fittings and syringe.
	Anode buffer valve does not open.	Open buffer valve. Note: the valve should depress easily when you push the top with your finger tip. After you release the pressure the valve should spring to the "open" position. If the valve is stuck, it should be cleaned.
	Capillary plugged, broken, or nonconducting capillary.	Replace the capillary.
	Poor quality water in buffer solutions.	Remake buffer with freshly autoclaved, deionized water.

Initials: RS Date: 5/16/00

Observation	Possible Cause	Recommended Action
5. Low current	Small bubble in capillary blocking current flow.	Replenish gel in capillary.
	Plugged, broken, or nonconducting capillary.	Replace the capillary.
	Poor quality water in buffer solutions.	Remake buffer with freshly autoclaved, deionized water.
	Old, defective, or incorrectly made buffer or polymer solution.	Replace buffer or polymer solutions.
6. Fluctuating current	Too little buffer in anodic chamber.	Replenish buffer jar.
	Small bubble in capillary blocking current flow.	Replenish gel in capillary.
	Broken or cracked capillary.	Replace the capillary.
	Arcing to conductive surface on the instrument.	Clean the hotplate and autosampler. Ensure that the ambient temperature is between 15 and 30°C and the humidity is below 80%. Check for excessive condensation on the instrument. Clean and dry the outside of the tube containing the genetic analyzer buffer (in position 1) including the lid thoroughly with dH_2O.

Initials: RS Date: 5/16/00

Observation	Possible Cause	Recommended Action
Analysis Trouble Shooting		
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 Formamide/standard master mix and forgot to add PCR product or added only oil supernatant.	Prepare samples again and rerun.
	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.	Prepare samples again and rerun.
	PCR Instrument System failure or wrong program.	Check thermocycler diagnostics and instrument calibration. Repeat test.
	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.

Initials: RG Date: 5/16/00

Observation	Possible Cause	Recommended Action
2. No peaks at all	Sample not at bottom of tube. Air bubble at bottom of sample tube.	Spin sample in micro centrifuge.
	Capillary bent out of sample tube.	Align capillary and cathode. Recalibrate autosampler. Note: to verify whether a bent capillary is the problem, watch the movement of the autosampler tray during run operation.
	Autosampler not calibrated correctly.	Calibrate autosampler in X,Y, and Z directions. The tip of the capillary should almost touch the Z calibration point.
	Sealed sample tube septum (that is, septum will not open to allow electrode into sample tube). Septum not placed in the sample tube properly.	Replace the septum.
3. Low peaks detected for all samples and red standard	Insufficient sample and size-standard added.	Make sure to add 1µL of product and 0.5 µL of red standard to formamide in each tube. Rerun.
	Samples added to formamide that has degraded to formic acid and formate ions (leading to insufficient sample injected).	Use freshly deionized formamide.
	Sample and size-standard not thoroughly mixed with formamide.	Mix sample and size-standard into formamide by pipetting up and down several times.

Initials: RG Date: Thu Lab

Observation	Possible Cause	Recommended Action
4. High baseline	Dirty capillary window.	Clean capillary window with 95% ethanol.
	Capillary moved out of position in front of laser window.	Position capillary in front of laser window.
	Incorrectly prepared and/or old buffer or polymer solutions.	Replace buffer and polymer with fresh solutions.
	Defective capillary.	Replace the capillary.
	Wrong or deteriorated matrix file used.	Select the correct matrix file. Notify QC to make a new matrix (QC210). Renalyze with the new matrix.
5. Noisy baseline	Incorrectly prepared and/or old buffer or polymer solutions.	Replace buffer and polymer with fresh solutions.
	Dirty capillary holder aperture.	Clean the capillary holder with deionized water.
	Defective capillary	Replace the capillary.
6. Spikes in baseline	Precipitate in the POP-4 polymer solution.	Allow polymer to equilibrate to room temperature before adding to capillary.
	Old polymer (POP-4)	Use fresh polymer.
7. Not all expected ladder fragments and/or only part of the allele peaks are visible.	Analysis range was too small.	Under **Analysis parameters** check Analysis Range, correct to appropriate range (3000-6000) and Analyze again.

Initials: RG Date: 5/16/00

Observation	Possible Cause	Recommended Action
	Wrong preparation of formamide/size-standard/PCR product, e.g. insufficient mixing.	Prepare samples again and rerun samples.
8. Positive signal from the positive controls, but no or below 100 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 starting with the amplification.
		Extract larger area of the stain to achieve higher DNA yield; repeat test.
		Repeat electrophoresis of the amplified sample with a longer injection time
	Test sample contains PCR inhibitor (e.g. heme compounds, certain dyes).	Any or all of the following actions may be taken: use more Taq Gold enzyme, dilute extract, purify using Microcon concentration.
	Test sample DNA is degraded.	Reamplify with an increased amount of DNA.
	Sample prepared improperly for capillary injection.	Prepare fresh sample and rerun.

Initials: RS Date: 5/16/00

Observation	Possible Cause	Recommended Action
9. 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** from the pop up menu, check peak assignment and entered sizes. Reanalyze project with correct standard, check each sample for proper peak assignment.
	Samples were not loaded with formamide or not properly heat denatured before loading.	Rerun samples.
10. Presence of unexpected or additional peaks in the amplified positive controls sample.	Contamination by amplified product or samples.	Reamplify samples.
	Non-specific priming	Check thermocycler settings for correct annealing and extension temp. and time. Reamplify samples.
	samples not fully denatured	Make sure the samples are heated at 95°C for five minutes prior to loading onto autosampler.
11. Peak positions off throughout size range.	Incorrect electrophoresis temperature.	Check the injection list for temperature setting. If correct on injection list, check the log for a recording of the actual electrophoresis temperature.

Initials: RCJ Date: 5/16/00

Observation	Possible Cause	Recommended Action
12. Runs get progressively slower (that is, size-standard peaks come off at higher and higher scan numbers	Leaking syringe: polymer is not filling capillary before every injection.	Clean thoroughly or replace syringe.
	Syringe out of polymer	Fill syringe with fresh polymer.
	Change in room temperature	Repeat run. Space additional allelic ladders.
13. Runs get progressively faster (that is, size-standard peaks come off at lower and lower scan numbers.	Water in the syringe.	Prime syringe with small amount of polymer, invert syringe to coat syringe walls, and discard polymer. Then fill syringe with fresh running polymer.
	Change in room temperature	Repeat run. Space additional allelic ladders.
14. More than two alleles present for the test samples at one or more of the loci.	Presence of a mixture of DNA, mixed sample or contamination.	See interpretation of Complex STR Results Section.
15. Some, but not all, loci are visible for the test samples.	Quantity of DNA test sample is below the assay sensitivity.	Make sure to add 1µL of product to formamide in each tube. Rerun.
	Test sample DNA is de-graded.	Reamplify with an increased amount of DNA.
	Test sample contains PCR inhibitor (e.g. heme compounds, certain dyes).	Any or all of the following actions may be taken: use more Taq Gold enzyme, dilute extract, purify using Microcon concentrators.

Initials: RCJ Date: 5/16/00

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.
16. 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)
17. Poor resolution	Poor capillary performance.	Replace capillary.
	Incorrectly prepared and/or old buffer or polymer solutions.	Replace buffer and polymer with fresh solutions.
	Syringe leaking and old polymer doesn't get totally replaced for each injection.	Replace syringe, or remove and clean syringe with hot water, reassemble and refill.
	Injection sheet settings wrong.	Check if settings were changed. (Default Settings: module: GS STR POP4(1mL) A; Inj. Secs: 5; inj kV:15; Run kV:15; Run °C:60; Run Time: 24; Matrix File: NED POP4 copy) If they were, change them back and rerun.
	Poor quality water.	Use freshly autoclaved, deionized water.

Initials: RC

Date: 5/16/00

Observation	Possible Cause	Recommended Action
18. Peaks which progressively decrease in height and definition (resembling a "sine wave") to no visible peaks at all.	"Death of the Capillary" caused by exceeding the recommended capillary count, or contamination of the inside of the capillary by an agent which has degraded the interior coating.	Change the Capillary, buffer, POP4, and formamide and rerun samples. Make sure the 10xTBE buffer being used contains EDTA.
19. Pull-up (bleed through) peaks visible for peak heights below 1000. **Raised, e.g. green, baseline between two high, e.g. blue, peaks.** **Indentations in, e.g. green, at the scan position of a high, e.g. blue, peak.**	Matrix artefacts caused by the application of a wrong or a deteriorated matrix.	Renalyze run with a different matrix. Notify QC that a new matrix should be run (QC210). Renalyze gel with new matrix.

Initials: RCS Date: 5/16 Loo

ABI 377 DNA Sequencer procedures

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 (QC167) 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: 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: RS Date: 5/16/00

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.

   NOTE: 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: QQ Date: 5/16/00

Gel Electrophoresis on ABI 377 Sequencer

Only the multiplexes Quad, YM1, and Profiler Plus have been validated for analysis using a gel and must be run on these instruments.

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 artifactual 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: RCS Date: 5/11 00

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: QCJ Date: 5/16/00

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.

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**.

Initials: RCS Date: 5/11/00

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 **Pause** then **Cancel** and then **Terminate** to end the plate check, then open the instrument door. Always press Pause first instead of directly canceling the run - this causes the laser to slow down first.

2. 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.

3. 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.

   IMPORTANT: Check for buffer leaks before you put the heat plate on.

4. 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.

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 can be added to the wells before pre-running the gel. 50µL of the loading buffer is applied by using a 100µL pipette tip and dragging the pipette across the gel while expelling the blue buffer (use it sparingly).

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

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.

Initials: RCS Date: 5/16 Loo

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!**

Samples from previous gels that need to be rerun may be added to a subsequent gel of the same type (evidence or exemplar).

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:
   System / year / instrument / gel #
   e.g.

QUAD multiplex gels	Q00-Men001, Q00-Mul001 and Q00-Jef001.
Y STR gels	Y00-Men001, Y00-Mul001 and Y00-Jef001
Profiler plus gels	P00-Men001, P00-Mul001 and P00-Jef001

   This reflects the STR system, 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.

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:

Initials: RCS Date: 5/16/00

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

Profiler Plus only: include one tube of allelic ladder in the above calculation.

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.
   Profiler Plus only: add 4µl of allelic ladder to the mastermix. Load the allelic ladder on the gel twice from that one tube.

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. Have another analyst witness the order of tubes in the ice box. 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.

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

Initials: KC Date: 8/30/00

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**
Electrophoresis voltage (kV):	3.00
Current (mA)	60.0
Power (W)	200
Gel Temperature (°C)	51
Laser Power (mW)	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.

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.

Initials: RG Date: 5/16 Loo

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.

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.

Initials: RCJ Date: 5/16/00

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 may stick 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: 5/16/00

H. 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:
   - while the instrument is running, 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

   Quit the data collection software program. Relaunch the data collection program. The firmware is automatically downloaded to the instrument in 60-90 seconds. (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 may have 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 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.

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.

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).

Initials: RCS Date: 5/16/00

6. **Weak fluorescence on one side of the gel only** can also be caused by improper alignment of the gel plates with the laser plane (see above).

7. **Weak fluorescence on the whole gel** can also be caused by improper alignment of the gel plates with the laser plane but is commonly caused by the laser being out of alignment. Notify QC and do not use the instrument in question until it has been serviced.

8. **Horizontal streaks** on a gel are a sign of the laser beginning to deteriorate. It must be replaced as soon as possible.

9. **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.

10. "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 (QC167).

11. **Loss of signal on certain gel areas** with inconsistent mobility like **smearing** up and down of bands ("black holes") may be caused by electrostatic charges caused by "over wiping" the glass plates with Kimwipes. Make a fresh gel and rerun the samples.

Initials: RCS Date: 5 Dec 00

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 make sure that these gels are properly archived 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 archiving procedure outlined in the General Guidelines for fluorescent STR analysis.

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 Collection Gel Processing

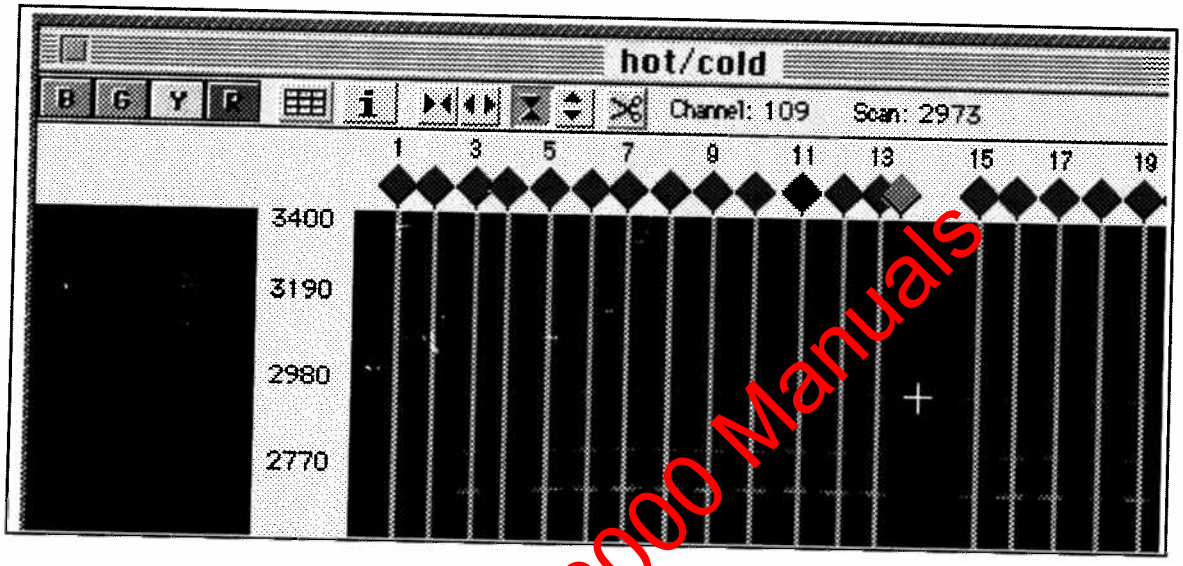
1. Open the **GS Analysis** software by clicking on the **377 analysis** icon.

