FORENSIC BIOLOGY PROTOCOLS FOR FORENSIC STR TYPING

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Body fluid identification

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

2. A positive and interpretable QuantiBlot and/or STR result can be considered human (primate) positive. Identification of the specific physiological fluid may be accomplished using the procedures described in the Biochemistry Manual. A positive screening test for blood followed by the detection of a positive QuantiBlot is reported as "human blood was found".

Laboratory organization

1. 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.

2. 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.

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

Microfuge tube and pipet handling

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

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

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

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

4. Always change pipet tips between handling each sample.

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

6. Discard pipet tips if they accidentally touch the bench paper or any other surface.

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

Sample handling

1. 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.

2. 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.

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

4. 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.

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

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

DNA Extraction Guidelines

Slightly different extraction procedures may be required for each type of specimen. Due to the varied nature of evidence samples, the user may need to modify procedures.

1. All tube set-ups must be witnessed **prior** to starting the extraction (**NOTE:** For differential extractions, the tube set-up should be witnessed after the incubation step.)

2. Use Kimwipes or a tube opener to open sample tubes; 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. For pipetting Chelex, the pipette tip used must have a relatively large bore – 1 mL pipette tips are adequate.

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

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

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

8. After extraction, the tubes containing the unamplified DNA should be transferred to a box and stored in the appropriate refrigerator or freezer. The tubes should *not* be stored in the extraction racks.

Controls for PCR analysis

The following controls have to be processed along side the sample analysis:

1. A positive control is a DNA sample where the STR alleles for the relevant STR loci are known. The positive control tests the success and the specificity of the amplification, and during the detection and analysis stage the electrophoretical separation and the correct allele calling.

2. An extraction negative control consists of all reagents used in the extraction process, and is necessary to detect DNA contamination of these reagents. A clean result - the absence of detectable DNA - in an extraction negative in one of the PCR multiplexes is sufficient to show the absence of contamination. **Note:** Since the Y STR system only detects male DNA, one cannot infer from a clean Y STR extraction negative the absence of female DNA.

3. An amplification negative control consists of only amplification reagents without the addition of DNA, and is used to detect DNA contamination of the amplification reagents.

   Failure of any of the controls does not automatically invalidate the test. Under certain circumstances it is acceptable to retest negative and positive controls. **See STR results interpretation for rules on retesting of control samples.**

Concordant analyses and "duplicate rule"

The general laboratory policy is to confirm DNA results by having concordant DNA results within a case - confirmation of a match or exclusion being the most common situation. Concordant and duplicate analysis is also used to detect sample mix-up and confirm the presence of DNA mixtures.

1. For evidence samples, the following guidelines apply:

   a. Identical DNA profiles among at least two items (two evidence samples or one evidence sample plus an exemplar) within a case are considered internally concordant results ("duplicate rule").

b. If a sample does not match any other sample in the case, it must be duplicated by amplification in either the same or a second PCR system. If the only result was obtained using Y-STRs, this must be duplicated in the Y system.

c. If after the first DNA analysis there is an indication that the sample consists of a mixture of DNA, several scenarios need to be considered. Further analysis steps have to be decided based on the nature of each case. Consult your supervisor if you encounter a situation that is not represented in the following examples:

1) If all alleles in a mixture are consistent with coming from any of the known or unknown samples in the case, e.g. a victim and a semen source, no further analysis is needed, unless one of the mixture components should be entered in CODIS.

2) If two or more mixtures in a case are consistent with each other and display the same allele combinations, they are considered duplicated.

3) If one or more alleles cannot be accounted for by other contributors in the case, the presence of the foreign component must be confirmed by amplification in either the same or a second PCR system.

4) If there is only one probative profile in a case and this happens to be a mixed sample , the results need to be confirmed by amplification in either the same or a second PCR system depending on the need to type all 13 loci for CODIS.

2. For exemplar samples, the following guidelines apply:

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

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

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

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

c. If the DNA profile of a **suspect's exemplar** (or other non-victim elimination exemplar) does not match any of the DNA profiles in the case, or in the local database, the exemplar does not have to be duplicated. *This is meant to streamline the process parallel to convicted offender testing.*

d. If the DNA profile of a **suspect's exemplar** matches any of the DNA profiles in the case, or in the Local database, the suspect's exemplar has to be duplicated to eliminate the possibility of an exemplar mix-up. *This is meant to streamline the process parallel to convicted offender testing.*

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

3. For evidence samples or exemplar samples analyzed in DNA systems containing overlapping loci, the DNA results for the overlapping loci must be consistent. If no or partial results were obtained for some of the overlapping loci, this amplification is still valid if consistent results were obtained for at least one complete locus. (Amelogenin is not considered an overlapping locus in this context.) If the partial amplification confirms a match or an exclusion of an exemplar or another evidence sample, it does not have to be repeated.

4. Partial profiles can satisfy the duplication policy. Consistent DNA typing results from at least one locus in a different amplification (same DNA system or a different DNA system) is considered a concordant analysis.

5. For Y-STR testing the sample does not have to be reamplified if the internal duplication rule applies or if the Y-STR results are concordant with the autosomal results: confirming an exclusion or inclusion, confirming the presence of male DNA, confirming the number of semen donors. Based on the case scenario it might be necessary to reamplify in order to confirm the exact Y-STR allele calls. There might not be sufficient autosomal data to establish concordance.

DNA storage

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

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

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

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

CHELEX DNA EXTRACTION FROM BLOOD AND BUCCAL SWABS

Sample sizes for Chelex extraction should be approximately 3µL of liquid blood or saliva, 1/3 of a swab, or a 3x3mm cutting of a bloodstain.

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. This can be done either in a shaker, or by occasionally inverting and 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 30 to 50 µL). If the sample is a bloodstain or swab, leave the substrate in the tube with pellet.
7. Add 175 µL of 5% Chelex (from a well-resuspended chelex solution).
8. Incubate at 56°C for 15 to 30 minutes.
9. Vortex at high speed for 5 to 10 seconds.
10. Incubate at 100°C for 8 minutes using a screw-down rack.
11. Vortex at high speed for 5 to 10 seconds.
12. Spin in a microcentrifuge for 2 to 3 minutes at 10,000 to 15,000 x g.
13. Pipet 20 µL neat and also a 1/10 dilution (using TE^{-4}) 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.

CHELEX DNA EXTRACTION FROM SOFT TISSUE (E.G. FETUS SAMPLES)

Sample sizes for this Chelex extraction should be approximately a 3x3mm cutting of tissue.

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 30 to 50 µL).

7. To each tube add: 200 µL of 5% Chelex (from a well-resuspended chelex solution).
   1 µL of 20 mg/mL Proteinase K

8. Mix using pipette tip.

9. Incubate at 56°C for 60 minutes.

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

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

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

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

14. Pipet 20 µL neat and also a 1/10 dilution and 1/100 (using TE^{-4}) into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration.

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

CHELEX DNA EXTRACTION FROM EPITHELIAL CELLS (AMYLASE POSITIVE STAINS OR SWABS, CIGARETTE BUTTS, SCRAPINGS)

Sample sizes for this Chelex extraction should be approximately a 5x5mm cutting or 50% of the scrapings recovered from an item.

1. Remove the extraction rack from the refrigerator.

2. To each tube add: 200 µL of 5% Chelex (from a well-resuspended chelex solution).
   1 µL of 20 mg/mL Proteinase K

3. Mix using pipette tip.

4. Incubate at 56°C for 60 minutes.

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

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

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

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

9. Pipet 20 µL neat and also a 1/10 dilution (using TE^{-4}) into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration.

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

NOTE: **Samples meant for this type of extraction should be placed on the "other evidence" or the Y-STR amylase sheet. Exemplars of this type must be extracted separately.**

For very large cuttings 200 µL of Chelex might not be enough to provide enough suspension of the sample. The reaction can be scaled up and re-concentrated using Microcon concentrators.

NON-DIFFERENTIAL CHELEX DNA EXTRACTION FROM SEMEN STAINS OR SWABS

Sample sizes for non-differential Chelex extractions depend on the circumstances of the case. Regularly 1/3 of a swab or a 3x3mm cutting of a stain should be used. For cases where semen is present but no sperm cells were detected, the sample size can be increased.

1. Remove the extraction rack from the refrigerator.

2. To each tube add: 200 µL of 5% Chelex (from a well-resuspended chelex solution).
   1 µL of 20 mg/mL Proteinase K
   7 µL of 1 M DTT

3. Use the pipette tip when adding the DTT to thoroughly mix the contents of the tubes.

4. Incubate at 56°C for approximately 2 hours.

5. Vortex at high speed for 10 to 30 seconds.

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

7. Vortex at high speed for 10 to 30 seconds.

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

9. Pipet 20 µL neat and also a 1/10 dilution (using TE^{-4}) into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration.

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

NOTE: For very large cuttings 200 µL of Chelex might not be enough to provide enough suspension of the sample. The reaction can be scaled up and reconcentrated using Microcon concentrators.

CHELEX DNA EXTRACTION FROM SEMEN STAINS OR SWABS

Approximately 1/3 of a swab or a 3x3mm cutting of a stain should be used for this type of extraction.

1. Remove the extraction rack from the refrigerator.

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

3. Mix by inversion or vortexing

4. Incubate at room temperature overnight or for a minimum of 1 hour using a shaking platform.

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

6. Label new tubes to hold the swab or substrate remains. Remove the swab or other substrate from the sample tube, one tube at a time, using sterile tweezers and close tube. Sterilize tweezers with ethanol before the removal of each sample. Store swab or substrate in a sterile labeled 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 and discard all but 50 µL of the supernatant.

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

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

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

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

13. During the incubation step do the following:

    a. Label a new tube for each sample, including the extraction negative control. Mark each tube as an epithelial cell fraction
    b. Add 50 µL of 20% Chelex (from a well-resuspended chelex solution) to each epithelial cell fraction tube
    c. Close tubes

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

15. Add 150 µL of the supernatant from each sample and the extraction negative to its respective epithelial cell fraction sample tube. Store at 4°C or on ice until step 21.

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

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

18. Resuspend the pellet by stirring with a sterile pipette tip.

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

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

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

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

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

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

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

26. Pipet 20 µL neat and also a 1/10 dilution (using TE^{-4}) into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration.

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

CHELEX DNA EXTRACTION FROM HAIR

Microscopic examination of hair by PD lab should be completed before attempts at DNA typing. If possible document state of root on photo. Be careful not to loose adhering sheath material prior to extraction.

A. Sample preparation for loose hairs:

1. 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.

2. Wash the hair containing sheath material to remove surface dirt and contaminants by immersing the hair in sterile, deionized water in a clean 15 or 50 mL Falcon tube.

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 and place in a 1.5 mL microcentrifuge tube.

B. Sample preparation for mounted hairs:

5. Remove cover slip by placing slide in freezer for 10 minutes and afterwards removing cover with a razor blade or clean forceps. Alternatively the slide can be submerged in fresh xylene for 1-2 minutes.

6. Add 10-20 µL of fresh Xylene to the embedded hair sample. Allow 10-20 seconds for Permount to dissolve, then tease out the hair sample with clean forceps. Check slide for tissue that was left behind.

7. Place hair on clean microscope slide and add more fresh Xylene to remove adhering Permount. Pipet off Xylene, add a few drops of water, repeat rinsing with water several times.

8. Put hair under dissecting microscope. Use a clean scalpel to cut a 1 cm portion from the root end of the hair and place in a 1.5 mL microcentrifuge tube.

ATTENTION: If not immediately starting Chelex extraction, do not place wet hair in 1.5mL tube. Perform additional 99% Ethanol rinse and let hair dry before placing in tube.

ATTENTION: Do not consume hair shaft. If possible leave at least 1cm for mtDNA typing.

C. Chelex extraction

9. To hair tubes add: 100 µL of 5% Chelex (from a well-resuspended chelex solution).
   1 µL of 20mg/mL Proteinase K.

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

10. Incubate at 56°C for at least 6 to 8 hours, or overnight.

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

12. 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.

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

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

15. Pipet 20 µL neat and also a 1/10 dilution (using TE^{-4}) into a microcentrifuge tube for QuantiBlot Analysis to determine human DNA concentration.

16. Store the remainder of the extract at either 2 to 8°C or frozen.

ORGANIC EXTRACTION PROCEDURE

A. Sample Preparation

Bone preparation and milling

A SPEX Certiprep 6750 Freezer mill is being used for this procedure. All parts that come into contact with the bone specimen need to be clean. See below for procedure.

Before extraction a bone specimen should be cleaned from soft tissue and dirt using a combination of scalpels, brushes and running water. Bone then will be dried and cut.

1. Using an autopsy saw or a Dremel tool equipped with a cut-off wheel cut the bone specimen into 5x5x5mm size pieces. Take enough cuttings for a end weight of approximately 2g. For older or compromised bones several aliquots of 2 grams can be extracted and combined during the Microcon step. **[Note: the cut off wheel should be disposed of after each use]. Protective eyewear, lab coats, cut resistant gloves and HEPA-filtered facial masks should be worn when cutting bone.**

2. Label 50mL Falcon tubes on the side and on the lid!!! Transfer cuttings to Falcon tubes and sonicate for 30-45 minutes in a 5% Terg-a-zyme solution (≈25mL). Repeat with fresh changes of 5% Terg-a-zyme until the dirt has been removed followed by the fresh changes of sterile dH_2O.

3. When the bone specimen has dried (use 56°C oven), record gross weight.

4. Assemble specimen vials (metal bottom, plastic cylinder and impactor). Add bone cuttings, close with metal top. Label the metal parts only.

5. Open cover of mill; add liquid nitrogen up to the fill line, place specimen vial into the round chamber.

6. The mill settings should be:

cycle	1
time	T1 (milling) 2 .0 min
	T2 (pause) 2.0 min
	T3 (precooling) 15.0 min
rate	10

7. Close cover and press run to start the mill. Precooling will start and milling will take place after that.

8. Remove vial (wear thermal gloves) and use opening device to remove metal top.

9. Using tweezers remove impactor from vial and submerge in 0.1% SDS.

10. Empty bone dust into labeled 50mL Falcon tube.

11. Soak metal end parts and plastic cylinder in 0.1% SDS.

12. If processing more than one bone sample it is possible to save precooling time by placing up to two vials in the mesh container inside the mill. Change cycle number to match total number of samples. During the 2-minute pause phase it is now possible to open the mill, remove the finished sample and place one of the precooled specimen in the round chamber.

    Add new sample to mesh container, close the lid and press Run again. Continue to rotate samples until all bones have been processed. Minimum precooling time for a sample should be 4 minutes.

13. Mill part clean up: Soak all parts in 0.1% SDS. Brush parts with a new toothbrush to remove any residual bone dust. Rinse with water. Soak parts in 10% bleach and brush each part in bleach individually. Rinse with water. ONLY the metal top, metal bottom, and compactor can be rinsed in 100% ethanol. DO NOT rinse the plastic cylinder in ethanol, as it will destroy it. Rinsing in ethanol may cause the plastic cylinder to break. Use isopropanol to remove any identifying marks made with a Sharpie on the tops or bottoms. As a final step, dry and expose the parts to UV light. Exposure time is minimum of 2 hours. The light in a biohood or a Stratalinker can be used.

14. Proceed to Section C – Incubation for bone, teeth and paraffin embedded tissue samples.

Laser Microdissection

This procedure can be used to separate maternal (decidual) tissue from fetal material (chorionic villi) in early stage abortions. The procedure can only be performed in cooperation with the OCME histology department and a trained pathologist. The instrument used, the PixCell® IIe Laser Capture Microdissection System (ARCTURUS Systems for Microgenomics, Carlsbad, CA), is owned by NYU Medical center and can only be operated by trained NYU staff.

1. Initial processing

The product of conception (POC) can be received in different stages of preparation:

a) POC scrapings in saline buffer:

Remove tissue from liquid either by filtration or centrifugation:

- Transfer liquid to 50mL falcon tube
- Spin sample in a bench top Eppendorf or IEC Centra CL3R at 1000 RPM for 5 minutes
- Discard liquid supernatant

Submit sample to the Histology department for tissue processing according to the OCME Histology Procedure Manual section E. Then proceed as for b.).

b) POC fixated and embedded in paraffin blocks:

Contact histology department and ask them to prepare microscope slides from the paraffin block using the following precautions:

- Use disposable blades for the microtome and discard after each case.
- Clean working surface on microtome by wiping with 10% bleach and alcohol before and after each case.
- Use individual floating chambers for each case
- Use uncharged microscope slides

The slides then should be stained with hematoxylin and eosin-phloxine (H&E technique) as described in the OCME Histology Procedure Manual. But again during the staining procedure separate sets of jars have to be used for each case.

c) Stained or unstained microscope slides from POC blocks:

If the slides are unstained, ask the histology department to stain them as described above. Otherwise proceed with the microdissection technique. **Attention:** for slides that were prepared by a histology laboratory outside of the OCME, foreign DNA not from the mother and the fetus might be present on the slide.

2. PixCell IIe Laser Capture Microdissection

The PixCell® IIe Laser Capture Microdissection System (LCM) utilizes a low-power infrared laser to melt a special thermoplastic film over the area of interest. Especially developed CapSure Caps that are coated with this thermoplastic film are placed on the tissue sample and are used to capture the cells of interest after the laser excitation. See the Arcturus instrument manual for a more detailed description.

A trained pathologist has to be present to distinguish decidual tissue from chorionic villi and operate the laser. After the slide has been placed on the microscope platform the pathologist will visually identify the area of interest, mark this area for the laser, and activate the laser. The laser setting is specified in the Arcturus instrument manual. The Forensic Biology Criminalist needs to be present during the complete procedure to maintain chain of custody of the evidence.

An area of chorionic villi and an area of maternal tissue should be collected on separate CapSure caps. The caps can be stored and transported in 50 ml Falcon tubes. A third unused CapSure cap should be extracted as an extraction negative control.

Use new scalpel and clean forceps to remove the film from the cap and transfer the film to a fresh 1.5mL reaction tube containing 500µL of organic extraction buffer, DTT, SDS and Proteinase K as described below.

B. Incubation for Liquid and Dry Blood, Bone Marrow and Tissue Samples

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

2. Use the following amounts of samples:

Sample type	Amount
Liquid blood	100 to 500 uL
Bone marrow	0.5 x 0.5 cm to 1.5 x 1.5cm
Oral swab	1/3 to a whole swab
Blood stain	0.5 x 0.5 cm to 1.5 x 1.5cm
Soft tissue	0.5 x 0.5 cm to 1.5 x 1.5cm

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

4. Prepare a master mix for N+2 samples and mix thoroughly:

	1 Sample	5 Samples	10 Samples
Organic extraction buffer	400 µL	2.0 mL	4.0 mL
20% SDS	10 µL	50 µL	100 µL
Proteinase K (20 mg/mL)	13.6 µL	68 µL	136 µL

5. Add 400 µL of the master mix to each tube. Vortex briefly. Make certain the fabric, tissue, or swab is totally submerged. The master mix volumes can be adjusted upwards for large samples.

6. Place tubes in a shaking 56°C heat block or water bath and incubate overnight.

Continue with Phenol Chloroform Extraction and Microcon Clean up.

C. Incubation for Bone, Teeth and Paraffin Embedded Tissue Samples (e.g. from Microdissection)

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

2. Use the following amounts of samples:

Sample type	Amount
Bone	Up to 2g per 50mL vial
Teeth	Whole root
Paraffin embedded tissue	0.3 x 0.3 cm to 1.0 x 1.0 cm

Prepare bone and microdissection samples as describe above.

3. Prepare a master mix for N+2 samples and mix thoroughly:

	1 tissue sample 0.5mL	1 tooth 1mL	1 bone sample 3mL
Organic extraction buffer	395 µL	790 µL	2370 µL
20% SDS	50 µL	100 µL	300 µL
0.39 M DTT	20 µL	40 µL	120 µL
Proteinase K (20 mg/mL)	35 µL	70 µL	210 µL

5. Use above volumes for the different tissue types.

6. Place tubes in a shaking 56°C heat block, a waterbath or the large incubation oven and incubate overnight.

7. After incubation centrifuge samples for 5 min at 1000RPM. Transfer clear supernatant into fresh tubes of the appropriate size.

Continue with Phenol Chloroform Extraction and Microcon Clean up.

D. Incubation for semen stains and swabs

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

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

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

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

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

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

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

	1 Sample	5 Samples	10 Samples
Digest buffer	400 µL	2.0 mL	4.0 mL
Proteinase K (20 mg/mL)	5 µL	25 µL	50 µL

6. Add 400 µL of master mix to each sample.

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

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

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

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

11. Without disturbing the pellet, pipet 350 µL of the supernatant in into a new labeled tube. This supernatant is called the epithelial cell fraction and is enriched for non-sperm DNA. Place the epithelial cell fraction on ice until step 17. In this step, the negative control is treated like a sample even though it should not have a pellet. The sperm negative is derived from the remaining lysate in the negative control and the epithelial negative is derived from the 350µL of supernatant, which was pipetted off.

12. Wash the sperm pellet with Digest buffer as follows:
    a. Resuspend the pellet in 0.5 mL Digest Buffer.
    b. Vortex briefly to resuspend pellet.
    c. Spin in a microcentrifuge at 10,000 to 15,000 x g for 5 minutes.
    d. Remove all but 50 µL of the supernatant and discard the supernatant.
    e. Repeat steps a-d for a total of 3 times.

13. Resuspend the pellet in the remaining liquid.

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

	1 Sample	5 Samples	10 Samples
Digest Buffer	400 µL	2.0 mL	4.0 mL
0.39 M DTT	40 µL	200 µL	400 µL
Proteinase K (20 mg/mL)	10 µL	50 µL	100 µL

15. Add 450 µL of the master mix to each pellet and optionally to the substrate remains.

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

Continue with Phenol Chloroform Extraction and Microcon Clean up.

E. Phenol Chloroform Extraction and Microcon Clean up

Eppendorf Phase Lock Gel (PLG) tubes make the phase separation easier and are optional. Prior to transferring incubated samples to 1.5mL PLG tubes, these must be centrifuged at maximum speed for 30 seconds. See below on how to prepare different size PLG tubes.

1. Insert Microcon 100 columns (blue) into labeled microcon tubes for each sample.

2. Prepare the Microcon 100 concentrators by adding 100µL of TE^{-4} to the filter side (top) of each concentrator.

3. Add equal volume (400 µL to 3mL) of Phenol:Chloroform:Isoamyl Alcohol (25:24:1). This step must be done in the fume hood. Shake the tube vigorously by hand or by inversion to form a milky colored emulsion. **Do NOT vortex the PLG tube.**

   <u>CAUTION:</u> Phenol:Chloroform:Isoamyl Alcohol is an irritant and is toxic. Its use should be confined to a hood. Gloves and a mask should be worn.

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

5. Carefully transfer the aqueous phase (top layer) to a newly labeled tube or to the prepared Microcon 100 concentrator. Do not disturb the layer of denatured protein that collects at the interface. Discard the organic layer into a waste bottle in the hood.

6. Repeat steps 3 to 5 with Phenol/Chloroform/Isoamyl Alcohol if the sample is discolored, shows a lot of particles in the aqueous phase, contains a lot of fatty tissue.

   <u>NOTE</u>: If it is not necessary to repeat the extraction step, the aqueous phase can be directly transferred to the upper Microcon chamber.

7. Carefully transfer the aqueous phase (top layer) to a newly labeled tube or to the prepared Microcon 100 concentrator. Do not disturb the layer of denatured protein that collects at the interface. Discard the organic layer into a waste bottle in the hood.

8. Spin the Microcon 100 concentrators for 15 minutes at 500xg (2500 rpm on the Eppendorf microfuge). (Note: additional spin time may be required to filter the entire volume.)

9. Discard the wash and return the filtrate cups to the concentrators.

10. Add 400µL of TE^{-4} to the filter side of each Microcon 100 concentrator.

11. Spin again at 2500 rpm for 15 minutes.

12. Add 40 µL of TE^{-4} to the filter side of each Microcon 100 concentrator.

13. Invert sample reservoir and place into a newly labeled tube. Spin at1000xg (3500 rpm Eppendorf microfuge) for 3 minutes to collect sample.

14. Discard sample reservoir and adjust sample volume depending on the starting amount and expected DNA content as follows using TE^{-4}:

Sample type	Final Volume
High DNA content (Large amounts of blood, fresh tissue, bone marrow, oral swabs, and dried bloodstains)	400 µL
Medium DNA content (Small amounts of blood, fresh tissue, bone marrow, oral swabs, and dried bloodstains); differential lysis samples	200 µL
Low DNA content (Formalin fixed tissue, dried bone)	100 µL

15. Transfer samples to 1.5mL reaction tubes for storage. Store at 2 to 8°C or frozen.

16. Submit aliquots for QuantiBlot analysis to determine human DNA concentration. Submit 1:10, 1:100 and higher dilutions for high and medium DNA content samples (use TE^{-4} to make the dilutions). Submit neat 20µL only for samples where the input was at the lower limit or where a low DNA content is expected.

NOTE: For larger volumes Centricon YM-100 concentrators (available in 2mL, 15mL and other sizes) can be used instead of Microcon 100. Use 500xg for concentration and 1000xg for recovery. Adjust RPM forces according to centrifuge used. Do not exceed 1000xg.

NOTE: See Microcon troubleshooting below.

F. Preparation of Phase Lock Gel (PLG) tubes

The purchase of Phase Lock gel (PLG) Syringes Heavy is economical and more convenient than having to stock all possible tube sizes. The gel in the syringe can be added to different disposable tubes. Make sure the plasticware being used is resistant to phenol and chloroform.

1. Without putting pressure on the plunger, twist off the **orange cab** and discard. Attach the **gray dispensing tip** (supplied) to the syringe and tighten securely. (NOTE: Use of gray tip is optional for a smoother application of PLG. Less force is necessary when gray tip is NOT used.)

2. Apply firm pressure on the plunger to dispense PLG until it reaches the end of gray tip. Add heavy PLG based on Table below.
   NOTE: 325µL = 3.25 cc corresponds to 3 lines on the syringe

Tube size	PLG heavy
0.5mL	100µL
1.5mL	325µL
2.0mL	325µL
15mL	3mL
50mL	5mL

3. Pellet the PLG by spinning the tubes prior to use. See table below.

Tube size	Centrifuge model	Speed	Time
0.5 to 2.0mL	Eppendorf 5415C Eppendorf 5415D	14 x 1000 RPM 13.2 x1000 RPM/16.1 x1000 RCF	30 seconds
15 and 50mL	Sigma 4-15 C	1500 RCF	2 minutes

MICROCON 100 DNA CONCENTRATION AND PURIFICATION

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

1. Label a sufficient number of blue Microcon 100 sample reservoirs and insert them into the vials.

2. Fill out Microcon worksheet. Process 50µL of TE^{-4} solution as an Microcon negative control. Make sure to use the same lot that is used to dilute the samples and don't forget to label the final negative control tube with the Microcon date and time and your initials.

3. Pipette 100 µL of TE^{-4} solution into each labeled sample reservoir including the negative control. 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!***

   Return the original Chelex tubes to their storage location. Do not discard empty tubes.

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

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

5. For **purification** of the DNA sample add 200 µL of TE^{-4} solution and repeat Step 4. Do this as often as necessary to generate a clear extract, and then continue with Step 6. When performing multiple wash steps it is necessary to empty the bottom vial intermittently.

   For **concentration** only proceed to step 6.

   NOTE: When purifying samples with a low DNA concentration it might be advantageous to use several wash steps and to also reduce the volume to achieve both, a cleaner sample and an increased DNA concentration.

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

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

8. Remove from centrifuge and separate sample reservoir. Measure resulting volume using an adjustable Micropipette, record volume on work sheet; adjust volume to desired level using TE^{-4}.

   Clean-up for high DNA concentrations: reconstitute starting volume.
   Low DNA samples (clean-up and concentration): adjust to 50µL

   Transfer the DNA extracts and the microcon negative control to new labeled 1.5mL Eppendorf tubes and store extract for later use. Note storage location on worksheet.

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

ATTENTION: **Do not store the DNA in the Microcon vials! The lids are not tight enough to prevent evaporation**.

Troubleshooting:

Lint, bone dust and other particles can clog the membrane. If the liquid does not go down, redistribute supernatant to multiple vials. Pipet off clear supernatant without disturbing particle pellet.

If the problem persists, the specific Microcon lot number might be faulty. Notify the QA Unit and try a different lot number.

