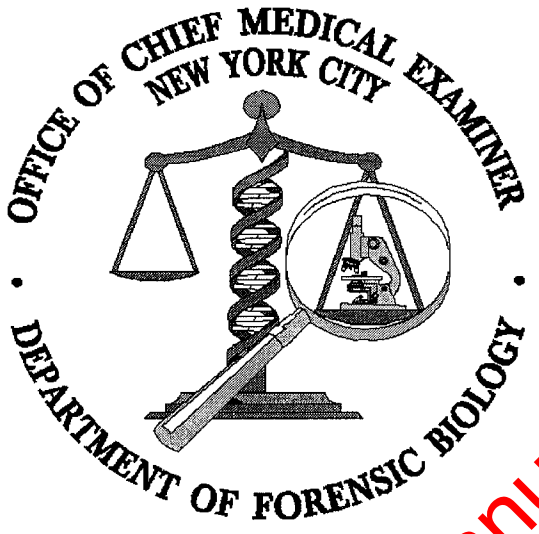

PROTOCOLS FOR HIGH SENSITIVITY TESTING
VERSION 1.0

Effective date: January 11, 2006

REVIEWED/APPROVED BY			
Title	**Print Name**	**Signature**	**Date**
Deputy Director/ Technical Leader	**Howard J. Baum, Ph.D.**	Howard J. Baum	**January 6, 2006**

FORENSIC BIOLOGY PROTOCOLS FOR HIGH SENSITIVITY TESTING

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REVISION

PROTOCOLS FOR HIGH SENSITIVITY TESTING
(SECTION 3)

Effective date: October 5, 2006

REVIEWED/APPROVED BY			
Title	**Print Name**	**Signature**	**Date**
Deputy Director / Technical Leader	**Howard J. Baum, Ph.D.**	Howard J. Baum	**October 5, 2006**

Cleaning

A. Decontamination of equipment

1. Surface decontamination of pipets, cap openers, racks, hoods, counters, centrifuges, robots, etc.
   a. Rinse with several times 10% bleach stored in the specialized containers that mix the bleach contents as they are used.
   b. Rinse with 70% Ethanol
   c. Rinse with distilled water.
2. Plates, tubes, swabs, and water are irradiated in a stratalinker as specified below.

B. Daily maintenance

1. Clean work area as specified in each section of the manual, before and after operation.
2. For hoods, apply UV light for ten minutes after working.

C. Weekly laboratory clean-up

1. Wipe down all benches, hoods, instruments, pipets, cap openers, scissors, and tweezers with bleach, water, and ETOH.
2. Maintain the sharpie waste.
3. Replenish laboratory supplies as needed according to the inventory lists.
   a. This includes, for example, tubes, plates, pipet tips, labcoats, masks, gloves, hair coverings, paper towels, kim wipes, and reagents.
   b. All tubes, plates, and water must be irradiated according to the method specified below.

D. Monthly laboratory clean-up

1. Wipe down all items specified on the monthly clean up list for each room. This includes, for example, wiping the benches, hoods, instruments, centrifuges, shakers, cap openers, scissors, tweezers, and pipets with bleach, water, and ETOH.
2. Wipe down the 3100 with distilled water only.
3. Soak the racks and cap openers in bleach, followed by two rinses with distilled water. Then, wipe down with ETOH and dry.
4. Maintain sharpie waste.
5. Replenish laboratory supplies as needed according to the inventory lists. Refer to the guidelines specified below for specifics on irradiation of water and labware.
6. Complete and review room clean up lists.

