

PROTOCOLS FOR FORENSIC STR ANALYSIS

VERSION 5

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

Initials: *RCI*

Date: *8/24/98*

General PCR Guidelines	6
DNA Extraction	9
DNA Extraction Guidelines	9
Protein and DNA Extraction of Stains and Swabs	11
Chelex DNA Extraction From Whole Blood, Bloodstains, or Blood Scraped or Swabbed Off a Surface, Tissue, or Saliva stains	12
Chelex DNA Extraction From Liquid Semen	13
Non-differential Chelex DNA Extraction from Semen Stains or Swabs	14
Chelex DNA Extraction from Semen Stains or Swabs	15
Chelex DNA Extraction From Hair	18
Chelex Extraction From Non-Organically and Organically Extracted Samples	19
Estimation of DNA Quantity from QuantiBlot Analysis	21
Sample Blotting	21
Hybridization	23
Color Development	24
Photography	25
Interpretation	25
Troubleshooting of QuantiBlot	27
References for QuantiBlot	30
Quad Multiplex System	31
Amplification of the Quad Multiplex System (VWA, THO1, F13A, FES/FPS)	31
Analysis of the Quad Multiplex System on the ABI 373 DNA Sequencer	35
Gel Casting for ABI 373 Sequencer	35
Troubleshooting	36
Gel Electrophoresis on ABI 373 Sequencer	37
Setting the Run Parameters	37
Preparing the Plates	38
Pre-Running the Gel	39
Sample Preparation	40
STR Gel Analysis Of Gels on Macintosh Terminals Connected to the ABI 373 Sequencer	42
STR Gel Analysis and Genotyper for 373 Gels on Separate Apple Macintosh Terminals	47
Quad Genotyper Analysis of 373 Gels on Macintosh Terminals Connected to the ABI 373 Sequencer	49
Troubleshooting	52
Analysis of the Quad Multiplex System on the ABI 377 DNA Sequencer	53

Initials:

EC

Date:

8/24/98

Gel Casting for the ABI 377 Sequencer	53
Troubleshooting	55
Gel Electrophoresis on ABI 377 Sequencer	56
Initial Preparation	56
Mounting the Gel Cassette in the Electrophoresis Chamber	56
Create a Run File	58
Checking the Plates	58
Pre-Running the Gel	59
Gel Naming and Sample Preparation	60
Sample Loading and Starting the Run	61
Removing the Used Gel and Clean-Up	63
Troubleshooting Electrophoresis	64
STR Gel Analysis of Gels Run on ABI 377	66
Gel File Copying and Archiving Procedure	66
Collection Gel Processing	67
Troubleshooting Collection Gel Processing	72
Project File Analysis	73
Troubleshooting Project File Analysis	76
QUAD Genotyper 2.0 Analysis of all ABI 377 Gels and those ABI 373 Gels	
Processed on Separate Apple Macintosh Terminals	78
Troubleshooting QUAD Genotyper	81
Y Chromosomal STR Analysis	83
Amplification of the Y STR Multiplex System 1 (DYS19, DYS390, DYS 389I and II)	
.....	83
Electrophoresis of Y Chromosomal STR Multiplex 1 (Y M1) on the ABI 377	87
Y Chromosomal STR Gel Analysis of Gels Run on ABI 377	87
Y STR Gel File Copying and Archiving Procedure	87
Collection Gel Processing	88
Troubleshooting	92
Project File Analysis	92
Troubleshooting Project File Analysis	95
Y M1 STR Genotyper 2.0 Analysis of all ABI 377 Gels	96
Genotyper troubleshooting see QUAD Genotyper section (page)	100
AmpFISTR Green and Blue STR's	101
Amplification of AmpFISTR Green and Blue STR	101
Analysis on the ABI Prism 310 Capillary Electrophoresis (CE) Genetic Analyzer ...	105
Instrument Preparation	106
Changing and Installing the Capillary	106
Calibrating the Autosampler	109

Initials:

LCJ

Date:

8/24/98

Filling and Installing the Glass Syringe and the Pump Block	110
Changing the Buffer and Baseline Check	111
Filling Out a Sample Sheet	113
Preparing a Genescan Injection List	115
Reinjecting a Sample	115
Preparing and Running the DNA Samples	117
STR Fragment Length Analysis for AmpFISTR Blue and Green	119
Capillary Electrophoresis Troubleshooting	122
AmpFISTR Blue and AmpFISTR Green Genotyper 2.0 Analysis of ABI 310	
Capillary Electrophoresis	133
Gel File Copying and Archiving Procedure	139
Genotyper Trouble Shooting	140
STR Results Interpretation	143
Autosomal STR Allele Calling Criteria	143
Y STR Allele Calling Criteria	146
Genotyper Results Table	148
Reporting Procedures	148
Comparison of Samples and Interpretation of Results in Report	150
Extraction Reagent, Amplification Negative and Substrate Controls	151
Amplification Positive Control	153
Interpretation of Complex Autosomal STR Results	154
Mixtures of DNA	154
Partial Profiles	156
Detection Of Previously Unreported Rare Alleles	157
Samples with High Background Levels	158
Additional Interpretations of Y STR Results and Complex Y STR Results	158
Mixtures of male DNA	158
Partial Profiles	159
Detection Of Previously Unreported Rare Alleles	159
Samples with High Background Levels	159
QUAD Genotyper Categories Table for ABI 373	160
QUAD Genotyper Categories Table for ABI 377	162
Y STR 1 Genotyper Categories Table for ABI 377	164
STR Troubleshooting	165

Initials: **RC**

Date: **8/24/98**

Quad and ABI DNA Sequencer References and STR References	170
Capillary Electrophoresis and AmpFISTR Blue and Green References	173
Population Frequencies for STR's	175
Autosomal STR's	175
Y STR's	176
Amplification Troubleshooting	177
Microcon Operation for DNA Concentration	178
Microcon Operation for DNA Concentration and Clean-up	179

Initials: RS

Date: 8/24/98

General PCR Guidelines

1. The general laboratory policy is to identify semen and perform presumptive blood analysis (see the Forensic Biochemistry and Hematology Laboratory Manual) before individualization is attempted. However, circumstances will exist when this may not be possible.
2. Duplicate analyses must be performed on all evidence samples as follows:
 - A. Concordant STR match results from at least one locus in a different amplification is considered a duplicate analysis.
 - B. Identical typing among at least three samples including exemplars in a case is considered internal duplication.
 - C. If after the first PCR analysis, there is no evidence of a mixture then the duplicate analysis may begin at the extraction stage (preferable) or the amplification stage. If there are additional alleles then either C or D (see below) apply.
 - D. If after the first PCR analysis, there are additional alleles less than 50 (or 100, if applicable), then the duplicate analysis may begin at the extraction stage (preferable) or the amplification stage
 - E. If after the first PCR analysis, there are three or more alleles greater than 50 (or 100, if applicable), then the duplicate analysis must begin at the amplification stage.

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

3. Duplicate analyses are performed to verify the typing results. At least one run (or the single run if there is only one) must have *no* visible peaks in the extraction reagent control, amplification negative control and no extraneous peaks in the positive control. The other run which is only used for verification, can have extraneous peaks less than 50 (or 100, if applicable) in the extraction reagent, amplification negative and positive controls.
4. The substrate control can have visible peaks. These peaks do *not* invalidate the results of the accompanying stains. The presence of peaks in the substrate control should be noted in the report.
5. To minimize the potential for carry-over contamination, the laboratory is organized so that the areas for DNA extraction, for PCR set-up, and for handling amplified DNA are physically isolated from each other. Each of the three areas is in a separate room. Dedicated equipment such as pipettors should not leave their designated areas. Only the samples in designated racks should move between areas.

Initials: *RC*

Date: *8/24/98*

6. Samples that have not yet been amplified should never come in contact with equipment in the amplified DNA work area. Samples that have been amplified should never come in contact with equipment in the unamplified work area.
7. Handle all samples aseptically to prevent contamination by extraneous DNA.
8. The DNA extraction and PCR setup of evidence samples should be performed at a separate time from the DNA extraction and PCR setup of exemplars. This precaution will help to prevent potential cross-contamination between evidence samples and exemplars.
9. Change gloves frequently to avoid sample-to-sample contamination. Change them whenever they might have been contaminated with DNA and whenever exiting a work area.
10. Always change pipette tips between handling each sample.
11. Never "blow out" the last bit of sample from a pipettor. Blowing out increases the potential for aerosols, which may contaminate a sample with DNA from other samples. The accuracy of liquid volume delivered is not critical enough to justify blowing out.
12. Use filter pipet tips for pipetting all DNA and use whenever possible for other reagents. Use the appropriate filter tips for the different sized Pipetmen. The tip of the Pipetman should never touch the filter.
13. Avoid splashes. Centrifuge all liquid to the bottom of the closed tube before opening it.
14. Avoid touching the inside surface of the tube caps.
15. Use disposable bench paper to prevent the accumulation of human DNA on permanent work surfaces. Bleach should be used periodically to decontaminate exposed work surfaces.
16. Limit the quantity of samples handled in a single run to a manageable number. This precaution will reduce the risk of sample mix-up and the potential for sample-to sample contamination.
17. Store evidence and unamplified DNA in a separate refrigerator or freezer from the amplified DNA.

Initials: **RCJ**

Date: **8/21/98**

18. 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.
19. Avoid exposing mineral oil to UV light. Exposure to UV light causes the mineral oil to inhibit PCR.
20. Use the Thermal Cycler only for amplification of DNA.
21. Make sure lab coat sleeves do not touch the caps of open tubes.
22. Discard pipette tips if they accidentally touch the bench paper or any other surface.
23. Wipe the outside of the Pipetman with 10% bleach solution if the barrel goes inside a tube.
24. During analysis, all evidence, unamplified DNA, and amplified DNA should be stored refrigerated or frozen. Freezing is generally better for long term storage.
25. After the report is issued, amplified DNA's are discarded. **The remainder of the DNA extract's must be retained.**
26. Make sure worksheets and logbooks are completely filled out.
27. A positive and interpretable, STR and/or QuantiBlot result can be considered primate positive. Identification of the specific physiological fluid may be accomplished using the procedures described in the Forensic Biochemistry and Hematology Laboratory Manual.

Initials: *RC*

Date: *8/24/98*

DNA Extraction

DNA Extraction Guidelines

Slightly different extraction procedures may be required for each type of specimen. Due to the varied nature of evidence samples, the user may need to modify procedures. See the trouble shooting guide (page 72, 177) for suggestions on how to modify procedures.

1. Use Kimwipes to open sample tubes.
2. Only one tube should be uncapped at a time.
3. When pouring or pipetting Chelex solutions, the resin beads must be distributed evenly in solution. This can be achieved by shaking or vortexing the tubes containing the Chelex stock solution before aliquoting.
4. Pour an aliquot of the Chelex solution from the stock tube into a sterile disposable working tube before adding to samples. The stock tube can be used multiple times. The working tube is discarded after each batch of extractions.
5. For pipetting Chelex, the pipette tip used must have a relatively large bore--1 mL pipetman tips are adequate.
6. Keep the Chelex extraction reagents and equipment separated from the rest of the laboratory equipment.
7. Be aware of small particles of fabric which may cling to the outside of tubes.
8. Include an extraction reagent control with each batch of extractions to demonstrate extraction integrity. The extraction reagent control contains water in place of biological fluids or stains. If DNA is found in the extraction reagent control by QuantiBlot analysis, the extraction of all the samples in the batch should be repeated and the samples should not be amplified. However, if no DNA is found then the extraction reagent controls are treated as normal samples and extracted, amplified and typed along with the test samples.

Initials: RC

Date: 8/24/98

9. Obtain substrate controls from unstained fabric or substrate as close to each stain as feasible. It is not always possible to find an unstained substrate control for each evidence sample. If no DNA is found in a substrate control by QuantiBlot analysis, the substrate control is discarded without amplification because no DNA is present. However if DNA is found in the substrate control either a new substrate control should be extracted and quantitated or the substrate control should be amplified and typed along with the test samples.
10. If a sample is found to contain <0.15 ng/20 μ L of DNA by QuantiBlot analysis the sample should not be amplified and it should be reported as containing insufficient DNA. 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/20 μ L) may also be concentrated and purified using a Microcon-100 if the DNA is suspected of being degraded or containing an inhibitor of PCR.
11. After extraction, the tubes containing the unamplified DNA should be transferred to a box and stored in the appropriate refrigerator or freezer. The microtube rack used to hold the DNA extraction tubes should be washed with 10% bleach. The tubes should *not* be stored in the extraction racks

Initials: *RC*

Date: *8/24/98*

Protein and DNA Extraction of Stains and Swabs

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

1. Suspend the sample in approximately 75-200 μL of sterile dH_2O (or 0.05 M DTT if no semen is present) in a 1.5 mL microcentrifuge tube.
2. Centrifuge the tube in the microcentrifuge for 2 minute at 10,000 to 15,000 $\times g$.
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: *RG*

Date: *8/24/98*

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

1. Remove the extraction rack from the refrigerator. Extract either evidence or exemplars. Do not extract both together.
2. Pipette 1 mL of sterile deionized water into each of the tubes in the extraction rack.
3. Mix the tubes by inversion or vortexing.
4. Incubate at room temperature for 15 to 30 minutes. Mix occasionally by inversion or vortexing.
5. Spin in a microcentrifuge for 2 to 3 minutes at 10,000 to 15,000 x g.
6. Carefully remove supernatant (all but 20 to 30 μ L). 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 μ L 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 μ L 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: *RD*

Date: *8/24/98*

Chelex DNA Extraction From Liquid Semen

1. Remove the extraction rack from the refrigerator.
2. Pipette 200 μ L of 5% Chelex into each tube in the extraction rack.
3. Add 1 μ L of 20 mg/mL Proteinase K and 7 μ L 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 12) beginning with Step 10.

Initials: *RC*

Date: *8/24/98*

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. Do not forget to process an extraction negative.
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 μ L of 5% Chelex, 1 μ L of 20 mg/mL Proteinase K, and 7 μ L 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: *RD*

Date: *8/24/98*

Chelex DNA Extraction from Semen Stains or Swabs

1. Remove the extraction rack from the refrigerator.
2. Pipette 1 mL of PBS into each tube in the extraction rack.
3. Mix by inversion or vortexing
4. Incubate at room temperature for 30 minutes.
5. Vortex or sonicate the substrate or swab for at least 2 minutes to agitate the cells off of the substrate or swab.
6. Remove the swab or other substrate from the sample tube, one tube at a time, using sterile tweezers (or with a hot needle punch a hole in the bottom of the tube and collect the supernatant in a piggy backed bottom tube) and close tubes. Sterilize tweezers with ethanol before the removal of each sample. Store swab or substrate in a sterile tube for the substrate remains fraction.
7. Spin in a microcentrifuge for 5 minutes at 10,000 to 15,000 x g.
8. Without disturbing the pellet, remove all but 50 μ L 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 μ L by stirring with a sterile pipette tip.
10. Remove about 3 μ L of the resuspended sample for a Christmas Tree Stain (see Biochemistry and Hematology Laboratory Manual). After staining, the slide should be labeled and saved as evidence. If sperm are not visible microscopically, the substrate may be put back into the PBS and vortexed more vigorously (step 6) to try to dislodge additional sperm.

Note: If epithelial cells are detected, proceed with the differential extraction procedure beginning with step 12. If no epithelial cells are observed, the swab remain fraction may either be combined with the resuspended sample or both fractions processed separately. If no epithelial cells are observed, the differential extraction procedure may be omitted and the samples may be processed beginning with step 21.

Initials: *RY*

Date: *8/24/98*

11. To the approximately 50 μ L of resuspended cell debris pellet, add 150 μ L sterile deionized water (final volume of 200 μ L).
12. Add 1 μ L of 20 mg/mL Proteinase K. Vortex briefly to resuspend the pellet.
13. Incubate at 56°C for about 60 minutes to lyse epithelial cells, but for no more than 75 minutes, to minimize sperm lysis.
14. During the incubation step do the following:
 - a. Label a new tube for each sample, including the extraction reagent control. Mark each tube as an epithelial cell fraction
 - b. Add 50 μ L 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 μ L of the supernatant from each sample and the extraction negative to its respective epithelial cell fraction sample tube. Store at 4°C or on ice until step 22.
17. Wash the sperm pellet with Digest Buffer as follows:
 - a. Resuspend the pellet in 0.5 mL Digest Buffer.
 - b. Vortex briefly to resuspend pellet.
 - c. Spin in a microcentrifuge at 10,000 to 15,000 x g for 5 minutes.
 - d. Remove all but 50 μ L 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 dH₂O as follows:
 - a. Resuspend the pellet in 1 mL sterile dH₂O.
 - 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 μ L of the supernatant and discard the supernatant.
19. Resuspend the pellet by stirring with a sterile pipette tip. Remove about 3 μ L 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 μ L resuspended sperm fraction and to the tubes containing the substrate remains and the sperm fraction extraction negative, add 150 μ L of 5% Chelex, 1 μ L of 20 mg/mL Proteinase K, and 7 μ L of 1M DTT. Mix gently.