Sample Blotting

1. Vortex DNA Standards and Calibrators 1 and 2. Centrifuge briefly to bring the contents to the bottom of the tube. If Chelex extracts are being used, centrifuge for 2 minutes.

2. While wearing gloves, label enough microfuge tubes for all standards. Labels can be made on the tops of the microfuge tubes, and should be made to reflect the wells that they will be loaded in (i.e. 10 ng standard - 1A, 5 ng standard - 1B, etc.).

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

   a. DNA Standards and Calibrators - 5 µL

   b. Negative (TE^{-4}) - 5 µL

3. While wearing gloves, label the tops of the sample tubes. Labels should be made in the same manner as for the standards. The samples were aliquoted ahead of time by the chelex rotation and are stored at 4°C in the QuantiBlot refrigerator.

4. Label a hybridization tray with the date, rack # of the samples, and your initials. Should the weekly allotment of trays be depleted, it is permissible to wash used trays containing membranes that have **already been reviewed** by the rotation supervisor. These trays should be thoroughly washed with ethanol and rinsed with deionized water. Labels may be removed with isopropanol. The trays should then be dried completely with a kimwipe prior to use.

5. Heat a shaking water bath to 50°C. The water level should be 1/4 to ½ inch above the shaking platform. The temperature should not go below 49°C or above 51°C. **It is essential to check the temperature with a calibrated thermistor probe or thermometer before the hybridization is performed. Also remember to record the temperature.**

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

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

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

7. Obtain a QuantiBlot membrane from the drawer and place it in a hybridization tray containing 50 mL of Pre-Wetting Solution and incubate at room temperature for 1-30 minutes. Note: Either side of the Biodyne B membrane can be used as the side onto which samples are pipetted. **Always wear gloves when handling the membrane**.

8. Inspect the gasket wells to make sure they are aligned correctly (improper alignment can lead to "thin" bands upon color development). Then, using forceps, remove the membrane from the Pre-Wetting solution. Place the membrane on the gasket of the slot blotter, then place the top plate of the slot blotter on top of the membrane. Turn on vacuum pump to a vacuum pressure of approximately 200 to 250 mm Hg. Turn off the sample vacuum and turn on the clamp vacuum on the slot blot apparatus. Push down to ensure a tight seal.

9. At this point have someone witness the tube setup.

10. Load the membrane as follows:

    Using a new pipet tip for each sample, apply all of each sample into a separate well of the slot blotter. For best results, slowly dispense each sample directly into the center of the wells, with the pipet tip approximately 5 mm above the membrane. **Note: Do not allow the pipet tip to touch the membrane since this may compromise the membrane at that spot.**

SLOT	SAMPLE
1A	10 ng Standard
1B	5 ng Standard
1C	2.5 ng Standard
1D	1.25 ng Standard
1E	0.625 ng Standard
1F	0.3125 ng Standard
1G	0.15625 ng Standard
1H	Plate negative Control (TE^{-4})
2A	3.5 ng Calibration 1 Standard
2B	0.5 ng Calibration 1 Standard
2C	0.15625 ng Standard
2D – 6F	Samples and Controls
6G	3.5 ng Calibration 1 Standard
6H	0.5 ng Calibration 1 Standard

11. 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.

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

    Clean the apparatus by soaking in enough 0.1% SDS to cover for 5-15 minutes. Following soaking in SDS, rinse the gasket and the side of the top plate that contacts the membrane **thoroughly** with H_2O (include a final rinse with deionized H_2O). Make sure that all the wells of the top plate are rinsed and clean. Rinse the bottom unit of the slot blotter with deionized H_2O and allow to dry at room temperature. **Never use bleach.** A toothbrush can be used to clean the surfaces if necessary.

13. Transfer the membrane to 100 mL of pre-warmed QuantiBlot Hybridization Solution in the hybridization tray. Add 5 mL of 30% H_2O_2. Place the lid on the tray. Put the tray into the 50°C shaking water bath. Place a weight (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

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

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

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

16. 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.

17. 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.

18. 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

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

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

    Mix thoroughly by swirling (do not vortex).

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

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

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

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

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

Photography

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

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

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

26. Photograph at 1/125 seconds and f8 for type 667 film. Photograph at 1/8 seconds and f8 for type 664 film.

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

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

28. Attach the photograph to the QuantiBlot worksheet. Once the QuantiBlot passes review (see below), make photocopies and distribute to the appropriate analysts. File the original QuantiBlot worksheet along with photograph in the appropriate binder. Discard the membrane.

Note: If an entire QuantiBlot or a portion of it does not pass review (see below), it is the responsibility of the analyst on the QuantiBlot rotation to resubmit those samples that are in question.

Quality Control

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

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

3. Estimate the quantity (ng) loaded for each sample by comparing band intensities of the unknown samples with those of the DNA standards. If the band intensity is between two DNA standards, **match the sample to the DNA standard with the lower amount of DNA.**

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

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

   The following guidelines must be followed:

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

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

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

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

NOTE: this scenario only applies if the 10ng and the 0.15ng standard fails. All other standards are required to read the calibrators.

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

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

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

Quantiblot interpretation

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

Neat	1/10 Dilution	1/10 Dilution
	Results OK	Review photo to determine best concentration
≥10	5, 2.5, 1.25	0.62
5	0.62	1.25, 0.31
2.5	0.31	0.62, 0.15
1.25	0.15	0.31, Blank
0.62	<0.15	0.15
0.31	Blank	<0.15
0.15	Blank	N/A

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

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

Modified QuantiBlot starting with extracts in microtiter plates

For this procedure the number of controls and calibrators has been reduced so that multichannel pipettes can be used for sample loading. All steps are performed as above with the following modifications:

1. Add 150 µL of Spotting Solution to the prepared standards and to each sample well. Do not vortex microtiter plate! Proceed without delay.

2. Prepare membrane and gasket as described above.

3. Load the membrane as follows:

Slot	Sample
1A	10 ng standard
1B	5 ng standard
1C	2.5 ng standard
1D	0.625 ng standard
1E	0.3125 ng standard
1F	0.15625 ng standard
1G	Plate negative control (TE^{-4})
1H	3.5 ng calibration standard

4. Load samples in columns 2A-H to 6A-H using an 8-channel 300 µL pipette. Proceed as described above.

5. Due to the reduced number of standards and calibrators the acceptance guidelines were changed as follows:

   a.) The intensity of the 3.5ng calibrator has to be between 2.5 and 5ng, otherwise the membrane must be repeated.

   b.) The membrane passes review if at least three standards in a row are correct and their intensities decrease in linear fashion.

Troubleshooting of QuantiBlot
(Taken from the Applied BioSystems QuantiBlot package insert, see QuantiBlot references)

Observation	Possible Cause	Recommended Action
1. No signal or low sensitivity.	Use of a membrane other than Biodyne B.	Use Biodyne B nylon membrane. Do not use membranes that have neutral charge.
	Incorrect NaOH or EDTA concentrations in Spotting Solution.	Prepare Spotting Solution correctly.
	Water bath temperature too high.	Water bath temperature should be 50°C (±1°C).
	DNA Probe was not added at hybridization step.	Add QuantiBlot D17Z1 Probe.
	Enzyme conjugate was not added.	Add Enzyme conjugate: HRP-SA at indicated step in protocol. Use 180 µL of Enzyme Conjugate: HRP-SA.
	Hydrogen peroxide was inactive.	Prepare a new Color Development Solution using a fresh bottle of hydrogen peroxide.

Observation	Possible Cause	Recommended Action
	Presence of $MgC1_2$ in the DNA sample.	Concentrations of $MgC1_2$ >0.3 mM can result in reduced sensitivity. Prepare all DNA dilutions in TE^{-4} Buffer. Any $MgC1_2$ can be removed from samples by micro dialysis using Microcon 100 spin units (follow manufacture's directions).
2. Areas of low sensitivity across the membrane.	Membrane slipped up onto the side of the Hybridization Tray during Hybridization or Stringent Wash steps.	Reduce the rotation rate of the water bath to 50-60 rpm. Check that the membrane is fully submerged in the bottom of the Hybridization Tray before shaking.
	Membrane dried-out significantly at some point in the protocol.	Do not allow the membrane to dry at any point in the protocol.
3. Non-uniform signal intensity within a slot.	Bubbles(s) in slot blot wells when sample was pipetted into well, or when vacuum was applied.	Slowly pipet the Spotting Solution directly over the center of the wells of the slot blot apparatus, with the pipet tip raised approximately 5 mm above the membrane.
		Turn on the sample vacuum slowly, not all at once. After being drawn through the membrane, the sample should appear as a uniform blue band on the membrane.

Observation	Possible Cause	Recommended Action
		If the entire sample is not drawn through the membrane, turn off the sample vacuum. Pipet the sample back into the pipet tip; then pipet the sample back into the well of the slot blot apparatus. Turn on the sample vacuum to draw the sample through the membrane.
4. Filter background	No or low SDS in the Hybridization Solution or in the Wash Solution.	Prepare solutions with proper concentrations of SDS.
	Membrane was not pre-wetted prior to slot blotting.	Pre-wet the membrane in Pre-Wetting Solution prior to slot blotting.
	Too much Enzyme conjugate: HRP-SA was added.	Use 180 µL of Enzyme Conjugate:HRP-SA.
	Lack of thorough rinsing.	Thoroughly rinse twice, for 1 minute each, using 100 mL of pre-warmed Wash Solution. These two rinse times can be extended beyond 1 minute if necessary.
	Slot blot apparatus not cleaned thoroughly after last use.	Immediately after each use, soak the slot blot apparatus in a large volume of 0.1% SDS solution. Never use bleach.
5. The DNA Calibrators do not quantitate cor-rectly with respect to the DNA Standards.	DNA Standard serial dilutions prepared incorrectly.	Prepare two-fold serial dilutions of DNA Standard in TE^{-4} Buffer as described. Add 5 µL of each dilution to 150 µL of Spotting Solution for slot blotting.

Observation	Possible Cause	Recommended Action
6. Signal obtained for non-human DNA samples.*	Water bath temperature too low.	Water bath temperature should be 50°C (±1°C).
	SSPE concentration too high in Wash Solution.	Check that the 20X SSPE solution and the Wash Solution were prepared correctly.

* DNA from primate species may give signals similar to those obtained from equivalent amounts of human DNA. In Roche Molecular Systems (RMS) laboratories, 30 ng to 300 ng quantities of non-primate DNA samples result in either no signals or signals that are less than or equal to the signal obtained for 0.15 ng of human DNA.

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

Do not forget to indicate on the DNA extract tracking worksheet that a sample was submitted.

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

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

A. Preparing DNA aliquots for the amplification

1. Do not amplify samples in which insufficient DNA was detected by QuantiBlot (0 or <0.15ng/20µL). These samples may be amplified after a Microcon concentration step.

2. The target amounts to be amplified are 1 ng of DNA for the Profiler Plus and Cofiler multiplexes, 0.5ng for Powerplex16, and 2ng for the Y-STR systems. Due to the varying volumes of reaction mix and magnesium chloride for the different multiplex reactions, the DNA aliquots have to be adjusted accordingly to achieve a final volume of 50µL, or 25µL respectively. Follow the appropriate tables when setting up the aliquots.

   ATTENTION: Powerplex16 is currently only validated for exemplars and human remains. Do not use for evidence specimen.

3. For each sample to be amplified, label a new tube. Add DNA and TE^{-4} and as specified in Tables 1A to 3A.

4. Remember the following general rules:

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

   When a dilution is made to determine DNA concentration, it is preferable to calculate the DNA concentration in the undiluted DNA extract. Then amplify the undiluted DNA extract, not the dilution. *If possible always amplify the optimal target amount.*

Powerplex 16 multiplex

Table 1A - Amount of DNA to be amplified for Powerplex 16

QuantiBlot DNA Concentration (ng/20µL)	Target Volume (µL) to be amplified	TE^{-4} (µL) for Target Volume	Range of Volumes (µL) which can be amplified (Corresponds to approx. 0.15 - 5 ng of DNA)
≥25	Dilute 1/10 and use the dilution for amplification		
12.5	1.6	18.4	0.3-8**
6.2	2.0	18.0	0.5-16**
5	3.2	16.8	0.6-20**
3.1	4.0	16.0	1.0 - 20**
2.5	6.6	13.4	1.2-20**
1.5	8.0	12.0	2.0 - 20**
1.25	16.0	4.0	2.4-20**
0.62	20	0	4.8-20**
0.31	20	0	9.6 -20**
0.15	20	0	19.2-20**
< 0.15	Do not amplify***	-	-

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

*** This does not apply to Missing Persons/Unidentified Body cases. Tissue samples and reference samples may be amplified with <0.15 Qblot value. Submit 20ul for PowerPlex 16 amplification only.

Table 1B - Control samples for the Powerplex 16 multiplex

Sample	DNA	TE^{-4}
Promega Kit positive control	10 µL	10 µL
Extraction negative control	20 µL	---
Amplification negative control	---	20 µL

Cofiler and Profiler Plus Kits

Table 2A - Amount of DNA to be Amplified for the Cofiler and Profiler Plus Kits

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

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

Table 2B - Control samples Cofiler and Profiler Plus Kits

Sample	DNA	TE^{-4}
ABI kit amplification positive control	20 µL	---
Extraction negative control	20 µL	---
Amplification negative control	---	20 µL

Y STR multiplex YM1

The amplification of exemplars, sperm cell fractions of samples extracted by differential lysis and semen stains, where no epithelial cells were seen during the differential lysis, is based on the QuantiBlot results (see Table VI). Semen positive swabs taken from female individuals that were extracted using the non-differential semen extraction and the swab remains fractions of differential lysis samples are amplified using the amounts specified in Table 3B.

Table 3A - Amount of DNA to be amplified for the Y M1 based on QuantiBlot results.

QuantiBlot DNA Concentration (ng/20µL)	Target Volume (µL) to be amplified	TE^{-4} (µL) for Target Volume	Range of Volumes (µL) which can be amplified (Corresponds to approx. 0.15 - 5 ng of DNA)
≥25	Dilute 1/10 and use the dilution for amplification		
12.5	3.2	22.8	0.3 - 8**
6.2	6.6	19.4	0.5 - 16**
5	8.0	18.0	0.6 - 20**
3.1	13.0	13.0	1.0 - 26**
2.5	16.0	10.0	1.2 - 26**
1.5	26.0	0	2.0 - 26**
1.25	26.0	0	2.4 - 26**
0.62	26.0	0	4.8 - 26**
0.31	26.0	0	9.7 - 26**
0.15	26.0	0	20 - 26**
< 0.15	Do not amplify	-	-

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

Table 3B - Amount of DNA extract from a non-differential semen extraction or from the swab/substrate remains fraction of a differential lysis sample to be amplified for YM1.**

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

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

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

Table 3C - Amount of DNA extract to be amplified for Amylase positive samples.**

Type of item		DNA Target Volume (µL)	TE^{-4} (µL)	Range (µL)
Vaginal swab	Initially try two amounts	10 26	16 0	1 - 26*
Anal or dried secretions swab	Based on Qblot result	see Table 3A.1		
Stain	Based on Qblot result			

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

** Qblot does not reflect male DNA especially for vaginal swabs. Try more or less if negative.

Table 3D - Control samples Y STR multiplex YM1

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

B. Amplification set-up

ATTENTION: Powerplex16 is currently only validated for exemplars and human remains. Do not use for evidence specimen.

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

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

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

4. For Powerplex 16 the reaction mix has to be prepared fresh. Prepare enough for N+1 samples and mix before using:

Reagent	1 sample	10 samples	30 samples
Powerplex 16 10x primer mix	2.5 µL	25 µL	75 µL
Gold Star 10 x buffer	2.5 µL	25 µL	75 µL
AmpliTaq Gold DNA Polymerase (5U/µL)	0.8 µL	8 µL	24 µL

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

	SYSTEM		
	Powerplex 16	**Y STR YM1**	**Cofiler, Profiler Plus**
Reagent	5.8 µL mastermix	4 µL of $MgCl_2$	10 µL of specific primer mix

6. Pipet carefully in the solution at the bottom of the tube. Use a fresh sterile pipette tip for each tube. Close all of the tubes. At this stage have another analyst witness the sample set-up.

7. **Note: Use a new sterile filter pipet tip for each sample addition. Open only one tube at a time for sample addition.** The final aqueous volume in the PCR reaction mix tubes will be 50µL for Profiler Plus, Cofiler and YM1, and 25.8µL for Powerplex 16. Transfer the DNA aliquot prepared earlier to the labeled PCR reaction mix tubes. After the addition of the DNA, cap each sample before proceeding to the next tube. **Do not vortex or mix.**

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

C Thermal Cycling

Turn on the ABI 9700 Thermal Cycler. (See manufacturer's instructions).

Choose the following files in order to amplify each system:

Powerplex16	YM1	Cofiler	Profiler Plus
user: casewk file: powerplex	user: casewk file: ym1	user: casewk file: profiler/cofiler	user: casewk file: profiler/cofiler

Tables 1 lists the conditions that should be included in each file. If the files are not correct, bring this to the attention of the Quality Assurance Manager and a supervisor.

Table 1 PCR Conditions for the Perkin Elmer GeneAmp PCR System 9700

9700 **Powerplex16** user: casewk file: powerplex	The Powerplex file is as follows: Soak at 95°C for 11 minutes Soak at 96°C for 1 minute Ramp 100% ▸Denature at 94°C for 30 seconds Ramp 29% ▸Anneal at 60°C for 30 seconds Ramp 23% ▸Extend at 70°C for 45 seconds for 10 cycles then Ramp 100% ▸Denature at 94°C for 30 seconds Ramp 29% ▸Anneal at 60°C for 30 seconds Ramp 23% ▸Extend at 70°C for 45 seconds for 22 cycles 30 minutes incubation at 60°C. Storage soak indefinitely at 4°C
9700 **Cofiler/ Profiler Plus** user: casewk file: cofiler/profiler	The Cofiler/Profiler Plus file is as follows: Soak at 95°C for 11 minutes ▸Denature at 94°C for 1 minute 28 Cycles: ▸Anneal at 59°C for 1 minute ▸Extend at 72°C for 1 minute 45 minute incubation at 60°C. Storage soak 90 minutes at 25°C Storage soak indefinitely at 4°C

9700 **YM1** user: casewk file: ym1	The YM1 file is as follows: Soak at 95°C for 10 minutes 30 Cycles: ▸Denature at 94°C for 45 seconds ▸Anneal at 58°C for 58 seconds ▸Extend at 72°C for 1 minute 15 seconds 30 minute incubation at 60°C. Storage soak indefinitely at 4°C

9700 Instructions

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

2. Start the run by performing the following steps:

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

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

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

   Select the RUN option (F1).

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

   Verify that the reaction volume is set to **50**µL (25µL for PowerPlex 16) and the ramp speed is set to **9600 (very important)**.

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

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

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

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

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

In case of an actual power failure, the 9700 thermal cycler will automatically resume the run if the power outage did not last more than 18 hours. The history file contains the information at which stage of the cycling process the instrument stopped. Consult the Quality Assurance Unit and/or the Technical Leader on how to proceed.

After the amplification process, the samples are ready to be loaded on the fluorescent instruments. They may be stored in the appropriate refrigerator at 2-8°C for a period of up to 6 months.

D. Amplification Troubleshooting

PROBLEM: No or only weak signal from both the positive control and the test samples

Possible Cause	Recommended Action
Mistake during the amplification set up such as not adding one of the components or not starting the thermal cycler	Prepare new samples and repeat amplification step
Thermal cycler defect or wrong program used	Check instrument, notify QA team, prepare new samples and repeat amplification step

PROBLEM: Positive control fails but sample signal level is fine

Possible Cause	Recommended Action
Mistake during the amplification set up such as not adding enough of the positive control DNA	Prepare new samples and repeat amplification step
Positive control lot degraded	Notify QA team to investigate lot number, prepare new samples and repeat amplification step

PROBLEM: Presence of unexpected or additional peaks in the positive control.

Possible Cause	Recommended Action
Contamination by other samples, contaminated reagents	Notify QA team to investigate the amplification reagents, prepare new samples and repeat amplification step
Non-specific priming	Notify QA team to check thermal cycler for correct annealing settings, prepare new samples and repeat amplification step

PROBLEM: Strong signal from the positive controls, but no or below threshold signal from DNA test sample.

Possible Cause	Recommended Action
The amount of DNA was insufficient or the DNA is severely degraded.	Amplify a larger aliquot of the DNA extract. Re-extract the sample using a larger area of the stain or more biological fluid to ensure sufficient high molecular DNA is present. Concentrate the extracted DNA using a Microcon 100 ultrafiltration device as described in the Microcon section.
Test sample contains PCR inhibitor (e.g. heme compounds, certain dyes).	Amplify using an additional 10 units of Taq Gold polymerase. Amplify a smaller aliquot of the DNA extract to dilute potential Taq Gold polymerase inhibitors. Re-extract the sample using a smaller area of the stain to dilute potential Taq Gold polymerase inhibitors. Re-extract the samples using the organic extraction procedure.. Purify the extracted DNA using a Microcon 100 ultrafiltration device as described in the Microcon section.

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

5. GENERAL GUIDELINES FOR FLUORESCENT STR ANALYSIS

Batch processing

1. Exemplars and evidence samples must be handled separately at all times. These samples must never be together on the same sample tray.

2. For the ABI 3100, it is acceptable to load one exemplar and one evidence tray in the same instrument. Two trays are the equivalent of two consecutive runs.

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

4. Use the correct worksheet for the specific sample type, and make sure the sample preparation set-up is witnessed properly.

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

   ATTENTION: Each capillary run that is performed must have at least one correct positive control.

Sample handling

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

2. Amplified samples that have been loaded on an instrument should be stored until the electrophoresis results are known. After it has been determined that the amplified samples do not require repeated testing, they may be discarded.

Instrument and computer maintenance

1. Be gentle with all instrument parts and instruments. Keep everything clean.

2. It is good practice to monitor initial instrument performance. This enables the user to detect problems such as leaks, air bubbles or calibration issues.

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

4. PCs need to be maintained through regular defragmentation.

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

6. Notify the Quality Assurance Unit if any problems are noted.

6. STR DATA ARCHIVING		
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STR DATA ARCHIVING ON JAZ DISKS FOR MACINTOSH BASED SYSTEMS (ABI 310)

In absence of a MAC network, JAZ disks are used as transport medium from the data collection instruments to the external analysis stations. A different set of JAZ disks are used as a final archive after analysis has been completed. Always transfer the run files onto the computer hard drive before processing; otherwise the JAZ disks will get corrupted.

Data transfer

1. Before copying a run, identify the **Run folder** containing the project file based on the date and time noted on the gel or sample sheet. At this point the folder can be renamed with the gel/sample sheet name, which will make it easier to find later.

2. Put a designated JAZ archiving disk in the JAZ drive. The disk icon will be visible on the desktop. 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!!!! Do not use paperclips!!!!

   Restarting the computer can help if the JAZ disk icon will not appear on the desktop.

3. Copy the run on external analysis station hard drive and delete it from the transfer disk.

Data archiving

1. After the gel or project has been analyzed and processed with Genotyper, the folder should contain the following items: An injection list, run log, a project file, sample files for all samples, and the Genotyper files

   Make sure the folder is complete.

2. The folder should have been renamed (see Genotyper instructions), according to the following format:

   Instrument name Files (e.g. CE1/04-002 files)

3. Save folder on instrument specific archiving disk or Y STR disk.

4. After the copy process is complete, delete the run folders from the collection instrument and the external analysis station computer hard drive.

5. JAZ disks are rewritable and the Genotyper files can be opened, reedited and saved. Reediting is documented on the hard copies.

STR DATA ARCHIVING ON CD'S FOR WINDOWS BASED SYSTEMS (ABI 3100)

The external analysis stations for 3100 data are networked together but CD's need to be burned to transfer data from the collection instruments to the analysis stations.

A. Formatting of transfer CD

When a blank CD-R or CD-RW disc is formatted by **DirectCD,** it may be used in the same way as any other removable media such as JAZ, ZIP, or floppy discs. A DirectCD formatted CD-R/CD-RW can have data added and deleted provided it is in a CD-RW drive. You may work and read off the disc in both CD-ROM and CD-RW drives.

To format a CD-R or CD-RW in DirectCD:

1. Insert a blank CD-R or CD-RW disc into the CD-RW drive.

2. Wait for the program **Easy CD Creator 4** to launch itself.*

3. Click **DATA**.

4. Click **DIRECTCD**. The **Adaptec DirectCD Wizard** will open.

   *If Easy CD Creator 4 does not open within 30 seconds, the Adaptec DirectCD Wizard may be opened directly from the **START** menu in the **Programs** category under the heading **DirectCD**.

5. Click **Next>**. The **Drive Information** window will open.

6. Click **Next>**. The **Format Disc** window will open.

7. Click **Next>**. The **Name Your Disc** window will open. Give the disc a name up to eleven characters long.

8. Click **Finish**. You will receive the message, "Disc in Drive _: has been successfully formatted."

9. Click **OK**. Your disc is now ready to write on and delete from (only in CD-RW drives), as well as read from and worked off of (in both CD-RW and CD-ROM drives). It behaves in the same way as the floppy discs, JAZ/ZIP.

10. You can save onto it from a program's file menu or you can add/delete files to/from the disc through **My Computer, Windows Explorer**, etc...

11. When a DirectCD disc is in the CD-RW drive, a small red spot appears on the DirectCD icon (a CD on top of a disc drive) in the bottom right-hand side of the screen.

To transfer casework runs, there are shortcuts on the desktops for faster access to the CD-RW drives. You can 'Drag-and-drop' files directly from the Current Runs folder onto the shortcut icons to copy them onto the disc. Double-click the shortcut icon to view the data on the disc.

B. Copy and transfer of files

1. When a run is finished, locate the injection folders on the 3100 instrumental computer that were generated during the course of the run. These folders are in the Current Runs folder that is located on the desktop of the instrumental computer.

2. Create and organize a new folder for each amplification system used (e.g., cofiler, profiler plus) to contain the injection folders. Name this folder(s) with the filename according to your sample sheet (e.g., Stripes04-009Co). This new folder will be referred to as the **common runs folder(s)**.

   Keep in mind that when two or more different amplification systems are used, several common runs folders will be needed (see examples below).

   Example #1: A Cofiler and a Profiler Plus amplification set were run on Stars with the following sample sheet name: Stars04-008Co-003Pro. Two common run folders will need to be created with the following names: Stars04-008Co and Stars 04-003Pro.

Example #2: Two Cofiler amplification sets were run on Stars with the following sample sheet name: Stars04-010Co. Only one common run folder needs to be created with the same name (Stars04-010Co) even though following analysis, two genotyper files will be generated with names corresponding to each amplification set as follows: Stars04-010CoA and Stars04-010CoB.

Note: Be sure to also include a copy of the run's sample sheet in the common runs folder. Sample sheets are located in the sample sheet folder on the desktop. It is necessary to save only the .xls file (**not** the .plt file). If more than one common runs folder is created, then each should contain a copy of the sample sheet.

3. Insert a CD-RW transfer disk into the CD-RW drive of the instrumental computer.

4. Copy the common runs folder(s) onto the CD-RW disk by clicking and dragging the common runs folder(s) onto the CD-RW drive icon located on the desktop.

5. Eject the CD-RW transfer disk from the instrumental computer and take it over to the analysis network for further analysis.

6. Insert the CD-RW transfer disk into the CD-R or CD-RW drive on analysis station #1, #2, or #3.

7. View the desired common runs folder(s) by double-clicking the CD icon that is located on the desktop. Copy the desired common runs folder(s) to the PC hard drive by clicking and dragging the common runs folder(s) from the transfer disk to the **Work folder** (located on the desktop).

   Note: This work folder is specific for each terminal and cannot be accessed from the other two terminals.

8. Proceed with the project and genotyper analysis according to the protocol described in the **STR Analysis on the 3100 Capillary Electrophoresis Genetic Analyzer** and **Genotyper** sections of the STR manual.

9. After analysis is completed and Genotyper files have been saved in the Common run folder, copy the Common run folder to the **Archive folder**. These folders are organized on each analysis station according to the following hierarchy:
   - **Archive folder**
   - Instrument folders (e.g., Stars, Stripes; located in the **Archive folder**)
   - Amplification system folders (e.g. Cofiler, Profiler Plus; located in the instrument folders)

   The **Archive folder** is physically present on analysis station #1 but is shared with analysis stations #2 and #3 on the analysis network. This means that even though the data is being copied from the **Work folder** to the **Archive folder** at analysis station #2 or #3, it is physically being copied to the hard drive of analysis station #1. Along the same lines, editing data (see Section E.) can be performed at any of the analysis workstations.