Irradiation with the Stratalinker 2400

A. Equipment to be irradiated

1. Tubes
2. Swabs
3. Water and buffers

B. Ensure that the Stratalinker is lined on all sides with foil.

C. Energy Mode (do not use)

1. Enter numbers in microjoules/cm^2 x 100.
2. The program completes when this energy has been delivered.

D. Time Mode

1. The 4000 mcirowatts/cm^2 is delivered for the specified time.
2. Lay tubes open on their sides in a single layer
   - **a. 50 mL tubes: 75 minutes**
   - **b. 15 mL tubes: 45 minutes**
   - **c. 1.5 mL tubes: 30 minutes**, store clean tubes in nalgene boxes.
   - **d. 0.2 mL tubes (PCR tubes): 10 minutes**, store clean tubes in tray labeled with date and your initials
   - **e. PCR plate raised within an inch of bulbs for 30 minutes**
   - f. Lay tubes filled with water or buffer closed on their sides.
     - **i. 50 mL tube: 75 minutes, fill to 15 mL**
     - **ii. 15 mL tube: 45 minutes, fill to 5 mL**
     - **iii. 1.5 mL tube: 45 minutes, fill to 0.5 mL**

Space

A. Evidence Sign In: Current evidence sign in room

1. Protective gear is not necessary in this room.
2. However, do not handle evidence or samples etc**.**

B. Evidence exam: High Sensitivity room 913a, evidence exam area

1. When prepared, sign the appropriate evidence into possession and enter the evidence exam area.
2. Don a gown, head covering, mask, and gloves in the changing area immediately outside room 916a prior to entering.
3. Examine evidence on the evidence exam tables under the hoods with the exception of very large items.
   a. Items potentially containing High Copy Number (HCN) DNA such as bottle swabs, will be examined under the exam hood near the extraction reagent freezer.
   b. Items potentially containing Low Copy Number (LCN) DNA, such as fingerprints, will be examined under the hood far from the door, away from foot traffic.
4. Following exam, return items and their packaging to the evidence unit for storage.
5. Remove protective gear in the changing area prior to traveling to the post-amplification or the office areas of the floor.

C. Extraction: High Sensitivity room 913a, extraction area

1. Separation of LCN and HCN samples
   a. All HCN samples will be processed under the hood or on the robot covered with a hood, and with the shakers and centrifuges proximal to the door.
   b. All LCN samples will be processed under the hood, on the robot covered by a hood, and with the shakers and centrifuges located distal to the door, away from foot traffic.
2. Store samples extracts in the extraction DNA freezer for long-term storage when applicable (extract remains).

3. Only items designated for pre-amplification procedures are permitted in the extraction area.
   a. The pre-amplification area includes room 916a and 916, rooms for extraction, quantitation, and amplification set-up.
   b. Once an item is placed in a post-amplification area, it may not return to the pre-amplification area without decontamination.
   c. Decontamination, as described above, includes a thorough washing with 10% bleach, followed by water and 70% ethanol. Tubes and plates also must be irradiated. (Refer to irradiation procedure.)
4. Remove protective gear in the changing area prior to traveling to the post-amplification or the office areas of the floor.
5. One may wear the same gear in room 916, however.

D. Aliquotting, quantitation, and preparing for amplification: High Sensitivity room 916

1. Separation of HCN and LCN samples
   a. All HCN samples will be processed under the hood and with the centrifuges near the pre-amp DNA refrigerator.
   b. All LCN samples will be processed under the hood with the centrifuge near the FX robot, away from foot traffic.
2. Only samples waiting for quantitation should be stored in the DNA refrigerator in room 916.
3. Quantitation products are discarded promptly upon removal from the Rotorgene 3000.

E. Amplification room: 937

1. 9700s are located in the opposite wing of the floor in the thermocycler room.
2. Exemplars and evidentiary samples must be amplified in separate thermocyclers.
3. Do not transfer items, including racks holding samples to be amplified, from the post-amplification area to the pre-amplification area without decontamination.

F. Sample Separation room: 934

1. Two ABI 3100 Genetic Prism Analyzers, "HS1" and "HS2" are located here.
2. Don gowns and gloves in the changing area immediately outside room
3. At the bench located near the post-amplification DNA refrigerator, prepare samples.
4. Examplars and evidentiary samples must be separated on different plates. However, they may be run sequentially, with exemplars on plate A and evidence on plate B, for example.
5. Do not transfer items, including racks holding samples to be amplified, from the post-amplification area to the pre-amplification area without decontamination.