Initials: *RC*

Date: *8/24/98*

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.
23. Vortex at high speed for 5 to 10 seconds.
24. Incubate in at 100°C for 8 minutes using a screw down rack.
25. Vortex at high speed for 5 to 10 seconds.
26. Spin in a microcentrifuge for 2 to 3 minutes at 10,000 to 15,000 x g.
27. Pipet 20 µL into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration.
28. Store the remainder of the supernatant at 2 to 8°C or frozen.

Initials: *RD*

Date: *8/24/98*

Chelex DNA Extraction From Hair

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

1. Fill out the extraction worksheet.
2. Handling hair with clean forceps, examine the hair under a dissecting microscope for the presence of sheath material. The hair may be placed on a clean piece of white paper. Note possible presence of body fluid on hair.
3. Wash the hair containing sheath material to reduce surface dirt and contaminants by immersing the hair in sterile, deionized water in a clean 50 mL beaker.
4. Return the hair to the dissecting microscope. Use a clean scalpel to cut a 1 cm portion from the root end of the hair. Because hair may contain cellular material on the surface which may or may not originate from the hair donor, it is advisable to cut off a 1 cm section of the shaft adjacent to the root portion for separate analysis as a control.
5. Add the root portion of the hair to 200 μ L 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 μ L 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: *RG*

Date: *8/24/98*

Chelex Extraction From Non-Organically and Organically Extracted Samples

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

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

Table I: Non-Organic or Organic Extract for Amplification

yield gel conc (ng/ μ L)	volume dH ₂ O (μ L)	volume (non-)organic extract (μ L)	volume 5% Chelex (μ L)
100	49.5	0.5	150
50	49	1.0	150
40	48.7	1.3	150
25	48	2.0	150
20	47.5	2.5	150
12.5	46	4.0	150
10	45	5.0	150
5.0	40	10	150
2.5	30	20	150
2.0	25	25	150
≤ 1.0	0	50	150

1. Fill out the extraction worksheet.
2. Vortex and briefly microfuge the tubes containing the (non-)organically extracted DNA samples.

Initials: *RU*

Date: *8/24/98*

3. Add in order, the appropriate amount of dH₂O, (non-)organic extract, and 5% Chelex solution to each sample tube (Table I) for a final volume of 200 μ L. The target amount of DNA to add to each Chelex Extraction Tube is 50 ng.
4. Add 50 μ L dH₂O and 150 μ L 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 μ L into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration.
11. Store the remaining sample at 2-8°C or frozen.

Initials: *RL*

Date: *8/24/98*

Estimation of DNA Quantity from QuantiBlot Analysis

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

Sample Blotting

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

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

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

- A. DNA Standards and Calibrators - 5 μ L
- B. Chelex extracts intended for HLA-DQA1 - 20 μ L
- C. Non-organic extracts - using the yield gel concentration, estimate the volume needed to apply 1-5 ng DNA; prepare a dilution with 1X TE⁻⁴ if necessary.

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

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

Heat a stationary water bath to between 37°C and 50°C. Warm the HLA DQA1 Hybridization Solution and the QuantiBlot Wash Solution in the water bath. All solids must be in solution before use.

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

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

Initials: *RS*

Date: *8/24/98*

5. While wearing gloves, cut a piece of Biodyne B membrane to 11.5 x 7.9 cm. Cut a small notch in the upper left corner to mark its orientation. Place the membrane in a container containing 50 mL of Pre-Wetting Solution and incubate at room temperature for 1-30 minutes.
6. Using forceps, remove the membrane from the Pre-Wetting solution. Place the membrane on the gasket of the slot blotter, then place the top plate of the slot blotter on top of the membrane. Turn on the vacuum source (house vacuum or vacuum pump) to a vacuum of approximately 8 to 10 inches Hg. Turn off the sample vacuum and turn on the clamp vacuum on the slot blot apparatus. Push down to ensure a tight seal.
7. Load the membrane as follows:

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

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

Initials: *RCJ*

Date: *8/24/98*

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

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

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

Clean the apparatus by soaking in enough 0.1% SDS to cover for 5-15 minutes. Using a Kimwipe, clean the gasket and the side of the top plate that contacts the membrane. Rinse the slot blotter in H₂O and allow to dry at room temperature. **Never use bleach.**

11. Transfer the membrane to 100 mL of pre-warmed HLA DQA1 Hybridization Solution in the hybridization tray. Add 5 mL of 30% H₂O₂. Place the lid on the tray. Put the tray into the 50°C shaking water bath. Place a weight (e.g. lead ring) on the covered tray to prevent the tray from sliding or floating.

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

Hybridization

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

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

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

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

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

Initials: **ACI**

Date: **8/24/98**

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

Repeat for a total two washes.

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

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

Color Development

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

60 mL of Citrate Buffer
3 mL Chromogen
60 μ L 3% H_2O_2 .

Mix thoroughly by swirling (do not vortex).

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

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

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

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

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

Repeat for a total of 3 washes.

Initials: *ps*

Date: *8/24/98*

Photography

21. Photograph the membrane while wet. Place the membrane on a dark, flat, non-absorbent surface.
22. Use a Polaroid MP4 camera system with type 667 (preferred) or 665 film and a Wratten 23A or 22 (orange) filter.
23. Turn on the flood lights. Adjust the height of the camera and focus so that the membrane fills the entire viewing frame.
24. Photograph at 1/125 seconds and f22 for type 667 film. Photograph at 1/2 second and f16 for type 665 film.
25. Develop the film for 30-60 seconds. If the photograph is out of focus, not exposed properly, or does not accurately record the bands on the membrane, vary the exposure conditions and re-photograph.

Discard the membrane once a good photograph is obtained.

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

Interpretation

27. At least one of the 0.15 ng standards must be visible. If not, the samples on the membrane with an amount of <0.31 ng must be repeated.
28. Compare the intensity of Calibrator 1 (3.5 ng/5 μ L) and Calibrator 2 (0.5 ng/5 μ L) to the DNA Standards. The intensity of Calibrator 1 should be between 2.5 ng and 5 ng; the intensity of Calibrator 2 should be between 0.31 ng and 0.62 ng. If not, the membrane must be repeated.

Initials: *RG*

Date: *8/24/98*

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

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

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

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

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

Initials: *RU*

Date: *8/24/98*

Troubleshooting of QuantiBlot

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
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 ($\pm 1^\circ\text{C}$).
	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: *pu*

Date: *8/24/98*

	<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
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.	<p>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.</p> <p>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.</p> <p>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.</p>
4.	Filter background	No or low SDS in the Hybridization Solution or in the Wash Solution	Prepare solutions with proper concentrations of SDS.

Initials: *RU*

Date: 8/24/98

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
	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 μ L 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 μ L 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 (\pm 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: *RC*

Date: *8/24/98*

References for QuantiBlot

1. Walsh, P.S., Valaro, J., and Reynolds, R., 1992. A rapid chemiluminescent method for quantitation of human DNA. *Nucleic Acids Research* **20**: 5061-5065.
2. Wayne, J.S. and Willard, H.F., 1986. Structure, organization, and sequence of alpha satellite DNA from human chromosome 17: evidence for evolution by unequal Crossing-Over and an ancestral pentamer repeat shared with the human X chromosome. *Molecular and Cellular Biology* **6**: 3156-3165.
3. Whitehead, T.P., Thorpe, G.H.G., Carter, T.J.N., Groucutt, C., and Kricka, L.J., 1983. Enhanced luminescence procedure for sensitive determination of peroxidase-labeled conjugates in immunoassay. *Nature* **305**: 158-159.
4. Miller, S.A., Dykes, D.D., and Polesky, H.F., 1988. A simple salting out procedure for extraction DNA from human nucleated cells. *Nucleic Acids Research* **16**: 1215.

Initials: *ED*

Date: *8/24/98*

Quad Multiplex System

Amplification of the Quad Multiplex System (VWA, TH01, F13A, FES/FPS)

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

1. For each sample to be amplified, label a new tube. Do not amplify samples in which no DNA was detected by QuantiBlot.
2. To each labeled tube, add TE⁻⁴ and DNA as specified in Table II.

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

3. Add the following amounts of TE⁻⁴ and DNA to new labeled tubes:

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

Initials: *RC*

Date: 8/24/98

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

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

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

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

** Add TE⁻⁴ to a final volume of 27 μ L.

Initials: *RD*

Date: *8/24/98*

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

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

Presoak at 95°C for 11 minutes; Presoak at 96°C for 1 minute

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

Denature at 94°C for 1 second; denature at 94°C for 45 seconds

Anneal at 54°C for 2 minutes; anneal at 54°C for 1 minute

Extend at 72°C for 1 second; extend at 72°C for 1 minute

Repeat the above for 29 cycles

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

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

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

5. Fill out the amplification worksheet and record the appropriate lot numbers.
6. Determine the number of samples to be amplified, including controls and label a PCR reaction mix tube for each sample.
7. Ensure that the solution is at the bottom of each PCR reaction mix tube by tapping the tube down onto a clean work surface or by centrifuging briefly. Label the caps of the PCR Reaction Mix tubes. Open caps using the microcentrifuge tube de-capping tool or a new Kimwipe. **Avoid touching the inside surface of the tube caps.**
8. Pipet 3 μ L of 25mM MgCl₂ into each reaction mix tube with a sterile pipet tip. Pipet carefully in the solution at the bottom of the tube. Use a fresh pipette tip for each tube.

Note: The samples should not sit for an extended period after adding the MgCl₂ solution to the PCR Reaction Mix. Proceed with the set up and begin the cycling processing as soon as possible.

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

Initials: *RU*

Date: *8/24/98*

10. Close all of the tubes.
11. **Note: Use a new sterile filter pipet tip for each sample addition. Open only one tube at a time for sample addition.** The final aqueous volume in the PCR reaction mix tubes will be 50 μ L. Add the content (27 μ L) of the sample DNAs and sterile H₂O tubes which were prepared above to each labeled tube by inserting the pipet tip through the mineral oil layer. After the addition of the DNA, cap each sample before proceeding to the next tube. **Do not vortex or mix.**
12. For each well of the Thermal Cycler heat block which will be used to amplify samples, add one drop of mineral oil to the well. Place the PCR Reaction Mix tubes into the Thermal Cycler. Push the tubes down completely into the heat block. Record the heat block position of each tube.
13. Start the Thermal Cycler amplification program (File #11). Verify the cycling parameters by monitoring the first cycle. The tubes should be checked after the first cycle and pressed further into the heat block so that they fit tightly.
14. Return the microtube rack used to set-up the samples for PCR to the PCR Set-Up Area.
15. After the amplification process, the samples are ready to be loaded on the Genescan gels. They may be stored in the appropriate refrigerator at 2-8°C for a period of up to 6 months, if they need to be stored further they should be transferred to a -20° freezer.

Initials: *pu*

Date: 8/24/98

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

Gel Casting for ABI 373 Sequencer

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

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

Add stir bar and place on stirrer with low heat.

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

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

3. Prepare lab bench with bench paper and square box. Place plain plate on box such that the etched letters "L" and "R" for left and right are readable and are on the correct sides. Place white spacers on each side of plate. Put notched plate on top of plain plate such that the etched letters "L" and "R" for left and right are readable and are on the correct sides. Place three clamps on each side of the sandwiched plates.
4. The urea in the gel solution should be completely dissolved before proceeding. Pour the gel solution into a 50 mL sterile tube and adjust the volume to 50 mL with deionized water. Filter the solution using disposable 0.2 μ m pore size filter units. Pour the filtered solution back into the 50 mL sterile tube. Let the solution cool down to room temperature.

Initials: *pu*

Date: *8/24/98*

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

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

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

Allow to polymerize for 1.5-2.0 hours.

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

Troubleshooting

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

Initials: *rd*

Date: *8/24/98*

Gel Electrophoresis on ABI 373 Sequencer

Setting the Run Parameters

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

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

The settings should be:

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

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

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

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

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

Enter the gel number in log book.

Initials: *RA*

Date: *8/24/98*

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

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

Place the gel in the Sequencer.

Preparing the Plates

5. Open the Sequencer door. Place lower buffer chamber in the bottom of the electrophoresis unit with the electrode to the front. Pull the beam-stop bar towards you until it stops.
6. Remove clamps from gel. Use a razor blade to remove gel crusts on the outside of the plate. Clean gel plates with low lint Kimwipes and deionized water, followed by isopropanol or methanol. **The laser read area must be lint free.** Use Kimwipe moistened with isopropanol or methanol for last wipe, and let dry.
7. Place gel in the electrophoresis unit with the notched plate facing **away** from you. The bottom edge of the plates should rest on the notched supports in the bottom of the lower buffer chamber. Press gel against the back of the chamber and push the beam-stop bar against the gel. Center the plates with relation to the beam-stop bar and then latch the bar in place. Also close clamps connecting gel with metal back plate.

Close door

8. Press the **Main Menu** button of the sequencer. Select **Start/Prerun** and then **Plate Check**. Select **Full Scan**.
9. On the computer screen, click on **Scan** button on the **GS Collection Controller Panel**. The scan window should show a relatively flat line across the screen in each of the four colors. Peaks appearing here indicate dirt on the glass or in the gel. If that is the case, reclean the plates as described below (step 10), and then scan the plates again.
10. On the Sequencer press **Main Menu** and **Abort Run**. Open the instrument door, unlatch the beam-stop bar, and slowly pull the bar towards you. Don't remove plate, first try to clean the laser read region with a Kimwipe moistened with isopropanol. Then perform another plate check. If the plate check still shows peaks, the gel must be removed and both sides of the read area cleaned. If peaks persist, it must be a gel problem. The lanes in this region of the gel cannot be used.

Initials: *RC*

Date: *8/24/98*

11. When the plates are clean according to the Scan, check the Y-axis values of the scan lines. The lowest line, usually the blue line, should be between 600 and 900. The highest line, usually the red line, should be between 1100 and 1400. If the values differ from this, the PMT voltage has to be adjusted. If the values are o.k. proceed with step 12.
12. To change the PMT setting, press **Main Menu** - press **Calibration** - press **Configure** - press **More** until "PMT voltage is --- volts." appears on the screen. Using the number keyboard to the right, raise or lower the value by steps of 10 and press **Change**. Observe the effect of these changes on the scan window. If desired height is reached press **Main Menu**.

Pre-Running the Gel

13. Open door and place upper buffer chamber in electrophoresis unit at the top of the gel. Place the clear plastic bar in front of the plate, and tighten the screws equally to maintain even pressure on the plates. Turn the screw just until the rubber gasket seal starts to flatten - **do not over tighten**.
14. Fill the upper buffer chamber with approximately 800 mL 1xTBE buffer. If the buffer chamber is leaking, tighten the screws some more. Gently remove the comb from the gel by pulling it straight up. If sample wells appear to be distorted, they can be put back into shape with a flat gel loading pipette tip. To remove excess polyacrylamide in the loading area clean the rim of the notched plate with gloved finger. Flush sample wells with the running buffer using a syringe or a pipette.
15. Fill the lower buffer chamber with approximately 600 mL 1xTBE buffer. Avoid splashing the buffer against the back of the chamber.
16. Attach the top and bottom electrode leads located on the left side of the electrophoresis chamber and gently close the door.
17. Press the **Main Menu** button of the Sequencer. Select **Start/Prerun** and then **Pre-Run Gel**.

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

Initials: *rd*

Date: 8/24/98

Sample Preparation

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

18. After filling out the gel sheet, label the appropriate number of GeneAmp Thin-walled Reaction Tubes with the numbers of the lanes that will be loaded.
19. For N+2 samples mix 3 μL of deionized formamide with 2 μL of GS500 standard in loading buffer. Consult the following table:

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

20. Aliquot 5 μL of the formamide/standard mix into each labeled tube. Add 2 μL of PCR product per tube according to the gel loading sheet.
21. Heat denature samples in the 95°C heat block for 2-3 minutes. Immediately afterwards place samples on ice, wait for 1 minute, then briefly spin down the samples using the microcentrifuge in the hybridization room.