10. Delete completed folders from Work folder and Transfer disk. Return the transfer disk back to the 3100 instrument area.

C. Temporary Archive (weekly)

At the end of each week it is the responsibility of the STR station supervisor(s) to do the following:

1. Copy all of the analyzed common runs folders present in the **Archive folder** to a backup folder present on analysis station #2. This folder (located in the D drive of analysis station #2) will not be accessed during routine run analysis and is maintained only for the purpose of having a backup copy. This backup procedure can only be done on analysis station #2.

2. Delete runs that have already been analyzed and backed-up on analysis station #2 from the 3100 instrumental computers

D. Permanent Archive (every three months)

At the end of each 6-month span (e.g. Jan-Jun, Jul-Dec) with a lag time of 3 months (e.g., Oct, Apr) it is the responsibility of the Forensic Biology computer support analyst(s) to do the following:

1. Two CD-Rs will each be burned to contain all of the analyzed common runs folders present on analysis station #1 for the given 6 month period. One set of copies will be stored at 520, the other off-site at Bellevue.

IMPORTANT: Never fill the CD-R disks with more than 675 Mb of data. This will insure that with the inclusion of future edits, all of the data will still fit on one CD-R disk during the reburning process.

2. After all of the analyzed common runs folders for the 6 month period have been burned and verified, copies of the burned files will be deleted the analysis network.

Note: *The end result in the storage of files is that at any given time there will be 2 copies of each run saved either on 2 separate hard drives or on 2 separate CD-R disks.*

E. Editing stored runs

If an edit(s) needs to be done on a previously analyzed run whose copy is still present in the Archive folder of analysis station #1, do the following:

1. Make all necessary edits to the relevant file(s) present in the **Archive folder** on the desktop of the analysis station.

Note: Due to file sharing on the analysis network, editing can be done from any of the analysis stations on the analysis network.

2. Any changes made in item 1 will be backed-up onto analysis station #2 according to the weekly backup procedure described above.

If an edit(s) needs to be done on a previously analyzed run that has already been burned on CD-R, do the following:

1. Find the desired CD-R disk at the analysis network area and copy all of its contents to the Temp Archive folder located on the desktop of the analysis workstation. This will be used as a temporary working file. Make edits to the desired file(s) present in the temporary working file.

2. After all of the necessary edits are done, re-burn two new CD-R disks to contain all of the contents of the temporary working file including the changes that were made. One of the CD-Rs will replace the one at the analysis network area and the other will replace the backup at Bellevue. After the original disks are replaced with the two new disks, the original disks should be destroyed (e.g. break into two or feed into an appropriate shredder) and discarded. After new copies of disks have been burned, delete all of the files from the Temp Archive folder on the desktop.

Only the Cofiler and Profiler Plus systems have been validated for analysis on the ABI 310 Genetic Analyzers and can be run on these instruments.

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. On the instrument level, it must be ensured that the capillary is properly installed and still valid and that the syringe is filled with the separation medium POP4. In order to have a hard copy of the run, a run control sheet documenting the sample names and the reagent lot numbers has to be filled out manually.

A. Preparing the instrument

1. Retrieve a fresh tube of deionized formamide from the freezer and the CE set up box containing the Genetic Analyzer TE buffer, the CXR size standard and the appropriate allelic ladders from the refrigerator. This way the reagents can adjust to room temperature during the instrument preparation.

2. Restart the computer by selecting Restart under **Special.**

3. Open the 310 collection software by double clicking on the **ABI Prism 310 Collection** icon.

4. Check the capillary count on the instrument and in the logbook.

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

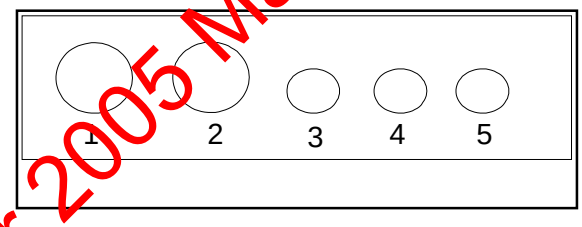
   IF THE CAPILLARY DOES NOT NEED CHANGING, CLICK **CANCEL**. **IMPORTANT**: DO NOT CLICK **OK**, BECAUSE THAT WILL RESET THE CAPILLARY COUNT TO ZERO.

   If the capillary needs changing click **OK** to reset the count to zero. Go to Changing and Installing the Capillary, section D. If the capillary does not need to be changed, click **Cancel**, enter your samples into the logbook, and proceed as follows:

5. Change the buffer and the deionized (DI) water as follows: Push the TRAY button next to the pump block. This action moves the autosampler forward and presents the tray. Remove the two glass vials and the Eppendorf tube. Also remove the anode buffer chamber from under the block on the left side by gently turning and pulling down. Rinse all containers with DI water. Shake to dry and place upside down on filter paper.

   To refill make 1X buffer by aliquoting 5mL of 10X Genetic Analyzer buffer with EDTA (use a 5mL pipette and the Pipetman to measure the buffer) into a 50mL Falcon plastic tube and filling the tube up to the 50mL graduation with deionized water. Do not use any other buffers. Label the tube with the lot number, the date of manufacture and your initials

   Autosampler tray positions



   Fill anode buffer chamber with 1X buffer up to the fill line and fit securely to its position on the pump block by gently turning and pushing up.

   Fill cathode buffer vial with 1X Genetic Analyzer EDTA buffer to the fill line, replace cap and septum, and place in position 1 on the autosampler platform

   Fill the other glass vial with deionized water, replace the cap, septum, and place in position 2 on the autosampler platform.

   The Eppendorf tube serves as the waste vial, should be 2/3 filled with DI water and placed in position 3. Positions 4 and 5 will remain empty.

   ATTENTION: Always make sure that the vials are completely dry on the outside. Otherwise electrical arching might occur and singe the autosampler.

6. Check the syringe for leaks and if the amount of POP4 is sufficient

   Leaks are indicated by the presence of crystallized POP4 solution at any of the ferrules or liquid POP4 in the syringe, alongside the plunger. The crystals can cause spikes and the leaking causes incomplete filling of the capillary. In that case, clean and refill the syringe and tighten or replace the ferrules.

For a full tray you should have at least 200µL POP4 in the syringe (0.2 mark). Remove syringe and refill if you have less. **Never add fresh polymer to old polymer.**

7. To refill the syringe it has to be removed from the instrument. Under **Window** go to **Manual Control Window**.

   Under the **Function** pop-up menu in the **Manual Control Window** select **Syringe Up** and click **Execute** multiple times until the syringe drive is clear of the end of the plunger of the glass syringe. Swing the pump toggle to the left. With caution, 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).

8. Do not remove the plunger but clean the syringe after expelling the remaining POP4 by repeatedly drawing up and expelling DI water from a small beaker. Before really refilling the syringe draw up a small amount of POP4 and expell it again to remove residual water.

   Always let the POP4 warm up to room temperature before using it. Make sure there are no crystals or other particles in the POP4. Manually fill 700µl (0.7 mark) of POP4 into the 1mL glass syringe Do this slowly to prevent the formation of air bubbles. **Important**: the presence of air bubbles may cause false leak alarms.

   If air bubbles are visible expel again, let POP settle and refill, or turn the syringe upside down and gently tap on the side to dislodge all air bubbles. Expel the air bubbles with some of the solution if necessary.

   Rinse exterior with deionized water and dry with a Kimwipe.

9. 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. Tighten firmly.

10. Remove any air bubbles from the block. Open the **Manual Control** menu and from the **Function** pop up menu select **Buffer Valve Close,** (click **Execute**). First open the bottom valve by unscrewing it. Slowly press down on the syringe plunger to remove any air bubbles. Tighten waste valve. Repeat process using the ferrule on top of the block.

    Then go back to the **Manual Control** menu; select **Buffer Valve Open** (click **Execute**). Manually push plunger to force any last air bubbles into the anode buffer chamber..

    Make sure there are no air bubbles left 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.

11. Tighten all valves. Under **Manual Control** choose **Buffer Valve Close**, click **Execute**. Do not forget this step.

12. 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 touches the top of the syringe plunger.

13. In order to check the color detection baseline (based on laser performance and state of the capillary window) do a **CCD four color** test.

    ATTENTION: a tray must be in the autosampler and the doors closed for this step.

    In the **current run folder** an icon should be named **CCD test**. Double click on this icon.

14. Click **Run**. Click **O.K.** to the instrument alert "ED current is Zero". The scan window should display all four color lines at about the same level; which should be above 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.

    A reading below 2048 indicates a dirty capillary, no reading might mean that the window is not placed in front of the CCD camera or that the laser is dead.

    Once the CCD test passes, close the scan window. Pause the run, then cancel the run, and click **terminate**. Close the injection list window, click **Don't save**. Also quit Genescan which was opened automatically.

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

    Set the temperature to 60°C, then click **Execute**. The instrument takes up to 30 minutes to reach the 60°C run temperature. Continue to fill out the sample information and prepare the samples while the instrument is heating.

    Under **Windows** open the **Status** window to observe the increasing temperature values.

B. Filling Out a the Sample information and Creating an Injection list

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 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 from an Amplification must stay together

   B. There should be an allelic ladder approximately every 12 samples.

   C. A full tray consists of two amplification sheets and two run control sheets. Evidence samples must to be run separately from exemplar samples. They cannot be in the same tray together.

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

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

   F. Samples that need to be rerun (e.g. because of a separation problem or the presence of a partial profile) must be placed with the appropriate sample type and amplification system. In order to improve the signal strength for samples that showed a partial profile, it is possible to increase the injection time from 5 to 10 seconds and the sample input to 2µL.

2. Double click on the 310 PRISM COLLECTION icon, if the collection program is not already open. Under **File** select **New.**, The Create New Dialogue box appears. Then click on **Genescan Sample Sheet, 48 tube** icon. A Genescan sample sheet appears.

#	Sample Name	Color	Std	Pres	Sample Info
A1		B		☐	
		G		☐	
		Y		☐	
		R	◆	☒	
A3		B		☐	

*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).

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

4. 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 check box in the column labeled "**pres**" (present) automatically becomes selected.

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

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

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

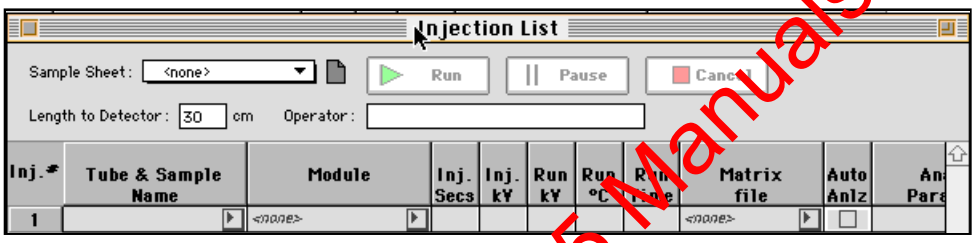
   CE1/04-001Pro, CE2/04-001Co, CE3/04-004Pro/002Co respectively,

   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 on the run control sheets.

6. Create an injection list by importing the sample sheet. 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 injection.

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

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



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

   The information recorded in the Sample Sheet will appear in the Sample Name Column of the Injection List. The Default module is "**GS STR POP4 1mL, F**" and should appear for each sample in the MODULE column. The run conditions are: Inj sec 5, Inj KV 15.0, Run KV 15.0, Run 60°C, Run time 24. Change the injection time to 10 seconds if necessary; do not touch the other settings. The matrix standard must be the most recent matrix made for the instrument. Size standard and Analysis Parameters are both preset analysis default settings.

   Auto Analyze should be checked. This means the Genescan Software will be launched and each sample will be automatically analyzed.

8. For each run it is advisable to inject the first ladder 2X. To accomplish this highlight injection #1, by clicking on #1 on the far left, 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**.

   This feature can be used to reinject any sample on the tray, e.g. if an intermediate check of the analysis results revealed a failed injection. Samples can also be reinjected with a longer injection time. Always reinject samples afer the last planned injection as to not alter the injection numbers for the previous samples.

C. Preparing and Running the DNA Samples

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

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

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

Mix thoroughly by inversion.

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

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

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

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

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

7. Close both instrument doors.

8. Click **Run** on the injection list. Enter the run date and time on the Run control sheet.

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

It is recommended to monitor the result for the first injection. This way any electrophoresis problems are detected early and can be fixed before all samples were injected in vain.

D. Changing and Installing the Capillary

1. Click on the TRAY button next to the pump block to present the autosampler tray. Remove the tray. Also remove the buffer and water vials, the waste vial from the autosampler and the anode buffer vial from the pump block. To remove the anode buffer chamber, the buffer valve needs to be open (Normally it should always be open, to double check go to the **Manual Control Window** select **Buffer Valve Open**, click **Execute**), twist and pull the chamber gently until it is released. Empty and rinse all vials with DI water and let sit to dry.

2. Open the heat block door and the laser detector door. Remove the capillary from the laser detector groove, remove the tape and pull the capillary out of the electrode thumb screw. Unscrew the ferrule on the right side of the pump block and pull the capillary out. Discard the capillary.

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

4. Under the **Function** pop-up menu, select **Syringe Up.** 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. Remove the pump block from the CE by firmly pulling the block away from the machine. Remove all ferrules and valves from the block and clean it using a squeeze bottle with deionized water. Thoroughly dry the pump block with Kimwipes

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

6. Make sure all the different valves and ferrules are thoroughly cleaned with water; especially make sure the hole in the ferrule (through which the capillary runs) is clear of any POP4. Also remove any dried POP4 from the threads of the screws. Replace all pieces to the proper positions on the block.

7. Before replacing the pump block verify that the gold electrode socket on the back of the block is dry. Align the steel shafts with the two large holes of the block, then push the block gently onto the shafts. Slide the pump block until flush against the instrument.

8. Choose the correct capillary. For STR separation use a 47cm x 50µm I.D. Capillary; this is distinguished from the sequencing capillary by a green mark on the window side of the capillary. Always handle the capillary carefully to ensure that the glass window is clean and unbroken.

9. Pull the capillary from the plastic storage tube and inspect and clean the capillary detection window with a Kimwipe and isopropanol or ethanol. **The window area is very fragile and breaks easily!**

10. Partially unscrew the plastic ferrule on the right side of the pump block. Insert the window end of the capillary through the center of the plastic screw through the cone-shaped ferrule until the end stops just before the cross hair section. While holding it in position firmly tighten the ferrule into the pump block to secure the capillary.

11. 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).

12. 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. **Make sure to use heat resistant tape!**

13. **Gently** close the door to the heat plate. Check that the capillary count was reset. Reset count by selecting under **Instrument** menu **Changing Capillary** and reset to 0 (zero) by clicking **OK**.

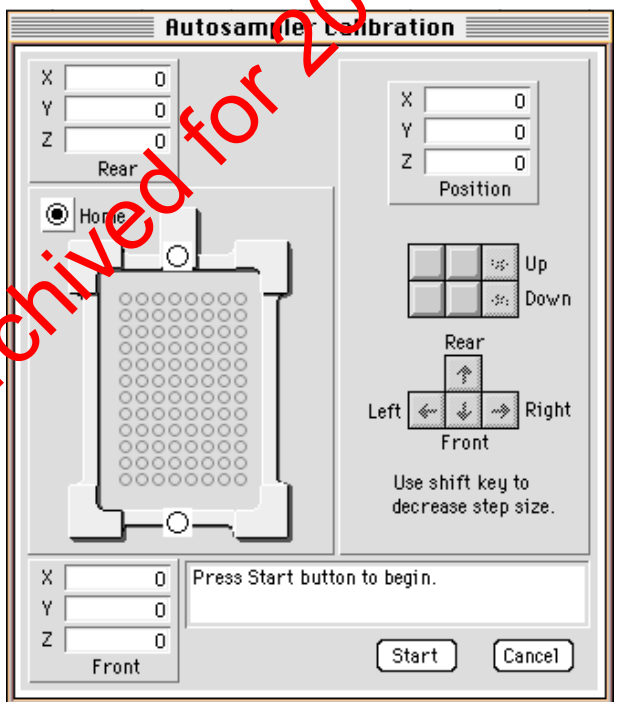
14. Go to **Calibrate Autosampler**, section E.

E. 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.

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**. Continue to set up the instrument.

F. Filling the Glass Syringe, the Pump Block and the Capillary

1. To refill the syringe it has to be removed from the instrument. Under **Window** go to **Manual Control Window**.

   Under the **Function** pop-up menu in the **Manual Control Window** select **Syringe Up** and click **Execute** multiple times 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).

2. Do not remove the plunger but clean the syringe after expelling the remaining POP4 by repeatedly drawing up and expelling DI water from a small beaker. Before really refilling the syringe draw up a small amount of POP4 and expel it again to remove residual water.

   Always let the POP4 warm up to room temperature before using it. Make sure there are no crystals or other particles in the POP4. Manually fill 700µl (0.7 mark) of POP4 into the 1mL glass syringe Do this slowly to prevent the formation of air bubbles. This is **important**: the presence of air bubbles might cause false leak alarms.

   If air bubbles are visible expel again, let POP settle and refill, or 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.

   Rinse exterior with deionized water and dry with a Kimwipe.

3. 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. Tighten firmly.

4. Remove any air bubbles from the block. Open the **Manual Control** menu and from the **Function** pop up menu select **Buffer Valve Close,** (click **Execute**). First open the bottom valve by unscrewing it. Slowly press down on syringe plunger to remove any air bubbles. Tighten waste valve. Repeat process using the ferrule on top of the block.

   Then go back to the **Manual Control** menu, select **Buffer Valve Open** (click **Execute**). Manually push plunger to force any last air bubbles into the anode buffer chamber..

   Make sure there are no air bubbles left 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.

5. Tighten all valves. Under **Manual Control** choose **Buffer Valve Close**, click **Execute**. Do not forget this step.

6. 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. Close the doors.

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

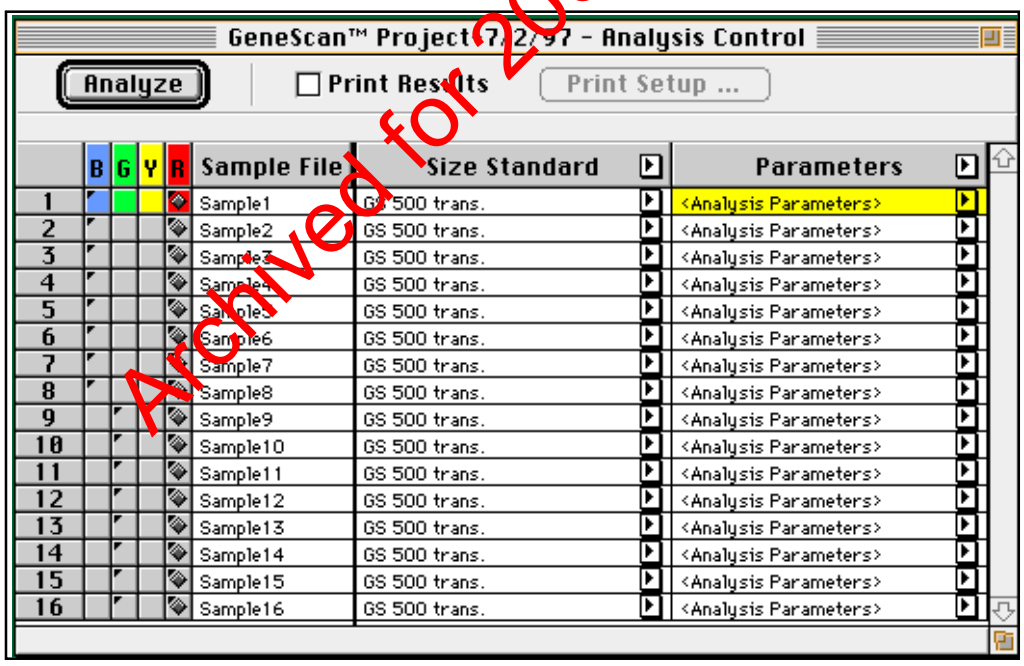
   It is possible to proceed with setting the temperature and filling out the sample sheet while the fill capillary module is running. The only item that cannot be accessed at this point is the injection list.

   Before running any samples it is recommended to perform a CCD color test.

310 Genescan Analysis

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

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



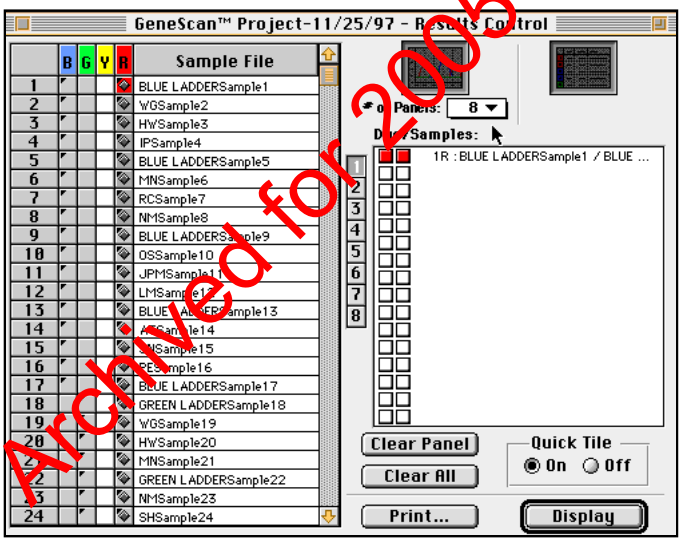
Under the **windows** menu at the top of the screen, select **Analysis Log**. In the Analysis Log window there should be messages indicating that the standard peaks have been sized from 80-400 bp. for each sample. This tells you that each peak has been sized. If this is not the case or you have some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

(the default setting is Promega CXR). To do this select the appropriate size standard from the Standard menu on the Results Control window.

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

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

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



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

The electropherogram results can be displayed as a table with the sizing results only (deselect the left electropherogram icon), as electropherogram only (deselect the right table icon) and as a combination of both (standard setting). Up to 8 display panels can be seen in parallel. Each color per lane can be separately assigned to a panel by clicking on it and choosing the next panel for the next color. All colors can also be viewed as

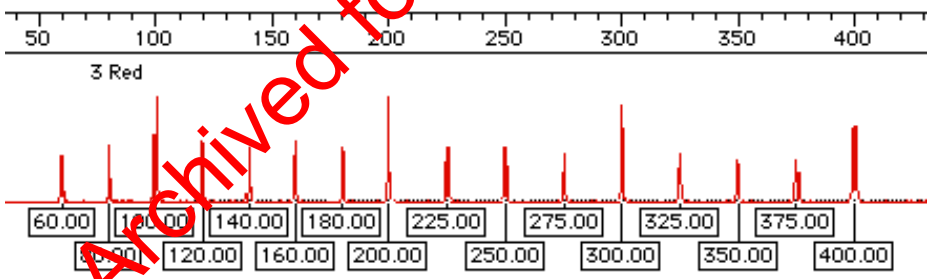
"stacked" electropherograms by assigning all colors to the same panel. It is optional to look at the allele peaks at this point.

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

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

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

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



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

   Under **Window** select **Analysis control.** Click on the arrow in the size standard column for the lane to be reanalyzed. Choose **define new**. The system displays an electropherogram of the size standard fragments in this lane. Highlight the 80bp peak by clicking on it. Enter the number, 80, and press return. Continue to enter the above values for the size standard peaks.

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

Reanalyze the corrected lane.

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

   Start: 3000 Stop: 6500

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

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

5. If some of the red peaks are visible but below 100fu's it is acceptable to change the threshold for the red dye only. Under **Settings** select **Analysis parameters.**

   Change the **peak amplitude threshold** to 25 for the red dye. **Do not change the setting for the other colors.** Reanalyze the samples. Change setting back to 100 before quitting Genescan analysis.

6. 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.

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

FOR GENOTYPER ANALYSIS GO TO THE GENOTYPER SECTION

310 Troubleshooting

See below for a 310 Capillary Instrument Electrophoresis and Analysis Troubleshooting Guide

INSTRUMENT/ELECTROPHORESIS TROUBLESHOOTING		
Observation	**Possible Cause**	**Recommended Action**
1. Error Message: "leak detected"	There is a leak in the pump block. There is a leak in the syringe.	Tighten all valves on the pump block including the ferrule in which the capillary is seated. Tighten, or remove and replace the syringe.
2. The 4 color bands are well above the 2048 mark in the raw data collection window.	There may be an air bubble in the pump block. There may be an air bubble or foreign particle in the capillary or on the capillary window.	Flush the pump block of any air bubbles. 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. Too little or no buffer in position 1 of the autosampler.	Replenish buffer jar. Replenish buffer in position 1 of the autosampler.

	Electrode bent.	Replace or straighten electrode and recalibrate autosampler.
	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.	Tighted valve fittings and syringe.
	Anode buffer valve does not open.	Open buffer valve. Note: the valve should depress easily when you push the top with your finger tip. After you release the pressure the valve should spring to the "open" position. If the valve is stuck, it should be cleaned.
	Capillary plugged, broken, or nonconducting capillary.	Replace the capillary.
	Poor quality water in buffer solutions.	Remake buffer with freshly autoclaved, deionized water.
5. Low current	Small bubble in capillary blocking current flow.	Replenish gel in capillary.

	Plugged, broken, or nonconducting capillary. Poor quality water in buffer solutions. Old, defective, or incorrectly made buffer or polymer solution.	Replace the capillary. Remake buffer with freshly autoclaved, deionized water. Replace buffer or polymer solutions.
6. Fluctuating current	Too little buffer in anodic chamber. Small bubble in capillary blocking current flow. Broken or cracked capillary. Arcing to conductive surface on the instrument.	Replenish buffer jar. Replenish gel in capillary. Replace the capillary. 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. Thoroughly clean and dry the lid and the outside of the tube containing the genetic analyzer buffer (position 1). Use DI water.

ANALYSIS TROUBLESHOOTING		
Observation	**Possible Cause**	**Recommended Action**
1. No peaks or only weak peaks from both the positive control and the DNA test samples at all loci, but the red ladder peaks are present	Prepared sample tubes with Formamide/standard master mix and forgot to add PCR product or added only oil supernatant. Insufficient PCR amplification.	Prepare samples again and rerun. See amplification trouble shooting
2. No peaks at all	Sample not at bottom of tube. Air bubble at bottom of sample tube. Capillary bent out of sample tube. Autosampler not calibrated correctly. Sealed sample tube septum (that is, septum will not open to allow electrode into sample tube). Septum not placed in the sample tube properly.	Spin sample in micro centrifuge. 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. Calibrate autosampler in X,Y, and Z directions. The tip of the capillary should almost touch the Z calibration point. Replace the septum.

ANALYSIS TROUBLESHOOTING		
Observation	**Possible Cause**	**Recommended Action**
3. Low peaks detected for all samples and red standard	Insufficient sample and size-standard added.	Make sure to add 1µL of product and 0.5 µL of red standard to formamide in each tube. Rerun.
	Samples added to formamide that has degraded to formic acid and formate ions (leading to insufficient sample injected).	Use freshly deionized formamide.
	Sample and size-standard not thoroughly mixed with formamide.	Mix sample and size-standard into formamide by pipetting up and down several times.
4. High baseline	Dirty capillary window.	Clean capillary window with 95% ethanol.
	Capillary moved out of position in front of laser window.	Position capillary in front of laser window.
	Incorrectly prepared and/or old buffer or polymer solutions.	Replace buffer and polymer with fresh solutions.
	Defective capillary.	Replace the capillary.
	Wrong or deteriorated matrix file used.	Select the correct matrix file. Notify QC to make a new matrix (QC210). Renalyze with the new matrix.