G. Exemplar processing room

1. Don a gown, hair coverings, masks, and gloves in the changing area immediately outside this room.
2. No evidentiary samples are permitted in this room; only exemplar samples are processed.
3. Examine exemplar samples under the hood located proximal to the door. Return packaging to the evidence unit after examination.
4. Extract samples under the large hood or on the Biomek 2000 robot under a hood according to the procedure for a DNA IQ exemplar extraction.
5. After a thorough cleaning with 10% bleach, 70% ethanol, and water, prepare samples for amplification under the large hood or on the Biomek 2000 robot under a hood.
6. Do not transfer any items to this area that that have not been decontaminated.

H. Office area

1. Analyze samples at the analysis computers located in the office area.
2. Alternatively, samples may be analyzed directly on the 3100 computers.
3. Do not wear any protective gear or transfer any items from the laboratory area in to the office area.
4. Food and drinks may only be consumed in the office area.

High Sensitivity Extraction

A. Personal Preparation

1. Don a mask, hair covering, lab coat, and gloves.
   a. Ensure that the cuffs of the lab coat completely cover arms.
   b. Lab coats may be re-worn in the pre-amp rooms only.
2. Do not enter room without appropriate attire. Moreover, never touch any apparatus, bench etc with bare hands.
3. Remove gloves when answering the phone and touching door handles in order to maintain a DNA free glove.

B. Paperwork preparation

1. Based upon the source of the sample, a supervisor will assign the sample to either the LCN or HCN extraction batch as indicated by the evidence intake data sheet from each day. The samples will be stored in 96 well racks with a cover.
   a. All touched object, and fingerprint samples as well as those homicide and sexual assault samples referred by casework will be deemed as potential low copy number samples.
   b. Cigarette butts and other possible amylase source samples, scrapings from clothing and gloves, and other samples with biological fluid such as blood or semen will be deemed as potential high copy number samples.
2. When no more than 47 or 55 samples have accumulated, begin a manual or an automated extraction, respectively.
   a. For a manual extraction, arrange the samples in a checkerboard fashion in rack: A1 (EN), A3, A5, A7, A9, A11, C2, C4, C6, C8, C10, C12, E1, E3, E5, E7, E9, E11, G2, G4, G6, G8, G10, G12, H1, H3, H5, and H7.
   b. For an automated extraction, fill two racks in the same fashion.
3. Open the G drive to the forensic biology folder and click on the “HIGHSENS” folder. Open the “Sheets” folder followed the “EXT” folder and the “EXTHCN” (high copy number DNA extraction) or “EXTLCN” (low copy number DNA extraction) files.
4. In cell K1, type in the name of this extraction assay as follows: “EL” for LCN Samples or “EH” for HCN Samples, “month, day, and year, “period”, hour and minute. For example, EL040905.1330.
5. Manually enter OR copy and paste the samples names from the evidence examination sheet to the appropriate extraction sheet.

6. Type in the number of samples on the upper right hand corner in the sample number box. The spreadsheet will automatically calculate the amount of each solution needed.
7. **Self Witness step**: Confirm the sample names with the names on the sample tubes.

C. Work Place Preparation

1. Apply 10% bleach followed by water and 70% ETOH to the entire work surface, cap opener, and pipets**.**
2. Retrieve clean racks and cap openers, and irradiated microcentrifuge tubes, and irradiated GIBCO™ ULTRA PURE™ distilled water from storage or the stratalinker. Refer to the irradiation protocol for the specific time to apply radiation for each object.
3. Arrange work place to minimize crossover. For example, the sharps waste for tips should be on one side whereas the fresh filter pipet tips and reagents should be on the opposing side.
4. Position gloves nearby with bleach/ETOH/water in order to facilitate frequent glove changes and cleaning.