Put samples back on ice.
22. Stop Pre-Run by pressing **Abort Run** on **Main Menu**. Open door and repeat flushing the sample wells.
23. Carefully load 7 μL of each sample per well.
24. On **Main Menu** select **Choose Run**. Then select **Genescan Run**.

Initials: Rd

Date: 8/24/98

25. If there are additional evidence samples to be loaded, allow the gel to run for 15 minutes and then click **Pause** to stop the run. Add 7 μ L of each sample to the wells that were originally skipped. Select **Resume** to continue the run.
26. On the screen click on the **Collect** button on the **GS Collection Controller Panel**. Data collection will begin.

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

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

Clean TBE buffer crusts from the sequencer electrophoresis unit.

Initials: *RC*

Date: *8/24/98*

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

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

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

After the gel run is complete, the **GS Collection** software automatically launches the **GS Analysis** program so that the preprocessed gel should be displayed on the computer screen and the top of the screen menu bar should display the **GS Analysis** commands. If the latter is not the case, the menu bar can be activated by clicking on the **GS Analysis** icon under **Finder**. A gel can also be opened and analyzed at a later time (see step 10).

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

Initials: *RS*

Date: *8/24/98*

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

top of gel —

—

—

—

—

bottom of gel —

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

1. View the gel in order to decide if the shorter or longer size standard fragments are missing, in what direction, and how much to extend the processed gel region. Click on left hand corner of the gel window to close the gel. Under **Analysis** select **Preprocess Parameters**. Under **Process Amount** change the Start at Scan, End at Scan parameters according to the above assessment. Click **OK**.
2. Under **Analysis** select **Preprocess Collection File**. Select the folder which contains the gel file (can be desktop, hard drive, or 672 software folder) by moving the cursor on the folder name and clicking open until correct gel file is highlighted. The gel files can be distinguished from the results file by two things: the results files have a date assigned to them and a horizontal page as an icon, while the gel files have no date and a vertical page as icon. Click **open**. The collected raw data will be preprocessed again; this may take a couple of minutes. The preprocessing is finished when the gel picture appears on the screen. Continue with step 3.

Initials: *RU*

Date: *8/24/98*

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

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

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

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

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

Initials: *RS*

Date: *8/24/98*

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

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

Required Sizes for the Peaks

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

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

6. Analyze all lanes that contain samples by clicking on the white number of these lanes and afterwards deselecting the color yellow by clicking on the yellow fields. This way the blue, green and red labels get analyzed. Don't analyze empty lanes. Click on **Analyze**.
7. Close the **Analysis Control** window. In order to look at the results after the analysis is complete, the **Results Control** window has to be opened. Under **Results** go to **Electropherograms Results**. The **Results Control** window shows the title of the gel run and the same lane number versus color display as the **Analysis Control** window. The analyzed colors per lane are shown in dark grey. The white squares mean that this color has not been analyzed.

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

Initials: *RU*

Date: 8/24/98

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

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

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

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

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

Initials: *RU*

Date: *8/24/98*

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

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

1. Copy the gel from the 373 to a JAZ transport disk. Then copy the gel from the transport disk to the Apple terminal hard disk, and delete the gel from the transport disk.
2. Open the **GS Analysis** software by clicking on the **373 / 377 analysis** icon.
3. Under **File** go to **Open**. From the listed possibilities click on existing **Collection gel**. Open the hard disk, go to the appropriate folders, and highlight the desired gel file. Gels are represented by vertical icons as opposed to folders. Click **open**.
4. To the computer message "This is an old gel format. Please verify that the number of lanes is 24" click **OK**

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

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

5. Continue with step 3 of **377 STR Gel Analysis and Allele Identification** section B "Collection Gel Processing". Follow all of the steps until the end of the **Quad Genotyper Ver 2.0 Analysis on Separate Macintosh Terminal** section.
6. **Since a 373 gel instead of a 377 Gel is being processed, note the following deviations from the listed procedures:**
 - A. Since the gel has been processed with a matrix, it is not necessary to install the matrix again for the sample files - skip **step 1** under **D "Project file analysis"**.
 - B. Due to sizing differences between both platforms, the Genotyper categories differ slightly between the 373 and the 377 runs. Use the **Quad 373 Genotyper** icon when performing **G "QUAD Genotyper" step 1**.

Initials: *RD*

Date: *8/24/98*

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

Initials: *RC*

Date: *8/24/98*

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

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

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

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

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

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

2. Under **File** select **Import Results**. The **GS Analysis** results files are on the **Hard drive** in Folder **672Software**. Choose correct results file by double clicking on the appropriate folders until the results file name and the correct version is highlighted. Then click **open** and then click **import**.
3. In order to preserve the template file, the current file has to be saved under a new name!!! Therefore, immediately after importing the results, change the name of the Genotyper template to your initials and the gel file. Under **File** select **Save As**. Enter your initials and the name of the gel you are processing. Click **Save**.

Initials: *RCJ*

Date: *8/24/98*

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

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

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

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

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

- 1 small constant bands of the following size and color
Blue 180-181 bp, 230-232 bp, and 246-248 bp
Green 128-131 bp and 211-220 bp
- 2 pull-ups of green peaks caused by very high blue peaks in the same lane
- 3 shoulder peaks approx. 1-3 bp bigger than main allele
- 4 peaks caused by overflow of sample in the adjacent lane
- 5 -4 and +4 bp stutter if there is no indication for the presence of a mixture.
(See interpretation section).

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

Initials: *RC*

Date: *8/24/98*

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

Close **Plot Window**.

6. Run the **Allele Designation Macro** by pressing the Apple key and the number 2. This macro automatically assigns allele names to the labeled peaks based on the size categories for the different fragments and writes a results table. The following steps are carried out: clear table, change labels to the categories name, add rows with one sample per row, containing sample info in column 1, up to 4 labels in columns 3-6, the text “check” in column 2 if number of labels >2 (titled “mixture.”); put column titles in first row.

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

7. Under **View** select **Show Plot Window**. Under **Analysis** select **Change Labels**. In the Change Labels Dialog box check both “with size in bp” and “the categories name”. Click **OK**.
8. The next step is to print the plot window. To achieve a more uniform format, the base pair range which should be printed has to be selected. Under **View** select **Zoom to**, type in 110 to 260. Click **OK**. The display on the screen will focus in on that range.

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

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

Initials: *PL*

Date: 8/24/98

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

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

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

Troubleshooting

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

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

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

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

Initials: *RC*

Date: *8/24/98*

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

Gel Casting for the ABI 377 Sequencer

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

1. When making **one** plate:

Pour 10.8 g of pre-weighed urea in 100 mL Erlenmeyer flask.

Add 10 mL of deionized water

3 mL of 10x TBE buffer

3 mL of 50% Long Ranger

For **two** plates:

Pour 18 g of pre-weighed urea in 100 mL Erlenmeyer flask.

Add 20 mL of deionized water

5 mL of 10x TBE buffer

5 mL of 50% Long Ranger

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

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

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

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

Initials: *RC*

Date: *8/24/98*

4. The urea in the gel solution must be completely dissolved before proceeding. Pour the gel solution into a 50 mL tube:

- for **one** plate adjust the volume to **30mL**,
- for **two** plates adjust the volume to **50 mL** with deionized water.

Filter the solution using disposable 100mL 0.2 μ m 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 **150 μ L** of 10% ammonium persulfate (APS) and **21 μ L** of TEMED to the Long Ranger/Urea solution.
- For **two** plates (**50 mL** volume) add **250 μ L** of 10% ammonium persulfate (APS) and **35 μ L** 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 with 1xTBE or deionized water on the bottom and the top ends of the gel, cover the moist paper towels with clear plastic wrap, fold the plastic wrap over and hold in place using gel clips.

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

Initials: *RCI*

Date: *8/24/98*

Troubleshooting

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

Initials: *RCW*

Date: *8/14/98*

Gel Electrophoresis on ABI 377 Sequencer

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

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

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

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

Initial Preparation

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

A Mounting the Gel Cassette in the Electrophoresis Chamber

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

Initials: *RU*

Date: *8/24/98*

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

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

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

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

5. Open the front door of the ABI Prism 377 and place the lower buffer chamber on the bottom shelf of the electrophoresis unit.
6. Check the amber colored positioning pins, and the back plate for buffer crusts and dirt. Remove any residue with deionized water.

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

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

Initials: *RD*

Date: *8/21/98*

B Create a Run File

1. Choose **New** from the **File** menu and click the **GeneScan™ Run** icon. This automatically creates a new Run file, and opens the Run window.

The default setting for the run parameters displayed in the run window are:

Plate check module - **Plate check A**

Prerun module - **GSPR36A-2400**

Run module - **GS Run36A-2400**

Collect time - **2.5h**

Sample sheet - **<none>**

Well to read distance - **36**

Gel's Matrix file - **appropriate Genescan matrix**

Lanes - **24**

These settings should not be changed.

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

2. Import the sample sheet named "empty" by clicking on the black arrow in the white field next to sample sheet, and selecting the file name "empty".
3. The **run folder** is automatically named after the instrument, and with the date and time of the creation of the run file. This name appears on top of the GS Run window.

C Checking the Plates

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

Proceed to Pre-running the Gel.

Initials: *RC*

Date: *8/24/98*

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

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

D Pre-Running the Gel

1. Click **Cancel** and then **Terminate** to end the plate check, then open the instrument door.
2. Un-clamp 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 is added to the wells before pre-running the gel. 50 μ L of the loading buffer are applied by using a 100 μ L pipette tip and dragging the pipette across the gel while expelling the blue buffer.
7. Place the lid on the upper buffer chamber, and close the instrument door.

Initials: *RC*

Date: *8/24/98*

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

The electrophoresis indicators should all be near zero.

The gray gel temperature indicator shows the temperature goal, the green indicator the actual temperature.

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

NOTE THE PRERUN PARAMETERS IN THE QC CHECK BOOK.

Proceed with naming the gel and sample preparation.

E. Gel Naming and Sample Preparation

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

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

The name for casework gels has to be in the following format:

98-Men001, 98-Mul001 and 98-Jef001.

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

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

Initials: *RC*

Date: *8/24/98*

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

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

3. Label a sufficient number of 0.5mL sample tubes. Have another analyst witness your tube set up. Add 5 μ l of the mastermix to each tube, then add 4 μ l of amplified product. The amplified product must be pipetted directly into the formamide, otherwise the sample will not be denatured correctly.
4. Heat the samples at 95°C for two minutes and put on ice immediately. The samples can stay chilled for a maximum of 30 minutes. If they have not been loaded in that time span, the samples must be reheated.

F. Sample Loading and Starting the Run

1. Remember: the run cannot be started before the gel temperature is at least 48°C. Check the status window for the temperature readings.
2. Interrupt the prerun by clicking **pause**.

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

3. Open the instrument door, remove the lid from the upper buffer chamber, and flush out the wells using a 10mL syringe equipped with a flat 0.2mm pipette tip.
4. Referring to the gel work sheet, load 4 μ l of the amplified product mix in the appropriate lanes.

Initials: *RA*

Date: *8/24/98*

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**.

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

NOTE THE ELECTROPHORESIS PARAMETERS IN THE QC CHECK BOOK.

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

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

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

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

Initials: *RC*

Date: *8/24/98*

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

After the gel run is finished the Collection Software quits automatically.

G. Removing the Used Gel and Clean-Up

1. Open the door, remove the lid from the upper buffer chamber, and remove the buffer from both buffer chambers before proceeding.
2. Disconnect all electrodes, the electrical wire for the heat plate and the tubings.
3. Un-clamp clamps #3, #4, #5 on each side to release the heat plate. Remove the heat plate. Put clamps back to hold the gel plates.

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

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

4. Un-clamp upper buffer chamber (clamps #2), remove it, and put clamps back to hold the gel plates.
5. Push the gel cassette towards the back plate, release the four outer clamps that hold the cassette, and remove the cassette/gel assembly from the instrument.
6. Place cassette/gel assembly on a bench top. Remove the gel plates from the cassette, pry open and clean the plates. Rinse the black gel cassette under running tap water to remove any electrophoresis buffer. Pay special attention to the beam stop bar and the clamps - these should not be allowed to be encrusted with dried buffer.
7. Go back to the instrument, and remove any TBE buffer from the amber positioning pins, the laser lens, the back plate, and the heat plate. Rinse the upper and lower buffer chambers with tap water.

Initials: *RU*

Date: *8/24/98*

F. Troubleshooting Electrophoresis

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

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

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

2. **Inconsistent mobilities from gel to gel**, and poor resolution are most likely caused by low quality, too old, or simply the wrong gel casting reagents. Check the age of e.g. the APS, remake the 1xTBE buffer, make a fresh gel, and try again. When making the gel check if the right urea aliquots, the right spacers, and the right comb were used.
3. **Inconsistent mobilities within a gel** are most likely caused by incomplete denaturation of single samples. There is no strong effect if the denaturation temperature is up to 10°C lower than 95°C, but the mobility changes if the sample was mistakenly not denatured at all, or if the sample was allowed to sit more than 30 minutes after the denaturation. Samples will not be properly denatured if the amplified product was not pipetted into the formamide, but streaked along the tube wall.

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

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

Initials: *RU*

Date: *8/24/98*

5. **Fuzzy bands and hazy gels** can be caused by improper alignment of the gel plates with the laser plane. Reasons can be buffer crusts on the amber alignment pins (see section A step 6), a mistakenly un-clamped lower gel cassette holding clamp, or a worn out gel cassette holding clamp. Always apply pressure on the gel cassette when releasing the outer clamps in order to avoid a loosening of the clamps (see section G step 5).
6. **Horizontal streaks** on a gel are a sign of the laser being on it's way out. It must be replaced as soon as possible.
7. **Vertical streaks** are less disruptive since they do not cause artificial peaks in an electropherogram. Vertical streaks are caused by scratches or lint on the glass plates in the laser read region.
8. "Red rain" is a phenomenon where **red vertical streaks** appear late into the run. These red streaks are caused by bubbles between the glass plate and the gel matrix. The problem occurs when a gel dried out after sitting without being wrapped. Another reason is the appearance of hydrophobic patches on the glass surface. This can be solved by repeating the treatment of the plates with sodium hydroxide (QC033).

Initials: *RC*

Date: *8/24/98*

STR Gel Analysis of Gels Run on ABI 377

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

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

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

A Gel File Copying and Archiving Procedure

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

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

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

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

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

Initials: *RC*

Date: 8/24/98

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

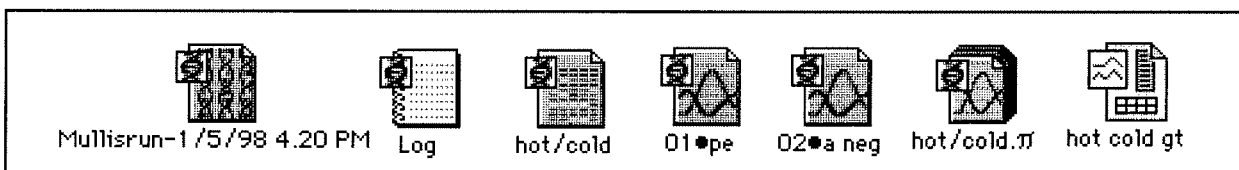
After the copy process is complete, delete the raw data folder from the JAZ transport disk. Eject the JAZ disk by dragging it onto the trash can icon.

4. After the gel has been analyzed and run through the Genotyper the run folder should contain the following items:
 - a run file (65K ABI Prism 377 Collection document)
 - the run log (65K ABI Prism 377 Collection document)
 - the gel file (ca. 10 MB Genescan document)
 - sample files for all samples (33K Genescan documents)
 - a project file (65K Genescan document)
 - the genotyper file (ca. 400K Genotyper document)

See picture below for appearance of file icons.