ANALYSIS TROUBLESHOOTING		
Observation	**Possible Cause**	**Recommended Action**
5. Noisy baseline	Incorrectly prepared and/or old buffer or polymer solutions. Dirty capillary holder aperture. Defective capillary	Replace buffer and polymer with fresh solutions. Clean the capillary holder with deionized water. Replace the capillary.
6. Spikes in baseline	Precipitate in the POP-4 polymer solution. Old polymer (POP-4)	Allow polymer to equilibrate to room temperature before adding to capillary. Use fresh polymer.
7. Not all expected ladder fragments and/or only part of the allele peaks are visible.	Analysis range was too small. Wrong preparation of formamide/size-standard/PCR product, e.g. insufficient mixing.	Under **Analysis parameters** check Analysis Range, correct to appropriate range (3000-6000) and Analyze again. Prepare samples again and rerun samples.
8. Positive signal from the positive controls, but no or below 100 signal from DNA test sample.	Quantity of DNA test sample is below the assay sensitivity or DNA quality is insufficient. Sample prepared improperly for capillary injection.	See amplification troubleshooting. Prepare fresh sample and rerun.

ANALYSIS TROUBLESHOOTING		
Observation	**Possible Cause**	**Recommended Action**
9. Allele peaks for the positive controls and the test samples are visible but not sized correctly.	Size standard was not defined correctly.	Under **Analysis** open **define size standard** from the pop up menu, check peak assignment and entered sizes. Reanalyze project with correct standard, check each sample for proper peak assignment.
	Samples were not loaded with formamide or not properly heat denatured before loading.	Rerun samples.
10. Presence of unexpected or additional peaks in the amplified positive controls sample.	Amplification problem	See amplification troubleshooting
	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.
	Change in room temperature	Repeat run. Space additional allelic ladders.

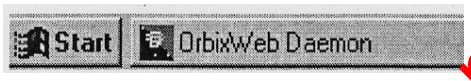
ANALYSIS TROUBLESHOOTING		
Observation	**Possible Cause**	**Recommended Action**
13. Runs get progressively faster (that is, size-standard peaks come off at lower and lower scan numbers.	Water in the syringe. Change in room temperature	Prime syringe with small amount of polymer, invert syringe to coat syringe walls, and discard polymer. Then fill syringe with fresh running polymer. Repeat run. Space additional allelic ladders.
14. Some, but not all, loci are visible for the test samples.	Quantity of DNA test sample is below the assay sensitivity or DNA quality is insufficient.	See amplification troubleshooting
15. Poor resolution	Poor capillary performance. Incorrectly prepared and/or old buffer or polymer solutions. Syringe leaking and old polymer doesn't get totally replaced for each injection. Injection sheet settings wrong.	Replace capillary. Replace buffer and polymer with fresh solutions. Replace syringe, or remove and clean syringe with hot water, reassemble and refill. 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) If they were, change them back and rerun.

ANALYSIS TROUBLESHOOTING		
Observation	**Possible Cause**	**Recommended Action**
16. Peaks which progressively decrease in height and definition (resembling a "sine wave") to no visible peaks at all.	"Death of the Capillary" caused by exceeding the recommended capillary count (150), or contamination of the inside of the capillary by an agent which has degraded the interior coating.	Change the Capillary, buffer, POP4, and formamide and rerun samples. Make sure the correct loading buffer is being used.
17. Pull-up (bleed through) peaks visible for peak heights below 1000. **Raised, e.g. green, baseline between two high, e.g. blue, peaks.** **Indentations in, e.g. green, at the scan position of a high, e.g. blue, peak.**	Matrix artifacts caused by the application of a wrong or a deteriorated matrix.	Renalyze run with a different matrix. Notify QC that a new matrix should be run. Renalyze run with new matrix.

Only the Cofiler, Profiler Plus, and YM1 (Y-STR) amplification systems have been validated for casework. PowerPlex 16 has been validated for Exemplar/Reference samples only, and can be run on this instrument.

How to start

Turn on the computer. Make sure computer is fully booted and the OrbixWeb Daemon is running. This is indicated by the presence of the following OrbixWeb Daemon icon and text next to the **Start** icon on the lower left portion of the desktop.



If the instrument is not on, turn it on. The status bar light will change from solid yellow (indicates instrument is booting) to blinking yellow (indicates machine is communicating with computer) and then to solid green (indicates instrument is ready for command).

NOTE: Collection cannot be launched unless the light on the instrument is green! Important phrase to remember, "If the light isn't green, the machine becomes mean!"

The program that is used to operate the run and collect the data is the ABI Prism 3100 Collection Software. To begin collection, double click on the **3100 Collection Software** icon.

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

1. If the number of injections you are running plus the capillary usage number will exceed 150 injections, proceed with **Section A "Changing and Installing the Capillary".**

2. If the amount of POP4 in the installed syringe is low (<600 µL/tray) or greater than one week old, you will have to change it. Start with **Section C "Changing\Filling Syringes with POP4".**

3. If the capillary and POP4 are fine at the first run of the day, start with **Section D "Changing the Buffer"** to change the buffer and water.

4. If the instrument has been run prior today and buffer and water had been changed then proceed to Section E "Creating a Plate Record Through Excel".

A. Changing and Installing the Capillary

The 3100 capillary array is a replaceable unit composed of 16 silica capillaries. The capillary usage number refers to the number of times the array has injected a set of 16 samples. Check the capillary array usage number by clicking on the **Status View** tab at the top of the collection screen. If this number, plus the number of your injection sets (6 for a 96 well plate) exceeds 150, the capillary array should be changed.

To replace a capillary array or to install a capillary array on an instrument, close the oven and instrument doors. From the **Tools** menu, select **Install Capillary Array Wizard**. Follow the directions given in the wizard to replace or install an array.

The steps involved in the **Capillary Array Wizard** are:

1. From the Capillary Array Wizard window, select the **Remove and Discard the Array** option. Click **Next**.

2. Push the tray button. Wait for the tray to stop moving before opening the instrument doors. The Capillary Array Wizard will then instruct you to "open instrument door, oven door, and detection block door".

3. Unclip the leading-end head combs of the array from the oven by pulling on the pins to the left and right of the comb.

4. To loosen the screw holding the end of the capillary inside the upper polymer block, unscrew towards you. Remove the syringe guard and pull the upper polymer block out towards you, gently but with a bit of force, until you feel the block reach a stop point. The capillary detection cell will pop out of position. Remove the capillary from the oven slots holding it in place.

5. Completely remove the screw and ferrule holding the end of the capillary inside the upper polymer pump block. Remove the capillary assembly and discard it.

6. Completely unscrew and remove syringes and tubing from upper and lower polymer blocks. Remove upper and lower polymer blocks from instrument. Clean the upper polymer block with hot, NOT BOILING, water using the cleaning syringe. Also clean the ferrule and screw. A final rinse of deionized water should be done. Vacuum or air dry the block and put the block back in the instrument without sliding either block all the way in. Click **Next**.

7. Select **Install a New Capillary Array** from the options. Enter the **Serial Number** of the capillary array located on the capillary array box. Also record the serial number of the capillary and the date in the 3100 Capillary Electrophoresis Log binder. Keep the capillary box on top of the instrument for future use. Click **Next**.

8. Slide the capillary leading-end head comb holder into the oven and snap the loading end of the array in place. To secure the capillary comb in place, push both buttons until they snap into place.

9. Place the capillary placement slots into their respective positions in the oven.

10. Remove the detection window protection clip from the capillary window. Make sure the window is clean (no dust or smudges). Slide the capillary assembly into the upper polymer block with the screw and ferrule and place the capillary detection cell in place. Push the polymer block back into place. Tightly screw the array assembly into the polymer block. Click **Next**.

11. Close the laser detection door and screw it shut. Close the oven door.

12. Fill the clean syringes with polymer and install them on the polymer block. Return the syringe guard. Click **Next**.

13. Push the pistons on both syringes slowly to remove any air bubbles. Continue to push the piston of the large syringe to fill both the upper and lower block with polymer.

    IMPORTANT: Pay close attention to any air bubbles trapped next to the end of the capillary assembly and remove them. Also, make sure that all air bubbles are removed from the assembly tubing connecting the upper and lower polymer block. This is a non-transparent tube and bubbles are not visible through it. Any bubbles left in it can cause electrophoresis problems.

14. Close the instrument doors and wait for the autosampler to stop moving. Click **No** if there is no air bubbles remaining. Click **Next**.

15. Click **Fill** to fill the new capillary array. This will take a few minutes. Wait for the **Finish** button to become active, and then click it.

16. Push the **Tray** button to present the tray. Once the autosampler has stopped moving, open the doors and change the buffer in the anode jar and the water and buffer in the reservoirs in the autosampler. Close the doors and Click **Finish.** The software will not accept the new information unless the Finish button is clicked.

17. Once the capillary array wizard is finished, a spatial calibration must be done as described in the following section.

18. Monitor the EP Current closely during the first injection. Make sure the EP Current is not fluctuating too much (ex. going from 20 to 160 then down again, etc.) Fluctuation of the EP Current is a good indicator that bubbles may be present and if the run is stopped immediately, the lower block can be saved. If, after 20 minute of the first run, the EP Current is constantly around 150 or 160, it's good.

B. Performing a Spatial Calibration

A spatial calibration provides information about the position of the fluorescence from each capillary on the CCD. It must be performed after each time a capillary array is replaced or temporarily moved from the detection block (as is done during the change of the POP4).

1. Select **Perform Spatial Calibration** from the **Tools** menu.

2. Click **Start.** The calibration will take 2 or 6 minutes (depending on if the capillaries are being filled.

3. Upon completion, a dialog box will indicate if the spatial calibration was successful. If successful, click **Details** to view the **Spatial Calibration Profile** window. Successful results yield 16 sharp peaks with similar heights having position values from 13 to 16 units (15 is optimal) higher for each subsequent capillary. A good spatial should be greater than 8000 units high.

4. If the peaks do not fit these specifications, click **OK** to return to the previous screen, press **Start** to perform another test. Follow step 3. If the spatial still does not fit the requirements, click **OK** to return to the previous screen. Select the **Fill capillaries** check box, then click **Start**.

5. If the peaks fit the specification in step 3, click **OK** to get back to the previous dialog box. Click **OK** and then **Yes** to accept the spatial calibration that was just performed.

6. If the spatial calibration is continuously unsuccessful, see the 3100 troubleshooting section.

Proceed with section E

C. Changing/Filling Syringes with POP4

Check the amount of POP4 that remains in the reservoir syringe (the large 5.0 mL syringe on the left). For a full tray (i.e. 96 samples or 6 injections), there should be at least 600 µL of POP4 in the reservoir syringe or the run will not start. The array fill syringe (smaller 250 µL syringe on the right) will be filled automatically by the reservoir syringe when it comes time to fill the capillary array. **Never add fresh polymer to old polymer.**

Check the 3100 Capillary Electrophoresis Log binder to see when the polymer was last changed. The polymer should be changed once a week. Document the change of polymer and its lot number in the 3100 Capillary Electrophoresis Log binder.

Completely unscrew and remove both syringes and tubing **(tubing damages easily!)** from upper and lower polymer blocks. Remove upper and lower polymer blocks from instrument. Clean the upper polymer block with hot **(not boiling!)** water using the cleaning syringe. Also clean the ferrule and screw. A final rinse of deionized water should be done. Vacuum or air dry the blocks and then put them back in the instrument **(gradually slide them back into place so as not to damage the tubing connecting them)** without sliding either block all the way in.

From the **Tools** menu, select **Change Polymer Wizard**. Follow the directions given in the wizard to put fresh polymer on the instrument.

The steps involved in the **Change Polymer Wizard** are:

1. From the Polymer Wizard window, click **Close** to close the anode buffer pin valve and **Home** to home the syringes. Click **Next**.

2. Click **Same Polymer Type**, the instrument uses only POP4 polymer at this time. Click **Next**.

3. Fill both syringes with new polymer (choose the volume to fill the larger syringe with based on the anticipated instrument use for the following week). Make sure the syringes are free of any air bubbles and install both syringes on the polymer block. Click **Next**.

4. Enter the polymer **lot number** and **expiration date**. Click **Open** to open the anode buffer. Click **Next.**

5. Push the pistons on both syringes slowly to remove any air bubbles in the block (the small syringe works best for removing bubbles stuck in the corner of the block nearest the capillary). Continue to push the piston of the large syringe to fill both the upper and lower block with polymer and insure all air bubbles have been flushed into the lower buffer chamber.

   IMPORTANT: Pay close attention to any air bubbles trapped next to the end of the capillary assembly and remove them. Also, make sure that all air bubbles are removed from the assembly tubing connecting the upper and lower polymer block. This is a non-transparent tube and bubbles are not visible through it. Any bubbles left in it can cause electrophoresis problems.

6. Click **No** when all of the air bubbles are gone. Click **Next.**

7. Replace the buffer in the anode jar (see section D) and Click **Finish.**

8. The capillary must be flushed of old POP4 with new POP4. From **Instrument** in the main menu, select **Manual Control.** Under the Command category select **Capillary**, in Command name select **Fill**, under value select **36cm.**

9. Monitor the EP Current (displayed in status window) closely during the first injection. Check and make sure the EP Current is not fluctuating too much (ex. going from 20 to 160 then down again, etc.) Fluctuation of the EP Current is a good indicator that bubbles may be present and, if the run is stopped immediately, the lower block can be saved. If, after 20 minute of the first run, the EP Current is constantly around 150 or 160, it's within the normal range.

NOTE: You must change the polymer weekly. The polymer is good at 25°C for about 7 days.

ATTENTION: IF the pump block was removed and the capillary window was disturbed, you must perform a spatial calibration (see B.) before running samples.

D. Changing the Buffer

The cathode buffer and water reservoirs located on the autosampler tray and anode buffer jar located below the lower pump block should be changed at the beginning of each day the instrument is in use.

1. To fill the water and cathode buffer reservoirs on the autosampler, close the instrument doors and press the tray button on the outside of the instrument to bring the autosampler to the forward position. **Wait until the autosampler has stopped moving and then open the instrument doors.**

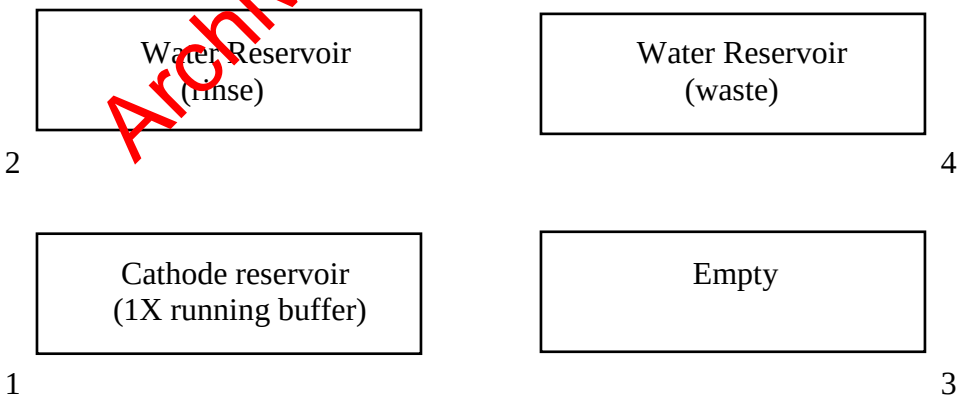
2. Remove all reservoirs and anode jar and dispose of the remaining fluid. Rinse and fill the water reservoirs to the line with deionized water and the cathode reservoir and anode with 1X running buffer, about 16mL each. Dry the outside and inside rim of the reservoirs using a Kimwipe and place a clean septa strip on each reservoir, as needed.

   Place the reservoirs in the instrument in their respective positions, as shown below.

3. Close the instrument doors.

NOTE: When using the 3100 for fragment analysis, it is not necessary to fill the water reservoir in position #3. This reservoir is used only for DNA sequencing applications.

Position of reservoirs in autosampler:



NOTE: Be sure that the septa fit snugly and flush on the tops of the reservoirs in order to prevent damage of the capillary tip. Also, it is important that there is no condensation on the inside of the reservoir. The septa strips must be dry!

E. Creating a Plate Record Through Excel

This excel sheet will be imported into the 3100 collection software.

1. Double click on the **Sample Sheet** icon on the desktop to open the sample sheet template.

2. Make sure that the "Sample Sheet" tab is selected (bottom left of the screen).

3. Fill in the sample sheet name in cell E3.

   IMPORTANT: When naming a plate/sample sheet, you may use letters, numbers, and the following characters only: **-_(){}#.+. Also, no spaces between characters are allowed. If an incorrect character is used in the sample sheet name, you will not be allowed to import the plate record into the 3100 collection software.**

   Casework sample sheets should be named indicating the instrument, the year and the consecutive run number for the multiplex, e.g.:

   Stars04-021Co-009Pro or Rudy04-004Co or Stripes04-015Y

4. Fill in the sample information in the **case number-sample description-tube label** columns. Room has already been reserved for the Allelic Ladders in wells A1, A3, A5, A7, A9, A11 (the grey cells), and the positive control in well B1 (the positive control may be edited, though the ladders may not, as it is mandatory that there be a ladder included with each injection set).

   IMPORTANT: As a result of a bug in Excel, the cut and paste function should not be used. Instead, highlight the cell(s) you wish to move and press <ctrl-c>. Right click on the first destination cell and choose "Paste Special...". Under "Paste" select "Values" and click OK.

5. In the "Sys." column, fill in the appropriate letter for the system in which the sample was amplified: 'P' for **P**rofiler Plus, 'C' for **C**ofiler, 'I' for **I**dentifiler, 'M' for Y**M**1, and 'X' for Powerple**X** 16. For reruns, use: 'PR' for **P**rofiler Plus **R**eruns, 'CR' for **C**ofiler **R**eruns, 'IR' for **I**dentifiler **R**eruns, 'MR' for Y**M**1 **R**eruns, and 'XR' for Powerple**X** 16 **R**eruns.

   IMPORTANT: If you do not specify the system, sample data will not be collected. If you fill in the wrong system, sample data will be collected incorrectly.

IMPORTANT: The 3100 uses the same injection conditions (time/voltage) for all 16 samples in a given injection. Therefore, samples that need to be run with non-standard conditions (such as most reruns) cannot be part of the same injection as normally run samples.

6. Save the sample sheet by selecting **Save As** from the File menu. Save in D:\appliedbio\3100\Bin\Sample Sheets (.xls files). This way you will have the file backed up in case further changes or additions are necessary.

7. If the sample sheet is full and/or you are ready to run the plate, print it out. If you only have filled out the first page, please set the printer to print only from page 1 to page 1.

8. Click on the **Second Tab** labeled "SaveAs.plt" or "text" on the bottom left of the screen. Select **Save As** from the File menu and do the following:

   - Change the file directory to D:\appliedbio\3100\Bin\PlateRecords(.plt).
   - Change **Save as file type** to "text(Tab-delimited)(*.txt)".
   - Change the file name so that its extension is **.plt** and there are no spaces, the file name should be of the format: *yourfilename.**plt***

9. Quit Excel. You will be prompted to save again by Excel, this is not necessary so select **NO**.

10. Open the 3100 Collection software. Select the **Plate View**. Click on the **Import** button. Open the Plate Records (.plt) folder and select your plate record file. Click **OK**.

F. Preparing and Running the DNA Samples

Before preparing the samples, turn on the oven. This saves time. The oven would turn on automatically after a run is started, but in order to monitor the first injection one would have to wait for the oven to reach 60°C.

1. Under **Instrument**, select **Manual Control**. Under the **Command category** select **Oven**. Make sure the **Set State** is selected under **Command Name** and under **Value** turn the oven **On.** Click **Send Command**.

2. Now change the **Set State** button to **Set Temperature** and under **Value** enter **60.** The oven will not heat unless it is turned on first, then the temperature can be set. Click on **Send Command.**

3. Arrange amplified samples in a 96-well rack according to how they will be loaded into the 96- well reaction plate. Sample order is as follows: A1, B1, C1, D1... G1, H1, A2, B2, C2... G2, H2, A3, B3, C3, etc. Thus the plate is loaded in a columnar manner where the first injection corresponds to wells A1-H2, the second A3-H4 and so on. Remember that an allelic ladder is run as the first sample of each injection. Therefore, leave room for the allelic ladders in A1, A3, A5, etc.

   Label the side of the reaction plate with the name used for the Plate Record with a sharpie and place the plate in the plate base (see diagram on page 102).

For Cofiler and Profiler:

4. For N+2 samples mix 9.5 µL of HiDi Formamide with 0.5 µL of GS500 Rox Standard per sample. Mix thoroughly by vortexing.

   Use the following volumes for the necessary number of samples:

# Samples +2	HiDi Formamide	GS500 Standard
18	171 µL	9 µL
34	323 µL	17 µL
50	475 µL	25 µL
66	627 µL	33 µL
82	779 µL	41 µL
98	931 µL	49 µL
114	1083 µL	57 µL
130	1235 µL	65 µL

5. Aliquot 10 µL of the formamide/standard mixture into each well being used on the 96-well reaction plate.

   IMPORTANT: If one injection has less than 16 samples add 12ul of either dH_2O or buffer or formamide/standard mix to all unused wells.

Have someone witness the tube setup by comparing the tube labels and positions indicated on the sample sheet with the tube labels and positions of the tubes themselves (tubes should be laid out to correspond with their respective positions in the 96 plate). Add 1 µL of allelic ladder to the respective tube(s). Add 2 µL of PCR product to each tube according to the sample sheet.

When samples are being rerun to obtain more information, use 4 µL of PCR product and 4 µL of a 1/10 dilution of the positive control and allelic ladder.

IMPORTANT NOTES FOR RE-RUN SAMPLES:

1. Re-runs "high" **cannot** be on the same injection as non-re-run samples.
2. Re-runs "high" **must always** be the last injection(s) of the run and **must always** have positive controls and allelic ladders run under the same "high" conditions.
3. Re-runs "normal" may be integrated with non-re-run samples.

When adding PCR product, make sure to pipet the solution directly into the formamide and gently flush the pipet tip up and down a few times to mix it.

Proceed to step 6.

For PowerPlex 16:

4. For N+2 samples mix 9 µL of HiDi Formamide with 1.0 µL of ILS600 Size Standard per sample. Mix thoroughly by vortexing.

Use the following volumes for the necessary number of samples:

# Samples +2	HiDi Formamide	ILS600 Standard
18	162 µL	18 µL
34	306 µL	34 µL
50	450 µL	50 µL
66	594 µL	66 µL
82	738 µL	82 µL
98	882 µL	98 µL
114	1026 µL	114 µL
130	1170 µL	130 µL

You might need more than N+2 if you are working with microtiter plates and a multichannel pipet.

Aliquot 10 µL of the formamide/size standard mixture into each well being used on the 96-well reaction plate.

<u>IMPORTANT</u>: If one injection has less than 16 samples add 12ul of either dH_2O or buffer or formamide/standard mix to all unused wells.

Have someone witness the tube setup by comparing the tube labels and positions indicated on the sample sheet with the tube labels and positions of the tubes themselves (tubes should be laid out to correspond with their respective positions in the 96 plate). If you are aliquoting from a 96 well tray, you do not need a witness. Add 1 µL of allelic ladder to the first well of each injection. Add 2 µL of PCR product to each tube according to the sample sheet.

When samples are being rerun to obtain more information, use 4 µL of PCR product and 4 µL of a 1/10 dilution of the positive control and allelic ladder.

IMPORTANT NOTES FOR RE-RUN SAMPLES:

1. Re-runs "high" **cannot** be on the same injection as non-re-run samples.
2. Re-runs "high" **must always** be the last injection(s) of the run and **must always** have positive controls and allelic ladders run under the same "high" conditions.
3. Re-runs "normal" may be integrated with non-re-run samples.

When adding PCR product, make sure to pipet the solution directly into the formamide and gently flush the pipet tip up and down a few times to mix it.

<u>Proceed to step 6.</u>

For YM1 STRs:

4. For N+2 samples mix 9.5 µL of HiDi Formamide with 0.5 µL of LIZ Size Standard per sample. Mix thoroughly by vortexing. Use the following volumes for the necessary number of samples:

# Samples +2	HiDi Formamide	LIZ Standard
18	171 µL	9 µL
34	323 µL	17 µL
50	475 µL	25 µL
66	627 µL	33 µL
82	779 µL	41 µL
98	931 µL	49 µL
114	1083 µL	57 µL
130	1235 µL	65 µL

5. Aliquot 10 µL of the formamide/standard mixture into each well being used on the 96-well reaction plate.

IMPORTANT: If one injection has less than 16 samples add 12ul of either dH_2O or buffer or formamide/standard mix to all unused wells.

Have someone witness the tube setup by comparing the tube labels and positions indicated on the sample sheet with the tube labels and positions of the tubes themselves (tubes should be laid out to correspond with their respective positions in the 96 plate). Add 2 µL of PCR product to each tube according to the sample sheet.

When samples are being rerun to obtain more information, use 4 µL of PCR product and 4 µL of a 1/10 dilution of the positive control and allelic ladder.

IMPORTANT NOTES FOR RE-RUN SAMPLES:

1. Re-runs "high" **cannot** be on the same injection as non-re-run samples.
2. Re-runs "high" **must always** be the last injection(s) of the run and **must always** have positive controls run under the same "high" conditions.
3. Re-runs "normal" may be integrated with non-re-run samples.

When adding PCR product, make sure to pipette the solution directly into the formamide and gently flush the pipette tip up and down a few times to mix it.

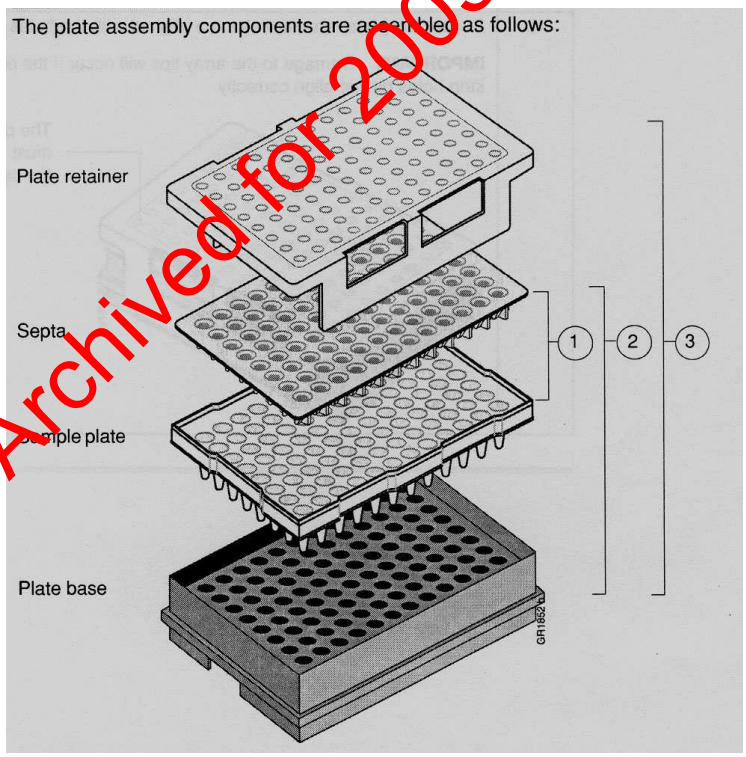
Proceed to step 6.

All Multiplexes:

6. Once all of the samples have been added to the master mix, place a new 96-well Septa over the reaction plate and firmly press the septa into place. Spin plate in centrifuge for one minute.

7. Remove the reaction plate from the base and heat denature samples at 95°C for 5 minutes followed by a quick chill at 4°C for 5 minutes using the denature/chill program on an ABI 9700 thermal cycler. **Make sure to keep the thermal cycler lid off of the sample tray to prevent the septa from heating up.** After removing tray from the heat block, check the wells for air bubbles. If there are any, centrifuge the tray to remove any air bubbles.

8. Once denatured, place the reaction plate into the plate base. Secure the plate base and reaction plate with the plate retainer.



IMPORTANT: Damage to the array tips will occur if the plate retainer and septa strip holes do not align correctly.

Do not write on the septa with pen, markers, sharpies, etc. Ink may cause artifacts in samples. Any unnecessary markings or debris on the septa may compromise instrument performance.

Placing the Plate onto the Autosampler

The Autosampler holds up to two, 96-well plates in tray positions A and B. To place the plate assembly on the autosampler, there is only one orientation for the plate, with the notched end of the plate base away from you.

1. Push the tray button on the bottom left of the machine and wait for the autosampler to move forward and stop at the forward position.

2. Open the doors and place the tray onto the autosampler in the correct tray position, A or B.

3. Ensure the plate assembly fits flat in the autosampler. Failure to do so may allow the capillary tips to lift the plate assembly off of the autosampler.

   When the plate is correctly positioned, the plate position indicator on the **Plate View** page changes from gray to yellow. Close the instrument doors and allow the autosampler to move back to the home position.