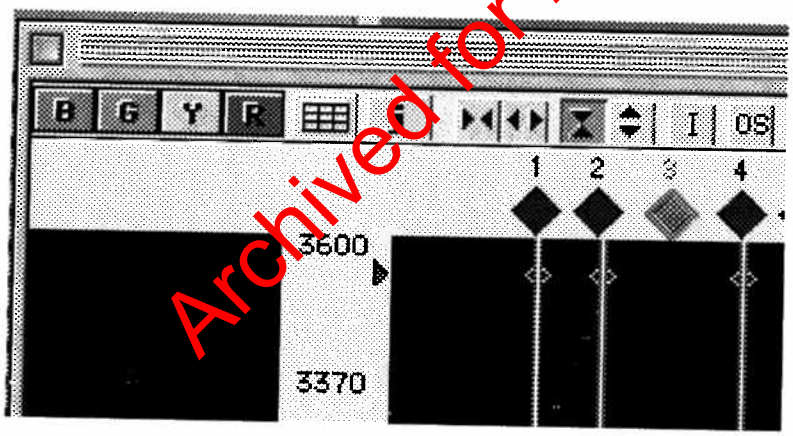
2. Under **File** go to **Open.** From the listed possibilities click on existing **Collection gel**. Open windows until the desired gel file (in the raw data folder) is displayed and highlighted. Gels are represented by vertical icons as opposed to folders.
   Click open.

Initials: RCS Date: 5/16/00

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. The standard setting displays all four colors.





Genescan version 3.0

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

Initials: RC Date: 5/16/00

For Genescan Version 3.0 with the new lane tracking module, the scissor tool has been replaced by the Interpolation button I. The OS button indicates oversaturation, if this function is selected only peaks higher than 8191fu's will be displayed on the gel image.

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.

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 all appropriate colors
   - for Profiler Plus only, copy and paste the sample info column also as **sample comments**

Initials: RG Date: 5/16/00

NOTE: It is important to enter the sample information in an identical fashion for all colors present in the amplification. The different STR multiplexes contain the following colors:

QUAD	blue and green
Y M1	blue and yellow
Profiler Plus	blue, green and yellow

The **edit copy, paste,** and **fill down** commands are used to fill out the sheet. Again a reminder the text for the sample info for the different colors must be identical for the same sample!! This is easily achieved by using the fill down function. 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.

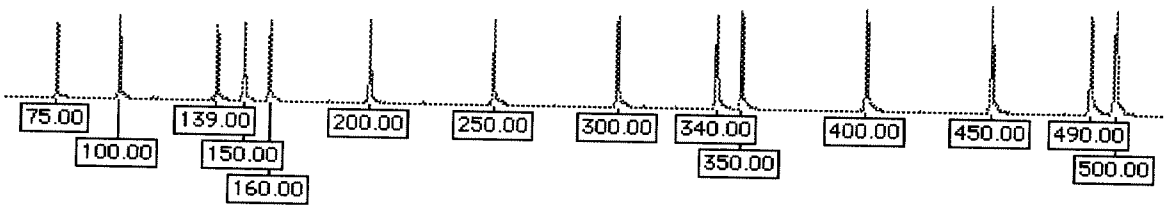
5. After entering all relevant information, close the sample sheet window. Choose **Save.** The gel image will display tracking lines for all used lanes.

6. The next step is to choose the appropriate scan area to analyze by looking at the red size standards:

   The size range for the different multiplex reactions varies as follows:

STR system	size range	necessary GS500 standard peaks
QUAD	127 to 238 bp	6 fragments from 100 - 250 bp
Y M1	188 to 382 bp	10 fragments from 139 - 450 bp
Profiler Plus	103 to 341 bp	12 fragments from 75 - 450 bp

The following picture shows the arrangement of the GS500 size standard peaks.



Initials: RCS Date: 5/15/00

7. Adjust the gel image so that it contains all of the red size standard fragments that are necessary for the specific multiplex. Determine which standards are present and 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.

8. The gel picture displayed on the screen should have a black background and according to the multiplex used distinct blue, green, yellow 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. If the autotracking results in fragmented lanes, this can be corrected by under **Gel** and **Track lanes**, selecting **Revert to straight tracking**.

10. Click on the **lane indicator diamond** for each used lane. The white line should drawn through the middle of each DNA fragment. All necessary 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.**

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 "**.3**". 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!

B 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.

Initials: RCS Date: 5/11/00

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 upper size standard fragment. The maximum number of scans after 2.5h is 5700 to 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 450bp fragment, the electrophoresis run was too slow.

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

C. 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**.

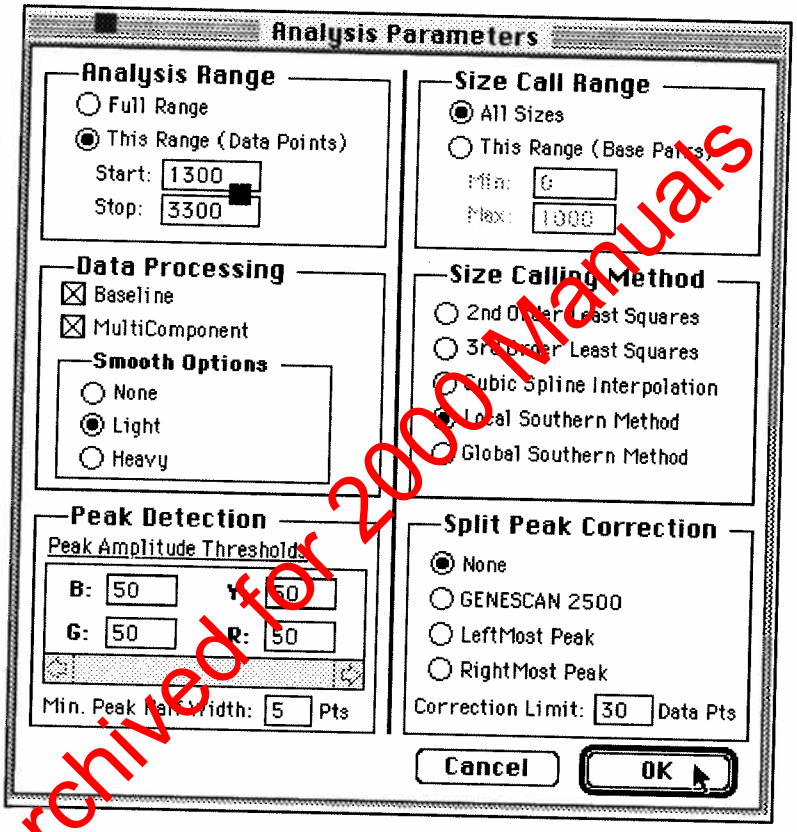
   After the matrix selection, the software should display a window stating that the matrix file was successfully installed within the selected sample files.

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**.

Initials: RO Date: 5/16/00

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:



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

4. Click on the top blue, green, yellow, and red boxes to select the necessary colors for the analysis for all lanes. Always select red!

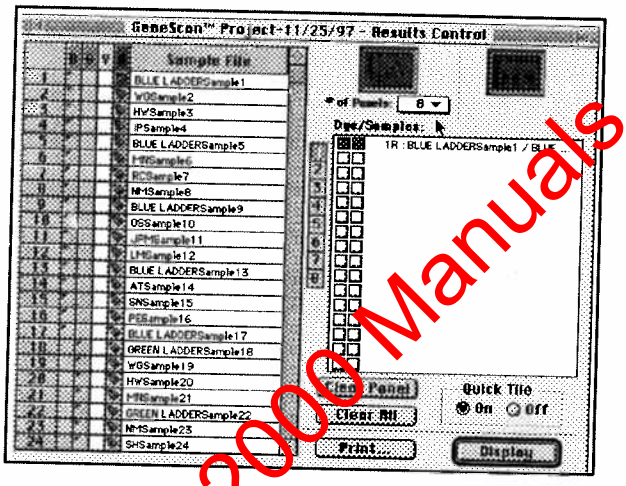
   Click on the **Analyze** button in the upper left corner. All selected samples will be analyzed. After the analysis a black triangle appears in the color boxes to indicate that this sample has been analyzed.

Initials: ACS Date: 5/16/00

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.



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. Check the size assignments to the red standard peaks. This can be done several ways:
Option 1: Overlay up to16 size standards in one panel by clicking on the top of the red data column. The red standards for 16 lanes should be highlighted.

Click **display**. 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. Repeat this step for the remaining lanes.

Initials: RG Date: 5/16 00

Option 2: Select 8 panels and check Quick Tile. Click on panel 1, hold the mouse button down and drag the highlight down to sample 8. Click on **Display**. Each sample standard will be displayed in its own window; to view all 8 you must scroll through all the windows. Continue checking your size standards for the entire tray by going back to the **Results Control** window, clicking on **Clear All** and selecting the next 8 samples.

Check sizes by clicking on each peak or by scanning the size table for intermediate sizes. You can also check the sizes by clicking on the leftmost column in the table window, the corresponding peak will automatically be highlighted. Continue until every lane with samples has been checked.

Required Sizes for the Peaks are:

QUAD		Y M1		Profiler plus	
peak 1	100	peak 1	139	peak 1	75
peak 2	139	peak 2	150	peak 2	100
peak 3	150	peak 3	160	peak 3	139
peak 4	160	peak 4	200	peak 4	150
peak 5	200	peak 5	250	peak 5	160
peak 6	250	peak 6	300	peak 6	200
		peak 7	340	peak 7	250
		peak 8	350	peak 8	300
		peak 9	400	peak 9	340
		peak 10	450	peak 10	350
				peak 11	400

7. 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 right size, and press return. Continue to enter the above values for the size standard peaks.

   After entering the largest size, 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.

Initials: RC Date: 5/16/00

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

D. 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 A collection gel processing.

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 A. 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 the wrong red size standard peaks are 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: RCS Date: 5/11/00

E. General STR failed amplifications and Gel 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.
	$MgCl_2$ or deionized water not added to PCR Reaction Mix.	Add $MgCl_2$ or deionized water; repeat test.
	Primer mix not added to mastermix.	Add primer mix; 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: RCS Date: 5/16/00

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.
3. Allelic ladder signal too low but amplified samples show normal intensity	Not enough or old allelic ladder used	Rerun gel with new allelic ladder preparation, use new lot if necessary
4. All peaks including allelic ladder, positive control, unknown samples and red size standard show low fluorescent intensity	Laser alignment problem	Notify QC and call service
	Gel not clamped in properly	Rerun all samples

Initials: RCS

Date: 5/16/00

Observation	Possible Cause	Recommended Action
5. 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 Microcon centrifugation; 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 Gold enzyme, dilute extract, purify using Microcon
	Test sample DNA is degraded.	Reamplify with an increased amount of DNA.
	Sample loss during the loading of the test sample.	Rerun sample on new gel.
6. 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 or APS was too old.	Prepare new APS, new Long Ranger solution, pour new gel and rerun samples.
	Samples were not loaded with formamide or not properly heat denatured before loading.	Rerun samples.

Initials: RCJ Date: 5/16/00

Observation	Possible Cause	Recommended Action
7. 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.	Reamplify samples.
8. Presence of additional peaks in the red size standard.	Samples were not loaded with formamide or not properly heat denatured before loading.	Rerun samples on new gel.
	Peaks caused by bleed through, wrong or deteriorated matrix applied.	Apply different matrix, make new matrix (QC120) and reanalyze.
9. More than two alleles (for Y-STRs more than one allele) present for the test samples at one or more of the loci.	Presence of a mixture of DNA, mixed sample or contamination.	See interpretation of Complex STR Results Section
10. 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 degraded.	(See Troubleshooting **3**.)
	Test sample contains PCR inhibitor (e.g. heme compounds, certain dyes).	(See Troubleshooting **3**.).
	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.

Initials: RCS Date: 5/16/00

Observation	Possible Cause	Recommended Action
11. 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)
12. Loss of signal on certain areas of the gel e.g. fading on one side only, or "black holes" round blots with no fluorescence	Gel not installed properly	(see section H "Trouble-shooting" under ABI 377 Sequencer procedures)
	Laser alignment problem	notify QC to call service
	Electrostatic charge on the gel plate, can be caused by "over wiping" the plates with Kimwipes	make new gel, rerun samples
13. Fluorescent artefacts such as vertical red lines, yellow or blue color spots, horizontal streaks	Laser performance or gel specific problem	(see section H "Trouble-shooting" under ABI 377 Sequencer procedures)
14. Pull-up (bleed through) peaks visible for peak heights below 1000.	Matrix artefacts caused by the application of a wrong or a deteriorated matrix.	Renalyze gel with a different matrix. Notify QC that a new matrix should be run (QC210). Renalyze gel with new matrix.
Raised, e.g. green, baseline between two high, e.g. blue, peaks.		
Indentations in, e.g. green, at the scan position of a high, e.g. blue, peak.		