D. Digestion

1. Prepare solution in a UV irradiated 15 mL tube.
2. Use the pipette aid and the 10mL sterile pipette to aliquot SDS.

Stock Solution	Concentration	1	28 + 2	56 + 2
0.01% SDS	0.01% (0.0096%)	192 µL	5760 µL	11 mL and 136 µL
Proteinase K 18 mg/mL	0.72 mg/mL	8 µL	240 µL	464 µL

3. Vortex solution well. Add 200 µL of the digestion buffer to each sample. Open only one sample tube at a time using the cap opener.
4. After adding the solution, move the tube to indicate addition of digestion buffer.
5. Incubate on the heat shaker at 56ºC for 30 minutes at 1400 rpm.
6. Incubate on the heat shaker at 99ºC for 10 minutes at 0 rpm.
7. Centrifuge samples at full speed briefly.
8. Ensure that the swabs in their entirety are submerged in the digestion fluid.
9. During the digestion period, one may label the Microcon®, elution, and storage tubes in advance.

E. Purification and Concentration

1. Arrange samples in the 96 well rack as described above.
2. Prepare Microcon® 100 tubes and label the membrane tube and filtrate tube cap. Use Microcon® 50 tubes as indicated, for example when degradation is suspected. Consult evidence exam schedule sheet or supervisor.
3. Align the Microcon® tubes in the same fashion as those of the extraction tubes in a separate rack as follows: A1 (EN), A3, A5, A7, A9, A11, C2, C4, C6, C8, C10, C12, E1, E3, E5, E7, E9, E11, G2, G4, G6, G8, G10, G12, H1, H3, H5, and H7.
4. **Witness step:** Confirm the sample names with the names on the sample and Microcon® tubes.
5. Pre-coat the Microcon® membrane
   a. Make a 1/10 dilution and then another 1/100 dilution of 1mg/mL of Poly A RNA as follows: add 2 µL of PA to 18 µL of irradiated water, mix the solution well, and then add 2µL of this dilution to198 µL of irradiated water (1ng/µL).
   b. Vortex mixture thoroughly and tap tube on bench to remove any reagent from the cap.
   c. Prepare the Poly A RNA mixture according to the table below in a 15 mL tube.

Reagent	1 sample	28 samples	56 samples
water	199 µL	5970 µL	11 mL and 542 µL
Poly A RNA 1ng/µL	1 µL	30 µL	58 µL

   d. Aliquot 200 µL of this mix to each Microcon®. Avoid touching the membrane.

6. Filtration
   a. Add the entirety of each sample to its pretreated Microcon® membrane. Aspirate the solution by placing the pipet within the swab, for example. The sample tubes may be discarded.
   b. Centrifuge at the Microcon® tube at 2400 rpm for 15 minutes.
   c. While samples are centrifuging, label the elution and storage tubes.
   d. If indicated on the evidence examination schedule sheet or by a supervisor, perform a second wash step applying 400 µL of water onto the membrane and centrifuge again at 2400 rpm for 15 minutes.
   e. Visually inspect each Microcon® membrane tube. If it appears that more than 5 µL remains above the membrane, centrifuge that tube for 5 more minutes at 2400 rpm.

7. Elution
   a. Line up samples as above and place concordant irradiated collection tubes adjacent to each Microcon® membrane tube.
   b. Open one Microcon® tube and its collection tube at a time.
   c. Add 20 µL of irradiated water to the Microcon® and invert the Microcon® over the collection tube. Avoid touching the membrane.
   d. Centrifuge at 3500 rpm for 3 minutes.
   e. Transfer the elutant to a labeled tube, and measure and record the approximate volume. If necessary, add water to each tube to bring the total volume to 20 µL for an Identifiler™ reaction. Discard the Microcon® membrane.
   f. If the elutant appears to be a dark color, it may be necessary to purify the sample again. Prepare a fresh Microcon® tube and repeat steps 4-6.

F. Clean up

1. Wipe the entire work area surface and pipets with 10% bleach followed by ETOH and water.
2. Soak and/or clean racks, pipets, the cap opener and other equipment that would not adequately cleaned with the stratalinker with 10% bleach, water and 70% ETOH.
3. If necessary, replenish the microcentrifuge tube and Microcon® collection tube supply. Place tubes in the stratalinker and apply ultraviolet radiation for 30 minutes. Do not irradiate the Microcon® membranes.
4. Turn on the UV lamp in the hood for ten minutes, and remember to turn off the light in order to preserve the bulb.