   NOTE: When removing a plate from the autosampler, be careful not to hit the capillary array. Plate B is located directly under the array, so be especially careful when removing this tray.

Linking the Plate Record to a Plate

1. On the **Plate View** screen, click on the plate record in the **Pending Plate Record** table that you are linking. Then click the plate position (A or B) that corresponds to the plate you are linking.

2. Once the plate has been linked, the:
   - **Run Instrument** button on the toolbar becomes highlighted, meaning that the instrument is ready to run.
   - Plate position indicator for the linked plate changes from yellow to green
   - Plate record moves from the **Pending Plate Records** table to the **Linked Plate Records** table

   NOTE: It may take a minute for the plate record to link to the plate depending on the size of the sample sheet.

   If two plates are being run, the order in which they are run is based on the order in which the plates were linked.

3. Click the **Run View** tab to view the run schedule. The **RunID** column indicates the folder number(s) associated with each injection in your run. These folder number(s) should be recorded in the **3100 Log** binder along with the run control sheet name. Click **Run** to start the run. NOTE: Before starting a run, check for air bubbles in the polymer blocks.

   To check the progress of a run, click on the **Array View** or **Capillary View** tab at the top of the collection screen. The **Array View** window will show the raw data of all 16 capillaries at once. The **Capillary View** window will show you the raw data of the capillaries you select to view.

   IMPORTANT: Always exit from the **Array View** and the **Capillary View** windows. During a run, do not leave these pages open for extended periods. This may cause unrecoverable screen update problems. Leave the **Status View** window open.

   The visible settings should be:
   EP voltage 15kV
   EP current (no set value)
   Laser Power Prerun 7.5mW
   Laser Power During run 15mW
   Laser Current (no set value)
   Oven temperature 60°C

   Expected values are:
   EP current constant around 120 to 150µA
   Laser current: 4.5A

   It is good practice to monitor the initial injections in order to detect problems.

Unlinking a Plate:

1. In the **Linked Plate Records** table of the **Plate View** page, select the plate record that you want to unlink.

2. Click **Unlink**. The plate record will return to the Pending Plate Record table and the plate position indicator will return to yellow.

3100 GENESCAN ANALYSIS

When a run is complete, it will automatically be placed in the current run folder, properly labeled with the *instrument name, date and runID* (e.g. **Run_3100_Sears_2004-1-31-85**). Before processing the samples, organize the folders for each injection into one common folder. This folder should be named the same as your run sample sheet/plate record. NOTE: If a run has 6 injections, you will have 6 folders for that run.

To view the data from the run, open the 3100 GeneScan Analysis Software for Windows NT by double-clicking on the **GeneScan** icon on the desktop. The analysis program will launch.

Creating A New Project

To create a new project, under **File** select **New**. The **Create New** dialog box appears.

1. Click the **Project** icon. An untitled **Analysis Control** window opens.

2. To add sample files to the open analysis control window, click on **Project** from the menu options and select **Add Sample Files**.

3. When the **Add Sample Files** dialog window appears, find the **Current Run** folder containing the injection folders with the samples that you want to add to the project. Find the specific injection folder(s) that you have previously documented on your sample sheet and open by double-clicking on the folder icon of interest. At this point you will see icons representing each individual sample, all belonging to one injection.

To add samples to a project, take the following action:

If you want to...	Then...
Select a single sample file	Double-click the file OR select the file and click **Add**
Select all the sample files	Click **Add All**
Add a continuous list of sample files	a. Click the first sample that you want to add. b. Press the **Shift key** and click the last sample you want to add. Click **Add**. All the files between the first and last file are selected.
Add a discontinuous list of samples	a. Click the first sample that you want to add b. Press the **Control key** and then click on the other sample(s) you want to add. Click **Add**. All the files you selected will be highlighted and selected.

4. Click **Finish** when you have added all of the samples.

Project File Analysis

The **Analysis Control** window shows in separate columns the dye lanes, sample file names, size standard options, and analysis parameters to choose for each lane. Boxes for the red dye lane should be marked with diamonds to indicate that red is the color for the size standard.

The samples have been auto analyzed and the matrix was installed during collection. The 3100 GeneScan Software does not place check marks in the dye lane boxes to indicate that the sample has been analyzed. Normally it is not necessary to reanalyze the samples.

The auto analysis should have been performed using the following predefined files:

System	Size Standard File	Analysis Parameter File
Cofiler	75-450.szs	GS500Analysis.gsp
Profiler Plus	75-450.szs	GS500Analysis.gsp
PowerPlex 16	ILS600.szs	ILS600.gsp
YM1	Ystr.szs	YM1.gsp

NOTE: A new or different matrix cannot be installed on sample files after a run has been completed because the matrix is applied to the samples during sample collection and becomes an integral part of the data. Although the 3100 Genescan Software for Windows NT has the option to "Install New Matrix", once the samples have been run a new matrix cannot be applied. If samples need a new matrix, a new spectral calibration (matrix) must be generated and the samples must be rerun using the new matrix.

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

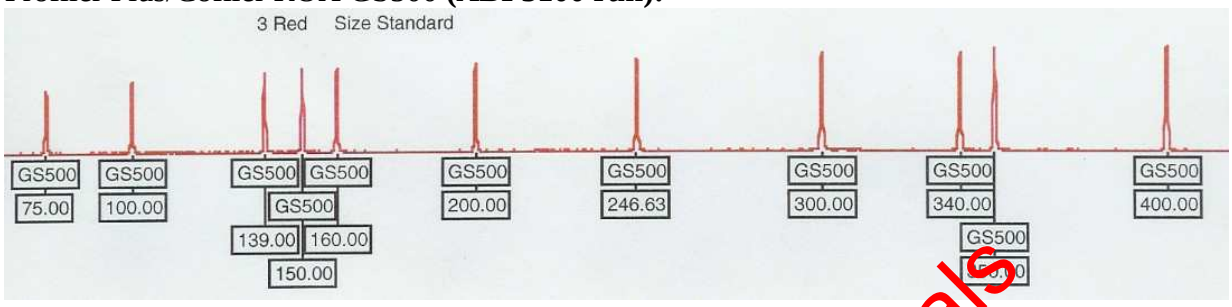
5. To view the analysis results, select **Windows** from the main menu and click on **Results Control**. The analyzed colors for each lane are shown in dark grey. The white squares mean that this color has not been analyzed.

6. The raw data can be seen in up to 8 display panels, by changing the **# of panels** to **8**. To view each color separately, check **Quick Tile** to **On**.

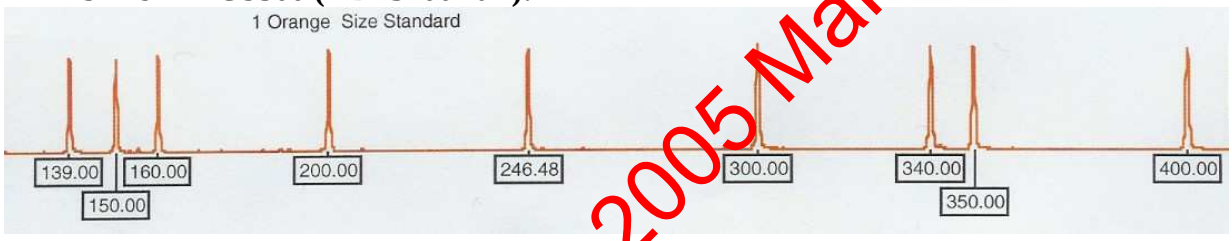
7. Select the first 8 red dye lanes by clicking on them and then click **Display**. Each sample standard will be displayed in its own window. To view all 8 standards, you must scroll through all of the windows. Continue checking your size standard for the entire tray by going back to the **Results Control** window, clicking on **Clear All** and selecting the next 8 samples. Repeat these steps until all of the sample size standards have been checked.

   IMPORTANT: For ABI 3100 runs, the 250bp fragment in GS500 may not be labeled as 250. However, for ILS600 and CXR standards (on the ABI 310), the 250bp fragment must be labeled as such.

Profiler Plus/Cofiler ROX GS500 **(ABI 3100 run):**

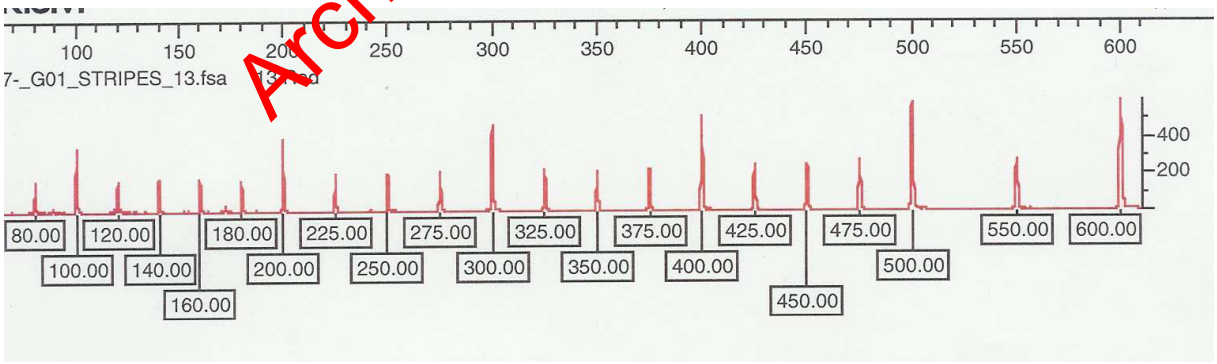


YM1 STRs LIZ GS500 **(ABI 3100 run):**



PowerPlex16 ILS600:

Before proceeding with the Genotyper analysis, under **File** select **Save Project As.** The project will be named according to the Sample Sheet name. Quit GeneScan Analysis by going to **File**



and Selecting **Exit**.

Analysis Problems

1. **Size standard peaks incorrect – redefine size standard**

   From the **Analysis control window**, go to the **Size Standard** column and click on the small arrow on the right side of the cell of one of your samples, click on it and go to **Define New**.

   From here a window displaying that samples size standard will appear. Label all necessary peaks as shown above.

   For ABI 3100 runs, skip the 250bp peak since it may not be labered as 250. If you label this peak with a value, your analysis will FAIL.

   When you are done defining the new size standard, click the "X" in the upper right corner of the box and save the new standard that you defined by clicking on "Save". Name the size standard whatever you wish. Select this size standard for the analysis of all the failed samples.

2. **Samples were not analyzed**

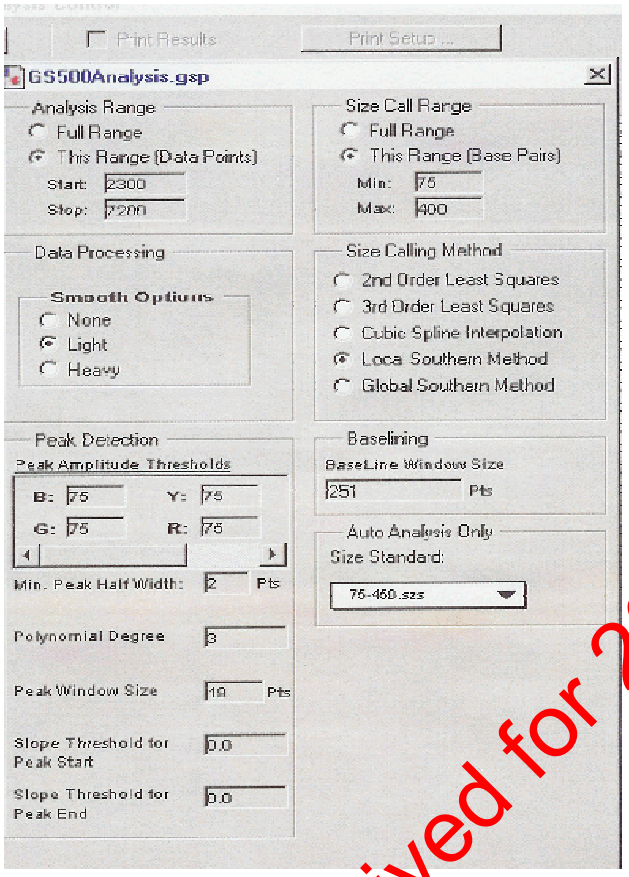
   If the auto-analysis did not work for the whole sample set it is possible that auto analysis was not selected before the collection or that the wrong .gsp file was attached. In the sample list, go to the **Parameter** drop down menu (click on arrow) and select **"GS500analysis.gsp** for Cofiler and Profiler Plus or **"LS600.gsp"** for Powerplex 16. Reanalyze samples.

   It is also possible, that the run was either to fast or to slow. The analysis range may need to be changed. Look at raw data by highlighting a sample and under **Sample** choose **Raw data**.

   Under **Settings** go to **Analysis Parameters**. The default analysis range for Profiler Plus and Cofiler is 2300 to 7200 data points (see picture below), for Powerplex 16 the setting is 1800 to 10000 data points.

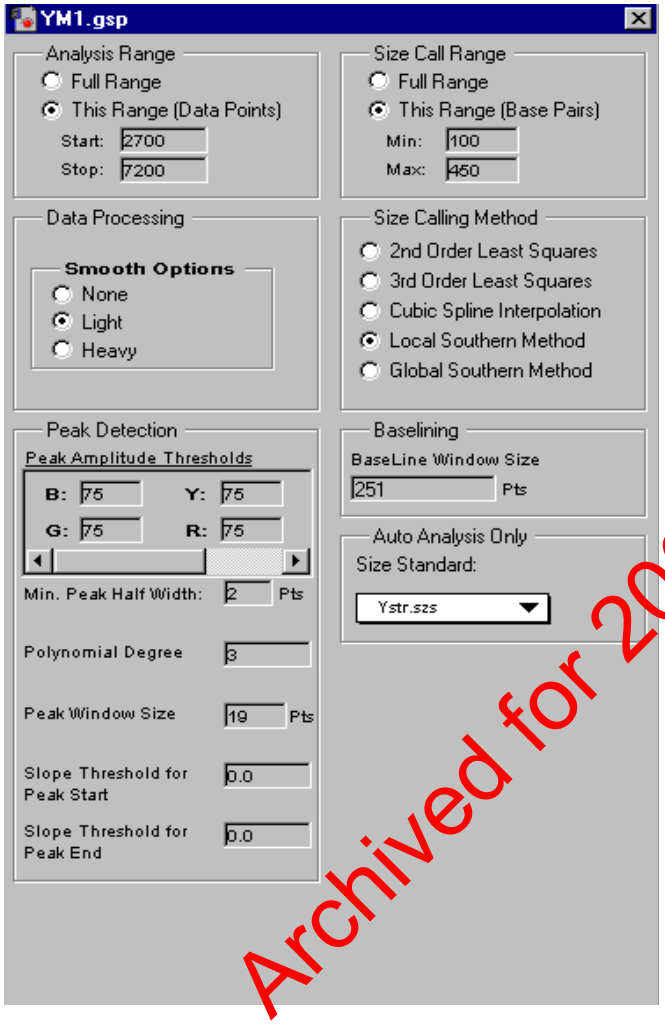
   Extend the range by choosing an earlier start point, e.g. 2100. Close window. In the sample list, go to the **Parameter** drop down menu (click on arrow) and select **"Analysis Parameters"** instead of the default GS500analysis.gsp. Reanalyze samples.

   Do not change the settings in the GS500analysis.gsp parameter file; do not create any new parameter files.



3100 Analysis default

Do not change any of the settings except the range or the peak amplitude threshold for Red.



3100 Analysis default
Do not change any of the settings except the range or the peak amplitude threshold for Orange (O).

3. **Samples still didn't analyze**

The error message for a failed analysis is: Analysis failed on Dye B, G, Y, R. Repeat the above choosing another scan range. Another possible problem is missing or to low red size standard. Look at **Raw Data** to find out. Access **Analysis Parameters** and lower threshold for red (R) to 25. Reanalyze samples.

<u>ATTENTION:</u> all re-analysis results and parameter changes are automatically written to the individual sample files, even if the changes to the project are not saved. Do not re-analyze casework data without a reason.

3100 Genetic Analyzer Troubleshooting

Instrument Startup

Observation	Possible Cause	Recommended Action
No communication between the instrument and the computer (yellow light is blinking).	Instrument not started up correctly.	Make sure the oven door is closed and locked and the front doors are closed properly. If everything is closed properly, start up in the following sequence: a. Log out of the computer b. Turn off the instrument. c. Boot up the computer. d. After the computer has booted completely, turn the instrument on. Wait for the green status light to come on. e. OrbixWeb Daemon should be launched, if not already. f. Launch Data Collection software
Red light is blinking	Incorrect start up procedure	Start up in the following sequence: a. Log out of the computer. b. Turn off the instrument. c. Boot up the computer. d. After the computer has booted completely, turn the instrument on. Wait for the green status light to come on. e. OrbixWeb Daemon should be launched, if not already. f. Launch the Data Collection Software**.**
Data Collection software will not launch.	Did not launch Orbixweb Daemon **first.**	Relaunch application following OrbixWeb Daemon

Observation	Possible Cause	Recommended Action
Computer screen is frozen	Communication error. This may be due to leaving the user interface in the Capillary View or Array View window.	There will be no loss of data. However, if the instrument is in the middle of a run, wait for the run to stop. Then, exit the Data Collection software and restart as described above.
Autosampler does not move to the forward position.	Possible communication error OR Oven or instrument door is not closed.	Restart the system, and then press the Tray button. OR a. close and lock the oven door. b. close the instrument doors. c. Press the Tray button.
Communication within the computer is slow.	Database is full.	Old files need to be cleaned out of the database. Follow proper manual procedures described in the ABI Prism 3100 Genetic Analyzer User's Manual.

Spatial Calibration

Observation	Possible Cause	Recommended Action
Unusual peaks or a flat line for the spatial calibration.	1. The instrument may need more time to reach stability. An unstable instrument can cause a flat line with no peaks in the spatial view. 2. Improper installation of the detection window. 3. Broken capillary resulting in a bad polymer fill. 4. Dirty detection window.	1. Check or repeat spatial calibration. 2. Reinstall the detection window and make sure it fits in the proper position. 3. Check for a broken capillary, particularly in the detection window area. If necessary, replace the capillary array using the Install Array Wizard. 4. Place a drop of METHANOL onto the detection window, and dry. Use only light air force.
Persistently bad spatial calibration results.	Bad capillary array.	Replace the capillary array, and then repeat the calibration. Call Technical Support if the results do not improve.

Spectral Calibration

Observation	Possible Cause	Recommended Action
No signal.	1. Incorrect preparation of sample. 2. Air bubbles in sample tray.	1. Replace samples with fresh samples prepared with fresh formamide. 2. Centrifuge samples to remove air bubbles.
If the spectral calibration fails, or if a message displays “No candidate spectral files found”.	1.Clogged capillary 2. Incorrect parameter files and\or run modules selected. 3. Insufficient filling of array. 4. Expired matrix standards.	1. Refill the capillaries using manual control. Look for clogged capillaries during capillary fill on the cathode side. 2. Correct the files and rerun the calibration. 3. Check for broken capillaries and refill the capillary array. 4. Check the expiration date and storage conditions of the matrix standards. If necessary, replace with a fresh lot.
Spike in the data.	1. Expired polymer. 2. Air bubbles, especially in the polymer block tubing assembly. 3. Possible contaminant or crystal deposits in the polymer.	1. Replace the polymer with fresh lot using the change Polymer Wizard. 2. Refill the capillaries using manual control. 3. Properly bring the polymer to room temperature; do not heat to thaw rapidly. Swirl to dissolve any solids. Replace the polymer if it has expired.

Data collection software problem

Observation	Possible Cause	Recommended Action
Cannot access injection folder, error message "Autoextraction failed"	AE server not active	Start AE server by going to Windows Start, Start up folder. Close Collection software. Select: Start > Applied Biosystems >3100 Utilities > Extractor Utility. Select the run to extract. Click Extract. The data will be extracted to the default location.

Run Performance

Observation	Possible Cause	Recommended Action
No data in all capillaries	Bubbles in the system.	Visually inspect the polymer block and the syringes for bubbles. Remove any bubbles using the Change Polymer Wizard. If bubbles still persist, perform the following: a. Remove the capillary array b. Clean out the polymer block and syringes. c. Replace polymer with fresh polymer. Make sure to draw the polymer into the syringe very slowly.
No signal.	1. Dead space at bottom of sample tube. 2. Bent capillary array. 3. Failed reaction. 4. Cracked or broken capillary	1. Centrifuge the sample tray. 2. Replace the capillary array 3. Repeat reaction. 4. Visually inspect the capillary array including the detector window area for signs of breakage.

Observation	Possible Cause	Recommended Action
Low signal strength.	1. Poor quality formamide. 2. Pipetting error, not enough sample. 3. Sample has high salt concentration. 4. Insufficient Mixing 5. Weak amplification of DNA	1. Use a fresh lot of formamide 2. Increase the amount of DNA added and check pipet calibration. 3. Dilute in high quality water, microcon. 4. Vortex the sample thoroughly, and then centrifuge the tube to condense the sample. 5. Re-amplify the DNA
Elevated baseline	1. Possible contamination in the polymer path. 2. Possible contaminant or crystal deposits in the polymer. 3. Poor spectral calibration. 4. Detection cell is dirty	1. Wash the polymer block with hot water. Pay particular attention to the upper polymer block, the ferrule, the ferrule screw, and the peek tubing. Dry the parts by vacuum pump before replacing them onto the instrument. 2. Bring the polymer to room temperature, swirl to dissolve any deposits. Replace polymer if expired. 3. Perform new spectral calibration. 4. Place a drop of methanol onto the detection cell window.

Observation	Possible Cause	Recommended Action
Loss of resolution.	1. Too much sample injected. 2. Poor quality water. 3. Poor quality or dilute running buffer. 4. Poor quality or breakdown of polymer. 5. Capillary array used for more than 150 injections. 6. Degraded formamide. 7. High salt concentration. 8. Improper injection and run conditions	1. Dilute the sample and reinject. 2. Use high quality, ultra pure water. 3. Prepare fresh running buffer. 4. Use a fresh lot of polymer. 5. Replace with new capillary array. 6. Use fresh formamide and ensure correct storage conditions. 7. Use a recommended protocol (e.g., microcon) for salt removal. Dilute salts with water. 8. Notify QA to check default settings.
Poor resolution in some capillaries.	Insufficient filling of array.	Refill array and look for cracked or broken capillaries. If problems persists contact Technical Support.
No current	1. Poor quality water. 2. Water placed in buffer reservoir position 1. 3. Not enough buffer in anode reservoir. 4. Buffer is too dilute. 5. Bubbles present in the polymer block and/or the capillary and /or peek tubing.	1. Use high quality, ultra pure water. 2. Replace with fresh running buffer. 3. Add buffer up to fill line. 4. Prepare new running buffer. 5. Pause run and inspect the instrument for bubbles. They may be hidden in the peek tubing.
Elevated current.	1. Decomposed polymer 2. Incorrect buffer dilution 3. Arcing in the gel block	1. Open fresh lot of polymer and store at 4^{o}C. 2. Prepare fresh 1X running buffer. 3. Check for moisture in and around the septa, the reservoirs, the oven, and the autosampler

Observation	Possible Cause	Recommended Action
Fluctuating current	1. Bubble in polymer block. 2. A slow leak may be present in the system. 3. Incorrect buffer concentration. 4. Not enough buffer in anode. 5. Clogged capillary. 6. Arcing	1. Pause the run, check the polymer path for bubbles, and remove them if present. 2. Check polymer blocks and syringes for leaks. Tighten all fittings. 3. Prepare fresh running buffer. 4. Add buffer up to the fill line. 5. Refill capillary array and check for clogs. 6. Check for moisture in and around the septa, the reservoirs, the oven, and the autosampler.
Poor performance of capillary array used for fewer than 150 runs	1. Poor quality formamide 2. Incorrect buffer 3. Poor quality sample, possible cleanup needed	1. Prepare fresh formamide and reprep samples 2. Prepare new running buffer 3. Desalt samples using a recommended purification protocol (e.g., microcon).
Migration time becomes progressively slower.	1. Leak in the system. 2. Improper filling of polymer block. 3. Expired polymer.	1. Tighten all ferrules, screws and check valves. Replace any faulty parts. 2. Check polymer pump force. If the force needs to be adjusted, make a service call. 3. If necessary, change the lot of polymer.
Migration time becomes progressively faster.	Water in syringe resulting in diluted polymer.	Clean the syringe, make sure it is completely dry OR replace syringe.
Peaks exhibit a shoulder effect in GeneScan application.	Sample renaturation.	Heat-denature the sample in good-quality formamide and immediately place on ice.
Purging of polymer from the polymer reserve syringe.	1. Arcing in the anode gel block. 2. Bubbles in syringes.	1. Replace the lower polymer block. 2. Remove bubbles.

Observation	Possible Cause	Recommended Action
Leaking polymer at the top of either syringe.	Insufficient seal around the tip of the syringe plunger.	Do not move the syringe plunger when it is dry. Make sure to wet the plunger before filling the syringe with polymer. Do not mix and match barrels and plungers.
Leaking polymer at the bottom of the polymer-reserve syringe.	Improper tightening of the array ferrule knob to the syringe and/or to the polymer block.	Ensure the array ferrule knob is tightened.
Error message, "Leak detected" appears. The run aborts	1. Air bubbles in the polymer path. 2. Pump block system is loose/leaking. 3. Lower pump block has burnt out. When there is condensation in the reservoir(s) this will cause electrophoresis problems and burn the lower block	1. Check for bubbles and remove if present, then check for leaks. 2. Make sure all syringes, screws tubing is tightly secure. Ferrule in capillary end of block may be positioned wrong or missing. Check for this ferrule. 3. Replace the lower block.
Buffer jar fills very quickly with polymer.	1. Air bubbles in the polymer path. 2. Lower polymer block is not correctly mounted on the pin valve.	1. Check for bubbles and remove if present. Then, look for leaks. 2. Check to make sure the metal fork is in between the pin holder and not on top or below it.
Detection window pops out while replacing the capillary array. Replacing the window in the correct orientation is difficult.	Tightening of the array ferrule knob at the gel block causes high tension.	Loosen the array ferrule knob to allow the secure placement of the window. Retighten and close the detection door.

Observation	Possible Cause	Recommended Action
Detection window stuck. It is difficult to remove when changing the capillary array.		To loosen the detection window: a. Undo the array ferrule knob and pull the polymer block towards you to first notch. b. Remove the capillary comb from the holder in the oven. c. Hold both sides of the capillary array around the detection window area, and apply gentle pressure equally on both sides. d. Release.

9. GENOTYPER ANALYSIS

For a run on the 3100 Genetic Analyzer, it is possible to run multiple sets of amplifications in one tray. IMPORTANT: Samples must be analyzed together with the controls from the same amplification sheet. Care has to be taken that the correct controls stay with each sample set.

A. YM1 GENOTYPER SECTION (3100)

To begin the YM1 Genotyper Software, double-click on the **YM1** icon.

1. Under **File** go to **Import** and select **From Genescan File**. This opens up the Add Sample Files window. Double-click on the folder containing the project that you created in Genescan.

2. To add the sample files, double-click on the project generated in the run. Click **Add** or double-click on the project icon to add the project for analysis.

3. When the project has been added, click **Finish**.

4. After importing the project, save the Genotyper as your initials and the run file name, e.g. STARS04–Y-001 CMK or STRIPES04-Y-001 JLS.

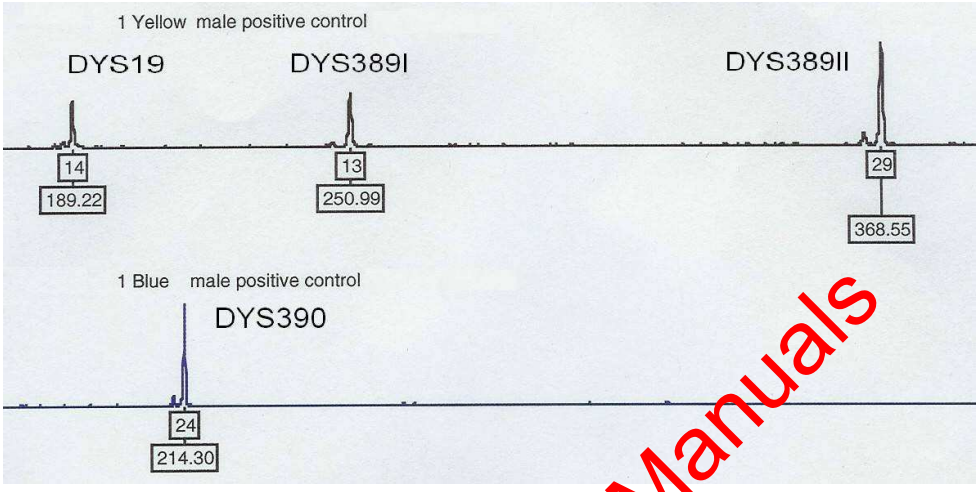
5. Run the Macro by pressing the **Control Key** and the **number 1** simultaneously, or by double-clicking on **POWER** in the lower left hand corner window. The plot window will appear automatically when the macro is completed. It will display orange size standard and all the peaks will be labeled with size in base pairs and allele names.