Initials: RG Date: 5/16/00

Genotyper Analysis

After the sizing of the fragments is completed, the project files 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 **appropriate Genotyper** icon. The Genotyper templates should have the following names:

QUAD	QUAD STR
Y STR	Y M1
Profiler Plus	ProPlus
Cofiler	Cofiler

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.), and highlight it.

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

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 into the same Genotyper file.

Initials: RCJ Date: 5/11/00

3. Continue with the appropriate section for the further steps:

QUAD	section A
Y STR	section B
Cofiler	section D
Profiler Plus	section E

Genotyper section A Quad

1. 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 - e.g. LP Q00-Men015. Click **Save**.

2. 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.

3. 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 (also see section STR results interpretation):

   1 Pull-ups of peaks in any color caused by a very high peak of another color in the same lane. Pull-ups are caused by the inability of the matrix file to remove all e.g. green light components from a blue signal.

Initials: RS Date: 5/16/00

2 Shoulder peaks approximately 1-4 bp smaller or bigger than the main allele. Shoulder peaks are mostly present on the right side of a peak if the peak shape shows a slope that is trailing out.

3 "N" bands where the main allele shows a split peak. "N" bands are caused by incomplete extra A addition and are characterized by either a jagged edge on the left side of the peak or a complete split on the top level.

4 -4 and +4 bp stutter peaks if there is not indication of the presence of a mixture. -4 bp stutter is common and will often be labeled if followed by a high allele peak. +4 bp stutter on the other side is extremely rare and has to be interpreted carefully. Defined peaks in a +4 bp position might indicate the presence of a mixture.

5 Non specific artifacts. This category should be used if a labeled peak is caused by a not previously categorized technical or amplification problem.

6 Labels placed on elevated or noisy baselines which do not resemble distinct peaks.

7 Peaks caused by overflow of sample in the adjacent lane.

4. Fill out the Genotyper Editing Sheet for each sample number and note the reason for removal of a peak using the number code above.

At this stage it is also necessary to make decisions about samples that should be rerun with either more or less amount of amplification product.

Inconclusive samples:
Over-amplified samples often have peak heights between 5000 and 7000 fu's and are characterized by a plateau shaped or misshaped peaks and often contain a lot of labeled stutter peaks and artifacts (also see section 4B. of Interpretation of Complex Autosomal STR Results). Instead of laboriously editing out all of these peaks, the sample should be deemed inconclusive and marked for re-running. **All DNA mixtures where peaks in at least one color are ≥ 6000 fu's have to be rerun with less.** Remove all labels from the lane in question, don't list all of the sizes, note "numerous" for peaks removed. The sample should be placed on a rerun sheet for re-running with 4µL of a 1/10 dilution of the amplification product for samples with peak heights between 5000 -7000 fu's. For problem samples were peak heights are less than 5000, re-running 1-2µL of amplified product is recommended.

DNA alleles visible but below threshold:
If a sample displays allele peaks just below the threshold of 50 fu's there is a distinct possibility that the alleles can be identified after a repeated run with a higher amount of amplified product.

Initials: RCS Date: 5/16/00

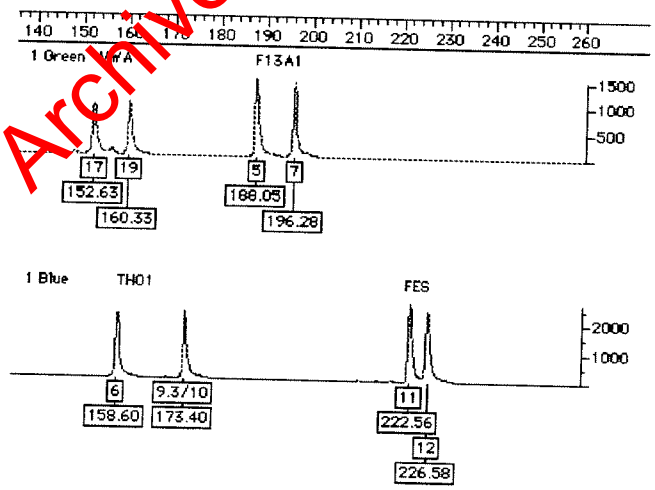
Place the sample on a rerun sheet. Acceptable amounts of amplification product are 6 - 8µL of amplified product.

New alleles:
If a locus displays only one peak label and a distinct same color peak is visible but is not labeled, or is labeled "OL allele?", it is because the unlabeled peak is outside the defined allele range or is not present in the allelic ladder. This peak might be a "new", previously unreported allele. This possibility must be considered, especially if the other loci show a proper allele profile. The presence of a possible "new" allele must be pointed out to a DNA supervisor for confirmation. The "new" allele will not be automatically reported in the Genotyper table, but will be visible on the electropherogram. Click on the unlabeled peak in order to label it with the size in bp, which is necessary for allele identification.

5. 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.

6. Check the positive control type.



Initials: RCS Date: 5/16/00

7. Scroll through the plot window again to check for artifacts 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**.

13. As already listed in the General Guidelines for fluorescent STR analysis 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. 12 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. 00-Men001 Files)

    This folder has to be archived on the appropriate disk..

14. Have a supervisor review the analyzed gel and get a signature on the editing sheet.

Initials: RCS Date: 5/14/00

Troubleshooting QUAD and YM1 Genotyper

A. 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).

B. 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.

C. 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.

D. 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**.

E. The Apple 1 Macro selects all blue and green (or blue and yellow) 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** (+select yellow). 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 lanes, this will re-label all edited peaks.

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

1. The peaks have not been sized. This occurs especially for the loci FES and DYS389II when the 250bp or for YM1 the 400bp size fragment were not present on the gel or had not been included in the analysis area. Quit Genotyper and correct any errors made during **Collection gel processing** or **Project file analysis**. If the collection time was over before the required size standard fragment data were measured, rerun the sample.

2. The peak is outside of the allele calling window because it is a new allele (see step 4 above).

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

Initials: RCJ Date: 5/16/00

4. The peak is outside of the allele calling window because of an ill defined size standard. Go to Genescan analysis, open the project and correct the size standard as described above.

H. The Genotyper printout for QUAD 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 Genotyper printout for YM1 also should have a standard format: yellow lanes, then blue lanes. The table should have 2 rows for each locus.

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 of the QUAD Genotyper section and step 3 of the YM1 Genotyper for correct setting.

I. NOTE: unsized peaks cannot be placed according to size on the electropherogram. Therefore, when comparing an unlabeled allele (unlabeled because it is too low to be sized, but high enough to be detected visually) to a labeled allele (e.g. of a duplicate) you cannot determine the allele type and size by visual comparison while the results are viewed by size. To be able to align an unlabeled allele with a labeled allele in the same run you must select **View by Scan** from under the **View** menu!

J. If you see the same sample listed several times in the dye/lanes window or you see more samples than you have imported, you have most likely imported your samples more than once or you have imported your samples into a Genotyper template that already contained sample information. Under **Analysis** select **Clear Dye/Lanes** window and also under **Analysis** select **Clear Table**. Re-import your file(s).

Initials: RCS Date: 5/16/00

Genotyper section B YM 1

1. 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 - e.g. MJS Y00-Men015. Click **Save**.

2. Run the **Y M1 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**.

   Y M1 Macro 1 selects peaks that meet allele calling criteria (see results interpretation) by automatically performing the following steps:
   select blue lanes + select yellow 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.

3. 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 (also see section STR results interpretation):

   1 Pull-ups of peaks in any color caused by a very high peak of another color in the same lane. Pull-ups are caused by the inability of the matrix file to remove all e.g. green light components from a blue signal.

   2 Shoulder peaks approximately 1-4 bp smaller or bigger than the main allele. Shoulder peaks are mostly present on the right side of a peak if the peak shape shows a slope that is trailing out.

   3 "N" bands where the main allele shows a split peak. "N" bands are caused by incomplete extra A addition and are characterized by either a jagged edge on the left side of the peak or a complete split on the top level.

   4 -4 and +4 bp stutter peaks if there is not indication of the presence of a mixture. -4 bp stutter is common and will often be labeled if followed by a high allele peak. +4 bp stutter on the other side is extremely rare and has to be interpreted carefully. Defined peaks in a +4 bp position might indicate the presence of a mixture.

   5 Non specific artifacts. This category should be used if a labeled peak is caused by a not previously categorized technical or amplification problem.

Initials: RG Date: 5/16/00

6 Labels placed on elevated or noisy baselines which do not resemble distinct peaks.

7 Peaks caused by overflow of sample in the adjacent lane.

4. Fill out the Genotyper Editing Sheet for each sample number and note the reason for removal of a peak using the number code above.

At this stage it is also necessary to make decisions about samples that should be rerun with either more or less amount of amplification product.

Inconclusive samples:
Over-amplified samples often have peak heights between 5000 and 7000 fu's and are characterized by a plateau shaped or misshaped peaks and often contain a lot of labeled stutter peaks and artifacts (also see section 4B. of Interpretation of Complex Autosomal STR Results). Instead of laboriously editing out all of these peaks, the sample should be deemed inconclusive and marked for re-running. **All DNA mixtures where peaks in at least one color are ≥ 6000 fu's have to be rerun with less.** Remove all labels from the lane in question, don't list all of the sizes, note "numerous" for peaks removed. The sample should be placed on a rerun sheet for re-running with 4µL of a 1/10 dilution of the amplification product for samples with peak heights between 5000-7000 fu's. For problem samples were peak heights are less than 5000, re-running 1µL of amplified product is recommended.

DNA alleles visible but below threshold:
If a sample displays allele peaks just below the threshold of 50 fu's there is a distinct possibility that the alleles can be identified after a repeated run with a higher amount of amplified product. Place the sample on a rerun sheet. Acceptable amounts of amplification product are 6 - 8µL of amplified product.

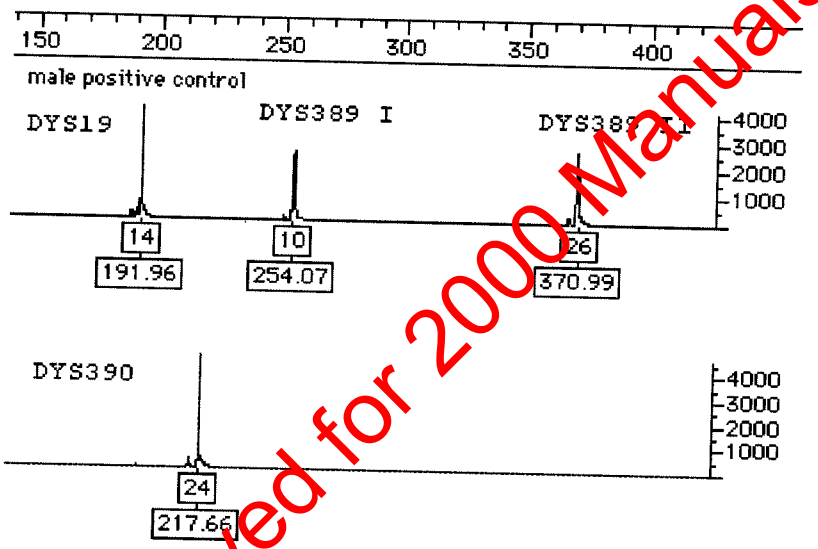
New alleles:
If a locus displays only one peak label and a distinct same color peak is visible but is not labeled, or is labeled "OL allele?", it is because the unlabeled peak is outside the defined allele range or is not present in the allelic ladder. This peak might be a "new", previously unreported allele. This possibility must be considered, especially if the other loci show a proper allele profile. The presence of a possible "new" allele must be pointed out to a DNA supervisor for confirmation. The "new" allele will not be automatically reported in the Genotyper table, but will be visible on the electropherogram. Click on the unlabeled peak in order to label it with the size in bp, which is necessary for allele identification.

Initials: RCJ Date: 5/16/00

5. 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 then 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 2 labels in columns 3-4, the text "check" in column 2 if number of labels >1 (titled "mixture."); put column titles in first row.

6. Check the positive control type. It is



7. Scroll through the plot window again to check for artifacts 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**.

Initials: RCf Date: 5/11/00

10. Next print the plot window. To achieve a uniform format, the base pair range to print must be selected. Under **View,** select **Zoom to.** Enter 140 to 400. 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. Under **File**, quit **Genotyper.**

12. As already listed in the General Guidelines for fluorescent STR analysis 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. 12 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. Y 00-Men001 Files)

    Archive this folder on the appropriate disk.

13. Have a supervisor review the analyzed gel and get a signature on the editing sheet.

Genotyper troubleshooting see QUAD Genotyper section (A). **For the YM1 all references to Green have to be applied to yellow.**

Initials: RG Date: 5/16/00

Genotyper section D Cofiler

Two sets of amplified samples can be run together on the CE in one sample tray, but it is not permitted to combine an evidence amplification with an exemplar amplification in one tray. The samples from one amplification sheet must be analyzed together with their controls. This means that when running a full tray you can remove the samples from one of the amplifications from the Dye/Lane list after the project was imported into Genotyper, but care has to be taken that the correct controls stay with the sample set.

1. Under **View** select **Show Dye/Lanes window** you will see a list of the samples you have imported from Genescan analysis. To remove one amplification set, hold down the shift key and highlight the lanes containing the samples you DO NOT want to genotype, select **Cut** from the **Edit** menu. Check the run sheet for the sample numbers for the separate amplifications.

2. After removing one set of samples, change the name of the Genotyper template to your initials and the casework run file name.