G. Sample storage

1. Place samples in a cryobox in the DNA refrigerator in the pre-amp room on the top shelf in the section labeled “samples waiting for quant”.
2. Place extraction sheet on the bulletin board in the preamp room.
3. Name the assay, for example, EL041405.1100, as follows:
   a. E = extraction
   b. H = High Copy Number DNA or L= Low Copy Number DNA samples
   c. Date and Time
4. To facilitate high throughput, enter the name of the extraction in the appropriate column for each sample in the database to indicate the status of the sample.

ALU-Based Quantitative Real Time PCR on the Rotorgene

A. Paperwork Preparation

The aim of the following steps is to set up an Excel spreadsheet with a list of samples that can be used for the quantitation and the PCR set up. LCN (low copy number) samples are contact DNA samples with low expected DNA yields, while HCN (high copy number) samples are body fluid samples.

1. Open the G drive and the "FBIOLOGY" folder and click on the "HIGHSENS" folder. Open the "SHEETS" folder followed by the "QUANT" folder and the "RGMANUAL" file.
2. Alternatively, in the pre-amp room on the Rotorgene computer, open the sheet entitled "RGMANUAL" located directly on the desktop.
3. In cell D3 type in the name of this quantitation assay as follows: "QL" for LCN Samples or "QH" for HCN Samples, month, day, and year, "period", hour and minute. For example, QL040905.1330. Exemplars are always considered HCN samples.
   a. LCN and HCN evidence samples may be quantitated simultaneously. However, the HCN extracts must be diluted prior to performing the assay. In other words, only the dilutions of the HCN samples may be present with the LCN extracts under the hood.
   b. Since LCN evidence samples should not be diluted and since LCN extract volume is limited such that only the two required microliters can be spared for quantitation, extreme care must be used in pipetting the LCN quantitation aliquot in its entirety.
4. Determine which samples require quantitation by examining the bulletin board for extraction sheets and sheets entitled "samples for quantitation/ amplification" in the pre-amp room. Prioritize samples by extraction date.
5. Copy sample names from the extraction sheet and/or "samples for quantitation" sheet located in the resolutions folder, and paste into the sample Rotorgene sheet (sheet two of the excel workbook).
6. Enter the initials of the analyst's to whom paperwork should be directed under the "INT" labeled columns.
7. Record the dilution factor for each sample in the column labeled "DIL" as follows: 1 for neat samples, 0.1 for a 1/10 dilution, and 0.01 for a 1/100 dilution.
8. On sheet one, in cell D4, enter the name of the extraction assay and in cell E4 type the Rotorgene well numbers of the appropriate samples. If more than two extraction sets were combined, enter this information in wells D4 and E4 and in wells G4 and H4.

9. Type in the number of samples that are being measured in the box directly below the box labeled “total Rx No” (E7). The spreadsheet will automatically calculate how much of each reagent to aliquot.

B. Personal Preparation

1. Put on a mask, lab coat, hair covering and gloves.
   a. Ensure that the cuffs of the lab coat completely cover arms.
   b. Lab coats may be recycled.
2. Do not enter room without appropriate attire. Moreover, never touch any apparatus, bench etc with bare hands.
3. Remove gloves when answering the phone and touching door handles in order to maintain a DNA free glove.

C. Work Place Preparation

1. Apply 10% bleach followed by water, and 70% ETOH to the entire work surface, cap opener, and pipets**.**
2. Retrieve clean racks, cap openers, Rotorgene 0.1 mL tubes and caps, microcentrifuge tubes, and irradiated GIBCO™ ULTRA PURE™ distilled water from storage or the stratalinker.
   a. The tube racks and the cap opener may be cleaned with 10% bleach followed by ETOH. Ensure that the racks are dry before use.
   b. The 1.5 mL microcentrifuge tubes and water aliquots in 1.5 mL tubes must be irradiated for 30 and 45 minutes, respectively.
   c. The Rotorgene gene tubes and caps are used as packaged.
3. Arrange work place to minimize crossover. For example, the sharps waste for tips should be on one side whereas the fresh filter pipet tips and reagents should be on the opposing side.
4. Position gloves nearby with bleach/ETOH/water in order to facilitate frequent glove changes and cleaning.