   ****Necessary GS 500 Standard Peaks: 9 Fragments from 139 to 400bp**

6. Check the results for the positive control.

   The genotype of the positive control is:

	DYS19	**DYS389 I**	**DYS389 II**
Yellow label	14	13	29
	DYS390		
Blue label	24		



7. Check all lanes. Labels for extra peaks can be manually deleted by placing the cursor on the peak above the baseline and clicking.

Shortcut: If you mistakenly delete a label, before you do anything else, press **crtl key + Z** and the allele name label will reappear (the command crtl 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.

Holding the left mouse click down draw a box around the desired area. Under **View** go to **Zoom**, select **Zoom In (selected area)**.

Shortcut: Zoom in by holding down the left mouse click button and dragging the cursor across the area you want to zoom in on. Then, simultaneously press the **Crtl** and **R** keys to zoom in on that region.

To revert to the correct scan range, go to **View, Zoom,** and choose **Zoom To**. Set the plot range to **120 to 410.** Click **OK**.

Peaks can be edited out if they meet one of the following criteria (also see section STR results interpretation):

1. *Pull ups of peaks in any color caused by a very high peak of another color in the same lane. Pull ups are caused by the inability of the matrix file to remove all of the overlap.*

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

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

4. *-4 and +4 stutter peaks if there is no indication of the presence of a mixture. A -4 bp stutter is common and will often be labeled if followed by a high allele peak. A +4 bp stutter on the other side is extremely rare and has to be interpreted carefully. Defined peaks in a +4 bp position might indicate the presence of a mixture.*

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

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

7. *Sharp peaks or spikes that do not resemble peaks but rather vertical lines and are caused by air bubbles or polymer crystals passing through the laser window. The presence of a spike can be verified by looking at the orange size standard in the same lane. A orange "spike" should occur at the same position.*

8. *Dye artifact occurring at a constant scan range.*

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

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

Samples without an orange size standard:
The lack of orange size standard will be first noted during Genescan analysis and can be confirmed on the Genotyper level. For 3100 runs these samples can be spotted easily since the Genotyper print out will state: "No Data Available." The lack of orange size standard can be either a failed injection or a post amplification sample prep mistake. These samples MUST be rerun under regular conditions.

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

DNA alleles visible but below threshold:
If a sample displays allele peaks just below the instrument detection threshold there is a distinct possibility that the alleles can be identified after a repeated run with increased amplification product. Place the sample on a rerun sheet. **Use 4µL of amplified sample with the Rerun Module (2 sec injection / 5KV).** Refer to Section E5 for appropriate letters for the system on a sample sheet)

New alleles:
If a locus displays a distinct unlabeled peak this could be a "new" allele that is outside the defined allele range. Especially if the other loci show a proper amplification signal, this possibility must be considered (but also see Troubleshooting F.). 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 and reporting.

A "new" allele that does not match an exemplar must be rerun to eliminate the possibility of an electrophoresis shift.

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

10. Create a table by running the **Table Macro**.

Simultaneously press **Crtl key** and the **number 2**, table will open.

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 or "sample info box"
- Place the cursor in the sample info box and correct the text
- Clear the table by going to **Analysis** on the main menu, select **Clear Table**
- Select the appropriate colors by shift clicking on the dye buttons or using edit
- Run **Table Macro** again

Continue to Step 12 and print the controls according to the directions. After the printing has finished, continue with Step 11.

11. Peak height labels are to be added to evidence-sample runs only. When processing, select the controls and case samples separately in order to add peak height labels to the evidence sample electropherograms. Follow the direction below for the different types of samples:

EVIDENCE SAMPLE RUNS

a. Open the **Dye Lane Window** (under **View**) and select **blue and yellow** for all lanes containing controls (allelic ladder, positive control, and all negative controls). In order to select multiple labels it is necessary to hold down the Ctrl-key while clicking on the lanes. The controls might not all be at the beginning of the run, do not forget any microcon samples placed elsewhere. Go to **View** and open the **Plot Window.**

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 **ok**. Continue to Step 12 and print the controls according to the directions. After the printing has finished, continue with b).

b. After the printing has finished, open the **Dye Lane Window** again (under **View**) and select **blue and yellow** for all lanes containing casework-samples. In order to select multiple labels it is necessary to hold down the Ctrl-key while clicking on the lanes. For a large sample sheet, it may be easier to select all samples and de-select the controls. Go to **View** and open the **Plot Window.**

Under **Analysis** select **Change Labels**. A dialogue box appears giving you the option of labels to assign to the peaks. Select **Size in bp, Peak Height,** and **Category name**. Click **ok**. Continue to Step 12 and print the samples according to the directions.

EXEMPLAR RUNS

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 **ok**. Continue to Step 12 and print according to the directions.

12. Before printing the results make sure the file is named properly, including initials. Set Plot window zoom range as shown below. The active window will be printed so open Table and Plot as needed.

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

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

14. WINDOWS: Go to **File > Print**. Click **OK**. Click **Properties**. Select orientation. Click **More**. Change scaling as indicated below. Click OK, OK, OK.

    YM1 Print out parameters:

	Table	Plot
Orientation	Portrait	Portrait
Scale	100% 2 per page	100%
Zoom range	n/a	120 - 410

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

    Normally the software will place the Genotyper file to the folder from which the data were imported. Make sure that the Genotyper is saved in the appropriate **Common runs folder**.

16. Archive data as described in the archiving section.

17. Initial all Genotyper pages. Pull the rerun samples. Have a Criminalist IV supervisor review the analyzed gel and get a signature on the editing sheet.

    The Criminalist IV supervisor must go back to the GenoTyper file to review the orange size standard. This review shall be documented on the Editing Sheet.

B. COFILER AND PROFILER PLUS GENOTYPER SECTION

For 310 and 3100 instruments, it is possible to run multiple sets of amplifications in one tray. If the amplifications were done in different multiplex systems it is necessary to perform the Genotyper analysis separately using the appropriate templates. For two amplifications in the same system it is optional to process them together or separately.

IMPORTANT: Samples must be analyzed together with the controls from the same amplification sheet. Care has to be taken that the correct controls stay with each sample set.

1. Under **File** go to **Import** and select **From GeneScan File**. If the Current Runs folder does not already appear in the window, scroll to find it from the pull-down menu and double-click on it. Double-click on the folder containing the project that you created in Genescan.

   MAC: Highlight project file, click **Import.**

   WINDOWS: Double-click on the project generated in the run. Click **Add** or double-click on the project icon to add the project for analysis. When the project has been added, click **Finish**.

   Under **View** select **Show Dye/Lanes window** you will see a list of the samples you have imported from Genescan analysis. If samples need to be removed, highlight the lanes for these samples and select **Cut** from the **Edit** menu.

2. Change the name of the Genotyper template to your initials and the casework run file name (under **File** select **Save As)**.

   e.g. Stripes04-Co001 LAR, Stars05-Pro001 EL

   Or

   CE3/04-005 Co LMS, CE1/05-002 Pro LAD

3. After importing the project and saving the Genotyper file run the first Macro.

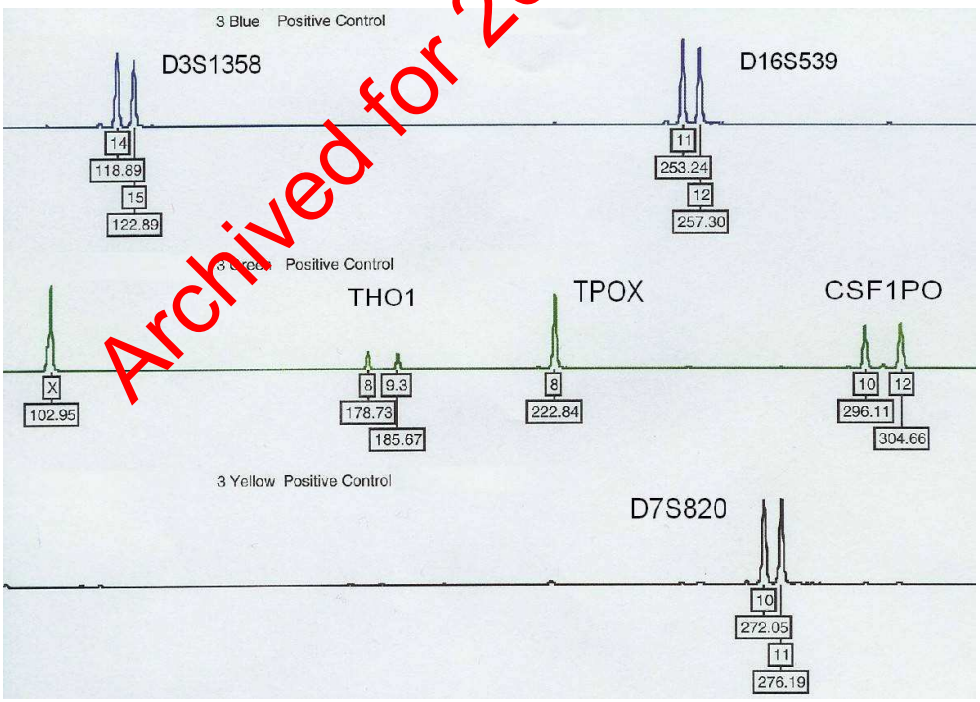
   MAC: simultaneously press **Apple key** and the **number 1**, or double click "**kazam**"
   WINDOWS: simultaneously press **Crtl key** and the **number 1**, or double click "**kazam**"

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

Multiplex System	**Necessary GS500 standard peaks**
Cofiler	10 fragments from 75 - 350 bp
Profiler Plus	11 fragments from 75 - 400 bp

The genotype of the Cofiler Positive Control is:

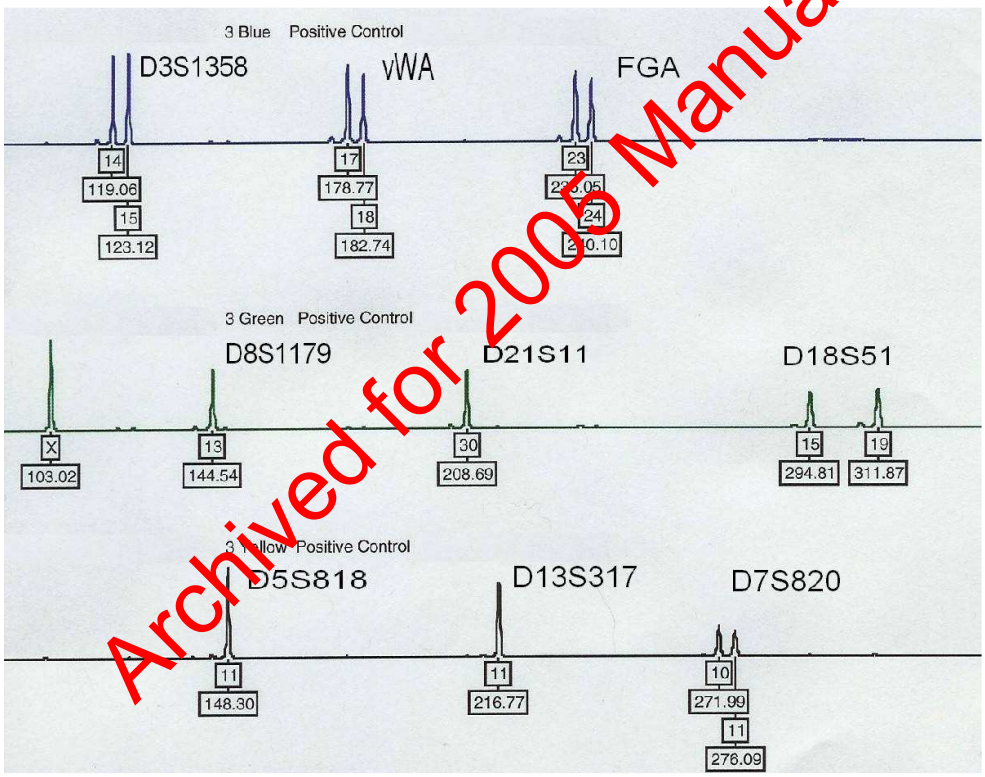
	D3S1358	**D16S539**		
Blue Label	14, 15	11, 12		
	Amelogenin	**THO1**	**TPOX**	**CSF1PO**
Green Label	X	8, 9.3	8	10, 12
	D7S820			
Yellow Label	10, 11			



(Basepair sizes in the figure above correspond to an ABI 3100 capillary run.)

The genotype of the Profiler Plus Positive Control is:

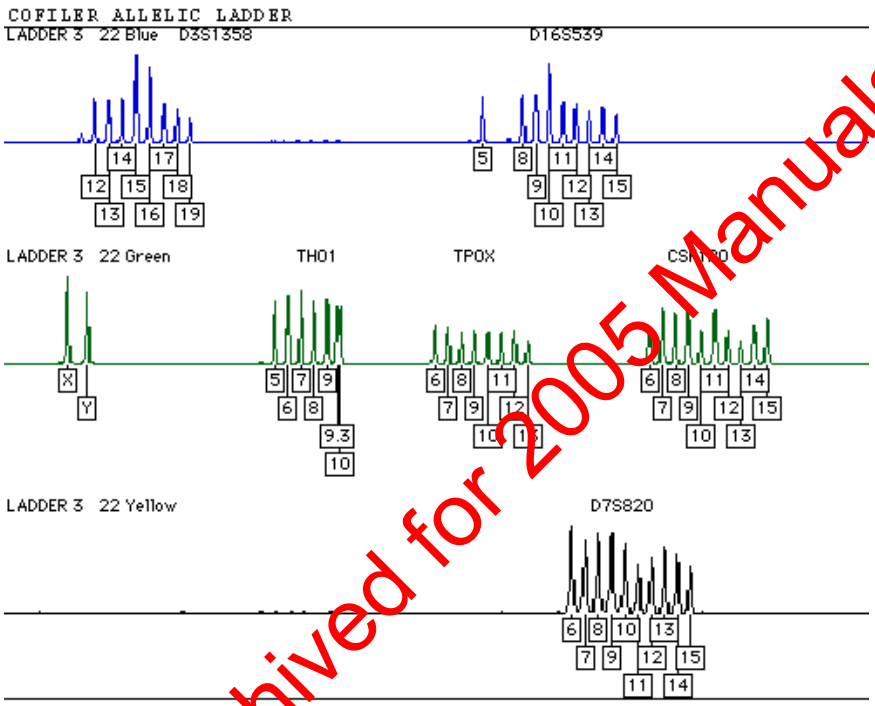
	D3S1358	**VWA**	**FGA**	
Blue Label	14, 15	17, 18	23, 24	
	Amelogenin	**D8S1179**	**D21S11**	**D18S818**
Green Label	X	13	30	15, 19
	D5S818	**D13S317**	**D7S820**	
Yellow Label	11	11	10, 11	



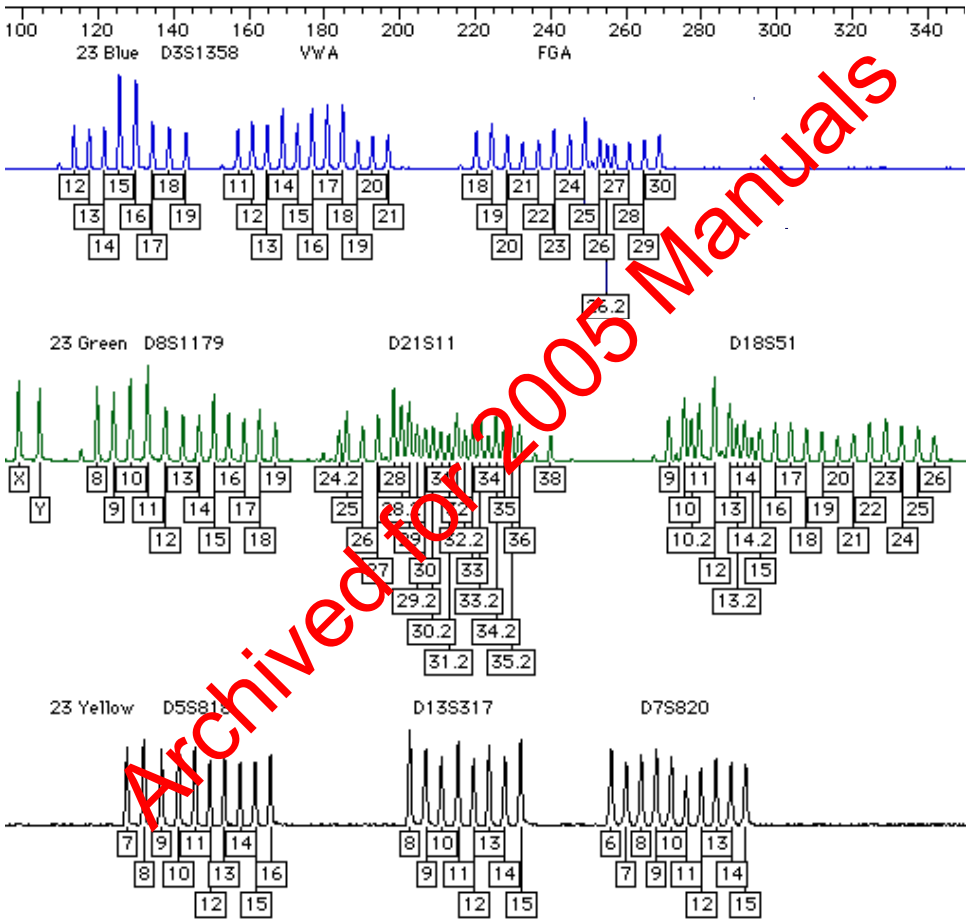
(Basepair sizes in the figure above correspond to an ABI 3100 capillary run.)

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

Cofiler Allelic Ladder:



Profiler Plus Allelic Ladder



5. Check all lanes. Labels for extra peaks can be manually deleted by placing the cursor on the peak above the baseline and clicking.

Shortcut: If you mistakenly delete a label, before you do anything else, press the **apple (or crtl) key + Z** and the allele name label will reappear (the command apple or crtl 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.

Holding the left mouse click down draw a box around the desired area. Under **View** go to **Zoom**, select **Zoom In (selected area)**.

> **Shortcut:** Zoom in by holding down the left mouse click button and dragging the cursor across the area you want to zoom in on. Then, simultaneously press the **apple (or Crtl)** and **R** keys to zoom in on that region.

To revert to the correct scan range, go to **View/ Zoom,** and choose **Zoom To**. Set the plot range to **90 to 330.** Click **OK**.

Peaks can be edited out if they meet one of the following criteria (also see section STR results interpretation):

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

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

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

4. *-4 and +4 bp stutter peaks if there is no indication of the presence of a mixture. -4 bp stutter is common and will often be labeled if followed by a high allele peak. +4 bp stutter on the other side is extremely rare and has to be interpreted carefully. Defined peaks in a +4 bp position might indicate the presence of a mixture.*

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

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

7. *Sharp peaks (spikes) that do not resemble peaks but rather vertical lines and are caused by air bubbles or POP4 crystals passing the laser window. The presence of a spike can be verified by looking at the red size standard for the same lane (see below). A red "spike" should occur at the same position.*

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

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

6. For all instrument platforms, fill out the Genotyper Editing Sheet for each sample number and note the reason for removal of a peak using the number code above.

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

   Red size standard:
   For the 310, the lack of red size standard will be noted during Genescan analysis and can be confirmed on the Genotyper level. For the 3100 runs, these samples can be spotted easily since the Genotyper print out will state: "No Data Available." The lack of red size standard can be either a failed injection or a post-amplification sample prep mistake. These samples MUST be rerun under regular conditions.

   Samples with compromised red size standards must either be re-analyzed with a re-defined size standard or designated for rerun.

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

DNA alleles visible but below threshold:
If a sample displays allele peaks just below the instrument detection threshold there is a distinct possibility that the alleles can be identified after a repeated run with either increased amplification product or a prolonged injection time. Place the sample on a rerun sheet.

310: use 2µL amplified sample and 10 seconds injection
3100: use 4 µL of amplified product with the Rerun Module (20 second injection/5 KVolts)

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 "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.

NOTE: OL alleles that were not duplicated in an overlapping system and do not match an exemplar or another sample in the case must be rerun in order to exclude the possibility of an electrophoresis shift.

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

8. Create a table by running the **Create Table Macro**.

   MAC: simultaneously press **Apple key** and the **number 2**, under **View** open the table.
   WINDOWS: simultaneously press **Crtl key** and the **number 2**, table will open.

   Compare the sample information in the table with the amplification and the run control sheet. If an error is detected at this point it can be corrected as follows:
   - Open the dye/lane window or "sample info box"
   - Place the cursor in the sample info box and correct the text
   - Clear the table by going to **Analysis** on the main menu, select **Clear Table**
   - Select the appropriate colors by shift clicking on the dye buttons or using edit
   - Run **Create Table Macro** again

   Continue to Step 10 and print the controls according to the directions. After the printing has finished, continue with Step 9.

9. Peak height labels are to be added to evidence-sample runs only. When processing, select the controls and case samples separately in order to add peak height labels to the evidence sample electropherograms. Follow the direction below for the different types of samples:

   EVIDENCE SAMPLE RUNS

   a. Open the **Dye Lane Window** (under **View**) and select **blue, green, and yellow** for all lanes containing controls (allelic ladder, positive control, and all negative controls). In order to select multiple labels it is necessary to hold down the Ctrl-key while clicking on the lanes. The controls might not all be at the beginning of the run, do not forget any microcon samples placed elsewhere. Go to **View** and open the **Plot Window.**

      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 **ok**. Continue to Step 10 and print the controls according to the directions. After the printing has finished, continue with b).

b. After the printing has finished, open the **Dye Lane Window** again (under **View**) and select **blue, green, and yellow** for all lanes containing casework-samples. In order to select multiple labels it is necessary to hold down the Ctrl-key while clicking on the lanes. For a large sample sheet, it may be easier to select all samples and de-select the controls. Go to **View** and open the **Plot Window.**

Under **Analysis** select **Change Labels**. A dialogue box appears giving you the option of labels to assign to the peaks. Select **Size in bp, Peak Height,** and **Category name**. Click **ok**. Continue to Step 10 and print the controls according to the directions.

EXEMPLAR RUNS

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 **ok**. Continue to Step 10 and print according to the directions.

10. Before printing the results make sure the file is named properly, including initials. Set Plot window zoom range as shown below. The active window will be printed so open Table and Plot as needed.

11. MAC: Under **file** change the **page set-up** to settings specified below. Then select **print**. WINDOWS: Go to **File** > **Print**. Click **OK**. Click **Properties**. Select orientation. Click **More**. Change scaling as indicated below. Click OK, OK, OK.

Cofiler print parameters:

	Table		**Plot**	
	MAC	WINDOWS	MAC	WINDOWS
Orientation	Landscape	Landscape	Letter	Portrait
Scale	65%	70%	90%	90%
Zoom range	n/a	n/a	90 - 330	90 - 330

Profiler Plus Print out parameters

	Table		Plot	
	MAC	WINDOWS	MAC	WINDOWS
Orientation	Landscape	Landscape	Letter	Portrait
Scale	65%	70%	90%	90%
Zoom range	n/a	n/a	90 - 360	90 - 360

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

    MAC: 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.

    WINDOWS: Normally the software will place the Genotyper file to the folder from which the data were imported. Make sure that the Genotyper is saved in the appropriate **Common runs folder**.

13. Archive data as described in the archiving section.

14. Initial all Genotyper pages. Pull the rerun samples and list on the appropriate rerun sheet. Have a supervisor review the analyzed gel and get a signature on the editing sheet.

    For **Troubleshooting** see Genotyper section **E Multiplex Kit Troubleshooting.**

C. POWERPLEX 16 GENOTYPER SECTION

For a run on the 3100 Genetic Analyzer, it is possible to run 85 samples (including PE and amp. negative). IMPORTANT: Samples must be analyzed together with the controls from the same amplification sheet. Care has to be taken that the correct controls stay with each sample set.

To begin the PowerPlex 16 Genotyper Software, double-click on the **PowerPlex 16** icon.

1. Under **File** go to **Import** and select **From Genescan File**. This opens up the Add Sample Files window. Double-click on the folder containing the project that you created in Genescan.

2. To add the sample files, double-click on the project generated in the run. Click **Add** or double-click on the project icon to add the project for analysis.

3. When the project has been added, click **Finish**.

4. After importing the project, save the genotyper as your initials and the run file name, e.g. STARS04–PP16-001 CMK or STRIPES04-PP16-001 JLS.

5. Run the Macro by pressing the **Control Key** and the **number 1** simultaneously, or by double-clicking on **POWER** in the lower left hand corner window. The plot window will appear automatically when the macro is completed.

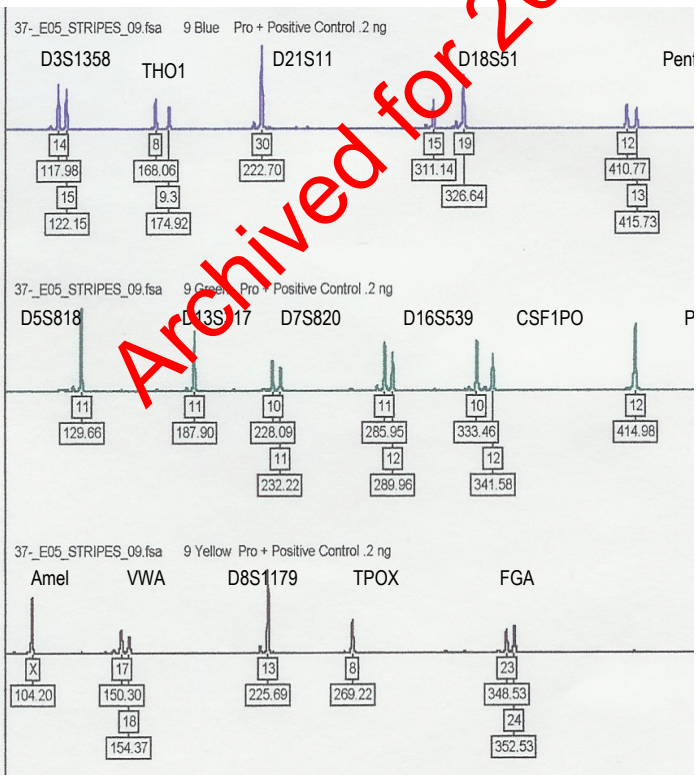
6. Check the allelic ladder (paying close attention to FGA alleles 18.2, 19.2, 20.2, 21.2, 22.2) and click on the 0.2 peaks that were not labeled by the macro. (The ladder has worked correctly but a default filter has removed those labels. You are just replacing those labels).

7. Go to **Change Labels** from **Analysis** in the main menu and check the boxes for the **Category Name** and the **Base Pair** values. Click **OK**.