It also should have been renamed (see Genotyper instructions), according to the following format:

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



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

B Collection Gel Processing

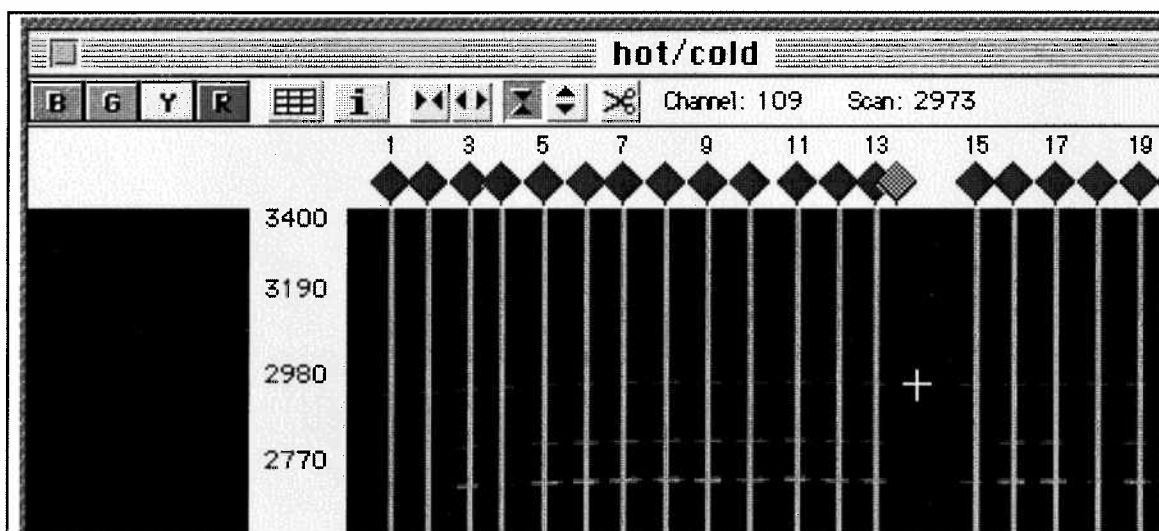
1. Open the **GS Analysis** software by clicking on the **373 / 377 analysis** icon.
2. Under **File** go to **Open**. From the listed possibilities click on existing **Collection gel**. Open windows until the desired gel file (in the raw data folder) is displayed and highlighted. Gels are represented by vertical icons as opposed to folders. Click open.

Initials: *RC*


Date: 8/24/88

WINDOW OUTLINE

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



Other functions displayed on top of the gel image are:

- grid icon for opening the sample sheet
- **i** icon to read run information
- different < > icons for the zoom options
-  icon to activate a lane tracking tool

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

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

Initials: *RC*

Date: *8/24/88*

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

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

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

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

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

Initials: *RCI*

Date: *8/24/98*

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

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

top of gel	—	250bp
	—	200bp
	—	160bp
	—	150bp
	—	139bp
bottom of gel	—	100bp

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

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

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

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

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

Initials: *RL*

Date: 8 / 24 / 98

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

Place a tracking line on one of the samples.

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

9. Under **Gel** select **Track lanes**. Click on **Autotrack lanes**. This will launch the automatic lane tracking. Afterwards the result has to be checked manually for each gel.
10. Click on the **lane indicator diamond** for each used lane. The white line should drawn through the middle of each DNA fragment. All 6 red standard fragments have to be visible in the left peak display. Make sure that the lane assignments correspond to the gel sheet.

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

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

11. After the tracking is satisfactory, click on the next lane. The changes for the previous lane will be saved automatically.
12. After the tracking for all lanes is satisfactory, the changes for the whole gel should be saved. Under **File** select **save**.

Initials: *RCJ*

Date: *8/24/98*

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

Under **Gel** select **Extract lanes**.

From the **extract lanes window** choose **extract from all used lanes!!**

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

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

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

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

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

C Troubleshooting Collection Gel Processing

1. If the automatic lane tracking results in completely fragmented tracking lanes, it might be easier to manually adapt the straight tracking lanes. Under **Gel** go to **Track lanes**, select **Revert to straight tracking**. Then move the tracking lanes appropriately.
2. If too many tracking lines (more lanes than samples loaded) are present, there must be a mistake on the sample sheet, e.g. an empty lane is marked as used. Click on the **sample sheet icon**, correct the sheet, close, and then click **save**.

Initials: *24*

Date: *8/24/98*

The default settings are shown in the following figure:

Analysis Parameters

Analysis Range

☐ Full Range

☒ This Range (Data Points)

Start:

Stop:

Size Call Range

☒ All Sizes

☐ This Range (Base Pairs)

Min:

Max:

Data Processing

☒ Baseline

☒ MultiComponent

Smooth Options

☐ None

☒ Light

☐ Heavy

Peak Detection

Peak Amplitude Thresholds

B: Y:

G: R:

Min. Peak Half Width: Pts

Size Calling Method

☐ 2nd Order Least Squares

☐ 3rd Order Least Squares

☐ Cubic Spline Interpolation

☒ Local Southern Method

☐ Global Southern Method

Split Peak Correction

☒ None

☐ GENESCAN 2500

☐ LeftMost Peak

☐ RightMost Peak

Correction Limit: Data Pts

Cancel OK

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

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

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

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

Initials: *RU*

Date: *8/28/98*

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

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

D. Project File Analysis

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

WINDOW OUTLINE

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

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

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

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

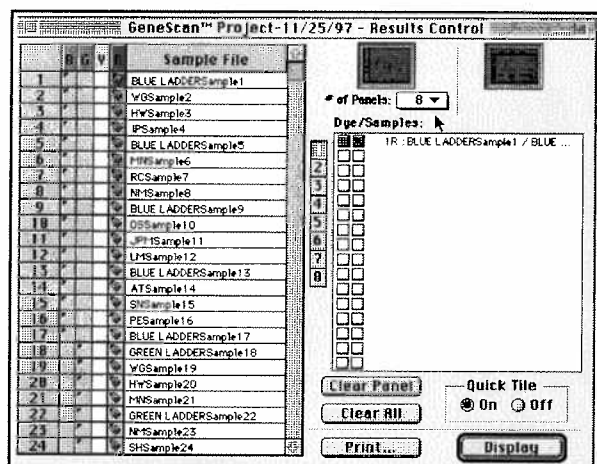
2. Click on the arrow of the top size standard box so that all lanes are highlighted, hold the mouse button down, and select **GS500 ROX**.
3. Under **Settings** choose **Analysis Parameters**. It is important that the **Analysis range** is consistent with the relevant scan range (see gel sheet) and contains all desired standard peaks. The settings for the remaining options are predefined and should not be changed.

Initials: *RCI*

Date: *8/24/98*

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

Click **display**.

Initials: *RC*

Date: *8/24/98*

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

Required Sizes for the Peaks are:

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

8. Repeat step 6 for the remaining size standards.
9. If an assignment is wrong the size standard must be redefined for this lane only. The lane must be reanalyzed with the newly defined standard.

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

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

Reanalyze the corrected lane.

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

E. Troubleshooting Project File Analysis

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

Initials: *RL*

Date: *8/24/98*

2. The assignment of the wrong sample information to a specific lane must be corrected on the sample sheet. Do not exit the project. Under **File**, go to **open** and select **Collection gel**. The collection gel belonging to the open project will highlight automatically. Correct the sample sheet as described in step 4 of section B. Close the sample sheet and re-extract all used lanes. Make sure that "overwrite original sample files" is checked and that the message "add to open project" is displayed. After re-extracting the lanes, all samples must be reanalyzed.
3. If an empty lane is extracted mistakenly, the sample file for the empty lane can be deleted by highlighting it. Click on **Project** and select **remove sample files**. Click **Remove** when asked "-- sample files are selected, are you sure you want to remove them."

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

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

Initials: *RC*

Date: *8/24/98*

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

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

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

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

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

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

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

Click **import**.

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

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

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

Initials: *RCI*

Date: *8/24/98*

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

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

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

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

- 1 small constant bands of the following size and color
Blue 180-181 bp, 230-232 bp, and 246-248 bp
Green 127-131 bp and 211-228 bp
- 2 pull-ups of green peaks caused by very high blue peaks in the same lane
- 3 shoulder peaks approx. 1-3 bp bigger than main allele
- 4 peaks caused by overflow of sample in the adjacent lane
- 5 -4 and +4 bp stutter if there is no indication for the presence of a mixture.
(See interpretation section)
- 6 other.

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

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

Initials: *RL*

Date: 8/24/98

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

This macro automatically assigns allele names to the labeled peaks based on the size categories for the different fragments and writes a results table. The following steps are carried out: clear table, change labels to the categories name, add rows with one sample per row, containing sample info in column 1, up to 4 labels in columns 3-6, the text "check" in column 2 if number of labels >2 (titled "mixture."); put column titles in first row.

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

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

8. Under **View**, select **Show Table**. Under **File**, select **Print**. Print Table. Close **Table Window**.
9. Under **View**, select **Show Plot Window**. Under **Analysis**, select **Change Labels**. In the Change Labels Dialog box check both "with size in bp" and "the categories name." Click **OK**.
10. The next step is to print the plot window. To achieve an uniform format, the base pair range, which should be printed, must be selected. Under **View**, select **Zoom to**. Enter 110 to 260. Click **OK**. The display on the screen will focus on that range.

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

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

Initials: *RC*

Date: *8/24/98*

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

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

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

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

Troubleshooting QUAD Genotyper

1. The sample information can be corrected for the Genotyper file by opening the dye lane list, highlighting the lane, and retyping the sample information. The sample name cannot be changed here. It can only be changed on the sample sheet level (section B, step 4).
2. If an incorrect peak has been removed accidentally, it can be re-labeled by placing the cursor above the baseline and clicking on it again. Re-labeling always labels the peak with the size in base pairs. To re-label with the category name, run Macro Apple 2 again.
3. If the table is inconsistent with the information displayed in the plot window, the most likely reason is that something was changed (corrected sample info, removed extra peak) after the Apple 2 Macro had been run. To rewrite the table, simply run the **Apple 2 Macro** again. This has to be repeated for all instances where anything was changed.
4. If a lane has been placed out of order at the top of the plot window, it probably has been marked accidentally with a black bullet after double clicking on the dye lane. This feature is meant for placing lanes next to each other for comparison purposes. Remove black bullets by double clicking on it again or, under **Edit**, selecting **unmark**.

Initials: *RCJ*

Date: *8/24/88*

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

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

6. **Unlabeled peaks** that are clearly higher than 50 fluorescent units, can be caused by several reasons:
- A) The peaks have not been sized. This occurs especially for the FES locus when the 250bp size fragment was not present on the gel or has not been included in the analysis area (see Troubleshooting sections C and E). Quit Genotyper and correct any errors made during **Collection gel processing** or **Project file analysis**. Rerun the sample if the 250bp fragment data are not in the collection data.
 - B) The peak is outside of the allele calling window because it is a new allele (see step 5, section F).
 - C) The peak is outside of the allele calling window because of an aberrant electrophoretic mobility. Rerun the sample.
7. The Genotyper printout should have a standard format: green lanes, then blue lanes. The table should have 4 rows for each locus. The order of the loci in the table should be VWA, F13A1, THO1 and FES. If this is not the case the defaults have been changed.

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

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

Initials: *ACS*

Date: *8/24/98*

Y chromosomal STR Analysis

Amplification of the Y STR Multiplex System 1 (DYS19, DYS390, DYS 389I and II)

A positive male control, a negative female control, an amplification negative control, and if applicable, an extraction reagent control and substrate controls must be included with each batch of samples being amplified to demonstrate procedural integrity. The male positive control is a control whose alleles are known to the analyst. The female control should have no detectable alleles. The STR PCR reaction mix already contains BSA. **Do not add additional BSA.**

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 III). 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 IV.

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

Quantiblot DNA Concentration* (ng/20 μ L)	Target Volume (μ L) to be amplified	TE ⁻⁴ (μ L) for Target Volume	Range of Volumes (μ L) 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**
2.5	16.0	10.0	1.2 - 26**
1.25	26.0	0	2.4 - 26**
0.62	26.0	0	4.8 - 26**
0.31	26.0	0	9.6 - 26**
0.15 and < 0.15	Do not amplify	-	-

* If the Quantiblot result is ≥ 10 ng/20 μ L, dilute the sample as described on page 64.

** Add TE⁻⁴ to a final volume of 26 μ L.

Initials: RCS

Date: 8/24/98

Table IV - 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 the Y STR multiplex 1.

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 (μ L) to be amplified	TE ⁻⁴ (μ L) for Target Volume	Range of Volumes (μ L) 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 μ L.

Initials: *LC*

Date: *8/24/98*

1. For each sample to be amplified, label a new tube.
2. To each labeled tube, add TE⁻⁴ and DNA as specified in Tables III and IV.

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

2 ng of the male positive control and 2 ng of the female positive control are already aliquoted, stored in the freezer, and do not have to be prepared at this step.

3. For the extraction reagent control use 20 μ L of the extract and add 6 μ L of TE⁻⁴.
4. Fill out the amplification worksheet and record the appropriate lot numbers.
5. Determine the number of samples to be amplified, including controls and label a PCR reaction mix tube for each sample.
6. 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.**
7. Pipet 4 μ L of 25mM MgCl₂ into each reaction mix tube with a sterile pipet tip. Pipet carefully in the solution at the bottom of the tube. Use a fresh pipette tip for each tube.
8. Carefully add 1 drop of sterile Mineral Oil to all reaction mixture tubes including the controls. **Do not touch tube.**
9. Close all of the tubes.
10. **Note: Use a new sterile filter pipet tip for each sample addition. Open only one tube at a time for sample addition.** The final aqueous volume in the PCR reaction mix tubes will be 50 μ L. Add the content (26 μ L) of the sample DNA tubes which were prepared above to each labeled tube by inserting the pipet tip through the mineral oil layer. After the addition of the DNA, cap each sample before proceeding to the next tube. **Do not vortex or mix.**

Initials:

Date:

11. Turn on the Perkin Elmer 480 Thermal Cycler. (See manufacturer's instructions).

Step Cycle File #24 on all 480 machines should include the following:

Taq Gold activation soak at 95°C for 11 minutes Link to file #23

23 Denature at 94°C for 1 minute
 Anneal at 56°C for 2 minutes
 Extend at 72°C for 2 minutes

Repeat the above for 30 cycles

Link to Time Delay File #22 for an additional 30 minutes incubation at 60°C.

Link to Soak File #20 for the 10°C storage soak.

If Files #24, 23, 22, or 20 are not correct, bring this to the attention of the QC/QA coordinator and a supervisor.

12. For each well of the Thermal Cycler heat block which will be used to amplify samples, add one drop of mineral oil to the well. Place the PCR Reaction Mix tubes into the Thermal Cycler. Push the tubes down completely into the heat block. Record the heat block position of each tube.
13. Start the Thermal Cycler amplification program (File #24). Verify the cycling parameters by monitoring the values for the first cycle after the initial denaturation. The tubes should be checked after the first cycle and pressed further into the heat block so that they fit tightly.
13. Return the microtube rack used to set-up the samples for PCR to the PCR Set-Up Area.
14. 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: *AC*

Date: *8/24/98*

Electrophoresis of Y Chromosomal STR Multiplex 1 (Y M1) on the ABI 377

Follow the procedure described under Gel Electrophoresis on ABI 377 Sequencer (page 56). There are no deviations in the electrophoresis settings or the sample preparation. The only difference is that when naming the gels, the gel name should have the prefix Y and be numbered independently from the Quad gels.

Do not run Y M1 gels on the 373 sequencer.

Y Chromosomal 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 as described for the QUAD STR system (page 66). 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 the Y-STR Genotyper templates. As with the QUAD gels, please adhere strictly to the following copying and archiving rules.

A. Y STR Gel File Copying and Archiving Procedure

1. Before copying or analyzing a gel, identify the **Run folder** containing the gel file based on the date and time noted on the gel sheet. Rename this run folder with the gel name according to the following format:
Y / year / instrument / gel # (e.g. Y 98 -Men001)
2. Put a designated Y STR gel archive JAZ 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!!!

3. Insert the transport disk in the JAZ disk drive at terminal #1 or #2. Analyze the gel on the disk.

Initials: *RC*

Date: *8/24/98*

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)
5. Leave the analyzed gel on the JAZ archive disk. Go back to the instrument and delete the run folder.

B. Collection Gel Processing

1. Open the **GS Analysis** software by clicking on the **373 / 377 analysis** icon.
2. Under **File** go to **Open**. From the listed possibilities click on existing **Collection gel**. Open windows until the desired gel file on the JAZ disk is displayed and highlighted. Gels are represented by vertical icons as opposed to folders. Click open.