   If two amplifications are being processed in separate Genotyper files the amplification set must be identified, e.g. AL CE1/00-00 semen A

3. Run the Macro (the program which assigns the correct allele names to the peaks, see appendix for complete details) by pressing the **Apple key** and the number 1 (simultaneously), or by highlighting "**kazam**" in the lower left-hand corner on the main window, and under **Macros** selecting **Run Macro**.

   This Macro contains the following filter functions:
   A 10% background filter for all loci and additionally a 15% filter for peaks in the -4bp stutter position for D3S1358, D16S539 and D7S820.

4. The plot window will appear automatically when the macro is completed. Check to make sure that the ladders that were run match the allele sequence shown below. Also check the results for the positive control.

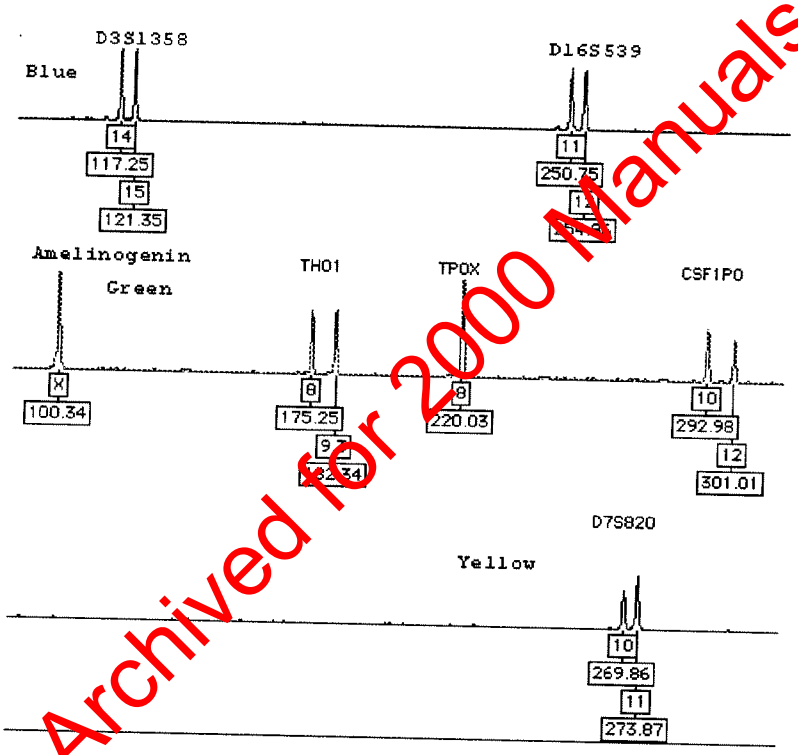
Initials: RES Date: 5/11/00

The genotype of the positive control is:

		D3S1358	D16S539
Blue label:	**14, 15**	**11, 12**	

		Amelogenin	THO1	TPOX	CSF1PO
Green label:	**X**	**8, 9.3**		**8**	**10, 12**

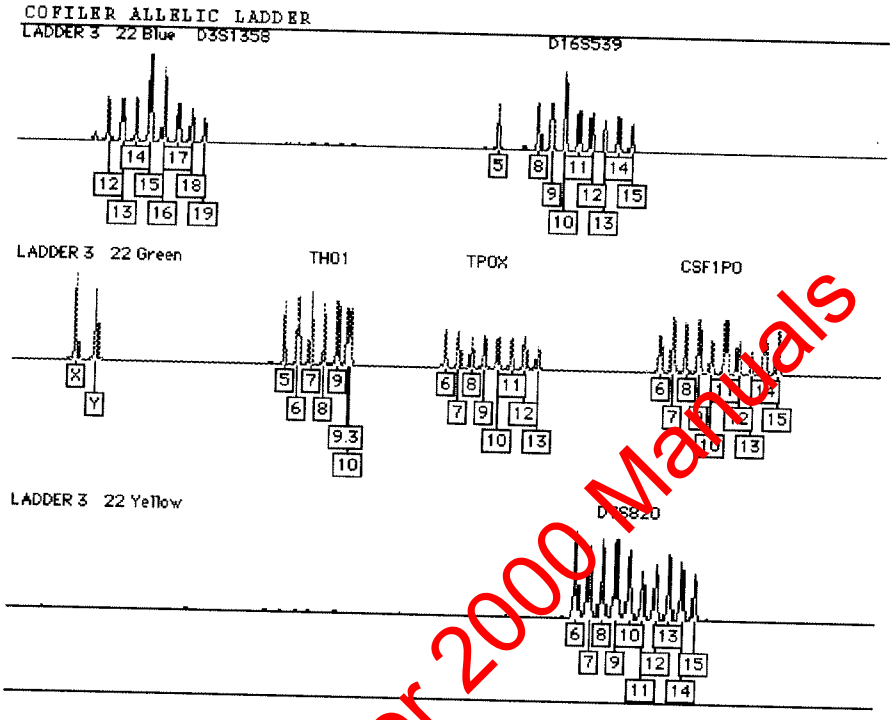
	D7S820
Yellow label:	**10, 11**



If the alleles for the positive control are shifted one step towards a higher allele number, this is an indication that the first allele for the allelic ladder has been assigned incorrectly (see step 7.).

Initials: AG Date: 5/16/00

Cofiler Allelic Ladder



5. If the first allele of the ladder has been assigned incorrectly in one of the systems, in most cases, this is because the preceding stutter peak is designated with the first allele name. If this is the case, you must raise the peak height in the categories window in order to force the software to skip the stutter peak.

   - First determine the height of the stutter peak by placing the cursor on the peak in question (as if you are editing). The information displayed on the top of the window refers to the peak where the cursor is located and contains the peak height. Make a note of the peak height.

   - Open the categories window (under views on the menu) and highlight the first allele in the offset category (e.g 18 o.s.) of the polymorphism that needs to be corrected.

   - In the dialogue box change the height for the minimum peak height to a few points above the determined height of the stutter.

   - Rerun the macro and then check to make sure everything is correct by looking at the first allele in each locus in the ladder and by comparing the result for the positive control

Initials: RCS Date: 5/16/00

If the ladder has been corrected but the genotype of the positive control is still incorrect see Genotyper Troubleshooting.

6. Check all lanes. Labels for extra peaks can be manually deleted by placing the cursor on the peak above the baseline and clicking. If you mistakenly delete a label, before you do anything else, press the **apple key + Z** and the allele name label will reappear (the command apple key +Z only undoes the last action).

   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 then click again to finally remove the label. Use the zoom functions to get a close look at certain peaks (for instance if you have an allele with two labels which are very close to each other) by using the **Zoom** submenu under the **Views** menu.

   Extra peaks are defined as peaks that meet one of the following criteria (also see section STR results interpretation):

   1 Pull-ups of peaks in any color caused by a very high peak of another color in the same lane. Pull-ups are caused by the inability of the matrix file to remove all e.g. green light components from a blue signal.

   2 Shoulder peaks approximately 1-4 bp smaller or bigger than the main allele. Shoulder peaks are mostly present on the right side of a peak if the peak shape shows a slope that is trailing out.

   3 "N" bands where the main allele shows a split peak. "N" bands are caused by incomplete extra A addition and are characterized by either a jagged edge on the left side of the peak or a complete split on the top level.

   4 -4 and +4 bp stutter peaks if there is not indication of the presence of a mixture. -4 bp stutter is common and will often be labeled if followed by a high allele peak. +4 bp stutter on the other side is extremely rare and has to be interpreted carefully. Defined peaks in a +4 bp position might indicate the presence of a mixture.

   5 Non specific artifacts. This category should be used if a labeled peak is caused by a not previously categorized technical problem.

   6 Labels placed on elevated or noisy baselines which do not resemble distinct peaks.

   7 Sharp peaks (spikes) that do not resemble peaks but rather vertical lines and are caused by air bubbles or urea crystals passing the laser window.

The presence of a spike can be verified by looking at the red size standard for the same lane (see below). A red "spike" should occur at the same position.

8 Dye artifact peak which is defined as a "blip", whose shape is not that of a regular peak, occurs at a constant scan position approximately between 3500 and 4000 and most frequently for the blue label. This peak is distinguishable from a normal allele by its indeterminate shape. If you are uncertain, use the zoom options in order to view the peak(s) more closely.

To compare the red electropherogram with the other color lanes, hold down the shift key and either click on the red "R" box in the upper left hand corner, or under **edit** go to **select** +red. Before printing the plots the red electropherograms must be deselected, and the other three colors re-selected as above.

7. Fill out the Genotyper Editing Sheet for each sample number and note the reason for removal of a peak using the number code above.

At this stage it is also necessary to make decisions about samples that should be rerun with either more or less amount of amplification product.

Inconclusive samples:
Over-amplified samples often have peak heights between 5000 and 7000 fu's and are characterized by a plateau shaped or misshaped peaks and often contain a lot of labeled stutter peaks and artifacts (also see section 4E of Interpretation of Complex Autosomal STR Results). Instead of laboriously editing out all of these peaks, the sample should be deemed inconclusive and marked for re-running. **All DNA mixtures where peaks in at least one color are ≥ 6000 fu's have to be rerun with less.** Remove all labels from the lane in question, don't list all of the sizes, note "numerous" for peaks removed. ATTENTION: Cofiler is characterized by extreme peak height differences between the different color labels. It is acceptable to only deem some of the loci inconclusive and keep the less over-amplified ones. The sample should be placed on a rerun sheet for rerunning with 1µL of a 1/10 dilution of the amplification product.

DNA alleles visible but below threshold:
If a sample displays allele peaks just below the threshold of 100 fu's there is a distinct possibility that the alleles can be identified after a repeated run with a prolonged injection time of 10 seconds. Place the sample on a rerun sheet. Do not use times longer than 10 seconds since this affects the peak shape. For very low peaks a combination of using 2µL amplified sample and 10 seconds injection time can be used.

New alleles:
If a locus displays only one peak label and a distinct same color peak is visible but is not labeled, or is labeled "OL allele?", it is because the unlabeled peak is outside the defined allele range or

is not present in the allelic ladder. This peak might be a "new", previously unreported allele. This possibility must be considered, especially if the other loci show a proper allele profile. The presence of a possible "new" allele must be pointed out to a DNA supervisor for confirmation. The "new" allele will not be automatically reported in the Genotyper table, but will be visible on the electropherogram. Click on the unlabeled peak in order to label it with the size in bp, which is necessary for allele identification.

8. After the editing has been finished scroll through the plot window to double check.

9. Then run the **Create Table 1 macro** by pressing the **Apple key** and the number **2** (simultaneously). Under **View** open the table window. Compare the sample information in the table with the amplification and the run control sheet. If an error gets detected at this point it can be corrected as follows:
   - open the dye/lane window
   - click on the lane in question
   - place the cursor in the sample info box and correct the text
   - under **Edit** select the appropriate color
   - run macro Apple 2 again

   Before printing the table under **file** select **page set-up** and choose 65% and landscape. Print the table by under **file** selecting **print**.

10. Under **Analysis** select **Change Labels**. A dialogue box appears giving you the option of labels to assign to the peaks. Select **Size in bp** and **Category name**. Click **okay**. The plot window will now show the peaks with labels indicating both size in bp and category name; there will not be enough room to scroll through the plot window at this point.

11. Before printing plots, check that the zoom range shows 90-330. Change the page set-up back to 100% and letter. Under **file** select **print**. Click **okay** in the print dialogue box.

12. After the printing is finished, under **file**, **quit** Genotyper. Click **save**.

    The Genotyper file will automatically be saved in the run folder from which you imported your data; it can be located there and re-edited at a later date.

13. As already listed in the General Guidelines for fluorescent STR analysis the run folder should now contain the following items:
    - a injection list (32K ABI Prism 310 Collection document)
    - the run log (32K ABI Prism 310 Collection document)
    - sample files for all samples (96K Genescan documents)
    - a project file (32K Genescan document)
    - the genotyper file (ca. 160K Genotyper document)

Initials: RG Date: 5/16 00

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

Files (e.g. CE2/00-001 Cofiler files)

Archive this folder on the appropriate disk.

14. Have a supervisor review the analyzed gel and get a signature on the editing sheet.

For **Troubleshooting** see Genotyper section **E** Profiler Plus.

Initials: RCS Date: 5/12/00

Genotyper section E Profiler Plus

1. After importing the project and saving the genotyper file, run the Macro by pressing the **Apple key** and the number 1 (simultaneously), or by highlighting "**kazam**" in the lower left-hand corner on the main window, and under **Macro**, selecting **Run**

   This Macro contains the following filter functions:
   A 10% background filter for all loci, and additionally for peaks in the -4bp stutter position a 11% filter for D13S317 and D7S820, a 13% filter for FGA, and a 15% filter for D3S1358, VWA, D8S1179, D21S11, D18S51, and D5S818.