8. Check the results for the positive control.

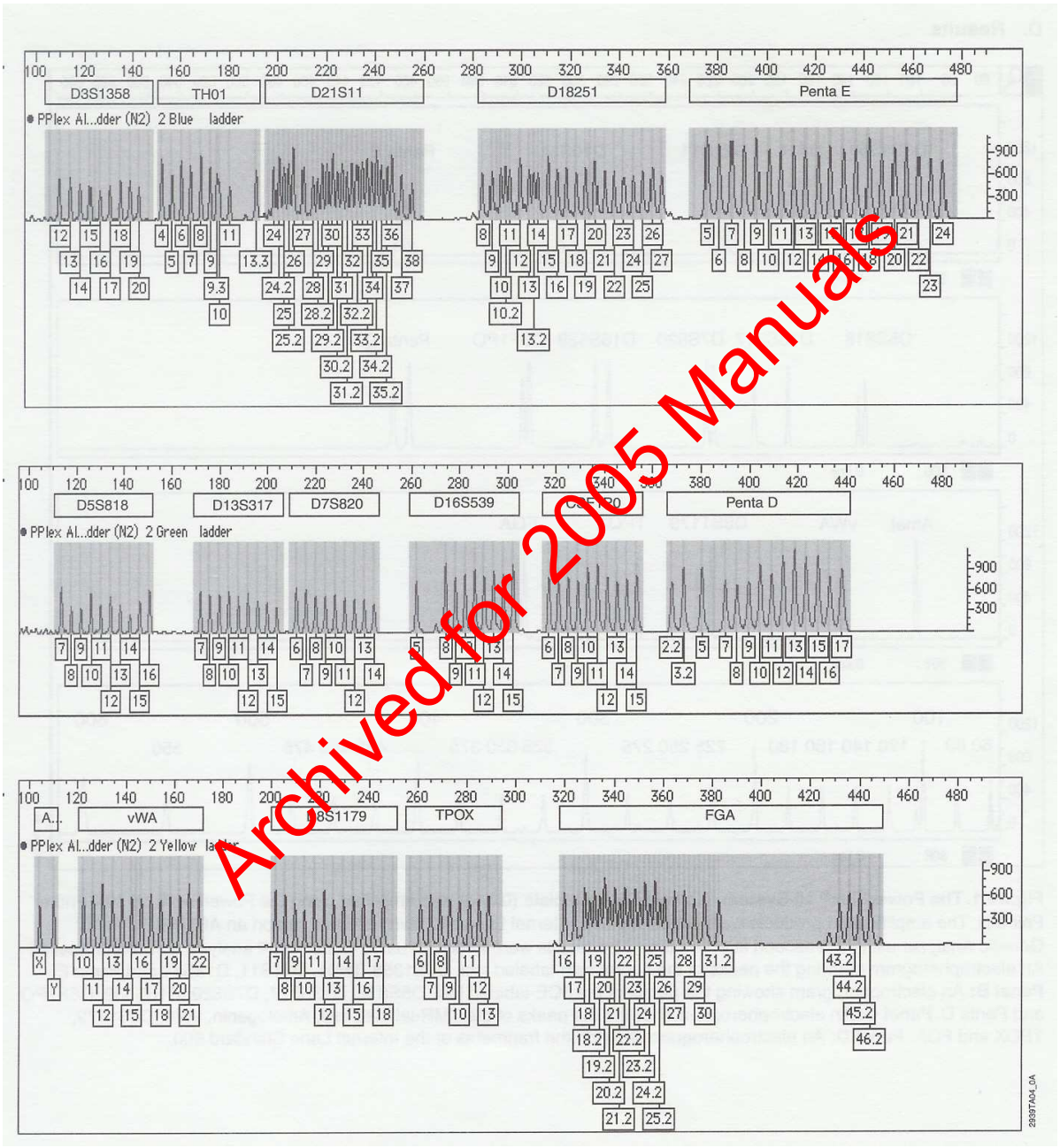
The genotype of the PowerPlex 16 Positive Control is:

	D3S1358	**THO1**	**D21S11**	**D18S51**	**Penta E**	
Blue Label	14, 15	8, 9.3	30	15, 19	12, 13	
	D5S818	**D13S317**	**D7S820**	**D16S539**	**CSF1PO**	**Penta D**
Green Label	11	11	10, 11	11, 12	10, 12	12
	vWA	**D8S1179**	**TPOX**	**FGA**		
Yellow Label	17, 18	13	8	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 troubleshooting section).

PowerPlex 16 Allelic Ladder



9. Check all lanes. Labels for extra peaks can be manually deleted by placing the cursor on the peak above the baseline and clicking.

Shortcut: If you mistakenly delete a label, before you do anything else, press the **apple (or crtl) key + Z** and the allele name label will reappear (the command apple or crtl 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.

Holding the left mouse click down draw a box around the desired area. Under **View** go to **Zoom**, select **Zoom In (selected area)**.

Shortcut: Zoom in by holding down the left mouse click button and dragging the cursor across the area you want to zoom in on. Then, simultaneously press the **apple (or Crtl)** and **R** keys to zoom in on that region.

To revert to the correct scan range go to **View, Zoom,** and choose **Zoom To**. Set the plot range to **90 to 480.** Click **OK**.

Peaks can be edited out if they meet one of the following criteria (also see section STR results interpretation):

1. *Pull ups of peaks in any color caused by a very high peak of another color in the same lane. Pull ups are caused by the inability of the matrix file to remove all of the overlap.*

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

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

4. *-4 and +4 stutter peaks if there is no indication of the presence of a mixture. A -4 bp stutter is common and will often be labeled if followed by a high allele peak. A +4 bp stutter on the other side is extremely rare and has to be interpreted carefully. Defined peaks in a +4 bp position might indicate the presence of a mixture.*

5. *Non specific artifacts. This category should be used if a labeled peak is caused by a not previously categorized technical problem. (This category should be chosen for the artifacts prevalent in vWA.)*

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

7. *Sharp peaks or spikes that do not resemble peaks but rather vertical lines and are caused by air bubbles or polymer crystals passing through the laser window. The presence of a spike can be verified by looking at the red size standard in the same lane. A red "spike" should occur at the same position.*

8. *Dye artifact occurring at a constant scan range.*

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

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

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

Samples without a red size standard:
The lack of red size standard will be first noted during Genescan analysis and can be confirmed on the Genotyper level. For the 3100 runs these samples can be spotted easily since the Genotyper print out will state: "No Data Available." The lack of red size standard can be either a failed injection or a post-amplification sample prep mistake. These samples MUST be rerun under regular conditions.

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

DNA alleles visible but below threshold:
If a sample displays allele peaks just below the instrument detection threshold there is a distinct possibility that the alleles can be identified after a repeated run with increased amplification product. Place the sample on a rerun sheet.

3100: use 4µL of amplified product with the Rerun Module (20 second injection/5 KVolts)

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 "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.

NOTE: OL alleles that were not duplicated in an overlapping system and do not match an exemplar must be rerun in order to exclude the possibility of an electrophoresis shift.

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

12. Create a table by running the **Create Table Macro**.

Simultaneously press **Crtl key** and the **number 2**, table will open.

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 or "sample info box"
- Place the cursor in the sample info box and correct the text
- Clear the table by going to **Analysis** on the main menu, select **Clear Table**
- Select the appropriate colors by shift clicking on the dye buttons or using edit
- Run **Create Table Macro** again

Continue to Step 14 and print the controls according to the directions. After the printing has finished, continue with Step 13.

13. Peak height labels are to be added to evidence-sample runs only. When processing, select the controls and case samples separately in order to add peak height labels to the evidence sample electropherograms. Follow the direction below for the different types of samples:

EVIDENCE SAMPLE RUNS

a. Open the **Dye Lane Window** (under **View**) and select **blue, green, and yellow** for all lanes containing controls (allelic ladder, positive control, and all negative controls). In order to select multiple labels it is necessary to hold down the Ctrl-key while clicking on the lanes. The controls might not all be at the beginning of the run, do not forget any microcon samples placed elsewhere. Go to **View** and open the **Plot Window.**

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 **ok**. Continue to Step 14 and print the controls according to the directions. After the printing has finished, continue with b).

b. After the printing has finished, open the **Dye Lane Window** again (under **View**) and select **blue, green, and yellow** for all lanes containing casework-samples. In order to select multiple labels it is necessary to hold down the Ctrl-key while clicking on the lanes. For a large sample sheet, it may be easier to select all samples and de-select the controls. Go to **View** and open the **Plot Window.**

Under **Analysis** select **Change Labels**. A dialogue box appears giving you the option of labels to assign to the peaks. Select **Size in bp, Peak Height,** and **Category name**. Click **ok**. Continue to Step 14 and print the controls according to the directions.

EXEMPLAR RUNS

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 **ok**. Continue to Step 14 and print according to the directions.

14. Before printing the results make sure the file is named properly, including initials. Set Plot window zoom range as shown below. The active window will be printed so open Table and Plot as needed.

15. WINDOWS: Go to **File > Print**. Click **OK**. Click **Properties**. Select orientation. Click **More**. Change scaling as indicated below. Click OK, OK, OK.

Powerplex 16 Print out parameters

	Table	Plot
Orientation	Landscape	Portrait
Scale	70%	90%
Zoom range	n/a	90 - 480

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

Normally the software will place the Genotyper file in the folder from which the data were imported. Make sure that the Genotyper is saved in the appropriate **Common runs folder**.

17. Archive data as described in the archiving section.

18. Initial all Genotyper pages. Pull the rerun samples. Have a Criminalist IV supervisor review the analyzed gel and get a signature on the editing sheet.

The Criminalist IV supervisor must go back to the GenoTyper file to review the red size standard. This review shall be documented on the Editing Sheet.

For **Troubleshooting** see Genotyper section **E Multiplex Kit Troubleshooting.**

D. Multiplex Kit Troubleshooting

1. 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.

2. 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 four possibilities:

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

      Solution: Close your Genotyper template, start the correct one and re-import your Genescan project.

   b. There may be peaks in the ladder that are too low to be recognized by the program.

      Solution: You have two options:

      One: If another ladder in the run is more intense, alter or delete 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.

      Two: You can lower the **minimum peak height** in the categories window. To do this, open the categories window by going under **Views** and selecting **Show Categories Window**. In the "offset" categories the first allele is defined with a scaled peak height of 200 or higher. The high value is meant to eliminate stutter and background.

Change this to 75 for the 3100, and 100 for the 310, by clicking on the first category that highlights it. In the dialogue box locate the **Minimum Peak Height** and change it to the appropriate value, click **Add,** and then click **Replace** when given the option. You must do this for each locus. Do not use values less than the instrument threshold.

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.

c. The first ladder peak of each locus is outside of the pre-defined size range window.

Solution: You can expand the search window 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 ceratin size +/- 7bp. Change the 7 to 10 or higher, by clicking on the first category which highlights it. In the dialogue box locate the **+/- box** and change the value, click **Add,** and then click **Replace** when given the option. You must do this for each locus that gives you the error message.

d. There are no peaks at all in any of the allelic ladders.

Solution: Rerun all samples with freshly prepared Allelic Ladders.

2. Off Ladder (OL) allele labels

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

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

a. If all or most of your samples have "OL allele?" labels it may be that your samples were automatically analyzed with an ill-defined size standard. In this case it is necessary to reanalyze your run using the correct or a redefined size standard (see Genescan analysis for 310 or 3100).

3. Incorrect positive control type:

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

Check the ladder and make sure the first assigned allele is assigned to the first real peak and not to a stutter peak, which may precede it. If the stutter peak is designated with the first allele name, you must raise the peak height in the categories window in order to force the software to skip the stutter peak and start with proper allele.

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

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

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

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

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

4. Lining up unlabeled peaks

In order to place samples next to each other for comparison purposes, mark them by double clicking. A black bullet appears in front of the lane number. If this happens accidentally, a lane can be unmarked by either double clicking on it again or, under **Edit**, selecting **unmark**. NOTE: unsized peaks cannot be placed according to size on the electropherogram. Therefore, when comparing an unlabeled allele (unlabeled because it is too low to be sized, but high enough to be detected visually) to a labeled allele (e.g. in the ladder) you cannot determine the allele type and size by visual comparison while the results are viewed by size. To be able to align an unlabeled allele with a labeled allele in the same run you must select **View by Scan** from under the **View** menu!

5. Too many samples

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 other samples. Under **Analysis** select **Clear Dye/Lanes** window and also under **Analysis** select **Clear Table**. Re-import your file(s).

6. Typographical error in the sample information and/or sample comment

If you detect a mistake in the sample information, this can be corrected for the Genotyper file by opening the dye lane list window, highlighting the lane, and retyping the sample information for all colors. The short sample name cannot be changed here. It can only be changed on the sample sheet level.

7. Less samples in Table than in Plots

Samples with the same sample information are only listed once in the Table. Add modifier to the sample information (see above) of one of the samples and rerun Macro.

8. Too many background peaks labeled

If peaks are still labeled in the plot even though they are listed as having been removed or they appear to be below the stutter filter threshold, the following mistake could have happened: instead of choosing **Change labels** from the **Analysis** drop down menu, the analyst clicked **Label peaks.** The **Change labels** command is supposed to label the valid peaks with the allele name and the size in basepairs prior to printing the plot. The **Label peaks** command will label all peaks above threshold independent of any Macro stutter and background filters. This command will also re-label peaks that were edited out. To fix this, rerun the macro, repeat the documented editing steps and reprint the Table and the Plot.

10. STR RESULTS INTERPRETATION		
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A. Allele Calling Criteria

Results are interpreted by observing the occurrence of electropherogram peaks for the loci that are amplified simultaneously. The identification of a peak as an allele is determined through comparison to the allelic ladder or for YM1 by the Genotyper categories. An allele is characterized by the labeling color of the locus specific primers and the length of the amplified fragment. See appendix for a listing of each locus in each multiplex.

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 intensity above the minimum threshold are labeled as alleles.

Based on validation data the different detection platforms have different minimum peak height thresholds:

ABI platform	Minimum threshold in fluorescent units
310	100 fu
3100	75 fu

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

- Recalculating fluorescence peaks using the instrument-specific matrix or spectral file 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 YM1 (a system without an allelic ladder) - labeling of all sized fragments that are >50 fu (fluorescent units), fall within the locus size range and match to an allele size average within a ± 1.0bp tolerance window. Labels are automatically removed from minor peaks based on the background and stutter filter functions outlined in the YM1 Genotyper section.

- For Cofiler, Profiler Plus and Powerplex 16 (systems with an allelic ladder) - comparing and adjusting the Genotyper allele categories to the sizing of the co-electrophoresed allelic ladder by calculating the off sets (the difference between the first allele in a category and the first allele in the allelic ladder at each locus).

- For Cofiler, Profiler Plus and Powerplex 16 - labeling of all sized fragments that are higher than the platform specific threshold and fall within the locus specific size range (see appendix). Removing the labels from minor peaks (background and stutter) according to the filter functions detailed in the appendix of this manual.

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

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

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

-4 stutter peaks that are caused by slippage of the Taq polymerase enzyme during copying of the STR allele. Under rare circumstances there may be a +4 stutter peak.

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

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

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

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

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

If the Genotyper program labels these additional non-allelic peaks, the labels may be removed manually. The removal is documented on the Genotyper editing sheet.

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

The Y chromosome allele nomenclature is also based on the number of 4bp core repeats and follows the nomenclature suggested in (Kayser et al 1997) and the one used in the European Caucasian Y-STR Haplotype database (Roewer et al 2001).

B. Interpretation of the Genotyper Print Out

Genotyper printouts of capillary electrophoresis runs containing the case specific samples are part of each casefile. The Genotyper results table reflects the number and allele assignments of the labeled peaks visible on the Genotyper Plot print out. **The printouts are the basis for results interpretation.** The plot will display peak height information, unlabeled peaks, intensity differences that indicate the presence of a mixture, and will show all peaks at one locus if the number of peaks exceeds the table's number of columns per locus. Looking at the plots also serves as a control for the editing process.

When to access the electronic Genotyper file:

1. No peak is above the minimum threshold but unlabeled peaks are visible.

   If these unlabeled peaks need to be compared to a previous amplification (see reporting rules) it is important to remember that peaks below the threshold are not sized by the software and cannot be displayed by size. They are randomly placed on the Genotyper plot and their types cannot be inferred! In order to make a visual allele interpretation one must reopen the corresponding Genotyper file and change the viewing mode from **View by Size** to **View by Scan** (also see Genotyper troubleshooting section E).

2. High peaks and very minor peaks present in the same color lane

   Since the fu scale of the electropherogram is based on the highest peak in each color, alleles at weak loci will not be clearly visible if the loci are imbalanced. For mixture interpretation or allelic dropout detection, it is helpful to access the archived Genotyper file and in the **View** menu enter a fixed y-scale for **Plot Options, Main Window Lower Panel**. Print pages. Do not save changes.

When the Genotyper print outs are not sufficient for proper interpretation and the Genescan Analysis results can be helpful:

1. Genotyper plot states “no size data”

   This means that none of the peaks was above threshold and it is possible that the original Genescan Analysis electropherogram displays visible peaks below the sizing threshold.

2. Distinct unlabeled peak in locus with similar height “homozygous” allele

   If a sample has only one allele at a locus but outside the previously reported size range a distinct peak is visible, this sample could have a “new” allele (see Interpretation of complex STR results). In this case the analyst might have to look at the original Genescan Analysis electropherogram in order to get the size in bp for this peak.

C. Reporting Procedures

Items listed in allele typing tables should be limited to samples that are used to draw important conclusions of the case. Genotypes are not reported and should not be inferred, i.e. if only a “7" allele is found; it should be reported as 7. Alleles and/or peaks are listed in the results tables regardless of intensity differences, based on the reporting criteria below.

A. If an allele meets the above reporting thresholds and fulfills the concordant analyses and the duplicate rule as stated in the General PCR Guidelines, then the allele is listed in the report.

B. Use * to indicate that an extract was not tested for this locus. This symbol is defined as “* = Typing not attempted.”

C. If extra peaks are visible that can't be accounted for by any editing categories, then the presence of this peak is reported as **. ** is defined at the bottom of the table as "** = additional peaks were detected which did not meet laboratory criteria for allele identification; therefore, these additional peaks are not reported."

D. In cases where a mixture sample was reamplified in the same multiplex system consult the table below about how to report the alleles:

Allele labeled for one amplification?	Results for the other amplification:	Report
Yes	also labeled	allele
Yes	not labeled but visible	allele
Yes	not present at all	**
No but visible	not labeled but visible	**
No but visible	not present at all	**

NOTE: the table above does not apply to reanalysis by repeated electrophoresis or to single source samples that are amplified in varying DNA amounts.

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

F. Off-ladder alleles should be reported using the CODIS nomenclature which incorporates their relative position to the alleles in the allelic ladder (see Interpretation of Complex Autosomal STR results, 3.B. reporting of previously unreported rare alleles).

G. New alleles observed for YM1 where no allelic ladder is available should be reported with their rounded basepair size. The basepair value should also appear in the footnotes, e.g. 128 = Allele is reported as size in basepairs.

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

D. 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, or a reproducible pattern of visible but unlabeled peaks.) 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.

C. Assuming a single physiological fluid donor, two samples could derive from a common biological source (inclusion) if all the alleles in the evidence sample are accounted for by the alleles in the exemplar sample. If however a mixture is possible in the evidence sample, there may be alleles that are not accounted for by the exemplar sample. If an inclusion requires the presence of more than one physiological fluid donor, this must be stated in the report. (i.e.. Hum THO1 (S) 7, (V) 6,9 vaginal swab sperm fraction 7, 8 (7 > 8); Assuming a single semen donor, the suspect can be eliminated as the semen donor. However if there is more than one semen donor, the suspect cannot be eliminated as a possible semen donor.

D. Statistics are calculated for evidence samples only where: (1) The sample is apparently unmixed. (2) The sample appears to be a mixture of two components and the source of one component is known. (i.e. when vaginal epithelial cells are present in the sperm fraction from a vaginal swab.). (3) There is a large difference in peak heights between the major and minor components and then the genotype of the major component is easily inferred. The minor component genotype can be determined if four alleles are present at a locus. If less than four alleles are present one has to be very careful because other alleles of the minor component may be masked by the major component alleles. A deduction may be possible based on peak height imbalances. See below for the calculation of statistics.

E. Statistics are not calculated for expected inclusions such as vaginal epithelial cells from a vaginal swab, panties or (for non blood only) the victim's own bedding.

NOTE: Do not forget to evaluate the significance of a match for epithelial cell fractions for items not connected to the victim such as condoms or the suspect's clothes.

E. Interpretation of controls

Extraction negative and Amplification Negative

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

Amplification Positive Control

The positive control DNA is used with each batch of samples typed to demonstrate that the kit is performing properly and that data analysis was performed correctly. The positive control results are shown in the specific Genotyper sections.

The failure of control samples can be caused by laboratory steps following the extraction and amplification that do not affect the quality of the sample data. In order to identify such problems and avoid unnecessary sample consumption, the control samples should be retested before failing a sample set. **See below for retesting strategies for control samples.**

Retesting strategies for control samples

Table 1 Amplification negative control and female negative control

If peaks attributed to DNA are detected in an amplification negative control, all samples in that amplification are inconclusive and have to be repeated. The only exception is, if the peaks might be have been caused by a problem during electrophoresis sample set-up or spill over from an adjacent sample and the amplification negative can be shown to be clean by repeating the electrophoresis step.

Result	Course of action
No red size standard in lane or "no data available" on 3100	Must be rerun, no data were analyzed and decision if control was clean cannot be made
Misshaped or a few missing red size standard peaks	STR data were not properly sized but have been analyzed, therefore: Control can pass if no blue/green/yellow peaks are present
Peaks detected	Very unlikely that peaks were introduced at run set up stage. In most cases a rerun will still show the same peaks. Only if the signal is very low and could be just background a reloading should be attempted. Otherwise the amplification fails and all samples must be resubmitted for amplification.
Run artifacts such as color blips or spikes	Can be edited out. A rerun of the control is necessary if the artifacts are so abundant that amplified DNA might be masked.
Rerun still displays peaks	Control cannot be tested for a third time. Amplification fails and all samples must be resubmitted for amplification.

Table 2 Extraction negative control

If peaks attributed to DNA are detected in an extraction negative control, the control must be retested to determine if the extract is truly contaminated or if something occurred during a later stage. This sample can either be retested by re-electrophoresis or re-amplification. If the peaks could just be background, it may be possible to just re-run the extraction negative in order to show that it was actually clean. Normally an extraction negative control should be reamplified as the first test. Only if DNA peaks are still present following the second amplification, then all samples have to be re-extracted.

Result	Course of action
No red size standard in lane or "no data available" on 3100	Must be rerun, no data were analyzed and decision if control was clean cannot be made
Misshaped red size standard peaks	STR data were not properly sized but have been analyzed, therefore control can pass if no blue/green/yellow peaks are present
Peaks detected	Very unlikely that peaks were introduced at run set up stage. In most cases a rerun will still show the same peaks. The extraction negative should be reamplified. The samples pass if the second amplification is clean.
Run artifacts such as color blips or spikes	Can be edited out. A rerun of the control is necessary if the artifacts are so abundant that amplified DNA might be masked.
Rerun still displays peaks	Reamplify control
Reamplification still displays peaks	Extraction fails and all samples must be reextracted.

Table 3 Positive control

The positive control has a double function. It serves as an amplification control to test the fidelity of the PCR reaction. Here it will indicate mistakes made during the set-up and thermocycling steps. It also serves as an electrophoresis control to test for proper separation and correct Genotyper performance. **An injection set consisting only of reruns still needs to have one positive control.** A set of samples with a failed positive control can only pass without repeating all samples, if a.) reloading could show that the positive control amplified correctly and if b.) the set of samples is accompanied by a second positive control, that can serve as the electrophoresis control. This is often the case for CE runs where two amplification sets are run and another positive control from the same tray can be imported into the Genotyper.

For runs with only one positive control the retesting strategy needs to include the samples. If it is unclear if the positive control will pass the rerun test, it is often easier to just retest the control alone first and then rerun the complete set later. If the control does show amplification product at a significant peak height and the problem is most likely a separation problem, the set should be run together. The separation problem might also have affected the samples.

Result	Course of action
No red size standard in lane	Will result in “No size data” message (310, 377) or “No Data available” on the 3100. Sample must be rerun to test if amplification yielded expected alleles
No amplification product but red size standard correct	Rerun separately to check for loading errors, if still no signal detected, all samples must be reamplified
Incorrect genotype - Could be caused by ill-defined size standard or other Genotyper problems	Reanalyze sample, if not able to resolve, rerun amplification product
Incorrect genotype - Could be caused by sample mix-up	Rerun amplification product, if type is still wrong all samples must be reamplified
OL alleles	Indicates a genotyper problem, rerun amplification product
Rerun fails to give correct type	All samples must be resubmitted to amplification.

If the positive control has been shown to give the correct type, this confirms the integrity of the amplification. If individual samples from this amplification set need to be re-analyzed on a second gel or CE run the positive control does not have to be repeated, if another positive control is included in the run.

11. INTERPRETATION OF COMPLEX AUTOSOMAL STR RESULTS

Occasionally typing results may appear markedly different from the standard patterns. Such results could be due to a procedural error, mixtures of DNA's (multiple contributors to the sample), or DNA degradation.

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

A. General Mixtures

Evidence samples may contain DNA from more than one individual either because of the nature of the sample or from contamination. The possibility of multiple contributors should be considered when interpreting STR typing results. For any typing system in which heterozygous genotypes are analyzed, the detection of more than two alleles indicates a mixed sample. The peak height ratio for the two alleles of a heterozygote locus has been shown to be >70% (Holt et al, 2002). Therefore the presence of a peak imbalance of more than 30% is indicative of the presence of a mixture. Holt et al (2002) also point out that degradation or primer binding site mutations are other possible causes for peak height ratio imbalance, and that low DNA amounts with weak signals (around 200 fu's) are more likely to show uneven heterozygote peak heights.

A single locus might not be helpful in detecting a mixture since even though a mixture is present a locus might only reveal two alleles. For example, in a 1:1 mixture there is a possibility that a phenotype, e.g. VWA 15,17, is a mixture of a homozygous 15,15 individual and a homozygous 17,17 individual. 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.

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

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 (Gill et al. 1995, Clayton et al. 1998). A VWA typing profile 18>16>14 can be caused by unequal amounts of 14,16 and 18,18 but also by a mixture of two individuals with 14, 18 and 16, 18. Here, different scenarios have to be considered:

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

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

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

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

   If the alleles foreign to the victim are the minor component and only one foreign allele is visible at a locus, it might not be possible to determine the complete allele composition for this STR. The foreign type might either be homozygous or heterozygous with one allele overlapping with the known component. For heterozygous types of the known component, peak height differences between the two alleles indicate the presence of an overlapping allele in the minor component. For homozygous patterns and very small peak height differences a decision cannot be made. In these cases it is possible to indicate that a second allele might be present without identifying the allele.

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

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

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

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

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

C. Possible mixture components masked by -4bp stutter

Due to enzyme slippage when replicating repetitive DNA stretches, an additional peak of a length exactly -4bp shorter than the main allele peak is a frequent occurrence for STR polymorphisms (Gill et al. 1995, Walsh et al 1996, Holt et al 2002). Some of the STR loci are very prone to stutter and almost always show stutter peaks e.g. DYS19 or VWA. The Amelogenin locus is not based on a repetitive STR sequence and doesn't show any stutter. Over all loci the average stutter peak height ranges from 2.5 to 9.5%, with maxima from 17.4% - 24.1% (in house validation). Therefore peaks in a -4bp position from a main peak and less than a certain percentage (differs per locus, see Appendix) of the main peak's height are not reported as true alleles. In a mixture the -4bp stutter could mask a real mixture component. Therefore individuals cannot be excluded from being a minor contributor to a mixture if their alleles are in the -4bp position of an allele from another individual.

2. Partial Profiles: not all loci display allele peaks

A. Degradation

DNA degradation is the process of the very long (>40,000 bp) DNA double strand being broken down into smaller pieces. With increasing degradation the DNA fragments get very short, until the target sequences for the PCR reaction which at least have to contain both primer annealing sites are also broken down. For the example the Profiler Plus multiplex contains alleles from 100bp (Amelogenin) to 337bp (D18S51). Other multiplexes contain even longer alleles e.g. Powerplex 16 with Penta E (440bp) and YM1 with DYS389II (362-386bp).

The longer alleles are more likely not to be present in partially degraded DNA (Gill et al. 1995, Sparkes et al. 1996, Holt et al 2002). A Profiler Plus result that displays only D3S1358 and Amelogenin but none of the higher molecular weight loci, can be explained as being caused by DNA degradation. A profile with no D3S1358 result but callable FGA 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 FGA type 19, 29, allele 19 (220 bp) can be present while allele 29 (260 bp) drops out. Parallel to the disappearing of the larger size allele, an imbalanced peak height with the larger allele peak being smaller, can be explained by DNA degradation.

The possibility of an allelic drop out has to be considered especially for low peak heights around 200 fu's.