See the QUAD STR gel analysis part (page 68) for a description of the gel window.

NOTE: Y STR multiplex 1 is labeled in blue (FAM) and yellow (NED).

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.

Initials: *rc*

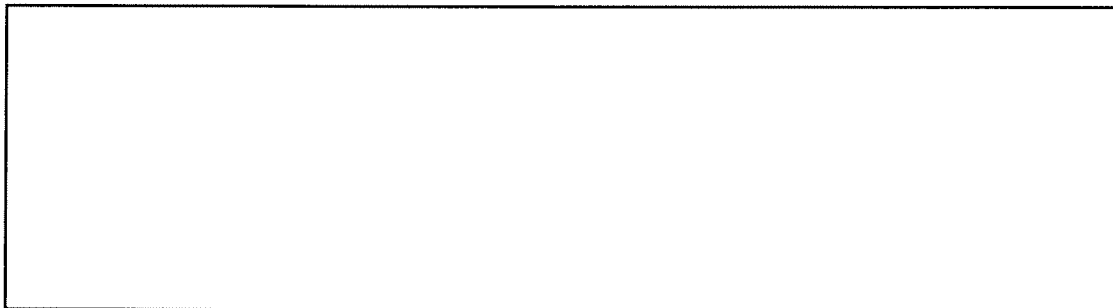
Date: *8/24/98*

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

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

5. After entering all relevant information, close the sample sheet window. Choose **Save**. The gel image will display tracking lines for all used lanes.
6. Next choose the appropriate scan area to analyse by looking at the red size standards:

Each lane must contain 10 red standard fragments from 139 - 450 bp. They are distributed as follows.



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

If the gel image contains less than these 10 fragments the gel image must be regenerated - go to step 7. If all fragments are present and the gel picture is as described in step 8, proceed with step 9.

Initials: *ACS*

Date: *8/24/98*

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

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

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

8. The gel picture displayed on the screen should have a black background and distinct blue, yellow, and red signals. If the background is colored 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**.

Initials: *RU*

Date: *8/24/98*

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

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

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

11. After the tracking is satisfactory, click on the next lane. The changes for the previous lane will be saved automatically.
12. After the tracking for all lanes is satisfactory, the changes for the whole gel should be saved. Under **File** select **save**.

Initials: *RL*

Date: 8/24/98

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

Under **Gel** select **Extract lanes**.

From the **extract lanes window** choose **extract from all used lanes!!**

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

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

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

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

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

- C. See the QUAD STR gel analysis section for **Troubleshooting** (page 72, 177) for this step.

D. Project File Analysis

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

WINDOW OUTLINE

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

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

Initials: *RC*

Date: *8/24/98*

1. Highlight all samples. Under **Samples** choose **Install new matrix**. To choose the correct matrix the fluorescent labels (here NED and 6-FAM) and the instrument on which the gel was run must be known. Open folders until the correct matrix file appears. Click **Open**.

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

2. Click on the arrow of the top size standard box so that all lanes are highlighted, hold the mouse button down, and select **Y GS500 ROX**.
3. Under **Settings** choose **Analysis Parameters**.

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

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

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

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

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

Initials: *pc*

Date: *8/24/98*

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

The results control window 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. See the QUAD STR section for a picture of the results control window.

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

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

Click **display**.

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

Required Sizes for the Peaks are:

peak 1	139
peak 2	150
peak 3	160
peak 4	200
peak 5	250
peak 6	300
peak 7	340
peak 8	350
peak 9	400
peak 10	450

8. Repeat step 6 for the remaining size standards.

Initials: *RC*

Date: *8/24/98*

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

Under **Window** select **Analysis control**. Click on the arrow in the size standard column for the lane to be reanalyzed. Choose **define new**. The system displays an electropherogram of the size standard fragments in this lane. Highlight the first peak by clicking on it. Enter the number, 139, and press return. Continue to enter the above values for the size standard peaks.

After entering the number 450, click **return**. The highlight should be back on peak 1. Click **Save**, to "save changes to "untitled," before closing, click **Save**, to "save this document as "untitled," click **replace**.

Reanalyze the corrected lane.

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

E. Troubleshooting Project File Analysis

1. If it is not possible to open a project because no project file can be highlighted in the desired run folder, it means that the lanes have not been extracted. Start with section B, 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 B. Close the sample sheet and re-extract all used lanes. Make sure that "overwrite original sample files" is checked and that the message "add to open project" is displayed. After re-extracting the lanes, all samples must be reanalyzed.

Initials: *RU*

Date: *8/24/98*

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

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

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

Y M1 STR Genotyper 2.0 Analysis of all ABI 377 Gels

After the gel analysis is completed, the file must be processed further using the Genotyper software. This program will assign allele names to the detected peaks and create the electropherogram output that goes into the case file. The following steps must be performed exactly as listed. **Do not change the order!**

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 **Y M1 STR 373 or 377 Genotyper** icon.

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

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

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

Initials: *RC*

Date: *8/24/98*

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, DO NOT combine samples from different gels into a single Genotyper file.

3. After importing the results, change the name of the Genotyper template to your initials and the gel file name. Under **File** select **Save As**. Enter your initials and the name of the gel you are processing. Click **Save**.
4. 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.

Initials: *RC*

Date: *8/28/98*

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

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

- 1 pull-ups of yellow peaks caused by very high blue peaks in the same lane
- 2 shoulder peaks approx. 1-3 bp bigger than the main allele
- 3 -4 and +4 bp stutter if there is no indication for the presence of a mixture.
(See interpretation section)
- 4 other.

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

If a locus displays a distinct peak which is not labeled, this might occur because its size is outside of the defined allele range, due to a mistake in the red standard, or to a new, previously unreported allele. This possibility must be considered especially if the other loci show a proper profile. The presence of a possible new rare allele must be pointed out to a DNA supervisor for confirmation. Click on the unlabeled peak in order to label it with the size in bp, which is necessary for allele identification.

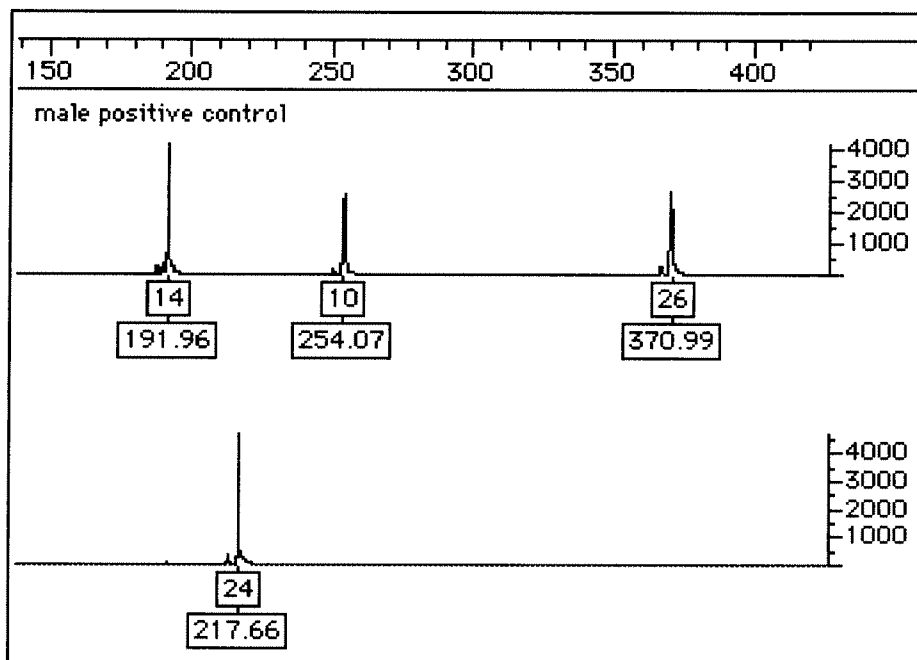
6. Run the **Allele Designation Macro** by pressing the Apple key and the number 2, or by highlighting the macro name in the lower left hand corner and, under **Macro**, selecting **Run Macro**.

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 column 1, 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.

7. Check the positive control type. It is
- DYS19 - 14
 - DYS390 - 24
 - DYS389 I - 10
 - DYS389 II - 26

Initials: *RD*

Date: *8/28/98*



8. 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.

9. Under **View**, select **Show Table**. Under **File**, select **Print**. Print Table. Close **Table Window**.
10. 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**.
11. 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**.

Initials: *RU*

Date: *8/24/98*

12. 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**.
13. As already listed in section A the run folder should now contain the following items:
 - a run file (65K ABI Prism 377 Collection document)
 - the run log (65K ABI Prism 377 Collection document)
 - the gel file (ca. 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 98-Men001 Files)

Refer to 377 STR Gel Analysis and Allele identification section A (page 66) for archiving.

Genotyper troubleshooting see QUAD Genotyper section (page 81). **For the YM1 all references to Green have to be applied to yellow.**

Initials: *RC*

Date: *8/24/98*

AmpFISTR Green and Blue STR's

Amplification of AmpFISTR Green and Blue STR

A positive control, an amplification negative control, an extraction reagent negative control and substrate controls, if applicable, must 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.

1. For each sample to be amplified, label a new tube. Do not amplify samples in which no DNA was detected by QuantiBlot.
2. To each labeled tube, add TE⁻⁴ and DNA as specified in Table V.

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

3. Add the following amounts of TE⁻⁴ and DNA to new labeled tubes:

Sample	TE ⁻⁴	DNA
amplification positive control-external	---	20 µL
extraction reagent control	---	20 µL
amplification negative control	20 µL	---

Initials: *RC*

Date: *8/24/98*

Table V- Amount of DNA to be Amplified for the AmpFISTR Blue and AmpFISTR Green Kits

DNA Concentration* (ng/20 μ L)	Target Volume (μ L) to be amplified	TE ⁻⁴ (μ L) for Target Volume	Range of Volumes (μ L) which can be amplified
25	Dilute 1:10 and use the dilution for amplification		
12.5	1.6	18.4	0.3-8**
6.2	3.2	16.8	0.5-16**
5	4	16	0.6-20**
2.5	8	12	1.2-20**
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	-	-

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

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

** Add TE⁻⁴ to a final volume of 20 μ L.

Initials: Ad

Date: 8/24/98

4. Determine the number of samples to be amplified, including controls and label a PCR reaction mix tube for each sample.
5. Fill out the amplification worksheet and record the appropriate lot numbers.
6. 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.
7. Pipet 10 μ L Blue or Green Primer Set into each reaction mix tube with a sterile pipet tip. Pipet carefully at a slight angle to avoid splashing. Use either a filter tip or a combi tip on the pipettor.
8. Carefully add 2 drops of the Mineral Oil from the dropper bottle provided in the kit to all tubes including the controls. **Do not touch tube with the tip of the oil dropper bottle.**
9. Close all of the tubes.
10. **Note: For sample addition, use a new sterile filter pipet tip for each sample and open only one tube at a time.** For both the AmpFISTR Blue and Green kits, the final volume is 50 μ L. Add the contents (20 μ L) of the sample DNAs and control tubes which were prepared above to each labeled tube by inserting the pipet tip through the mineral oil layer. After the addition of the DNA, cap each sample before proceeding to the next tube. **Do not vortex or mix.**
11. Carry samples and amplification worksheet to the thermocycler area.

Initials: *RC*

Date: *8/24/98*

12. Turn on the Perkin Elmer 480 Thermal Cycler. (See manufacturer's instructions).

For the **Blue STR system step File #30** on all 480 machines should include the following:

Soak at 95°C for 11 minutes Link to file #31

#31: Denature at 94°C for 1 minute
 Anneal at 59°C for 1 minute
 Extend at 72°C for 1 minute

Repeat the above file #31 for 28 cycles

Link to Time Delay File #32 for an additional 30 minutes incubation at 60°C.

Link to Soak File #21 for a 25°C soak.

For the **Green STR system step File #34** on all 480 machines should include the following:

Soak at 95°C for 11 minutes Link to file #33

#33: Denature at 94°C for 1 minute
 Anneal at 59°C for 1 minute
 Extend at 72°C for 1 minute

Repeat the above for file #33 for 29 cycles

Link to Time Delay File #32 for an additional 30 minutes incubation at 60°C.

Link to Soak File #21 for a 25°C soak.

13. For each well of the 480 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 480 Thermal Cycler. Push the tubes down completely into the heat block. Record the heat block position of each tube.
14. Start the 480 Thermal Cycler amplification program (File #30 for Blue or File #34 for Green). Verify the cycling parameters by monitoring the first cycle. The tubes should be checked after the first cycle and pressed further into the heat block so that they fit tightly.
15. Return the microtube rack used to set-up the samples for PCR to the PCR Set-Up Area.

Initials: *PC*

Date: 8/24/98

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

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).

BEFORE STARTING CHECK THE CAPILLARY COUNT!

Double click on the PRISM 310 Collection icon on the desktop. Under the INSTRUMENT menu select **changing the capillary**. 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 ($<200\mu\text{L}$) you will have to refill it. Start with section **C** of instrument preparation.
- 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.

Initials: *ACS*

Date: 8/24/98

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: *RCJ*

Date: *8/24/98*

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 50 μ m 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 50 μ m 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.

Initials: *AC*

Date: *8/24/98*

14. Insert the window end of the capillary through the center of the plastic screw and through the cone-shaped ferrule.
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.
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.

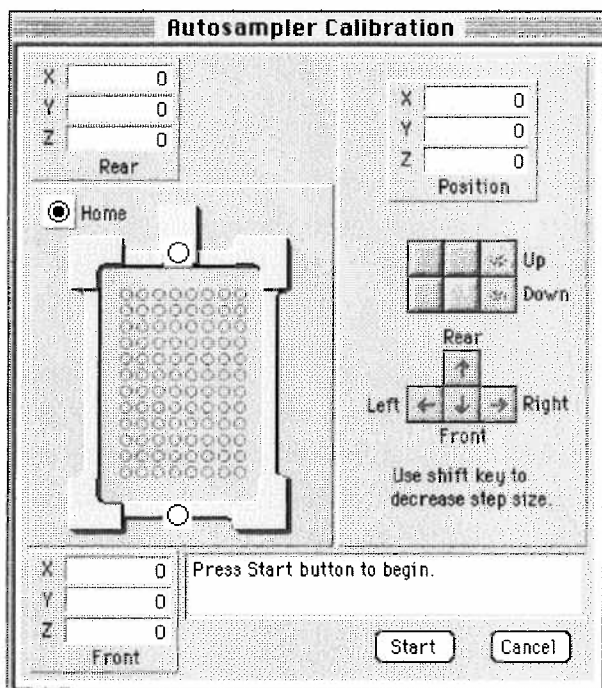
Initials: *RA*

Date: *8/24/98*

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: 24

Date: 8/24/98

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 1 μ L 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 not 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 WATER THROUGH IT SEVERAL TIMES. CHECK THAT THERE IS NO OLD POP4 RESIDUE.

2. Prime the syringe with a low amount of POP4 in order to remove remaining water. Discard the POP4 used for priming. 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. Manually draw 600 μ l (for three full trays of samples) of POP4 into a 1mL Hamilton 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 1 μ L of polymer, plus extra to fill the pump block and eradicate any air bubbles which may be present).

Initials: *ACI*

Date: *5/24/98*

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.

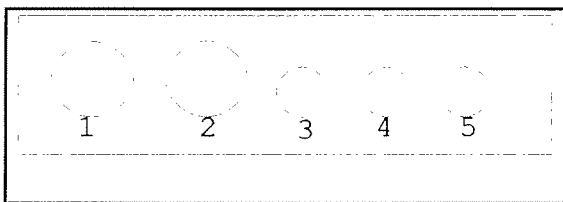
D. Changing the Buffer and Baseline Check

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

Initials: *Ad*

Date: *8/24/98*

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



4. 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.

5. Under **File** go to **Open**, and open the Injection list stored in the current run folder named **Injection list - CCD 4 color test**.
6. 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.
7. Close the scan window. Cancel the run, and click **terminate**. Close the injection list window, click **Don't save**.

Initials: *AC*

Date: *8/24/98*

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:
 - 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 Blue and Green 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.
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.

Initials: *AC*

Date: *8/24/98*

4. A Genescan sample sheet appears.

	Sample Name	Color	Std	Pres	Sample Info
A1		B		<input type="checkbox"/>	
		G		<input type="checkbox"/>	
		Y		<input type="checkbox"/>	
			◆	<input checked="" type="checkbox"/>	
A3		B		<input type="checkbox"/>	

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).