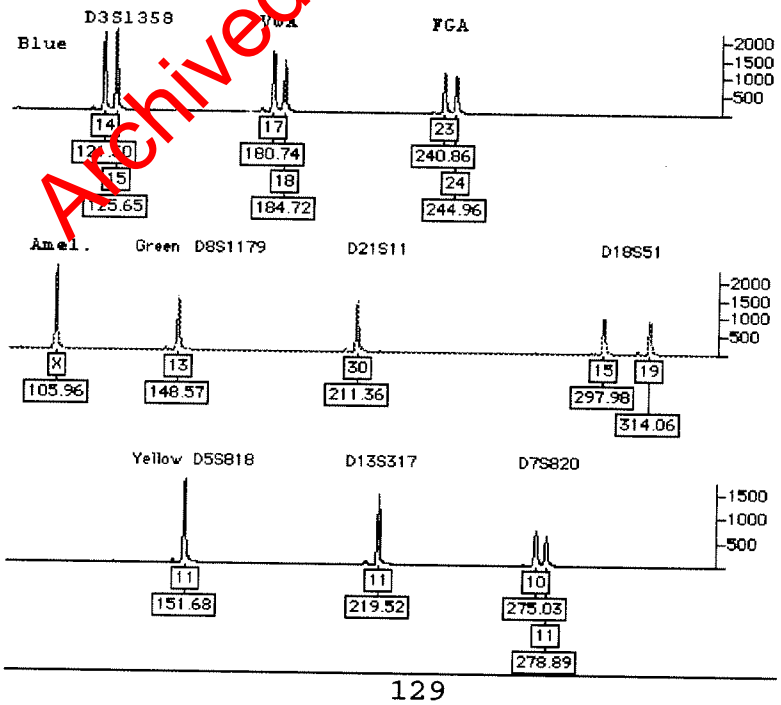
2. The plot window will appear automatically when the macro is completed. Check to make sure that the ladders that were run match the allele sequence shown below. Also check the results for the positive control.

   The genotype of the positive control is:

	D3S1358	VWA	FGA
Blue label:	**14, 15**	**17, 18**	**23, 24**

	Amelogenin	D8S1179	D21S11	D18S818
Green label:	**X**	**13**	**30**	**15, 19**

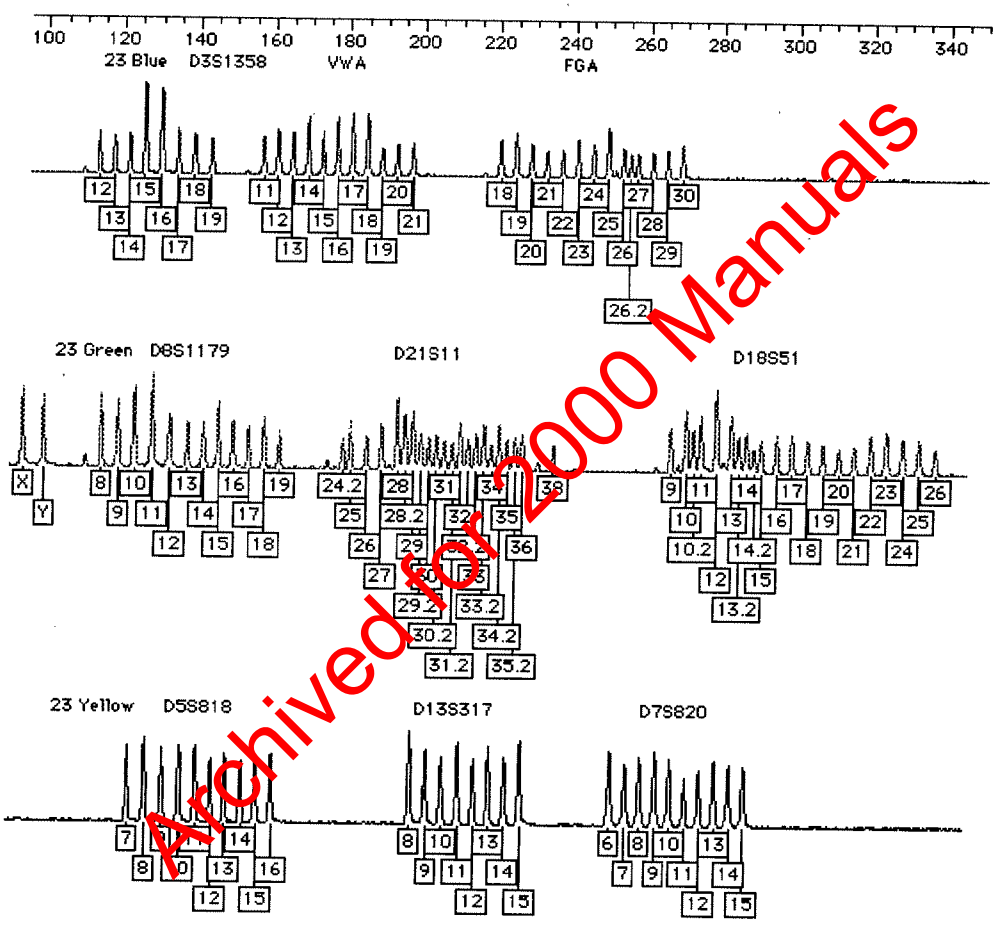
	D5S818	D13S317	D7S820
Yellow label:	**11**	**11**	**10, 11**



Initials: RG Date: 5/16/00

If the alleles for the positive control are shifted one step towards a higher allele number, this is an indication that the first allele for the allelic ladder has been assigned incorrectly (see step 7.).

Profiler Plus Allelic Ladder



3. If the first allele of the ladder has been assigned incorrectly in one of the systems, in most cases, this is because the preceding stutter peak is designated with the first allele name. If this is the case, you must raise the peak height in the categories window in order to force the software to skip the stutter peak.

Initials: RG Date: 5/11/00

- First determine the height of the stutter peak by placing the cursor on the peak in question (as if you are editing). The information displayed on the top of the window refers to the peak where the cursor is located and contains the peak height. Make a note of the peak height.

- Open the categories window (under views on the menu) and highlight the first allele in the offset category (e.g 18 o.s.) of the polymorphism that needs to be corrected.

- In the dialogue box change the height for the minimum peak height to a few points above the determined height of the stutter.

- Rerun the macro and then check to make sure everything is correct by looking at the first allele in each locus in the ladder and by comparing the result for the positive control

If the ladder has been corrected but the genotype of the positive control is still incorrect see Genotyper Troubleshooting.

4. Check all lanes. Labels for extra peaks can be manually deleted by placing the cursor on the peak above the baseline and clicking. If you mistakenly delete a label, before you do anything else, press the **apple key + Z** and the allele name label will reappear (the command apple key +Z only undoes the last action).

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 then click again to finally remove the label. Use the zoom functions to get a close look at certain peaks (for instance if you have an allele with two labels which are very close to each other) by using the **Zoom** submenu under the **Views** menu.

Extra peaks are defined as peaks that meet one of the following criteria (also see section STR results interpretation):

1 Pull-ups of peaks in any color caused by a very high peak of another color in the same lane. Pull-ups are caused by the inability of the matrix file to remove all e.g. green light components from a blue signal.

2 Shoulder peaks approximately 1-4 bp smaller or bigger than the main allele. Shoulder peaks are mostly present on the right side of a peak if the peak shape shows a slope that is trailing out.

3 "N" bands where the main allele shows a split peak. "N" bands are caused by incomplete extra A addition and are characterized by either a jagged edge on the left side of the peak or a complete split on the top level.

4 -4 and +4 bp stutter peaks if there is not indication of the presence of a mixture. -4 bp stutter is common and will often be labeled if followed by a high allele peak. +4 bp stutter on the other side is extremely rare and has to be interpreted carefully. Defined peaks in a +4 bp position might indicate the presence of a mixture.

5 Non specific artifacts. This category should be used if a labeled peak is caused by a not previously categorized technical or amplification problem.

6 Labels placed on elevated or noisy baselines which do not resemble distinct peaks.

7 Peaks caused by overflow of sample in the adjacent lane.

5. Fill out the Genotyper Editing Sheet for each sample number and note the reason for removal of a peak using the number code above.

At this stage it is also necessary to make decisions about samples that should be rerun with either more or less amount of amplification product.

Inconclusive samples:
Over-amplified samples often have peak heights between 5000 and 7000 fu's and are characterized by a plateau shaped or misshaped peaks and often contain a lot of labeled stutter peaks and artifacts (also see section 4B of Interpretation of Complex Autosomal STR Results). Instead of laboriously editing out all of these peaks, the sample should be deemed inconclusive and marked for re-running. **All DNA mixtures where peaks in at least one color are ≥ 6000 fu's have to be rerun with less.** Remove all labels from the lane in question, don't list all of the sizes, note "numerous" for peaks removed. The sample should be placed on a rerun sheet for re-running with 4µL of a 1/10 dilution of the amplification product for samples with peak heights between 5000 -7000 fu's. For problem samples were peak heights are less than 5000, re-running 1-2µL of amplified product is recommended.

DNA alleles visible but below threshold:
If a sample displays allele peaks just below the threshold of 50 fu's there is a distinct possibility that the alleles can be identified after a repeated run with a higher amount of amplified product. Place the sample on a rerun sheet. Acceptable amounts of amplification product are 6 - 8µL of amplified product.

New alleles:
If a locus displays only one peak label and a distinct same color peak is visible but is not labeled, or is labeled "OL allele?", it is because the unlabeled peak is outside the defined allele range or is not present in the allelic ladder. This peak might be a "new", previously unreported allele. This possibility must be considered, especially if the other loci show a proper allele profile. The

Initials: RO Date: 5/14/00

presence of a possible "new" allele must be pointed out to a DNA supervisor for confirmation. The "new" allele will not be automatically reported in the Genotyper table, but will be visible on the electropherogram. Click on the unlabeled peak in order to label it with the size in bp, which is necessary for allele identification.

6. After the editing has been finished scroll through the plot window to double check.

7. Then run the **Create Table 1 macro** by pressing the **Apple key** and the number **2** (simultaneously). Under **View** open the table window. Compare the sample information in the table with the amplification and the run control sheet. If an error gets detected at this point it can be corrected as follows:
   - open the dye/lane window
   - click on the lane in question
   - place the cursor in the sample info box and correct the text
   - under **Edit** select the appropriate color
   - run macro Apple 2 again

   Before printing the table under **file** select **page set-up** and choose 65% and landscape. Print the table by under **file** selecting **print**.

8. Under **Analysis** select **Change Labels**. A dialogue box appears giving you the option of labels to assign to the peaks. Select **Size in bp** and **Category name**. Click **okay**. The plot window will now show the peaks with labels indicating both size in bp and category name; there will not be enough room to scroll through the plot window at this point.

9. Before printing plots, check that the zoom range shows 90-350. Change the page set-up back to 100% and letter. Under **file** select **print**. Click **okay** in the print dialogue box.

10. After the printing is finished, under **file**, **quit** Genotyper. Click **save**.
    The Genotyper file will automatically be saved in the run folder from which you imported your data; it can be located there and re-edited at a later date.

11. As outlined in the General Guidelines for fluorescent STR analysis after running the Genotyper the run folder should contain the following items:
    - a run file and the run log (ABI Prism 377 Collection documents)
    - the gel file (ca. 12 MB Genescan document)
    - a project file and sample files for all samples (Genescan documents)
    - the genotyper file (ca. 400K Genotyper document)

Archive data as described in the General Guidelines for fluorescent STR analysis.

12. Have a supervisor review the analyzed gel and get a signature on the editing sheet.

Initials: RCJ Date: 5/16/00

Genotyper Trouble Shooting for Profiler Plus and Cofiler

A. If you get an Error Message when you try to run the Genotyper Macro 1 that reads: **"Could not complete your request because no dye/lanes are selected"**.

Make sure you have actually imported the ladder from the project. Make sure "ladder" is spelled correctly in the **dye/lanes window**. If there is a mispelling or the sample information for the ladder is absent, the macro will not recognize the ladder and will not be able to complete the procedure. Correct the spelling and rerun the macro.

B. If you get an Error Message when you try to run the Genotyper Macro 1 that reads: **"Could not complete your request because the labeled peak could not be found"**.

This message indicates that the ladder cannot be matched to the defined categories. There are three possibilities.

1. The wrong ladder is being typed, (i.e. You are trying to type a Cofiler ladder in the Profiler Plus Genotyper or vice versa).

2.. There may be peaks in the ladder which are too low to be recognized by the program. There are two possible remedies.

First: alter the name of the first ladder in the Genotyper Dye/Lane window and rerun **Macro 1**. Now the macro will use the first backup ladder for the off-set calculation.

Second: You can lower the **minimum peak height** in the categories window. To do this, open the categories window by going under **Views** and selecting **Show Categories Window**. In the "offset" categories the first allele is defined with a scaled peak height of 200 or higher. Change this to 50 for gels and 100 for the capillary, by clicking on the first category which highlights it. In the dialogue box locate the **Minimum Peak Height** and change it to 50 or 100, click **Add,** and then click **Replace** when given the option. You must do this for each locus. Do not use values less than 50 and 100.

DO NOT CHANGE THE MINIMUM PEAK HEIGHT FOR ANY OTHER CATEGORY EXCEPT THE OFF-SET.

It is important, after you rerun the macro, to make sure the ladder begins with the correct allele and that the first allele is not assigned to a stutter which might precede the first peak.

3. There are no peaks at all in both allelic ladders. If this is the case rerun all samples with freshly prepared Allelic Ladders.

Initials: RCS Date: 5/16/00

C. Off Ladder (OL) allele labels

1. If you have a run with a large number of samples you may find that the samples toward the end of the run have a high incidence of OL allele labels. This is due to a shift during the run.

   In order to improve the number of correctly called alleles, try to reanalyze the run by using the second allelic ladder as the off-set reference. This is done by removing the word "ladder" from the name of the first ladder in the dye lane window. This way this ladder is not recognized by the macro program. Rerun **Macro 1** and evaluate the results. Determine which one of both allelic ladders causes fewer "OL allele?" labels. Complete the genotyping process using this ladder. Any remaining samples displaying OL alleles have to be rerun.

2. If all or most of your samples have "OL allele?" labels it may be that your samples were automatically analysed with an ill-defined size standard. In this case it is necessary to reanalyze your run using the correct size standard.