B. Detection limit

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

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 can be rerun with more amplification product or if possible should be re-amplified using a higher amount of DNA. 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 >75 (or 100 if applicable) and does not show artifacts,
- The same color locus closest to the new size peak does not have more than one allele peak, and
- The new size peak is also detected in the duplicate run.

For overlapping loci such D3S1358 and D7S820 the presence of a previously unreported rare allele can be confirmed by the typing results in the second multiplex. Also, if multiple evidence samples and/or the matching exemplar show the same OL allele this allele can be considered duplicated. Alleles for non-overlapping loci must be confirmed by rerunning the amplified extract to eliminate the possibility of an electrophoresis shift.

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. The new allele should not be included in the statistical evaluation of a match.

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

STR systems with an allelic ladder (Profiler Plus, Cofiler, Powerplex 16): In order to be consistent with the CODIS reporting requirements, all alleles that are not present in the allelic ladder should be identified by their relative position to the alleles in the allelic ladder. The Genotyper peak label should show the length in basepairs and this value can be used to determine the proper allele nomenclature, e.g. a FGA allele of 322bp is longer than the longest allele in the FGA allelic ladder (30 - 269bp) and has to be designated >30. A TPOX allele with a size of 208bp is shorter than the smallest ladder allele (6 - 212 bp) and has to be designated <6. A D7S820 allele of the length 276 bp is located between alleles 10 and 11 and has to be designated 10.x. The off-ladder allele should be reported using this nomenclature.

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

4. Samples with High Background Levels

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

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

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

5. Discrepancies for overlapping loci in different multiplex systems

The primer-binding site of an allele may contain a mutation, which renders the annealing phase of its amplification less efficient, or if the mutation is near the 3' end completely blocks the extension (Clayton et al. 1998). This may result in a pseudo-homozygote type, which is reproducible for the specific primer pair. These mutations are extremely rare, approximately estimated between 0.01 and 0.001 per locus (Clayton et al. 1998). A comparison between evidence and exemplar samples based on a locus where both samples were amplified with the same primer sequence is no problem. If the same locus is amplified using different multiplex systems (especially Powerplex 16 versus the AmpFlSTR kits) it is possible to obtain a heterozygote type in one multiplex and the pseudo-homozygote in the second, because the primer sequences for the same loci may differ. The heterozygote type should be the correct type and should be reported. It is important to have typing results for evidence and exemplars based on the same multiplex.

12. ADDITIONAL INTERPRETATIONS OF Y-STR RESULTS AND COMPLEX Y-STR RESULTS

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

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

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

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

   A. General Mixtures

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

   B. Mixtures with different level of starting DNA

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

C. Possible mixture component masked by -4bp stutter

Peaks in a -4bp position from a main peak and less than 20% of the 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. 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**

13. POPULTION FREQUENCIES FOR STR's

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

If the subject and evidence samples have the same alleles, then the subject is "included", and could be the source of the evidence sample. The probability that another, unrelated, individual would also match the evidence sample is equal to the frequency of the alleles in the relevant population. Population frequencies are calculated separately for the Asian, Black, Caucasian and Hispanic populations and each population's frequency is listed in the laboratory report regardless of the population group of subject(s) in the case. Additional population frequencies may be used for other population groups. If a source contains more than one frequency for a single population group, than the highest frequency is used for calculations. Allele frequencies are used for all calculations. Locus frequencies are calculated according to the National Research Council report entitled *The Evaluation of Forensic DNA Evidence* (National Academy Press 1996, pp. 4-36 to 4-37).

The overall frequency for each racial group is reported as occurring in 1 out of x individuals (i.e. 1/overall frequency). The overall frequency is reported to two significant digits for autosomal STR systems. For example, 1 out of 12,345 (spread sheet value 1.2E + 04) would be reported as 1 out of 12,000. Values that are greater than 1 out of 1,000,000,000,000 (spread sheet value 1.0 + 12) are not reported in their order of magnitude. The report should state "one in greater than a trillion".

A Quattro Pro Spreadsheet is used to automate the calculation of the racial specific loci and overall frequencies. The spreadsheet is located in the popstat subdirectory on the network and explanations for its use are included with the spreadsheet.

The population frequencies are derived from the OCME Database.

Autosomal STR's

In the standard scenario, for each group, homozygotes are calculated using the formula $p^2+p(1-p)\theta$ for $\theta = 0.03$ and heterozygotes are calculated using the formula $2p_ip_j$. The overall frequency for each group is calculated by multiplying the individual locus frequencies if the loci are unlinked. If the loci are linked then only the locus with the lowest locus frequency is used in the calculation. In addition, locus frequencies are calculated for "evidence and subject from the same subgroup (isolated village)" and for relatives using the formulas in the National Research Council Report and $\theta = 0.03$. Overall frequencies are calculated as described above.

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 θ=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".

e.g. A haplotype that has been seen 4 times in the Asian database is reported as "1 in 29 Asians"

A haplotype that has been seen 1 time would be reported as "1 in 116 Asians," or for samples that have not been previously observed in the database "less than 1 in 116 Asians."

The haplotype frequency can also be calculated for partial profiles. This calculation, however, can only be done manually. The Quattro Pro spreadsheet is not accurate with partial Y profiles.

See page 2 of the QuattroPro spread sheet for the exact Y database values. Print this page for the casefile.

If both autosomal and Y STR's are typed for a sample, than the overall frequency can be calculated by multiplying the overall autosomal frequency for each racial group by the larger of either a) the Y haplotype frequency or b) the Y haplotype frequency assuming 1 count in the database.

14. KINSHIP ANALYSIS

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

The DNA from the subject/stain in question is compared to the DNA of close biological relatives. For parent(s)/child comparisons, the loci are first evaluated to determine whether the individual in question can be excluded as a biological relative of the other individual(s) (see below). If the individual cannot be excluded, or for comparisons not involving a parent(s)/child relationship, a PI (traditionally called a paternity index, but this could be a maternity or kinship index), is calculated for each locus using the DNAVIEW program of Dr. Charles Brenner. The formulas for parent/child comparisons are listed in Appendices 6 and 11 of Parentage Testing Accreditation Requirements Manual, 3rd edition, AABB.

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

> μ (locus specific mutation rate) is obtained from Appendix 14 of Parentage Testing Accreditation Requirements Manual, Fourth Edition, AABB and
>
> **PE** = $h^2 (1-2hH^2)$ where H is the frequency of homozygosity and h is the frequency of heterozygosity. PE is calculated by the DNAVIEW program.

An overall CPI (combined paternity index) is calculated by multiplying all of the individual PIs. A probability of paternity (maternity/kinship) is then calculated using Bayes' theorem and assuming a prior probability of 50%. The individual loci PI, the CPI, and probability of paternity (W) are calculated by the DNAVIEW program **(see Appendix)**. The report printed out from DNAVIEW should be included in the case file as the statistics sheet. The DNAVIEW calculations should be performed for each race.

The Forensic Biology case report should report the results for ONE race, preferably the race of the individual in question (eg, the race of the tested man in a paternity case). The case report must list the PI for each locus, the race used for the calculations, the CPI, the probability of paternity, and the assumed prior probability. It must also state the final conclusion. The three possible final conclusions are exclusion, inconclusive, or inclusion, of the tested hypothesis of kinship.

Exclusions occur when either 2 or more loci exclude in a parent/child comparison, or when the CPI < 0.1.

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

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

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YM1 Genotyper Categories Table for ABI 3100

DYS19

12	Highest peak at 180.70 ± 1.00 bp in yellow with height ≥75
13	Highest peak at 184.70 ± 1.00 bp in yellow with height ≥75
14	Highest peak at 188.80 ±1.00 bp in yellow with height ≥75
15	Highest peak at 192.60 ±1.00 bp in yellow with height ≥75
16	Highest peak at 196.70 ±1.00 bp in yellow with height ≥75
17	Highest peak at 200.50 ±1.00 bp in yellow with height ≥75
18	Highest peak at 204.50 ±1.00 bp in yellow with height ≥75

DYS389 I

10	Highest peak at 238.60 ±1.00 bp in yellow with height ≥75
11	Highest peak at 242.60 ±1.00 bp in yellow with height ≥75
12	Highest peak at 246.50 ±1.00 bp in yellow with height ≥75
13	Highest peak at 250.70 ±1.00 bp in yellow with height ≥75
14	Highest peak at 254.70 ±1.00 bp in yellow with height ≥75
15	Highest peak at 258.70 ±1.00 bp in yellow with height ≥75

DYS389 II

26	Highest peak at 356.60 ±1.00 bp in yellow with height ≥75
27	Highest peak at 360.60 ±1.00 bp in yellow with height ≥75
28	Highest peak at 364.60 ±1.00 bp in yellow with height ≥75
29	Highest peak at 368.50 ±1.00 bp in yellow with height ≥75
30	Highest peak at 372.40 ±1.00 bp in yellow with height ≥75
31	Highest peak at 376.40 ±1.00 bp in yellow with height ≥75
32	Highest peak at 380.50 ±1.00 bp in yellow with height ≥75
33	Highest peak at 384.40 ±1.00 bp in yellow with height ≥75

DYS390

20	Highest peak at 197.90 ±1.00 bp in blue with height ≥75
21	Highest peak at 201.90 ±1.00 bp in blue with height ≥75
22	Highest peak at 205.80 ±1.00 bp in blue with height ≥75
23	Highest peak at 209.90 ±1.00 bp in blue with height ≥75
24	Highest peak at 213.90 ±1.00 bp in blue with height ≥75
25	Highest peak at 217.90 ±1.00 bp in blue with height ≥75
26	Highest peak at 221.90 ±1.00 bp in blue with height ≥75
27	Highest peak at 225.90 ±1.00 bp in blue with height ≥75

Profiler Plus loci and size range

Profiler Plus	Color	Size Range 310 CXR Std.	Size Range 3100 GS500 Std.	Allele range in Ladder
D3S1358	Blue	108±0.5bp to 137±0.5bp	111±0.5bp to 140±0.5bp	12 to 19
vWA	Blue	151±0.5bp to 191±0.5bp	154±0.5bp to 195±0.5bp	11 to 21
FGA	Blue	213±0.5bp to 262±0.5bp	216±0.5bp to 264±0.5bp	18 to 30
Amelogenin	Green	X:100±0.5bp; Y:106±0.5bp	X:103±0.5bp; Y:109±0.5bp	X and Y
D8S1179	Green	121±0.5bp to 166±0.5bp	123±0.5bp to 170±0.5bp	8 to 19
D21S11	Green	183±0.5bp to 238±0.5bp	187±0.5bp to 240±0.5bp	24.2 to 38
D18S51	Green	268±0.51bp to 337±0.5bp	270±0.51bp to 341±0.5bp	9 to 26
D5S818	Yellow	129±0.5bp to 166±0.5bp	131±0.5bp to 169±0.5bp	7 to 16
D13S317	Yellow	202±0.5bp to 230±0.5bp	205±0.5bp to 233±0.5bp	8 to 15
D7S820	Yellow	253±0.5bp to 290±0.5bp	256±0.5bp to 292±0.5bp	6 to 15

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

Cofiler loci and size ranges

Cofiler	Color	Size Range 310	Size Range 3100 GS500 Std	Allele range in Ladder
D3S1358	Blue	109±0.5bp to 138±0.5bp	111±0.5bp to 140±0.5bp	12 to 19
D16S539	Blue	226±0.5bp to 267±0.5bp	229±0.5bp to 270±0.5bp	5 to 15
Amelogenin	Green	X:100±0.5bp; Y:106±0.5bp	X:103±0.5bp; Y:109±0.5bp	X and Y
THO1	Green	163±0.5bp to 183±0.5bp	166±0.5bp to 187±0.5bp	5 to 10
TPOX	Green	212±0.5bp to 240±0.5bp	215±0.5bp to 243±0.5bp	6 to 13
CSF1PO	Green	277±0.51bp to 313±0.5bp	280±0.51bp to 317±0.5bp	6 to 15
D7S820	Yellow	254±0.5bp to 290±0.5bp	256±0.5bp to 292±0.5bp	6 to 15

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

Powerplex 16 loci and size ranges

Powerplex 16	Color	Size Range 3100 ILS 600 Std.	Allele range in Ladder
D3S1358	Blue	109±0.5bp to 142±0.5bp	12 to 20
THO1	Blue	152±0.5bp to 190±0.5bp	4 to 13.3
D21S11	Blue	198±0.5bp to 255±0.5bp	24 to 38
D18S51	Blue	284±0.5bp to 358±0.5bp	8 to 27
Penta E	Blue	375±0.5bp to 472±0.5bp	5 to 24
D5S818	Green	113±0.5bp to 150±0.5bp	7 to 16
D13S317	Green	172±0.5bp to 204±0.5bp	7 to 15
D7S820	Green	212±0.5bp to 244±0.5bp	6 to 14
D16S539	Green	262±0.5bp to 302±0.5bp	5 to 15
CSF1PO	Green	317±0.5bp to 354±0.5bp	6 to 15
Penta D	Green	168±0.5bp to 439±0.5bp	2.2 to 17
Amelogenin	Yellow	X:104±0.5bp; Y:110±0.5bp	X and Y
vWA	Yellow	122±0.5bp to 170±0.5bp	10 to 22
D8S1179	Yellow	202±0.5bp to 246±0.5bp	7 to 18
TPOX	Yellow	261±0.5bp to 289±0.5bp	6 to 13
FGA	Yellow	320±0.5bp to 444±0.5bp	16 to 46.2

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

Genotyper Macro Filter functions

Cofiler	Allele filters		Ladder lanes only
Locus	Stutter filter 310 (in house values)	Stutter Filter 3100 (ABI default)	Background Filter (All platforms ABI default)
D3S1358	15%	11%	20%
D16S539	15%	13%	15%
Amelogenin	None	None	25%
CSF1PO	None	9%	25%
THO1	None	6%	25%
TPOX	None	6%	25%
D7S820	15%	9%	25%

Profiler Plus	Allele Filters		Ladder lanes only
Locus	Stutter Filter 310 (in house values)	Stutter Filter 3100 (ABI default)	Background Filter (All platforms ABI default)
D3S1358	15%	11%	20%
vWA	15%	11%	25%
FGA	13%	11%	30%
Amelogenin	none	none	40%
D18S51	15%	16%	10%
D21S11	15%	13%	20%
D8S1179	15%	12%	30%
D5S818	15%	10%	25%
D13S317	11%	10%	25%
D7S820	11%	9%	25%

The Amelogenin category for both Cofiler and Profiler Plus does not have a stutter filter because it is not a repeat. However, it does have a locus filter of 3%. For the 310, the Macro additionally applies a 10% background filter.

The **PowerPlex 16** Macro contains the following filter functions described in table format (all values Promega default settings):

Locus	Stutter Filter	Ladder Filter
D3S1358	11%	30%
THO1	5%	20%
D21S11	18%	30%
D18S51	11%	30%
Penta E	10%	20%
D5S818	9%	30%
D13S317	10%	20%
D7S820	8%	20%
D16S539	10%	30%
CSF1PO	8%	20%
Penta D	5%	20%
Amel	none	20%
VWA	12%	20%
D8S1179	10%	20%
TPOX	5%	20%
FGA	12%	30%

A 15% background filter was added to the Macro based on in house validation.

See Y M1 Genotyper section for **Y M1** filter functions.

Kinship and Paternity Analysis Using the DNAView Program
01/24/05

DNAVIEW version 25.68 is loaded analysis computers at 520. DNAVIEW 24.96 is loaded on computers on the second floor of 520, WTC Identification. This version of the DNAVIEW program has some differences but can be used for analysis. The DNAVIEW programs do not communicate, even on the analysis local network. Information entered at one computer is not accessible on the others.

INSTRUCTIONS FOR DNAVIEW

Open DNAVIEW by double-clicking on the desktop DNAVIEW Icon. If a DNAVIEW message appears, hit "Enter". If the DNAVIEW main menu does not appear, be patient, it can take a minute or two.

(Alternatively, you can enter MS_DOS by selecting the MS_DOS prompt, desktop, or programs. The MS_DOS screen will appear showing C:\WINDOWS>. Type **\dnaview\dnaview** , and hit "Enter". The main menu for DNAVIEW will be displayed.)

YOU CAN ALWAYS RETURN TO THE MAIN MENU FROM ANY STAGE OF THE PROGRAM (AND WITHOUT LOSING MUCH INFORMATION) BY HITTING **CONTROL** AND **PAUSE/BREAK** KEYS SIMULTANEOUSLY. THIS MAY COME IN HANDY IF YOU MISTYPE ANY ENTRY OR COMMAND OR IF YOU WANT TO MOVE BACK TO A PREVIOUS SCREEN.

USING THE MOUSE TO SELECT FROM THE MENU IS VERY PROBLEMATIC. SCROLL USING KEYBOARD ARROWS OR TYPE IN COMMANDS.

The analysis consists of four discrete programming steps:
1) Define a "case".
2) Define the individuals in the case
3) Enter their STR DNA alleles
4) Link the case to the individuals and calculate statistics.

You will have to return to the main menu of the program between each step.

A) FOR PATERNITY OR MATERNITY CASES (the DNA STR type of a child and at least one (possible) parent is known:

Select Casework, and hit Enter.

In the casework menu select Paternity case, and hit Enter.

In the paternity menu select case, and hit Enter.

Enter a case #. To define a new case, enter a new, unique, case#. The case # must consist of a number, of up to seven digits, with no letters, symbols, spaces, etc., therefore "FB01-1234" is not an acceptable case number, but 0101234 is. (Do not use 0101234, it is already taken, and case numbers must be unique.) Make sure you keep track of the unique case number you select, since you will need to know it later, when you link this case to the individuals and their DNA STR types. After typing the case number you select, hit Enter. If you enter zero, or zeroes, as the first number(s), DNAVIEW will drop them (i.e., 0123456 will be saved by DNAVIEW as 123456).

The standard paternity scenario will be displayed. The screen will ask you to identify three individuals,

Mother

Child

First tested man

Designations are the underlined capital letter. If additional people are tested, the program will assign them other capital letters (e.g., a second tested man will be assigned capital letter "G").

The assigned capital letter will remain the identifier, regardless of later possible kinship scenarios.

In order to identify these individuals and link them to STR DNA data, you will enter an "accession number" for each. Accession numbers must be in the format "four digit number - five digit number", eg, 1234-00001 (case number-individual 1). Keep track of the accession number you select for each individual, since they will be needed to link the correct DNA STR types to each individual.

After entering the accession number for M(other), hit the spacebar on the keyboard, NOT Enter. The program will then ask you to enter the race of the individual. The choices are **b** (black), **c** (caucasian), **a** (asian), **h** (hispanic), **-** (unknown). YOU MUST ENTER A RACE. If you do not know the race of the individual, you can enter a race on the race list after defining the people.

You will eventually perform a separate calculation for each race, entering the different races one at a time, using menu choice "edit race list", and calculate a separate PI for each possible race.

Type the appropriate response and hit Enter. A line for comments will appear. Type any comments, for example the name of the individual, (optional), and hit Enter.

The program will now advance to the next individual, C(hild), and request an accession number.

Enter an accession number for C, eg, 1234-00002, and hit the spacebar.

The race of the child should always be entered as "-".

Type the appropriate response and hit Enter. A line for comments will appear. Type any comments (optional), and hit Enter.

The program will advance to the third individual in the case scenario, T(ested man). Follow the same proceedure to provide an accession number and a race for this individual.
If you need to go back to a previous individual, hit Shift-Tab.

If you have more individuals tested, for example more than one child, or more than one tested man, select add role from the menu, and add these people to the case.

If you have not entered a race for the mother and father, select "edit race list" from the menu, select one race for the first calculation, and hit Enter.

When you are finished defining the case, making sure you have recorded the case number, and all the individuals' accession numbers, return to the main menu by hitting quit, Q-Enter.

You should perform a separate calculation for each race, entering the different races one at a time, using menu choice "edit race list", and calculate a separate PI for each possible race.

From the main menu choices, select Casework, and hit Enter.

From the Casework menu select Membrane, and hit Enter.

To define a new membrane, hit End, then hit Enter.

Hit Enter to accept date.

The program will ask for a new membrane ID. A membrane ID can have any format, e.g. FB02-1234. Type in your membrane ID and hit Enter. Record the membrane ID you select.

The program will ask "Are you inputting allele calls or bp sizes?" Answer "no" by typing "n".

From the displayed choices, select 10 lanes, no ladder, and hit Enter. (If your case scenario could have more than ten individuals typed, select 80 lanes, no ladder.)

The program will ask you to verify if the information is correct. Hit Enter.

Your membrane should now be highlighted, and you should select it.

You will now be in the program to define the "lanes" of your 10-lane membrane. This is done by assigning an accession number to each selected lane. You will assign each of the (three) accession numbers from your case to a lane. Note that the cursor is on "1", meaning it is ready to assign an accession number to lane 1. At this point hit the spacebar. This means that you have selected lane 1. (If you hit a number, the cursor will advance to that lane !) Now enter the accession number for individual M(other), from your case. Remember, the exact format for accession numbers is four digit-five digit. (If you are entering the defined roles, Mother, Child, and alleged Father, from your case, you can also hit "M" at lane 1, "C" at lane 2, and "F" at lane 3, instead of the individual accession numbers.)
Verify that the case ID and accession number for each individual is correct.

After entering the accession number, hit spacebar, NOT Enter, to select the next lane.

The program will now advance to the next lane. Remember to hit the spacebar before assigning the next accession number to each lane. Also remember to hit the spacebar, not Enter, immediately after typing the accession number.

When the lanes have been assigned, hit End, then select File and Quit, and hit Enter.

Return to the main menu by hitting Control-Pause/Break.

The next step is to type in the DNA alleles at each STR locus for each individual on the membrane.

From the main menu, select Casework, and hit Enter.

From the casework menu, select Type in a Read, and hit Enter.

When asked to identify the reader, select Genotyper.

The program will ask you to type in the membrane ID, (your membrane ID should be the default selection), and hit Enter.

The program now asks you to select a locus. Select the locus, and hit Enter.

The program should now display a screen for entering alleles, with the locus shown on the top right of the screen, and spaces next to each "lane" on the membrane, with the appropriate accession number next to each lane. Enter the alleles.
Enter the genotype at each locus (ie, enter both alleles for homozygotes). Off-ladder alleles can be entered as eg Tho1, 10+1, or entered as the actual size in base pairs, eg, Tho1 184. Do not enter as the lab report protocol, eg 10.x.

When finished entering the alleles, hit Escape. The program asks you to choose file this image. Select this option and hit Enter.

The program now asks if you want to enter the next locus. Type "y", and hit Enter.

Choose the next locus, and type in the alleles as before. When all the loci have been entered, type "n" when asked if you would like to type a new locus.
This will return you to the main menu.

From the main menu, select Casework, and hit Enter.

From the casework menu, select Paternity case, and hit Enter. Select case and choose your case ID. Depending on the details of the kinship analysis you want to perform, select these options:

If your case is a standard paternity or maternity case, with one known parent, a known child, and one questioned parent, then from the paternity menu, Select Calculate Report, and hit Enter. After the statistics have been calculated, select Print Report, and hit Enter. Local printer will print the report.

If no values are shown on the printed report, go back to the membrane, and refile the loci by hitting File and Quit. Then return to the case, and Calculate Report again.

Now you need to calculate the statistics for each of the three remaining races:
Follow previous instructions to open DNAVIEW, casework, paternity case, and select your case. Choose "edit race list" and select the next race to calculate, and hit "enter". Go to calculate report , and hit "enter". After calculations are finished, follow previous instructions to print the report, and calculate the next races.

When finished, hit Control-Pause/Break to go to the main menu.

Select "Exit from DNAVIEW to exit the program. If question about error message appears type "n".

B) For kinship cases (involves body identification, missing persons, non-paternity cases):

1. Select **Casework**.
2. Select **Membrane**. Hit Enter. Hit End.
3. Select **Month.** The current month and day should be selected. Hit Enter.
4. Type in a brief ID for the case. For example, if your case if FB04-1079, enter 1079 or 041079 #. To define a new case, enter a new, unique, case#. The case # must consist of a number, of up to seven digits, with no letters, symbols, spaces, etc..., therefore "FB01-1234" is not an acceptable case number, but 0101234 is. (Do not use 0101234, it is already taken, and case numbers must be unique.) If you enter zero, or zeroes, as the first number(s), DNAVIEW will drop them (i.e., 0123456 will be saved by DNAVIEW as 123456). Make sure you keep track of the unique case number you select, since you will need to know it later, when you link this case to the individuals and their DNA STR types. After typing the case number you select, hit Enter.
5. Are you inputting allele calls? **No**
6. Select a configuration: **80 lanes**
7. Is the above information correct? **Yes or No.**
8. Highlight the membrane you created. Hit Enter.
9. Press the spacebar to open Lane #1.
10. Create a sample label for each of the samples typed and that you will be entering a profile for. You will be designating a number to each person or sample. For example: if you have a mother for case FB04-1079, then the sample label could be **1079-00001**.
    - Type up to 4 digits, hit the spacebar ONLY, and enter the last 5 digits.
    - Hit spacebar. Enter "**–**" for Race. Hit spacebar. Hit Delete. You do not want the data you just entered repeated or any info except what you just typed.
    - Once you hit Delete you should be automatically routed to the next #.
11. If entering more than one profile, repeat Step 10.

12. Hit End when finished. You must do this to save the information you just entered.
13. Select **File and Quit.**
14. Press **ESC.**

Entering a Profile (Type in a Read)

1. Select **Casework**
2. Select **Type in a Read**.
3. For "Who are you? ", select **Student** or any other reader. It does not affect the analysis.
4. Select the membrane you created.
5. Select the first locus (start with D3S1358)
6. Type in alleles using the spacebar or tab to move from box to box. Only one allele per box is allowed. Remember the sample labels that you assigned each person and type in the alleles corresponding to each person.
7. If you see a possible false homozygote (e.g. unlabeled peaks of substantial height), DO NOT enter any alleles for that locus. Hit Esc.
8. Hit Esc when done entering the alleles for the locus.
9. Select **File this Image**
10. Select **Y** to enter more loci or **N** when all loci are entered.
11. Repeat steps F-I as needed. The next locus will automatically be highlighted. If a locus is NEG, hit Esc to move to the next locus.
12. Hit Ctrl + Break to go to the **Main Menu**.
13. Select **Casework**
14. Select **Paternity**
15. Select **Case**. Hit Enter.
16. Type in the membrane ID number you created. Hit Enter.
17. Define a role for each person (each sample label you created). For each relationship type, you will enter the sample label you created above, such as 1079-00001. Hit spacebar. Enter " – " for race. Hit spacebar. Hit Del to move to the next role. Example of defining a role-**M** stands for Mother. Next to "M", you will assign 1079-00001. Next to **F,** type in 1079-00002.
18. If you need to add or change a role, select **language is:** Change it from "paternity" to "kinship". You can "add role" or "change role" after entering case number and before "immigration".
19. After entering roles, enter race. Enter "**chba"** lowercase, no spaces. These letters stand for Caucasian, Hispanic, Black, and Asian. Hit Enter.
20. Select **immigration/kinship**

21. Select **Type in (or edit) scenario**
    Enter the appropriate kinship formula. If your case is not the standard scenario, type in the scenario you would like to test. DO NOT USE LOWERCASE FOR SCENARIOS. SINGLE LETTERS REPRESENT DEFINED ROLES and IT MEANS YOU ENTERED A GENOTYPE. WORDS REPRESENT UNDEFINED ROLES OR SAMPLES UNTESTED OR NOT AVAILABLE.
    - The standard paternity scenario is **C: M + F/?**
    - Missing Child: **C/?: M + F**
    - Questioned mother of fetus (unknown father): **C: M/? + Father**
    - Mother not available or typed: **C: Mother + F/?**
    - Sibling (parents not available or typed): **U/?, A: Mother + Father**
    - Siblings and parents: **U/?, A:M + F**
22. After typing in the appropriate scenario, hit Esc.
23. Hit Esc after entering the kinship formula.
24. Check formula and overall kinship. Select **Estimate Likely Relationships.**
25. **Add Relationship Estimate to Report.** This should be highlighted already.
26. Check to make sure that:
    Prior prob=0.5
    Do NOT "restrict" data.
    DO consider mutation.
27. Select **Calculate & Report LRs, 4 races.**
28. After calculations are complete, hit Enter.
29. If question about error messages or data appear, type "n".
30. Select **Quit immigration**
31. Select **Print Report**
32. Type "q" to quit paternity.
33. Select **Exit from DNA View**