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. 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** and **paste** functions can 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.

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

CE1/98-001
CE2/98-001 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.

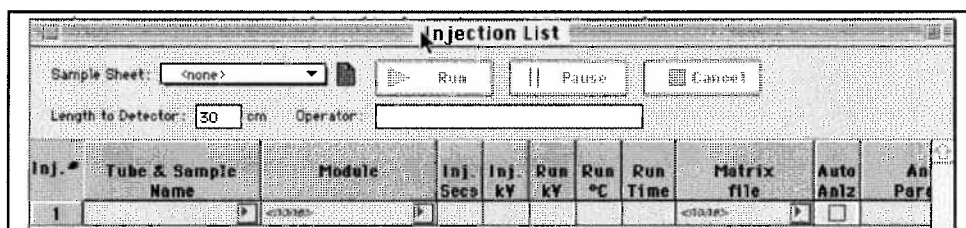
Initials: *RC*

Date: *8/24/98*

F. Preparing a Genescan Injection List

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.



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, A" 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. Do not touch these settings. The matrix standard must be NED POP4. 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.

Initials: *RCJ*

Date: *8/24/98*

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: *AL*

Date: *8/24/98*

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 24.5 μ L deionized formamide with 0.5 μ L CXR standard per sample.

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

Mix thoroughly by inversion.

3. Aliquot 25 μ L of the formamide/standard mixture into each labeled tube. Have someone witness tube set-up and sign the run sheet. Add 1 μ L 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.
6. When the instrument has reached the 60°C run temperature (the temperature is displayed in the Status window), 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.

Initials: *RU*

Date: *8/28/98*

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

To check the progress of the run, open the **Status** window under the **Windows** menu. 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.

Initials: *RL*

Date: 8/24/98

STR Fragment Length Analysis for AmpFISTR Blue and Green

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.

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.

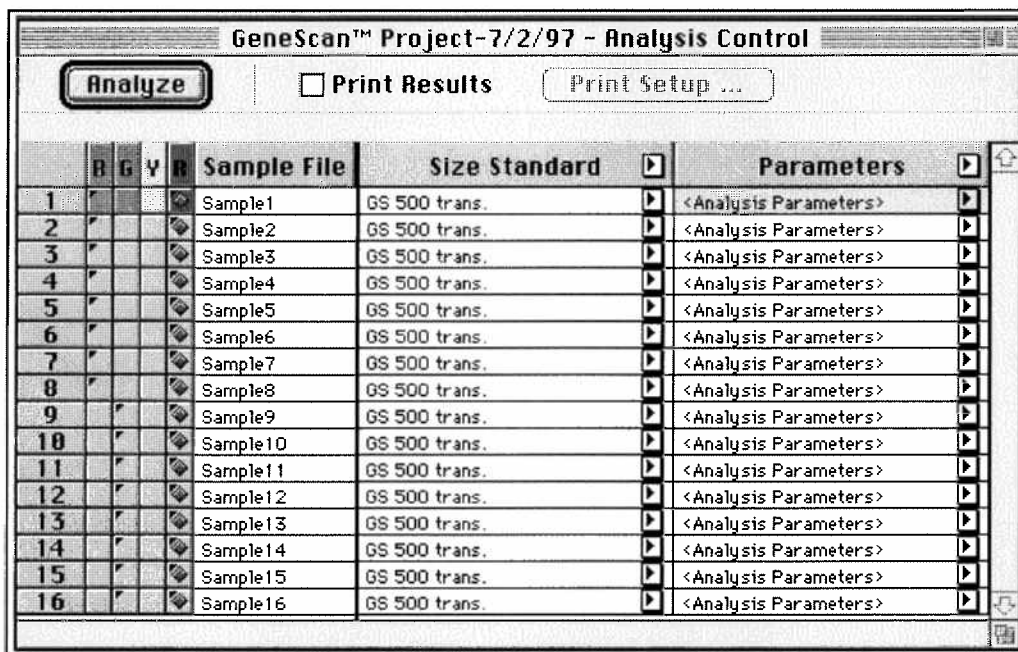
Under the **windows** menu at the top of the screen, select **Analysis Log**. In the Analysis Log window there should be messages indicating that the standard peaks have been sized from 80-400 bp. for each sample. This tells you that each peak has been sized. If this is not the case or you have some warning message (e.g. "size standard skipped peak defined as 220bp") 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**.

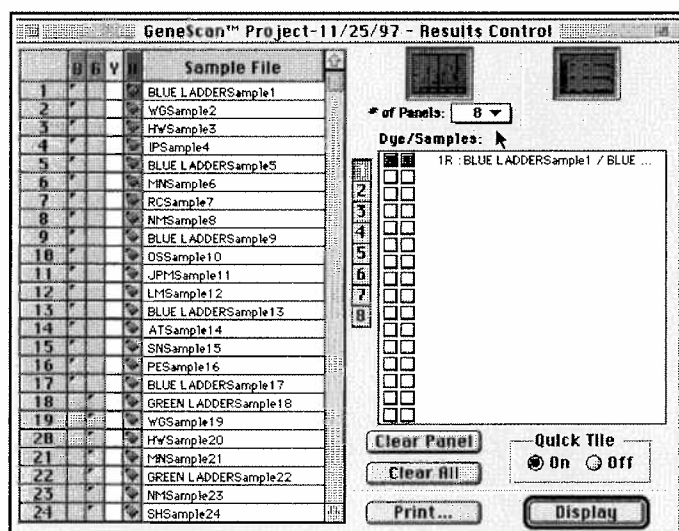
To ensure that all the sizing results are correct, check the labeling of the size standard peaks for each sample.

Initials: *Rel*

Date: *8/24/98*



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

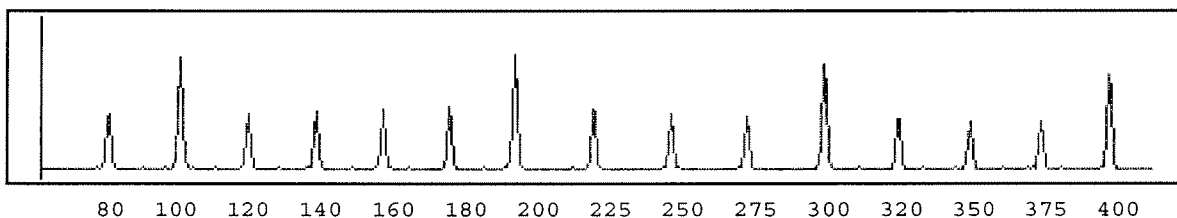


Initials: *RC*

Date: *8/24/98*

2. You will be given a choice of how many panels of data you would like to display (8 maximum at a time). In the **# of Panels** pop-up menu choose the number you'd like. The electropherogram and table icons in the upper right-hand corner have to be dark. Quick Tile should be **on**.
3. To check the size standard select 8 panels. Click on panel 1 then click on the red box corresponding to the sample you want to view, click on panel 2 and the next red box corresponding to the next sample and so on until all 8 panels are filled. 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.
4. 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.

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



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

Initials: *RC*

Date: *8/24/98*

5. 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.

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.

6. 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.

7. 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.
8. 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.

Capillary Electrophoresis Troubleshooting

See below for a Capillary Instrument Electrophoresis and Analysis Troubleshooting Guide

Initials: *RU*

Date: *8/28/98*

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
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 airbubble in the pump block.	Flush the pump block of any air bubbles.
	There may be an airbubble 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: *RC*

Date: 8/24/98

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
	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, distilled, deionized water.

Initials: *KU*

Date: *8/24/98*

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
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, distilled, 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.

Initials: *RC*

Date: *8/24/98*

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
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.	Quantitate DNA and add 1-10 ng DNA; repeat test.
	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: *RC*

Date: *8/24/98*

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
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 1 μ L of product and 0.5 μ L 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: *pcj*

Date: *8/24/98*

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
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 matrix file used.	Select the correct matrix file.
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.
	Wrong preparation of formamide/size-standard/PCR product, e.g. insufficient mixing.	Prepare samples again and rerun samples.

Initials: *RC*

Date: *8/28/98*

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
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. Extract larger area of the stain to achieve higher DNA yield; repeat test. Repeat sample electrophoresis 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 enzyme, dilute extract, use BSA, purify using Centricon (see page 63 of the manual for detailed procedures and more options).
	Test sample DNA is degraded.	Reamplify with an increased amount of DNA.
	Sample prepared improperly for capillary injection.	Prepare fresh sample and rerun.
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.

Initials: *RCJ*

Date: *8/24/98*

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
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.
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.
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.
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.

Initials: *RCJ*

Date: *8/24/98*

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
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 1 μ L of product to formamide in each tube. Rerun.
	Test sample DNA is degraded.	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 enzyme, dilute extract, use BSA, purify using Centricon.
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).	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.
	Presence of a DNA mixture, DNA degradation (see Interpretation section).	(see Interpretation section)

Initials: *PCJ*

Date: 8/24/98

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
17. Poor resolution	Poor capillary performance.	Replace capillary.
	Incorrectly prepared and/or old buffer or polymer solutions.	Replace buffer and polymer with fresh solutions.
	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.
	Oil in sample.	Carefully pipette PCR product without oil carryover. Or, pipette 5µL from amplification tube onto a piece of Parafilm. Roll the sample around until the oil (hydrophobic) is completely separated from PCR product (hydrophilic) and pipette 1µL of PCR product into the sample tube.
18. Peaks which progressively decrease in height and definition (resembling a "sine wave") to no visible peaks at all.	Poor quality water.	Use freshly autoclaved, distilled, deionized water.
	"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.

Initials: *RCJ*

Date: *8/24/98*

AmpFISTR Blue and AmpFISTR Green Genotyper 2.0 Analysis of ABI 310 Capillary Electrophoresis

After the run analysis is completed the file must be further processed using the Genotyper software. This program assigns allele names to the detected peaks and creates 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 the troubleshooting section for help.

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 AmpFISTR Blue or Green icon on the desktop.

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

2. Under **file**, select **Import Genescan File(s)**. The analysis result files are in the run folder. Choose the correct run folder (it is named with the date and time of your run) by double clicking on the appropriate folders until the sample file names appear. Go to the project file, and highlight it by clicking once on the project name.

IMPORTANT: Always deselect the option to import the raw data!!! This makes the Genotyper files much too large. Leave all colors checked. We will need the data for the red size standard!

Click **import**

Always import the complete project.

3. Under **View** select **Show Dye/Lanes window**. you will see a list of the samples you have imported from Genescan analysis. For casework gels it is not acceptable to combine samples from different gels in one Genotyper file. When running a full tray with two sets of amplified samples you must remove the samples from one of the amplifications (only genotype the samples which belong to a particular amplification sheet). If you have run both Blue and Green samples in one project and you are genotyping Blue, you will need to cut Green samples. Conversely, if you are genotyping Green, you will need to cut away the Blue samples. This reformatting also applies if you have run two Blue amplifications. To do this hold down the shift

Initials: *RC*

Date: *8/24/98*

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.

4. After importing the results, change the name of the Genotyper template to your initials and the casework file name. Under **File** select **Save As....** Enter your initials, the name of the run you are processing and a description to indicate Blue or Green and the sample type. Click **Save**.

If two amplifications of the same color and sample type were run together the amplification set must be identified.

e.g. AL CE1/98-001 Blue semen A

5. 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 **Macro**, selecting **Run Macro**.
6. 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 genotypes of the positive control for both systems are as follows:

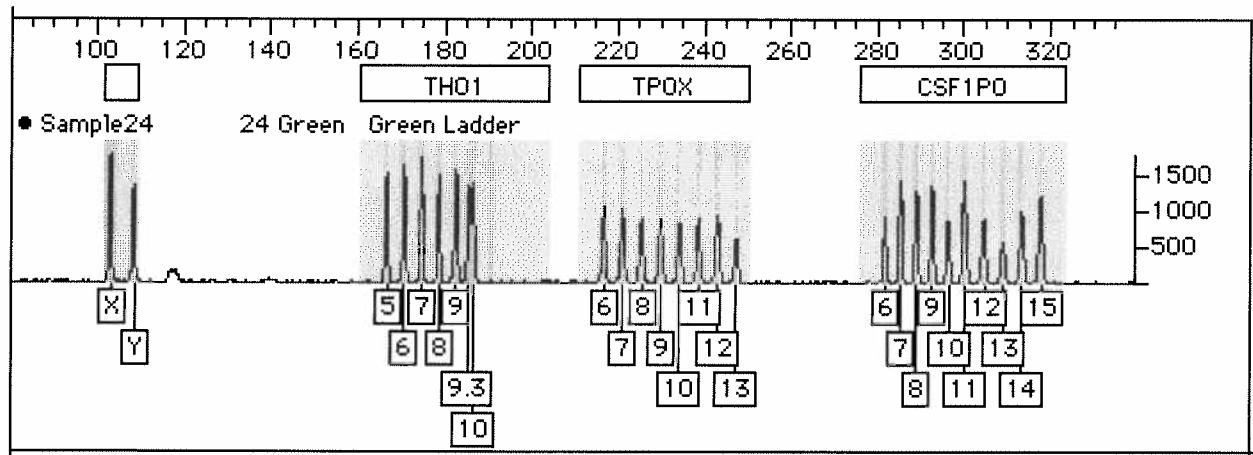
	Amelogenin	THO1	TPOX	CSF1PO
AmpFISTR Green:	X	8, 9.3	8	10, 12
	D3S1358	vWA	FGA	
AmpFISTR Blue:	14, 15	17, 18	23, 24	

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.).

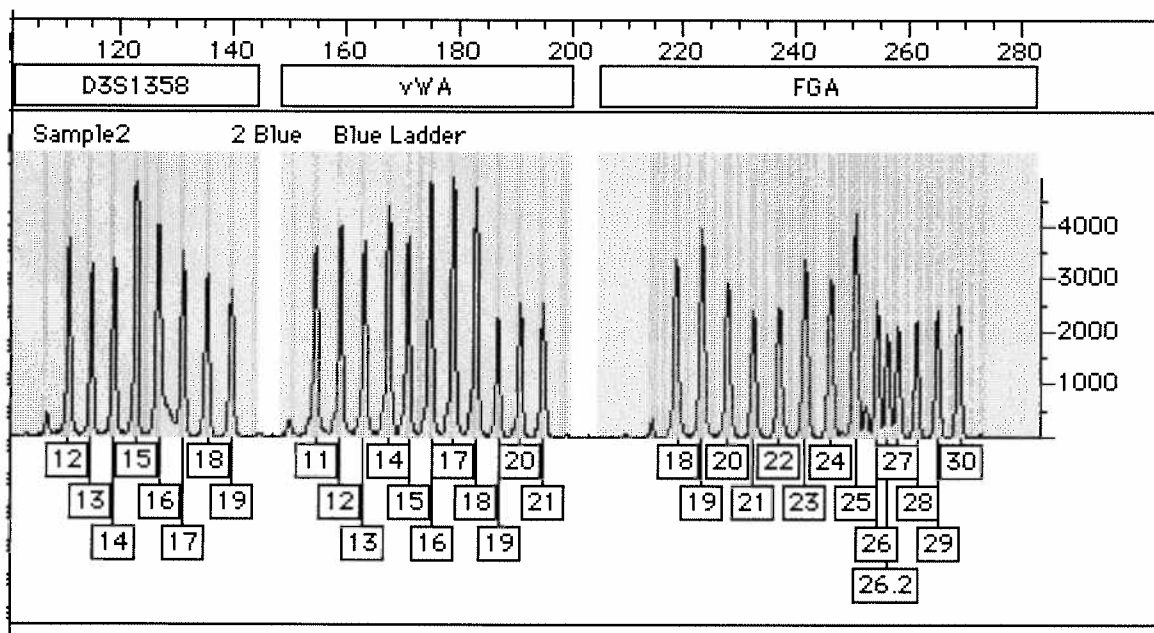
Initials: *RCJ*

Date: *8/24/98*

AmpFISTR Green Allelic Ladder



AmpFISTR Blue Allelic Ladder



August 21, 1998

Initials: *RC*

Date: *8/24/98*

7. If the first allele of the ladder has been assigned incorrectly, 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 first polymorphism contained in the allelic ladder.

- 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.