   I. Open the **Genscan Analysis** Program on the Desktop. Open the appropriate project. In the **Analysis Control Window** select the appropriate size standard (CXR or GS500) from the grey **size standard** pop-up menu at the top of the list.

   II. Select all colors from the dye boxes on the left hand side of the window.

   III Click **Analyze**.

   IV Proceed with the project file analysis as described for the ABI 377 and 310.

D. If the genotype of the positive control (PE) does not match the known type for the positive control:

1. The Genotyper has shifted allele positions during the category assignment to the ladder.

   Check the ladder and make sure the first assigned allele is assigned to the first real peak and not to a stutter peak which may precede it. If the stutter peak is designated with the first allele name, you must raise the peak height in the categories window. See Genotyper Analysis section, step 6.

2. You have a sample mix-up and you have to reamplify and/or to rerun your samples.

E. In order to place samples next to each other for comparison purposes, mark them by double clicking. 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**. NOTE:

unsized peaks cannot be placed according to size on the electropherogram. Therefore, when comparing an unlabeled allele (unlabeled because it is too low to be sized, but high enough to be detected visually) to a labeled allele (e.g. in the ladder) you cannot determine the allele type and size by visual comparison while the results are viewed by size. To be able to align an unlabeled allele with a labeled allele in the same run you must select **View by Scan** from under the **View** menu!

F. If you see the same sample listed several times in the dye/lanes window or you see more samples than you have imported, you have most likely imported your samples more than once or you have imported your samples into a Genotyper template that already contained sample information. Under **Analysis** select **Clear Dye/Lanes** window and also under **Analysis** select **Clear Table**. Re-import your file(s).

G. If you detect a mistake in the sample information, this 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.

Initials: RC Date: 5/16/00

STR Results Interpretation

Allele Calling Criteria

Results are interpreted by observing the occurrence of electropherogram peaks for the loci that are amplified simultaneously. 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:

Quad Multiplex	Color	Size Range GS500 Std.	Allele range
HumVWA	green	127±1.5bp to 172 ±1.5bp	10 to 22
HumTHO1	blue	150±1.5bp to 179 ±1.5bp	4 to 11
HumF13A1	green	181±0.75bp to 247 ±1.5bp	3.2 to 17
HumFES/FPS	blue	206±1.5bp to 238 ±1.5bp	7 to 15

Profiler Plus	Color	Size Range GS500 Std.	Allele range
D3S1358	Blue	114±0.5bp to 143±0.5bp	9 to 20
vWA	Blue	157±0.5bp to 197±0.5bp	10 to 22
FGA	Blue	220±0.5bp to 268±0.5bp	16 to 31.2
Amelogenin	Green	X:106±0.5bp;Y:112±0.5bp	X and Y
D8S1179	Green	127±0.5bp to 172±0.5bp	8 to 19
D21S11	Green	189±0.5bp to 244±0.5bp	24.2 to 38
D18S51	Green	274±0.51bp to 342 ±0.5bp	9 to 26
D5S818	Yellow	134±0.5bp to 172±0.5bp	7 to 16
D13S317	Yellow	207±0.5bp to 236±0.5bp	5 to 15
D7S820	Yellow	259±0.5bp to 294±0.5bp	6 to 15

Initials: RG Date: 5/16/00

Cofiler	Color	Size Range CXR Std.	Allele range
D3S1358	Blue	108±0.5bp to 137±0.5bp	9 to 20
D16S539	Blue	226±0.5bp to 267±0.5bp	5 to 15
Amelogenin	Green	X:100±0.5bp;Y:106±0.5bp	X and Y
THO1	Green	163±0.5bp to 183±0.5bp	4 to 11
TPOX	Green	212±0.5bp to 240±0.5bp	6 to 13
CSF1PO	Green	277±0.51bp to 313 ±0.5bp	6 to 15
D7S820	Yellow	253±0.5bp to 290±0.5bp	6 to 15

Y M1 multiplex	Color	Size Range GS500 Std.	Allele range
DYS19	yellow	184 ±1.5bp to 208 ±1.5bp	12 to 18
DYS390	blue	201 ±1.5bp to 230 ±1.5bp	20 to 27
DYS389I	yellow	243 ±1.5bp to 262 ±1.5bp	7 to 12
DYS389II	yellow	363 ±1.5bp to 387 ±1.5bp	24 to 30

The above values might expand if additional alleles are discovered for the various loci.

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 fu's (fluorescent units) 377 instruments: Quad, Profiler Plus, YM1], [>100fu's CE instruments: Cofiler], and have a peak height >7-10% of the major peak are called alleles by the instrument. For several of the loci, 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 11 to 20% are also disregarded (see Genotyper sections).

Initials: RCS Date: 5/16/00

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.

- calculating the fragment length for the detected peaks using the known in-lane standard fragments.

- for the Quad and YM1 (systems without an allelic ladder) - labeling of all sized fragments that are >50 fu (fluorescent 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). Allele identification window is ± 1.5bp.

-for Cofiler and Profiler Plus (systems with allelic ladder) - comparing and adjusting the categories listed above to the sizing of the co-electrophoresed allelic ladder by calulating the off sets (the difference between the first allele in a category and the first allele in the allelic ladder at each locus).

-for Cofiler (run on 310) - labeling of all sized fragments that are >100 fu and removing the labels from minor peaks according to the filter functions detailed in the Cofiler Genotyper section of this manual.

-for Profiler Plus (run on 377) - labeling of all sized fragments that are >50 fu and removing the labels from minor peaks according to the filter functions detailed in the Profiler Plus Genotyper section of this manual.

Additional **non-allelic peaks** may occur under the following instances (Clark 1988, Walsh et al. 1996, Clayton et al. 1998):

"Pull-ups" of peaks in one color caused by very high peaks in another color. This occurs only for multiplexes employing more than one labeling color and is caused by the inability of the software to compensate for the spectral overlap between the different colors, if the peak height is too high. Pull ups are matrix artifacts.

Shoulder peaks approximately 1-4 bp smaller but mostly larger than main allele, caused by a flat decline of main peak fluorescence. Shoulders are easily recognized because they do not have the shape of an actual peak.

-4 and +4 bp stutter peaks that are caused by slippage of the Taq polymerase enzyme during copying of the STR allele.

Initials: RCS Date: 5/16/00

Non-specific artifacts caused by non-specific priming in a multiplex reaction. These artifacts are usually easily recognized due to their low peak height and their position outside of the allele range.

Labels placed on elevated or noisy baselines which do not resemble distinct peaks. Noisy and elevated baselines are matrix artifacts.

"N" bands, where the main peak is split into two peaks caused by the Taq polymerase activity that causes the addition of a single A to the terminus of the amplified product ("N+1" band). The allele calling is based on the N+1 bands, therefore complete extra A addition is desired.

For the gel systems only:
Overflow of sample that has been loaded in the adjacent lane

For capillary electrophoresis runs only:

A constant peak at 3500-4000 scans. This peak is caused by fluorescent dye that is not attached to the primers anymore. These "color blips" can occur in all colors. The "color blip" falls into the allele range of D3S1358 and is therefore labeled if it occurs in blue.

Sharp spikes caused by power surges or crystals or airbubbles traveling by the laser detector window. Spikes might look like a single vertical line or a peak; they can easily be distinguished from DNA peaks by looking at the other fluorescent colors, including red; the spike should be present in all colors.

If these additional non-allelic peaks are labeled by the Genotyper program, remove the labels manually. Then record the removal on 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. Subtypes displaying incomplete repeat units are labeled with the number of complete repeats and a period followed by the number of additional bases.

The Y chromosome allele nomenclature is also based on the number of 4bp core repeats and follows the suggestions from Dr. Peter de Knijff, Forensic Laboratory for DNA Research, University of Leiden, (submitted).

Initials: RCJ Date: 5/16/00

Genotyper Print Out

Genotyper print outs of all gels or capillary electrophoresis runs containing the case specific samples are part of each casefile. The Genotyper results table reflects the number and allele assignments of the labeled peaks visible on the Genotyper Plot print out. These print outs are the basis for results interpretation. Any interpretation should be based on the plots rather than on the table. Only the plot reveals intensity differences that indicate the presence of a mixture, shows all peaks at one locus if the number of peaks exceeds 2 for YM1, and 4 for QUAD, Profiler Plus and Cofiler. Looking at the plots also serves as a control for the editing process.

IMPORTANT:
Especially if a sample's DNA concentration is at the lower detection limit, it may occur that when a sample gets duplicated, previously labeled peaks are now below the 50 or 100 flourescent units minimum. If the peak is unlabeled but visible this allele would be reported in brackets. It is important to remember that on the Genotyper plot unlabeled peaks are **not** displayed by size and that their types cannot be inferred! In order to make a visual allele interpretation one must reopen the corresponding Genotyper file and change the viewing scale from **View by Size** to **View by Scan** (see Genotyper troubleshooting section E)

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 <50fu for a gel and <100fu for the capillary. If only one peak is >50 or>100 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 (or <100) were visible for this sample.

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.

Initials: RCS Date: 5/11/00

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 and fulfills the concordant analyses and the triplicate rule as stated in the General PCR Guidelines, then the allele is listed in the report.

B. An allele from a locus that is present in more than one DNA system (overlapping loci: VWA, THO1, D3, D7, and amelogenin) must meet the above reporting criteria in at least one DNA system to be listed in the report.

C. If an allele meets the above reporting criteria in one run and is visible on the electropherogram but does not meet the criteria in at least one additional run (in the same DNA system) 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.

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

E. If an allele meets the above reporting criteria in one run and is not visible in at least one additional run (in the same DNA system) then the allele is reported as **.

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 peak height 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. Off-ladder alleles should be reported using their relative position to the alleles in the allelic ladder (see Interpretation of Complex Autosomal STR results, 3.B. reporting of previously unreported rare alleles). The alleles should be marked with ◊. The ◊ should be defined at the bottom of the table as "◊ = The observed allele is not represented in the allelic ladder".

J. New alleles observed for Quad and YM1 where no allelic ladder is available should be reported with their rounded basepair size. The alleles should be marked with ◊. The ◊ should be defined

at the bottom of the table as "◇ = This allele has not been previously observed for this locus. Allele is reported as size in basepairs ".

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

Initials: RCJ Date: 5/16/00

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 fraction 7, 8 (7 > 8); 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.). (3) 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 locus (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.

Initials: RCJ Date: 5/16/00

Extraction negative, Amplification Negative and Substrate Controls

The extraction negative control and amplification negative control are a check for the possible contamination of the reagents in the STR test by other human DNA or by amplified STR alleles. The extraction negative 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 an apparently unstained substrates (e.g. piece of fabric) located as close to a stain or group of stains as possible.

The extraction negative 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 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 negative, amplification negative or substrate controls show very weak peaks below 50 (or 100 fluorescent units for the capillary) and the test samples show distinct peaks that meet the reporting criteria, the contamination problem is not serious. If the extraction negative, amplification negative or substrate controls show peaks above 50 (or 100), the contamination problem is more serious. **See Table VI for interpretation guidelines.**

The appearance of signals in extraction negative, 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.

Initials: RCS Date: 5/11 00

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 Peaks in the Extraction Negative and Substrate Controls

Control	above 50/100	Interpretation of Test Sample
Extraction negative/ Amplification Neg.	yes	All test samples inconclusive
	no	Test samples are conclusive if there is a duplicate run with no visible peaks.
Substrate	yes/no	Sample is conclusive.

Initials: RCS Date: 5/16/00

Amplification Positive Control

The positive control DNA is used with each batch of samples typed to demonstrate that the kit is performing properly. Positive control DNA has the following types:

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

Y multiplex 1	
DYS 19	14
DYS 390	24
DYS 389 I	10
DYS 389 II	26

Cofiler	
D3S1358	14, 15
D16S539	11, 12
Amelogenin	X,X
THO1	8, 9.3
TPOX	8, 8
CSF1PO	10, 12
D7S820	10, 11

Profiler Plus	
D3S1358	14, 15
VWA	17, 18
FGA	23, 24
Amelogenin	X,X
D8S1179	13, 13
D21S11	30, 30
D18S51	15, 19
D5S818	11, 11
D13S317	11, 11
D7S820	10, 11

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: RCS Date: 5/16 00

Interpretation of Complex Autosomal 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.

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. Results for all tested loci should be interpreted in order to determine a mixture.

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 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 18%. With including a standard deviation a difference in peak heights of more than 30% 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, Clayton et al. 1998). 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. Here different scenarios have to be considered:

(1) Mixture has a known component, e.g. a vaginal swab

After identifying the alleles that could have come from the victim, it can be stated that the remaining alleles must have come from the unknown DNA source. To deduce the complete allele combination of the foreign DNA, the results and allele peak heights must be taken into consideration for each locus:

If two foreign alleles of similar peak height are present at a locus, these two alleles are likely to comprise the genotype of the unknown contributor.

If the alleles foreign to the victim constitute the major component of a mixture, the allele combination can be deduced by combining all major allele peaks (also see section (2) below). All peak height inconsistencies for heterozygote loci should be accounted for by overlap with the known component.

If the alleles foreign to the victim are the minor component and only one foreign allele is visible at a locus, it might not be possible to determine the complete allele composition for this STR. The foreign type might either be homozygous or heterozygous with one allele overlapping with the known component. For heterozygous types of the known component, peak height differences between the two alleles indicate the presence of an overlapping allele in the minor component. For homozygous patterns and very small peak height differences a decision cannot be made. In these cases it is possible to indicate that a second allele might be present.