8. 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:

1. Dye artifact peak which is defined as a Blue or Green "blip", whose shape is not that of a regular peak, occurs at a constant scan position approximately between 3700 and 4000 for Blue and Green respectively. In the Blue system

Initials: *RCJ*

Date: *8/24/98*

this “blip” will be labeled with “OL allele?” (= off ladder allele). 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. In the Green system, the blip normally falls outside the defined category size and will not be labeled.

2. Shoulder or split peaks approximately 1-3 bp smaller or bigger than the main allele.
3. -4 and +4 bp stutter peaks if there is not indication of the presence of a mixture.
4. 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.
5. Other

To compare the red electropherogram with the blue 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 by simply clicking on the blue “B” box in the upper left hand corner.

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

Inconclusive samples:

Over-amplified samples are characterized by a plateau shaped peak and often contain a lot of labeled stutter peaks and artifacts. Instead of laboriously editing out all of these peaks, the sample should be deemed inconclusive and marked for re-running. Remove all labels from the lane in question, don’t list all of the sizes, note “numerous” for peaks removed.

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.

9. After the editing has been finished scroll through the plot window to double check.
10. 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 againBefore printing the table under **file** select **page set-up** and choose 65% and landscape. Print the table by under **file** selecting **print**.
11. 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.
12. Before printing plots, check that the zoom range shows 90-300 for Blue and 90-330 for Green. Change the page set-up back to 100% and letter. Under **file** select **print**. Click **okay** in the print dialogue box.
13. 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.

Initials: *RCJ*

Date: *8/24/98*

Gel File Copying and Archiving Procedure

1. 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:

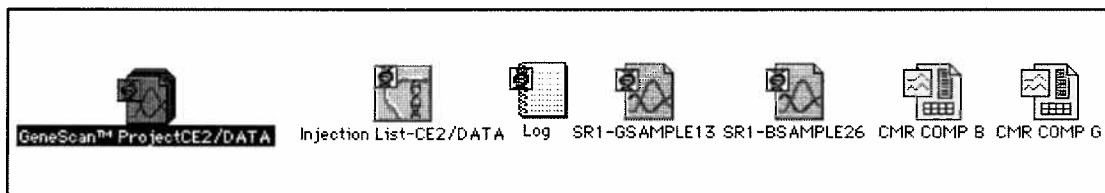
CE1/98-001 files

CE2/98-001 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.



2. 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.

3. After the copy process is complete, delete the run folder from the CE computer harddrive.

Initials: *RC*

Date: 8/24/98

Genotyper Trouble Shooting

- 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 misspelling 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.

- C. The wrong ladder is being typed, (i.e. You are trying to type a Green ladder in the Blue Genotyper or vice versa).

- D. 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 450 or higher. Change this to 200, by clicking on the first category which highlights it. In the dialogue box locate the **Minimum Peak Height** and change it to 200, click **Add**, and then click **Replace** when given the option. You must do this for each locus. Do not use values less than 200.

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.

Initials: *RC*

Date: *8/24/98*

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

E. Off Ladder (OL) allele labels

- I. 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. The remaining samples displaying OL alleles have to be rerun.

- II. 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.

1. Open the **Genscan Analysis** Program on the Desktop. 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.
2. Select the colors you wish analyzed from the dye boxes on the left hand side of the window (make sure you select the standard color (RED) in addition to Blue and/or Green).
3. Click **Analyze**.
4. Proceed from STR fragment length analysis for AmpFI STR Blue and Green.

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

- I. 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

Initials: *pu*

Date: *8/24/98*

with the first allele name, you must raise the peak height in the categories window. See Genotyper Analysis section, step 6.

- II. 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 are randomly placed 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) (AmpFISTR Blue and AmpFISTR Green Genotyper analysis step 2).

Initials: *RC*

Date: 8/24/98

STR Results Interpretation

Autosomal STR Allele Calling Criteria

Results are interpreted by observing the occurrence of electropherogram peaks for the loci that are amplified simultaneously - ([Quad: HumVWA, HumTHO1, HumF13A1, and HumFES]; Green: [Amelogenin, Tho1, TPOX, CSF1PO]; Blue: [D3S1358, vWA, FGA]). 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 Locus	Color	Size Range GS500 Std.
HumVWA	green	127±1.5bp to 172 ±1.5bp
HumTHO1	blue	150±1.5bp to 179 ±1.5bp
HumF13A1	green	181±0.75bp to 247 ±1.5bp
HumFES/FPS	blue	206±1.5bp to 238 ±1.5bp

Blue Locus	Color	Size Range CXR Std.
D3S1358	Blue	108±0.5bp to 137±0.5bp
vWA	Blue	151±0.5bp to 191±0.5bp
FGA	Blue	213±0.5bp to 262±0.5bp

Green Locus	Color	Size Range CXR Std.
Amelogenin	Green	X:100±0.5bp;Y:106±0.5bp
THO1	Green	163±0.5bp to 183±0.5bp
TPOX	Green	21±0.5bp to 240±0.5bp
CSF1PO	Green	277±0.51bp to 313 ±0.5bp

Initials: RU

Date: 8/24/98

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 (fluorescent units) Quad], [>100 Blue/Green];, and have a peak height $>7-10\%$ of the major peak are called alleles by the instrument. For the Quad, due to the systematic occurrence of stutter peaks smaller than 4bp of an allele peak, peaks at this position that are lower than the empirically determined stutter threshold of 20% are also disregarded.

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

- recalculating fluorescence peaks using the instrument specific matrix in order to correct for the overlapping spectra of the four fluorescent dyes.
- calculating the fragment length for the detected peaks using the known in-lane standard fragments.
- For the Quad- labeling of all sized fragments that are >50 (arbitrary units) and $>10\%$ of the highest peak at that locus, and are $>20\%$ of a following 0-5bp bigger peak (see the Genotyper section of this manual).
- For Blue/Green - Comparing and adjusting the categories listed above to the sizing of the coelectrophoresed allelic ladder and calculating the off sets (the difference between the first allele in a category and the first allele in the allelic ladder at each locus).

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

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

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

For the Quad:

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

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

Overflow of sample that has been loaded in the adjacent lane

Initials: *RU*

Date: *8/24/98*

For Blue/Green:

A constant peak at 3700-4000 scans

Sharp spikes caused by crystals traveling by the laser detector window.

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 the number of additional bases.

NOTE: 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 fluorescent units minimum. If the peak is unlabeled but visible this allele would be reported in brackets. It is important to remember that 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)

Initials: *RD*

Date: *8/24/98*

Y STR Allele Calling Criteria

Results are interpreted by observing the occurrence of electropherogram peaks for the four loci that are amplified simultaneously - (Y multiplex 1: DYS19, DYS390, DYS389I and DYS389II). 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:

Y M1 Locus	Color	Size Range GS500 Std.
DYS19	yellow	187 \pm 1.5bp to 204 \pm 1.5bp
DYS390	blue	201 \pm 1.5bp to 230 \pm 1.5bp
DYS389I	yellow	246 \pm 1.5bp to 258 \pm 1.5bp
DYS389II	yellow	365 \pm 1.5bp to 382 \pm 1.5bp

The raw data collected by the Genescan Software undergo the same computer processing steps as described for QUAD and AmpFISTR Blue and Green.

In order to eliminate possible background and stutter peaks, only peaks that display an intensity >50 (fluorescent units), and have a peak height >10% of the major peak are called alleles by the instrument. Also for the Y M1 multiplex, due to the systematic occurrence of stutter peaks (as seen during validation) smaller than 4bp of an allele peak, peaks at this position that are lower than the empirically determined stutter threshold of 20% are also disregarded.

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

Peaks approx. 1-3 bp smaller or larger than the main allele, caused by a split main peak or a flat decline of main peak fluorescence

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

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

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.

Initials: *RU*

Date: *8/24/98*

For a Y chromosome specific polymorphism a male individual will display one allele, a female individual no allele peaks. The allele nomenclature is 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).

The reporting procedures follow the manual as described for the autosomal STRs, except that the presence of more than one allele constitutes the criterion for the presence of a mixture.

Initials: *RC*

Date: *8/24/98*

Genotyper Results Table

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

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

1.) If a sample other than a control sample has no alleles listed for all four loci in the Genotyper results table and the Genotyper plot states "no size data" it is possible that the original Genescan Analysis electropherogram displays visible peaks <50 (or <100 for blue/green)). If only one peak is >50 (or >100 for blue/green) 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.

Reporting Procedures

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

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

Initials: *RC*

Date: *8/24/98*

- C. If an allele peak is visible but does not meet the above reporting criteria in at least one run and does not fit into categories A and B above then the allele is reported as **. The ** is defined at the bottom of the table as “ ** = additional alleles were detected which did not meet laboratory criteria for allele identification; therefore, these additional alleles are not reported.”
- D. If an allele meets the above reporting criteria in one run and is not visible during the duplication, then the allele is reported as **.
- E. If no alleles are detected in a sample then the sample is reported as “Neg= no alleles detected.”
- F. If DNA below the minimum threshold is found on QuantiBlot Analysis then the sample is reported as “INS =Insufficient human DNA was detected; therefore, this sample was neither amplified nor typed.”
- G. If there is a large intensity difference between alleles from a locus, the intensity difference should be noted on the report with ***. The *** is defined at the bottom of the table as “***= Large intensity difference between alleles suggests a mixture of DNA.”
- H. Other symbols or reporting procedures will be used if necessary depending on the details of the case.

Initials: *RL*

Date: *8/24/98*

Comparison of Samples and Interpretation of Results in Report

- A. Determine whether it is likely that a sample contains a mixture of DNA (i.e. more than two alleles for a locus, intensity differences between alleles within a locus, reproducible pattern of visible but weak peaks, or the facts of the case suggest a potential mixture.) State in the report whether a sample contains a mixture or possible mixture of DNA, determine the minimum number of individuals who could have contributed to a mixture and the likely source of each component of the mixture
- B. Compare all possible evidence and exemplar pairs and all possible evidence pairs to determine inclusions and exclusions. For inclusions look at the examination table in the report and compare all alleles reported regardless of whether they are in brackets.
- C. Assuming a single physiological fluid donor, two samples could derive from a common biological source (inclusion) if all the alleles in the evidence sample are accounted for by the alleles in the exemplar sample. If however a mixture is possible in the evidence sample, there may be alleles that are not accounted for by the exemplar sample. If an inclusion requires the presence of more than one physiological fluid donor, this must be stated in the report. (i.e.. Hum THO1 (S) 7; (V) 6,9 vaginal swab sperm fract. 7, 9.3/10 ($7 > 9.3/10$); Assuming a single semen donor, the suspect can be eliminated as the semen donor. However if there is more than one semen donor, the suspect can not be eliminated as a possible semen donor.
- D. Statistics are calculated for probative inclusions only where: (1) The sample is apparently unmixed. (2) The sample appears to be a mixture of two components and the source of one component is known. (i.e. when vaginal epithelial cells are present in the sperm fraction from a vaginal swab.) or if there is a large difference in peak heights between the major and minor components and then the genotype of the major component is easily inferred. The minor component genotype can only be determined if four alleles are present at a 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: *ad*

Date: 8/24/98

Extraction Reagent, Amplification Negative and Substrate Controls

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

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

The extraction reagent control, amplification negative control and substrate control (only if the presence of DNA has been shown by QuantiBlot) are amplified and typed along with the test samples. The appearance of signals in the typing of these controls indicates any or all of the following:

- a. The sample preparation reagents may have been contaminated.
- b. Cross-contamination between samples may be occurring during preparation.
- c. The substrate might have an undetected stain containing biological material.
- d. Human DNA or amplified STR DNA may be getting into the samples from some other source.
- e. Sample overflow from the adjacent lane might have happened during gel loading.

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

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

Initials: *RCJ*

Date: *8/24/98*

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

- a. The contamination might be due to a single event limited to the control.
- b. The level of contamination might be inconsequential compared to the amount of DNA being amplified and typed in the test samples.
- c. The contamination might be easily distinguished from the test samples because the contamination and test samples do not have any alleles in common.

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

See Table VI for interpretation guidelines.

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

Control	above 50	Interpretation of Test Sample
Extraction Reagent/ Amplification Neg.	yes	All test samples inconclusive
	no	Test samples are conclusive if there is a duplicate run with no visible peaks.
Substrate	yes	Sample is conclusive and alleles matching the control are not attributed to the stain.
	no	Sample is conclusive. The presence of additional alleles is noted in the report.

Initials: *RC*

Date: *8/28/98*

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

AmpFISTR BLUE

D3S1358	14, 15
VWA	17, 18
FGA	23, 24

AmpFISTR GREEN

Amelogenin	X,X
THO1	8, 9.3
TPOX	8, 8
CSF1PO	10, 12

Y multiplex 1

DYS 19	14
DYS 390	24
DYS 389 I	10
DYS 389 II	26

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

Initials: *RCJ*

Date: *8/24/98*

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. It can be advisable to repeat the test with increased or decreased amounts of DNA, in order to help with the interpretation. This option should be discussed with a supervisor.

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

A. General Mixtures

Evidence samples may contain DNA from more than one individual either because of the nature of the sample or from contamination. The possibility of multiple contributors should be considered when interpreting STR typing results. For any typing system in which heterozygous genotypes are analyzed, the detection of more than two alleles indicates a mixed sample. Furthermore, there is a possibility that a phenotype, e.g. VWA 15,17, is a mixture of approximately equal contributions from a homozygous 15,15 individual and a homozygous 17,17 individual. Such mixtures are not detected from typing results alone, as they reveal only two alleles which could have similar peak heights.

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

Initials: RC

Date: 8/24/98

B. Mixtures with different level of starting DNA

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

A VWA typing profile 18>16>14 can be caused by unequal amounts of 14,16 and 18,18 but also by a mixture of two individuals with 14, 18 and 16, 18. Only in cases where it is known that two individuals contributed to the mixture and four alleles are present at a locus with two each matching in height, a genotype can be inferred. If there is a large difference in peak heights, then the genotype of the major component can be inferred without four alleles present at the locus.

C. Possible mixture components masked by -4bp stutter

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

Initials: *RC*

Date: 8/24/98

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

A. Degradation

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

The longer alleles are more likely not to be present in partially degraded DNA (Lygo et al. 1994, Gill et al. 1995). A QUAD result that displays only VWA and TH01 but no F13A1 and FES/FPS alleles, can be explained as being caused by DNA degradation. A profile with no VWA result but callable FES/FPS alleles cannot be caused by degradation but must have other reasons (e.g. see the following paragraph B).

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

B. Detection limit

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

Initials: *RC*

Date: *8/24/98*

C. Reporting partial profiles

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

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

3. **Detection Of Previously Unreported Rare Alleles**

A. Definition

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

- the overall amplification for the other loci displays distinct peaks >50 (or 100 if applicable) and does not show artefacts,
- the same color locus closest to the new size peak does not have more than one allele peak, and
- the new size peak is also detected in the duplicate run.

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

B. Reporting previously unreported rare alleles

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

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

Initials: *RC*

Date: *8/24/98*

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.

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 can therefore not 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 peaks 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.