(2) The major and the minor component of the mixture can clearly be distinguished

Using a locus where four alleles are present, it is possible to determine the ratio of the two DNA components in a mixture. This ratio can then be used to interpret the amount of copies of each allele that must be present at other loci with less than four alleles. Therefore, if there is a large difference in peak heights, the genotype of the major component can be inferred without having one known contributor and without four alleles being present at each locus. Be careful to eliminate the possibility of more than two contributors before interpreting the mixture.

It might not be possible to unambiguously deduce the DNA type for the minor component. See above for a discussion of the limitations.

(3) Very small additional allele peaks are detected at only a few loci

The major DNA profile can be interpreted. The presence of additional alleles should be noted, but it should not be attempted to deduce a type for the minor component.

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). Some of the STR loci are very prone to stutter and almost always show stutter peaks e.g. DYS19 or VWA. The Amelogenin locus is not based on a repetitive STR sequence and doesn't show any stutter. Overall over all loci the average stutter peak height ranges from 2.5 to 9.5%, with maxima from 17.4% - 24.1%. Therefore peaks in a -4bp position from a main peak and less than a certain percentage (differs per locus, see Genotyper sections) of the main peak's height are not reported as true alleles. 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. The occurrence of +4bp stutter is rare, but the above considerations also apply if peaks are present in this position.

2. Partial Profiles: not all 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. For the example the four QUAD STRs have different allele size ranges, with FES/FPS being the longest (210-238bp) and VWA being the shortest (127-172bp) locus. Other multiplexes contain even longer alleles e.g. Profiler Plus with D18S51 (274-342bp) and YM1 with DYS389II (362-386bp).

The longer alleles are more likely not to be present in partially degraded DNA (Lygo et al. 1994, Gill et al. 1995, Sparkes et al. 1996). 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 3.2, 17, allele 3.2 (182 bp) can be present while allele 17 (237 bp) 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.

B. Detection limit

Due to the different detection sensitivity of the dyes, the yellow peaks are generally lower than the blue and green peaks. If the DNA sample is at the lower limit of the testing sensitivity it is therefore possible to get a partial profile where one or all of the yellow loci are missing. Also blue is slightly more sensitive than the green dye, so that it is possible to see only the blue peaks, or the blue and one of the green systems in Quad.

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.

Initials: RG Date: 5/16/00

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 (or 100 if applicable) and does not show artifacts,
- 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 allele should be confirmed by an additional typing, if possible in a different multiplex system containing the same locus.

B. Reporting previously unreported rare alleles

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 of 5 counts per database. In order to do that enter the allele size in basepairs which should be on the genotyper print out in the QuattroPro spreadsheet.

STR systems without an allelic ladder (Quad, YM1): The new size allele should be reported using the size in bp rounded up or down to the whole number and a footnote stating the fact that this allele has not been observed for this locus (see reporting procedures J.).

STR systems with an allelic ladder (Profiler Plus, Cofiler): In order to be consistent with the CODIS reporting requirements, all alleles that are not present in the allelic ladder should be identified by their relative position to the alleles in the allelic ladder. The Genotyper peak label should show the length in basepairs and this value can be used to determine the proper allele nomenclature, e.g. a FGA allele of 322bp is longer than the longest allele in the FGA allelic ladder (30 - 269bp) and has to be designated >30. A TPOX allele with a size of 208bp is shorter than the smallest ladder allele (6 - 212 bp) and has to be designated <6. A D7S820 allele of the length 276 bp is located between alleles 10 and 11 and has to be designated 10.x. The off-ladder allele should be reported using this nomenclature and a footnote stating the fact that this allele is not represented in the allelic ladder and has not been observed for this locus (see reporting procedures I.).

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.

Initials: RCS Date: 5/16/00

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.

A. If all peak heights in a sample with a high background level are low, the background is probably caused by degradation artifacts (Sparkes et al. 1996). In this case it might not be possible to ever obtain the true genotype. A degraded sample should be amplified with more DNA e.g. after Microcon concentration.

B. Sometimes high background is caused by overamplification where the main allele peaks are so high that they reach fluorescence saturation level. Common observations for overamplified samples are: peaks are not pointed but rather resemble narrow plateaus, peaks are not pointed but show multiple jagged edges and split peaks, peaks cause major pull-ups in other colors, peaks resemble plateaus and show an elevated stutter peak in the -4bp position. All samples with a fluorescence level ≥ 6000 fu's in at least one of the colors, have to be considered overamplified. **DNA mixtures with peak heights ≥ 6000 fu's in at least one of the colors must be repeated because it is not possible to reliably determine the ratio of two DNA components with one component being close to the saturation limit.** This reanalysis is not always necessary for clean DNA samples if, in spite of the peak heights, all peaks show the proper shape and no major background is present. If the above described background is present the sample should be repeated. The repeat analysis does not have to start with the amplification but can be achieved by rerunning a lower amount or a 1/10 dilution of the amplified product.

5. Discrepancies for overlapping loci in different multiplex systems

The primer-binding site of an allele may contain a mutation which renders the annealing phase of its amplification less efficient, or if the mutation is near the 5' end completely blocks the extension (Clayton et al. 1998). This may result in a pseudo-homozygote type, which is reproducible for the specific primer pair. These mutations are extremely rare, approximately estimated between 0.01 and 0.001 per locus (Clayton et al. 1998). A comparison between evidence and exemplar samples based on a locus where both samples were amplified with the same primer sequence is no problem. If the same locus is multiplied using different multiplex systems (especially Quad versus the AmpFlSTR kits) it is possible to obtain a heterozygote type in one multiplex and the pseudo-homozygote in the second, because the primer sequences even for the same loci may differ. The heterozygote type should be the correct type and should be reported. It is important to have typing results for evidence and exemplars based on the same multiplex.

Initials: RCJ Date: 5/16 100

Additional Interpretations of Y STR Results and Complex Y STR Results

For a Y chromosome specific polymorphism, a male individual will display one allele for DYS19, one allele DYS390, and two allele peaks for DYS389. For DYS389 one primer pair amplifies two polymorphic STR stretches in this region, the shorter fragment (DYS389I) is the product of an internal second annealing site of the forward primer and the reverse primer, the longer fragment (DYS389II) includes the DYS389I stretch and a second polymorphic tetrameric STR stretch. The true number of STR repeats for the DYS389II stretch can be determined by subtracting DYS389I from DYS389II. This is only necessary for the determination of the separate allele frequencies, and does not change the frequencies of the allele combinations (Kayser et al. 1997).

All STRs in Y Multiplex 1 are located outside of the pseudoautosomal region, the alleles are therefore not subject to recombination (ref Jobling and Tyler-Smith 1995, Kayser et al. 1997). The allele combination is a haplotype, that is inherited through the paternal germline. **The frequency of a specific Y STR allele combination cannot therefore be assessed by the product rule.**

Since no allele will be amplified for female DNA , a DNA mixture with female and male contributors will only display the alleles of the male components (Prinz et al. 1997.). Mixtures of more than one male contributor are likely to display more than one allele peak for at least one locus. It has been observed that an allele duplication at DYS19 or DYS390 creates a two allele pattern for these systems for a single male individual (Kayser et al. 1997, Santos et al. 1996). In this case the two allele peaks will be of similar height.

1. **Mixtures of male DNA**: more than one haplotype present in the DNA sample.

   A. General Mixtures

   The occurrence of more than one allele peak of similar height at one or more loci of the Y M1 haplotype indicates the presence of a mixture of male DNAs, where the different components are present in equal ratios. If only either DYS19 or DYS390 displays two alleles, and the other three loci show single peaks, the presence of an allele duplication event has to be considered.

   B. Mixtures with different level of starting DNA

   Mixtures of male DNAs with different levels of starting DNA will lead to unequal peak heights for the different alleles for one system. If the ratio of the lower peak to the higher peak is consistent for all loci with two allele peaks, the haplotypes of the major and minor component can be inferred. If this is not the case, the possible presence of three contributors must be considered.

   C. Possible mixture component masked by -4bp stutter

   As for the autosomal QUAD STR systems the peaks in a -4bp position from a main peak and less than 20% of this peak height are not reported as true alleles. In a mixture the -4bp stutter could mask a real mixture component. Therefore individuals cannot be excluded from being a

Initials: QCS Date: 5/16/00

minor contributor to a mixture if their alleles are in the -4bp position of an allele from another individual.

For the following see the Interpretation of Complex Autosomal STR Results section. Follow the procedures outlined in the appropriate section.

2. **Partial Profiles**: not all four loci display allele peaks
3. **Detection Of Previously Unreported Rare Alleles**
4. **Samples with High Background Levels**

Initials: RCS Date: 5/11/00

Population Frequencies for STR's

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 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 Asian, 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).

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 autosomal STR systems. 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.

The population frequencies are derived from the OCME Database.

Autosomal STR's

In the standard scenario, for each group, homozygotes are calculated using the formula $p^2+p(1-p)$ for = 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 (isolated village)" and for relatives using the formulas in the National Research Council Report and = 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 =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.

Initials: ECS Date: 5/16/00

Y STR's

Since all the loci are linked on the Y chromosome, they are not independent and only a haplotype frequency can be calculated. The frequency for the Y STR haplotype is calculated by counting the number of times the haplotype occurs in each of the racial databases. This is commonly referred to as the "counting method". If both autosomal and Y STR's are typed for a sample, than the overall frequency can be calculated by multiplying the overall autosomal frequency for each racial group by the larger of either a) the Y haplotype frequency or b) the Y haplotype frequency assuming 1 count in the database.

Kinship Analysis

Kinship analysis tests alternate or competing hypotheses of kinship. In the forensic context, it is useful for determining familial relationships, the identification of unknown bodies, identification of the donor of blood stains when the donor/body is missing or unavailable, and the identification of the biological father of products of conception/babies which result from a sexual assault. All calculations are performed according to the Parentage Testing Standards of the American Association of Blood Banks.

The DNA from the subject/stain in question is compared to the DNA of close biological relatives. For parent(s)/child comparisons, the loci are first evaluated to determine whether the individual in question can be excluded as a biological relative of the other individual(s) (see below). If the individual can not be excluded or for comparisons not involving a parent(s)/child relationship, a PI (traditionally called a Paternity Index but this could be a Maternity or Kinship Index) is calculated for each locus using the DNAVIEW program of Dr. Charles Brenner. The formulas for parent/child comparisons are listed in Appendices 6 and 11 of Parentage Testing Accreditation Requirements Manual, Third Edition, AABB. The race of the individual in question is used for all of the calculations. If the race is unknown than all races must be used.

If there is an exclusion at a single locus in a parent/child comparison, the PI is calculated according to the formula in Appendix 11 (PI= /PE) where

μ (locus specific mutation rate) is obtained from Appendix 14 of Parentage Testing Accreditation Requirements Manual, Fourth Edition, AABB and

PE = h^2 $(1-2hH^2)$ where H is the frequency of homozygosity and h is the frequency of heterozygosity. PE is calculated by the DNAVIEW program.

An overall CPI (Combined Paternity Index) is calculated by multiplying all of the individual PI's. A probability of paternity (maternity/kinship) is then calculated using Bayes Theorem and assuming a prior probability of 50%. The formula for calculating the probability of paternity with a 50% prior probability is CPI/(CPI +1). Both the CPI and probability of paternity are calculated using the Quattro Pro spreadsheet kinship.wb2. If due to the facts of the case, other prior probabilities are necessary, the probability of paternity can be manually calculated using the formula CPI x P/[CPI x P + (1-P)] where P is the prior probability.

Initials: RCS Date: 5/16/00

The case report must list the PI for each locus, the race used for calculation, the CPI, the probability of paternity and the assumed prior probability. It also must state the final conclusion. The three possible final conclusions are exclusion, inconclusive or inclusion of the tested hypothesis of kinship.

Exclusion occurs when either 2 or more loci exclude in a parent/child comparison or the CPI < 0.1.

Inconclusive occurs when the CPI is between 0.1 and 10 and for individual loci in mixtures of parent/child combinations when there are other peaks visible which could potentially exclude or include but can not be genotyped by the software.

Inclusions occur when either 0 or 1 loci exclude in parent child combinations and when for all cases the CPI >10. The analyst should bear in mind the strength of the inclusion based on the CPI. When the CPI is greater than 2000 (Prob of paternity >99.95%, 50% prior prob.) the hypothesis of kinship should be accepted (considered proven). When the CPI is between 100 and 2000, the hypothesis is supported by the data. When the CPI is between 10 and 100, the hypothesis should not be rejected and should be considered a weak inclusion.

Initials: RA Date: 5/16/00

QUAD Genotyper Categories Table for ABI 377

VWA

11	Highest peak at 127.41 ± 1.50 bp in green with height ≥50
12	Highest peak at 131.40 ±1.50 bp in green with height ≥50
13	Highest peak at 135.40 ±1.50 bp in green with height ≥50
14	Highest peak at 139.27 ±1.50 bp in green with height ≥50
15	Highest peak at 143.84 ±1.50 bp in green with height ≥50
16	Highest peak at 148.23 ±1.50 bp in green with height ≥50
17	Highest peak at 152.45±1.50 bp in green with height ≥50
18	Highest peak at 156.28±1.50 bp in green with height ≥50
19	Highest peak at 160.23 ±1.50 bp in green with height ≥50
20	Highest peak at 164.20 ±1.50 bp in green with height ≥50
21	Highest peak at 168.25 ±1.50 bp in green with height ≥50
22	Highest peak at 172.25 ±1.50 bp in green with height ≥50

THO1

4	Highest peak at 151.00 ±1.50 bp in blue with height ≥50
5	Highest peak at 155.00 ±1.50 bp in blue with height ≥50
6	Highest peak at 158.71 ±1.50 bp in blue with height ≥50
7	Highest peak at 162.66 ±1.50 bp in blue with height ≥50
8	Highest peak at 166.64 ±1.50 bp in blue with height ≥50
9	Highest peak at 170.61 ±1.50 bp in blue with height ≥50
9.3/10	Highest peak from 172.50 to 176.00 bp in blue with height ≥50
11	Highest peak at 178.65 ±1.50 bp in blue with height ≥50

Initials: RCJ Date: 5/11 00

QUAD Genotyper Categories Table for ABI 377 continued:

F13A1

3.2	Highest peak at 181.93 ±0.75 bp in green with height ≥50
4	Highest peak from 183.00 to 185.25 bp in green with height ≥50
5	Highest peak at 187.92 ±1.50 bp in green with height ≥50
6	Highest peak at 192.04 ±1.50 bp in green with height ≥50
7	Highest peak at 196.05 ±1.50 bp in green with height ≥50
8	Highest peak at 200.26 ±1.50 bp in green with height ≥50
9	Highest peak at 204 43 ±1.50 bp in green with height ≥50
10	Highest peak at 208.50 ±1.50 bp in green with height ≥50
11	Highest peak at 212.66 ±1.50 bp in green with height ≥50
12	Highest peak at 216.78 ±1.50 bp in green with height ≥50
13	Highest peak at 220.90 ±1.50 bp in green with height ≥50
14	Highest peak at 225.04 ±1.50 bp in green with height ≥50
15	Highest peak at 229.19 ±1.50 bp in green with height ≥50
16	Highest peak at 233.33 ±1.50 bp in green with height ≥50
17	Highest peak at 237.46 ±1.50 bp in green with height ≥50
18	Highest peak at 241.40 ±1.50 bp in green with height ≥50
19	Highest peak at 245.40 ±1.50 bp in green with height ≥50
20	Highest peak at 249.40 ±1.50 bp in green with height ≥50

FES

7	Highest peak at 206.50 ±1.50 bp in blue with height ≥50
8	Highest peak at 210.56 ±1.50 bp in blue with height ≥50
9	Highest peak at 214.69 ±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 222.63 ±1.50 bp in blue with height ≥50
12	Highest peak at 226.52 ±1.50 bp in blue with height ≥50
13	Highest peak at 230.68 ±1.50 bp in blue with height ≥50
14	Highest peak at 234.60 ±1.50 bp in blue with height ≥50
15	Highest peak at 238.60 ±1.50 bp in blue with height ≥50

Initials: RCJ Date: 5/16/00

Y STR 1 Genotyper Categories Table for ABI 377

DYS19

12	Highest peak at 184.00 ± 1.50 bp in yellow with height ≥50
13	Highest peak at 188.00 ± 1.50 bp in yellow with height ≥50
14	Highest peak at 192.00 ±1.50 bp in yellow with height ≥50
15	Highest peak at 196.00 ±1.50 bp in yellow with height ≥50
16	Highest peak at 200.00 ±1.50 bp in yellow with height ≥50
17	Highest peak at 204.00 ±1.50 bp in yellow with height ≥50
18	Highest peak at 208.00 ±1.50 bp in yellow with height ≥50

DYS389 I

7	Highest peak at 242.80 ±1.50 bp in yellow with height ≥50
8	Highest peak at 246.80 ±1.50 bp in yellow with height ≥50
9	Highest peak at 250.40 ±1.50 bp in yellow with height ≥50
10	Highest peak at 254.00 ±1.50 bp in yellow with height ≥50
11	Highest peak at 258.00 ±1.50 bp in yellow with height ≥50
12	Highest peak at 262.00 ±1.50 bp in yellow with height ≥50

DYS389 II

24	Highest peak at 362.80 ±1.50 bp in yellow with height ≥50
25	Highest peak at 366.80 ±1.50 bp in yellow with height ≥50
26	Highest peak at 370.50 ±1.50 bp in yellow with height ≥50
27	Highest peak at 374.50 ±1.50 bp in yellow with height ≥50
28	Highest peak at 378.50 ±1.50 bp in yellow with height ≥50
29	Highest peak at 382.20 ±1.50 bp in yellow with height ≥50
30	Highest peak at 386.50 ±1.50 bp in yellow with height ≥50

DYS390

20	Highest peak at 201.20 ±1.50 bp in blue with height ≥50
21	Highest peak at 205.20 ±1.50 bp in blue with height ≥50
22	Highest peak at 209.40 ±1.50 bp in blue with height ≥50
23	Highest peak at 213.50 ±1.50 bp in blue with height ≥50
24	Highest peak at 217.50 ±1.50 bp in blue with height ≥50
25	Highest peak at 221.80 ±1.50 bp in blue with height ≥50
26	Highest peak at 226.00 ±1.50 bp in blue with height ≥50
27	Highest peak at 230.00 ±1.50 bp in blue with height ≥50

Initials: RCJ Date: 5/16/00

References for Extraction and QuantiBlot

1. Perkin Elmer Corp. 1996. QuantiBlot: Human DNA Quantitation Kit. Package insert (Part No. N808-0114). Foster City, CA: Applied Biosystems Division.

2. 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.

3. Walsh, P.S., Metzger, D.A., Higuchi, R. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR based typing from forensic material. Biotechniques 10(4):506-513.

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

5. 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.

6. 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.

References for ABI 377 and 310 instruments and STR typing and interpretation

1. American Association of Blood Banks, Standards for Parentage Testing Laboratories. AABB 3rd edition, 1998.

2. American Association of Blood Banks, Parentage Testing; Accreditation Requirements Manual. AABB, 3rd edition, 1998.

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

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

5. Applied Biosystems, ABI Prism 310 Genetic Analyzer User's Manual, The Perkin Elmer Corporation 1995

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

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

8. Applied Biosystems, AmpFlSTR Profiler Plus User's Manual, The Perkin Elmer Corporation 1997.

9. Applied Biosystems, AmpFlSTR Cofiler User's Manual, The Perkin Elmer Corporation 1998.

10. ABI PRISM™ Genescan Analysis Software 3.1 User's manual. The Perkin Elmer Corporation 1998.

11. Anker, R.; Steinbrueck, T.; Donis-Keller, H. Tetranucleotide repeat polymorphism at the human thyroid peroxidase (HTPO) locus. Hum. Mol. Genet. 1:137 (1992)

12. Buel, E.; Schwartz, M.B.; LaFountain, M.J.; Capillary electrophoresis STR analysis: Comparison to gel based systems. J. Forensic Sci. 43:164-170, 1998

13. Buoncristiani, M.; Wallin, J.; Lee, M.; Tonkyn, J.; Lee, S.; TWGDAM validation of AmpFlSTR Blue. Abstract, Symposium on Human Identification, Scottsdale, 1996

14. Butler, J.M.; McCord, B.R.; Jung, J.M.; Lee, J.A.; Budowle, B.; Allen, R.A. Application of dual internal standards for precise sizing of polymerase chain reaction products using capillary electrophoresis. Electrophoresis 16:974-980, 1995

15. Clark, J.M. Novel non-templated nucleotide addition reactions catalyzed by prokaryotic and eukaryotic DNA polymerases. Nucleic Acids Res. 16:9677-9686, 1988

16. Clayton, T.M., Whitaker, J.P, Sparkes, R., Gill, P. Analysis and interpretation of mixed forensic stains using DNA STR profiling. Forensic Sci. Int. 91:55-70, 1998

17. De Knijff P, Kayser M, Prinz M, et al (1997) Chromosome Y microsatellites: population genetic and evolutionary aspects. Int. J. Leg. Med. 110: 134-140.

18. 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.

19. DNA View User's Manual. Brenner, C.H., Berkeley, 2000

20. 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.

21. Füredi, S.; Budowle, B.; Woller, J.; Padar, Z.; Hungarian Population Data on Six STR Loci - HUMVWFA31, HUMTHO1, HUMCSF1PO, HUMFES/FPS, HUMTPOX and HUMHPRTB- Derived Using Multiplex PCR Amplification and Manual Typing. Int J Legal Med 109:100-101 (1996)

22. Gehrig C, Hochmeister M, Sajantila A, Borer UV, Dirnhofer R (1996) Allele frequency of 3 chromosome Y specific STR loci and their application to forensic casework. Abstract, Proc. Sixth Int. Symp. on Human Identification 1995, Scottsdale, Promega, pp.170-171

23. 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.

24. 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.

25. Hochmeister, M.N.; Use of *Geneprint*™ CSF1PO, TPOX, THO1 Multiplex System (CTT) for the Analysis of DNA Evidence in a serial Killer Case. Profiles in DNA 3-6 (1997)

26. Isenberg, A.R.; McCord, B.R.; Koons, B.W., Budowle, B., Allen, R.O. DNA typing of a polymerase chain reaction amplified D1S80/amelogenin multiplex using capillary electrophoresis and a mixed entangled polymer matrix. Electrophoresis 17:1505-1511, 1996

27. Jobling MA, Tyler-Smith C (1995) Fathers and sons: the Y chromosome and human evolution. TIG 11: 449-456

28. Kayser M, Prinz M, et al (1997) Evaluation of Y chromosomal STRs: a multicenter study. Int. J. Leg. Med. 110:125-133.

29. Kimpton, C.; Walton, A.; Gill, P. A Further Tetranucleotide Repeat Polymorphism in the vWF Gene. Human Molecular Genetics 1:287 (1992)

30. 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

31. 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.

32. 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.

33. 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.

Initials: RS Date: 5/12/00

34. Li, H.; Schmidt, L.; Wei,M.; Hustad, T.; Lerman, M.I.; Zbar, B.; Tory K. Three Tetranucleotide Polymorphisms for Loci: D3S1352; D3S1358; D3S1359 Human Molecular Genetics 2:1327 (1993)

35. 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.

36. Mannucci, A.; Sullivan, K.M.; Ivanov, P.L.; Gill, P. Forensic Application of a Rapid and Quantitative DNA Sex Test by Amplification of the X-Y Homologous Gene Amelogenin. BioTechniques 15:636-641 (1993)

37. Mills, K.; Even, D.; Murray, J. C; Tetranucleotide Repeat Polymorphism at the Human Alpha Fibrinogen Locus (FGA). Human Molecular Genetics, 1:779 (1992)

38. National Research Council. The evaluation of forensic DNA evidence. National Academy Press, Washington DC 1996.

39. Polymeropoulos, Michael H.; Xiao, Hong; Rath, Denise S.; Merril, Carl R.; Tetranucleotide Repeat Polymorphism at the Human Tyrosine Hydroxylase Gene (TH). Nucleic Acids Res. 19:3753 (1991)

40. Prinz M, Roewer L, Baum H (1996) Performance of a Y-chromosome specific STR for mixed samples. Abstract, Proc. Sixth Int. Symp. on Human Identification 1995, Scottsdale, Promega, pp.156-157

41. Prinz M, Boll K, Baum H, Shaler R (1997) Multiplexing of Y chromosome specific STRs and performance for mixed samples. Forensic Science International 85:209-218

42. Prinz, M.; Coleman, A.; Baum, H.J.; Shaler, R.C. Practical aspects of switching to a capillary electrophoresis system. Abstract and hand out, 23rd NEAFS meeting, White Plains 1997

43. Roewer L, Arnemann J, Spurr NK, Grzeschik KH, Epplen JT (1992) Simple repeat sequences on the human Y-chromosome are equally polymorphic as their autosomal counterparts. Human Genetics 89: 389-394

44. Santos FR, Gerelsaikhan T, Munkhtuja B, Oyunsuren T, Epplen JT, Pena SDJ (1996) Geographic differences in the allele frequencies of the human Y-linked tetranucleotide polymorphism DYS19. Hum Genet 97:309-313

45. Sparkes, R., Kimpton, C., Watson, S., Oldroyd, N., Clayton, T., Barnett, L., Arnold, J., Thompson, C., Hale, R., Chapman, J., Urquhart, A., Gill, P. The validation of a 7-locus multiplex STR test for use in forensic casework (I) Mixtures, ageing, degardation and species studies. Int J. Leg. Med. 109:186-194, 1996

Initials: RCS Date: 5/16/00

46. Sparkes, R., Kimpton, C., Gilbard, S., Carne, P., Andersen, J., Oldroyd, N., Thomas, D., Urquhart, A., Gill, P. The validation of a 7-locus multiplex STR test for use in forensic casework (I) Artefacts, casework studies and success rates. Int J. Leg. Med. 109:195-204, 1996

47. Sullivan, K.M.; Mannucci, A.; Kimpton, C.P.; Gill, P. A rapid and quantitative DNA sex test: Fluorescence based PCR analysis of X-Y homologous gene amelogenin. BioTechniques 15:636-642, 1993

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

49. Yoshida, K.; Sekiguchi, K.; Kasai, K.; Sato, H.; Seta, S.; Sensabaugh, G.F.;Evaluation of New Primers for CSF1PO. Int J Legal Med 110:36-38 (1997)

50. Wallin, J.; Buoncristiani, M.; Lazaruk, K.; Fildes, N.; Holt, C.D.; Walsh, P.S. TWGDAM validation of AmpFlSTR Blue PCR amplification kit for forensic casework analysis. J. Forens. Sci. 43:117-121, 1998

51. Walsh, P.S., Fildes, N.J., Reynolds, R. Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus VWA. Nucleic Acids Res. 24:2807-2812, 1996.

52. 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.676-677.