Initials: *RCJ*

Date: *8/24/98*

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 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 beginning on page 154, 156. 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: *RU*

Date: *8/24/98*

QUAD Genotyper Categories Table for ABI 373

VWA

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

TH01

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

Initials: *RU*

Date: *8/24/98*

F13A1

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

FES

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

Initials: *RU*

Date: 8/24/98

QUAD Genotyper Categories Table for ABI 377

VWA

11	Highest peak at 127.21 ± 1.50 bp in green with height ≥ 50
12	Highest peak at 131.74 ± 1.50 bp in green with height ≥ 50
13	Highest peak at 135.74 ± 1.50 bp in green with height ≥ 50
14	Highest peak at 139.70 ± 1.50 bp in green with height ≥ 50
15	Highest peak at 143.70 ± 1.50 bp in green with height ≥ 50
16	Highest peak at 147.70 ± 1.50 bp in green with height ≥ 50
17	Highest peak at 151.70 ± 1.50 bp in green with height ≥ 50
18	Highest peak at 156.55 ± 1.50 bp in green with height ≥ 50
19	Highest peak at 160.55 ± 1.50 bp in green with height ≥ 50
20	Highest peak at 164.50 ± 1.50 bp in green with height ≥ 50
21	Highest peak at 168.50 ± 1.50 bp in green with height ≥ 50
22	Highest peak at 172.45 ± 1.50 bp in green with height ≥ 50

THO1

4	Highest peak at 150.25 ± 1.50 bp in blue with height ≥ 50
5	Highest peak at 154.25 ± 1.50 bp in blue with height ≥ 50
6	Highest peak at 159.25 ± 1.50 bp in blue with height ≥ 50
7	Highest peak at 163.25 ± 1.50 bp in blue with height ≥ 50
8	Highest peak at 167.25 ± 1.50 bp in blue with height ≥ 50
9	Highest peak at 171.25 ± 1.50 bp in blue with height ≥ 50
9.3/10	Highest peak from 173.0 to 176.75 bp in blue with height ≥ 50
11	Highest peak at 179.25 ± 1.50 bp in blue with height ≥ 50

Initials: *RCS*

Date: *8/24/98*

F13A1

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

FES

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

Initials: *RC*

Date: *8/28/98*

Y STR 1 Genotyper Categories Table for ABI 377

DYS19

- | | |
|----|--|
| 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 |

DYS390

- | | |
|----|--|
| 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 |

DYS389 I

- | | |
|----|--|
| 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 |

DYS389 II

- | | |
|----|--|
| 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 |

Initials: *RCJ*

Date: *8/24/98*

STR Troubleshooting

<u>Observation</u>	<u>Possible Cause</u>	<u>Recommended Action</u>
1. No peaks or only weak peaks from both the positive control and the DNA test samples at all loci, but the red ladder peaks are present.	Prepared sample tubes with loading buffer and forgot to add PCR product or added only oil supernatant.	Rerun samples on a new gel
	No PCR amplification or insufficient PCR amplification of all markers, which can be caused by:	
	No DNA added or insufficient DNA added to PCR Reaction Mix.	Quantitate DNA and add 1-10 ng DNA; repeat test.
	Mg Cl ₂ or deionized water not added to QUAD PCR Reaction Mix.	Add Mg Cl ₂ or deionized water; repeat test.
	GeneAmp PCR Instrument System failure or wrong program.	See GeneAmp PCR Instrument System Manual and check instrument calibration.
	Tubes not seated tightly in the DNA Thermal Cycler 480 block during amplification.	Push tubes firmly into contact with block after first cycle; repeat test.
	MicroAmp Base used with tray and tubes in GeneAmp PCR System 9600.	Removed MicroAmp Base; repeat test.

Initials: *RC*

Date: *8/24/98*

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

Initials: *RV*

Date: *8/24/98*

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

Initials: *RC*

Date: *8/24/98*

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

Initials: *RC*

Date: *8/24/98*

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

Initials: *RC*

Date: *8/24/98*

Quad and ABI DNA Sequencer References and STR References

1. Applied Biosystems, Genescan 672 Software User's manual. The Perkin Elmer Corporation 1993.
2. ABI PRISM™ Genescan Analysis 2.1 User's manual. The Perkin Elmer Corporation 1996.
3. Applied Biosystems, Genotyper DNA fragment Analysis Software User's Manual Set. The Perkin Elmer Corporation, 1994.
4. ABI PRISM™ Genotyper 2.0 User's Manual. The Perkin Elmer Corporation, 1996.
5. Applied Biosystems, 373 DNA Sequencing System User's Manual. The Perkin Elmer Corporation, 1994.
6. ABI PRISM™ 377 DNA Sequencer User's Manual. The Perkin Elmer Corporation, 1995.
7. Lygo, J.E.; Johnson, P.E.; Holdaway, D.J.; Woodroffe, S.; Whitaker, J.P.; Clayton, T.M.; Kimpton, C.P.; Gill, P. The Validation of Short Tandem Repeat (STR) Loci for Use in Forensic Casework. *Int. J. Leg. Med.* (1994) 107: 77-89.
8. Kimpton, Colin; Fisher, Deborah; Watson, Stephanie; Adams, Maia; Urquhart, Andy; Lygo, Joan; Gill, Peter. Evaluation of an automated DNA Profiling System Employing Multiplex Amplification of Four Tetrameric STR Loci. *Int. J. Leg. Med.* (1994) 106: 302-311.
9. DNA recommendations-1994 report concerning further recommendations of the DNA Commission of the ISFH regarding PCR-based polymorphisms in STR (short tandem repeat) systems. *Int. J. Leg. Med.* (1994) 107: 159-160.
10. Whitaker, J.P.; Clayton, T.M.; Urquhart, A.J.; Millican, E.S.; Downes, T.J.; Kimpton, C.P.; Gill, P. Short Tandem Repeat Typing of Bodies from a Mass Disaster: High Success Rate and Characteristic Amplification Patterns in Highly Degraded Samples. *Biotechniques*, Vol.18 No. 4 (1995) p.671-677.
11. van Oorschot, R.A.H.; Gutowski, S.J.; Robinson, S.L. HUMTH01: Amplification, species specificity, population genetics and forensic applications. *Int. J. Leg. Med.* (1994) 107: 121-126.
12. Kimpton, C.; Gill, P.; D'Aloja, E.; Andersen, J.F.; Bar, W.; Holgersson, S.; Jacobsen, S.; Johnsson, V.; Kloosterman, A.D.; Lareu, M.V.; Nellesmann, L.; Pfitsinger, H.; Phillips, C.P.; Rand, S.; Schmitter, H.; Schneider, P.M.; Sternerssen, M.; Vide, M.C. Report on the second EDNAP collaborative STR exercise. *Forensic Science International* 71 (1995) 137-152.

Initials: *RU*

Date: *8/24/98*

13. Kimpton, C.P.; Gill, P.; Walton, A.; Urquhart, A.; Millican, E.S.; Adams, M. Automated DNA Profiling Employing Multiplex Amplification of Short Tandem Repeat Loci. PCR Methods and Application, Cold Spring Harbor Laboratory Press, 1993,3: 13-22
14. Gill, P.; Ivanov, P.L.; Kimpton, C.; Piercy, R.; Benson, N.; Tully, G.; Evett, I. Hagerlberg, E.; Sullivan, K. Identification of the remains of the Romanov Family by DNA analysis. Nature Genetics Vol. 6, February 1994, p.130-135.
15. Gill, P.; Kimpton, C.P.; Urquhart, A.; Oldroyd, N.; Millican, E.S.; Watson, S.K.; Downes, T.J. Automated short tandem repeats (STR) analysis in forensic casework - a strategy for the future. Electrophoresis 1995. 16: 1543-1552.
16. Leclair, B.; Carmody, G.; Fourney, R.M. Automated fluorescent STR technology: An update on troubleshooting and analytical precision issues. Poster presented at the Eight International Symposium on Human Identification, Promega Corp., Scottsdale, 1997.
17. Frazier, R.R.E.; Millican, E.S.; Watson, S.K.; Oldroyd, N.J.; Sparkes, R.L.; Taylor, K.M.; Panchal, S.; Bark, L.; Kimpton, C.P.; Gill, P.D. Validation of the Applied Biosystems Prism™ 377 automated sequencer for forensic short tandem repeat analysis. Electrophoresis (1996) 17: 1550-1552.
18. 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
19. Jobling MA, Tyler-Smith C (1995) Fathers and sons: the Y chromosome and human evolution. TIG 11: 449-456
20. Kayser M, Prinz M, et al (1997) Evaluation of Y chromosomal STRs: a multicenter study. Int. J. Leg. Med. 110:125-133.
21. 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.
22. 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
23. 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

Initials: *RC*

Date: *8/24/98*

24. 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
25. 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

Initials: *RC*

Date: *8/24/98*

Capillary Electrophoresis and AmpFISTR Blue and Green References

1. Applied Biosystems, ABI Prism Genotyper 2.0 User's Manual, The Perkin Elmer Corporation 1996.
2. Applied Biosystems, ABI Prism Genescan Analysis 2.1 User's Manual, The Perkin Elmer Corporation 1996.
3. Applied Biosystems, ABI Prism 310 Genetic Analyzer User's Manual, The Perkin Elmer Corporation 1995
4. Applied Biosystems, AmpFISTR Blue User's Manual, The Perkin Elmer Corporation 1996.
5. Applied Biosystems, AmpFISTR Green User's Manual, The Perkin Elmer Corporation 1996.
6. Kimpton, C.; Walton, A.; Gill, P. A Further Tetranucleotide Repeat Polymorphism in the vWF Gene. Human Molecular Genetics 1:287 (1992)
7. Polymeropoulos, Michael H.; Xiao, Hong; Rath, Denise S.; Merrill, Carl R.; Tetranucleotide Repeat Polymorphism at the Human Tyrosine Hydroxylase Gene (TH). Nucleic Acids Res. 19:3753 (1991)
8. 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)
9. 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)
10. 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)
11. Hochmeister, M.N.; Use of *Geneprint*TM CSF1PO, TPOX, THO1 Multiplex System (CTT) for the Analysis of DNA Evidence in a serial Killer Case. Profiles in DNA 3-6 (1997)
12. Mills, K.; Even, D.; Murray, J. C; Tetranucleotide Repeat Polymorphism at the Human Alpha Fibrinogen Locus (FGA). Human Molecular Genetics, 1:779 (1992)
13. 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)

Initials: *RC*

Date: *8/24/88*

14. Anker, R.; Steinbrueck, T.; Donis-Keller, H. Tetranucleotide repeat polymorphism at the human thyroid peroxidase (HTPO) locus. Hum. Mol. Genet. 1:137 (1992)
15. Wallin, J.; Lazaruk, K.; Buoncristiani, M.; Walsh, P.S.; Development and TWGDAM validation of AmpFI STR Blue PCR amplification kit. Hand out from the presentation at the AAFS meeting, New York, 1997
16. Buoncristiani, M.; Wallin, J.; Lee, M.; Tonkyn, J.; Lee, S.; TWGDAM validation of AmpFI STR Blue. Abstract, Symposium on Human Identification, Scottsdale, 1996
17. Buel, E.; Schwartz, M.B.; LaFountain, M.J.; Capillary electrophoresis STR analysis: Comparison to gel based systems. J. Forensic Sci. 43:164-170, 1998
18. 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
19. 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
20. 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
21. 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

Initials: *RC*

Date: *8/24/98*

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

If the subject and evidence samples have the same alleles, then the subject is "included", and could be the source of the evidence sample. The probability that another, unrelated, individual would also match the evidence sample is equal to the frequency of the alleles in the relevant population. Population frequencies are calculated separately for the 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, then 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 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 following sources:

<u>System</u>	<u>Reference</u>
QUAD, Blue, Green, Y1	OCME Database

Autosomal STR's

In the standard scenario, for each group, homozygotes are calculated using the formula $p^2 + p(1-p)\theta$ for $\theta = 0.03$ and heterozygotes are calculated using the formula $2p_i p_j$. 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 $\theta = 0.03$. Overall frequencies are calculated as described above.

Initials: *RU*

Date: *8/24/98*

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

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, then 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 5 counts in the database.

Initials: *RW*

Date: *8/24/88*

Amplification Troubleshooting

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

- (1) Amplify using an additional 10 units of Taq or Taq Gold polymerase.
- (2) Amplify a smaller aliquot of the DNA extract to dilute potential Taq polymerase inhibitors or amplify a larger aliquot of the DNA extract because the sample is highly degraded.
- (3) Re-extract the sample using a smaller area of the stain or less biological fluid to prevent saturation of the Chelex and to dilute potential Taq polymerase inhibitors.
- (4) Re-extract the sample using a larger area of the stain or more biological fluid to ensure sufficient high molecular DNA is present.
- (5) Re-extract the samples following the appropriate extraction procedure in the RFLP manual followed by a Chelex extraction.
- (6) For PM + DQA1 only, add BSA to a final concentration of 160 $\mu\text{g/mL}$ in the amplification reaction.
- (7) Purify the extracted DNA on a Centricon-100 ultrafiltration device as follows:
 - a) Assemble the filter unit according to the manufacturer's directions and label each unit.
 - b) Add 1.5 mL TE^{-4} Buffer to the upper Centricon-100 reservoir.
 - c) Add the entire extracted DNA sample to the top of the TE^{-4} Buffer. Cover the tube with ParafilmTM. Use a sterile needle to punch a pinhole in the Parafilm being careful not to touch the solution with the needle. Centrifuge in a fixed-angle rotor (see Centricon instructions) at 1000 x g at room temperature for 20 minutes. The DNA sample will remain concentrated in about 20 to 40 μL of TE^{-4} Buffer in the bottom of the upper Centricon reservoir and molecules with molecular weights of less than about 100,00 Daltons will pass through the filter. Discard the effluent in the lower reservoir.

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

- d) Add 2 mL of TE^{-4} Buffer to the concentrated DNA solution in the upper Centricon reservoir.

Initials: *AG*

Date: *8/24/98*

- e) Repeat the centrifugation and wash steps in c through d twice for a total of 3 washes. After the last wash cycle collect the approximately 40 μ L concentrated DNA sample (as per Centricon instructions) by inverting the upper reservoir into the provided retentate cup, and centrifuging at 500 x g for 2 minutes to transfer concentrate into cup. Label retentate cup. QuantiBlot 5 μ L. The sample is now ready for the PCR amplification process. Store the sample at 2 to 8°C or freeze at -20°C until ready to perform PCR.
- (8) Purify the extracted DNA on a Microcon ultrafiltration device as follows:
- I. Microcon Operation for DNA Concentration
- a) Insert Microcon sample reservoir into vial.
- b) Fill out Microcon worksheet. Process 20 μ L of the appropriate extraction negative as an Microcon negative control.
- c) Pipette 100 μ L of TE⁻⁴ solution into labeled sample reservoir. Add DNA sample (0.4 mL maximum volume) to buffer. Don't transfer any Chelex beads, or in case of an organic extraction sample any organic solvent! Seal with attached cap. ***Avoid touching the membrane with the pipette tip!***
- d) Place the assembly into an variable speed microcentrifuge. Make sure all tubes are balanced! ***To prevent failure of device, do not exceed recommended g-forces.***
- i) Microcon-100 (blue colored top)-Spin at 500 x g (2500 RPM, Eppendorf) for 15 minutes at room temperature.
- ii) Microcon-50 (rose colored top)-Spin at 14,000 x g (13,000 RPM, Eppendorf) for 4 minutes at room temperature.
- e) For additional purification of the DNA sample add 200 μ L TE⁻⁴ and repeat step d), for concentration proceed to step f).
- f) Remove assembly from centrifuge. Open attached cap and add 20 μ L TE⁻⁴. ***Avoid touching the membrane with the pipette tip!*** Separate vial from sample reservoir.
- g) Place sample reservoir upside down in a new labeled vial, then spin 3 minutes at 1000 x g (3500 RPM Eppendorf) for 3 minutes. Make sure all tubes are balanced!

Initials: *AG*

Date: *8/24/98*

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

II. Microcon Operation for DNA Concentration and Clean-up

- a) Place a Micropure-EZ into a Microcon sample reservoir and insert both into a vial.
- b) Pipette solution into sample reservoir ($250\mu\text{L}$ maximum volume). Seal with attached cap. ***Avoid touching the membrane with the pipette tip!***
- c) Place the assembly into an variable speed microcentrifuge. Make sure all tubes are balanced! Spin at for 30 sec at room temperature at the following speeds. ***To prevent failure of device, do not exceed recommended g-forces.***
 - i) Microcon-100 (blue colored top)-Spin at $500 \times g$ (2500 RPM, Eppendorf)
 - ii) Microcon-50 (rose colored top)-Spin at $14,000 \times g$ (13,000 RPM, Eppendorf).
- d) Add $100\mu\text{L}$ TE^{-4} to the Micropure-EZ reservoir.
- e) Microcon -100- Spin for 15 minutes at room temperature at the above speed.
Microcon -50- Spin for 4 minutes at room temperature at the above speed.
- f) Remove and discard Micropure-EZ
- g) Add $500\mu\text{L}$ of sterile water or TE^{-4} to the Microcon and spin at the appropriate speed listed in section c and the appropriate time listed in section e.
- f) Remove assembly from centrifuge. Open attached cap and add $20\mu\text{L}$ TE^{-4} . ***Avoid touching the membrane with the pipette tip!*** Separate vial from sample reservoir.
- g) Place sample reservoir upside down in a new labeled vial, then spin 3 minutes at $1000 \times g$ (3500 RPM Eppendorf) for 3 minutes. Make sure all tubes are balanced!
- h) Remove from centrifuge and separate sample reservoir. Measure resulting volume using an adjustable Micropipette, adjust volume to $50\mu\text{L}$ using TE^{-4} . Calculate resulting concentration. Snap sealing cap shut to store product for later use.