D. Sample Dilution

If necessary, for HCN DNA samples for example, dilute the sample extracts.

1. Label microcentrifuge dilution tubes with sample name and “1/10”.
2. Place each dilution tube directly behind the corresponding extract tube in a rack.
3. Add 18 µL of irradiated water to each dilution tube. Pipet tips do not need to be changed to add water to empty tubes. Close all caps.
4. Open only one sample and its corresponding dilution tube at one time.

5. To thoroughly mix each extract, pipet sample extract up and down place 2 µL of this sample into each dilution tube.
6. Immediately following each dilution, return the original sample extract tube to its cyrobox, which is under the hood. Return the original samples to 4ºC storage.
7. Once the dilutions are completed, evidentiary samples may join exemplar dilutions under the hood, for example.

E. Remove reagents for the master mix from the reagent freezer/refrigerator

1. Retrieve $MgCl_2$, 10X PCR buffer, BSA, dNTPs, TAQ GOLD, unlabeled "EB1 and EB2" primers, and SYBR Green I from the freezer, irradiated GIBCO™ ULTRA PURE™ distilled water from the refrigerator, and DMSO from the cabinet.
2. Store reagents except DMSO and water in a Nalgene cooler on the bench.
3. Record lot numbers of reagents.
4. Just before initiating "sample preparation", place $MgCl_2$, 10X PCR buffer, BSA, dNTPs, unlabeled "EB1 and EB2" primers, and SYBR Green I on a 48-position microcentrifuge rack in order to thaw these reagents.

In order to ensure accuracy and to minimize contamination, focus on the task and avoid unnecessary conversations.

F. Standard Curve Preparation

1. Retrieve standard DNA from the freezer labeled 1600 pg/µL and record lot #.
2. Centrifuge the 1600 pg/µL standard DNA tube at full speed briefly.
3. Label tubes as follows: 400, 100, 25, 6.25, 1.56, 0.39, and NTC (no template control or 0 pg/µL).
4. Add 7.5 µL of irradiated water to tubes 400, 100, 25, 6.25, 1.56, 0.39, and the NTC. Pipet tips to not need to be changed to add water to empty tubes. Close all caps.

5. 0.25 Serial dilution
   a. Open only two consecutive standard DNA tubes at once starting with the 1600 and the 400 pg/µL tubes. (Do not open the 0 pg/µL tube for this dilution.)
   b. Pipette the DNA solution in the 1600 pg/µL tube up and down several times to thoroughly mix the contents. Take 2.5 µL of standard DNA at 1600 pg/µL and add to the 400 pg/µL tube. Pipette the DNA solution in the 400 pg/µL tube up and down several times to thoroughly mix the contents.
   c. With a new pipet tip, take 2.5 µL of standard DNA at 400 pg/µL and add to the 100 pg/µL tube. Pipette the DNA solution in the 100 pg/µL tube up and down several times to thoroughly mix the contents.
   d. With a new pipet tip, take 2.5 µL of standard DNA at 100 pg/µL and add to the 25 pg/µL tube. Pipette the DNA solution in the 25 pg/µL tube up and down several times to thoroughly mix the contents.
   e. With a new pipet tip, take 2.5 µL of standard DNA at 25 pg/µL and add to the 6.25 pg/µL tube. Pipette the DNA solution in the 6.25 pg/µL tube up and down several times to thoroughly mix the contents.
   f. With a new pipet tip, take 2.5 µL of standard DNA at 6.25 pg/µL and add to the 1.56 pg/µL tube. Pipette the DNA solution in the 1.56 pg/µL tube up and down several times to thoroughly mix the contents.
   g. With a new pipet tip, take 2.5 µL of standard DNA at 1.56 pg/µL and add to the 0.39 pg/µL tube. Pipette the DNA solution in the 0.39 pg/µL tube up and down several times to thoroughly mix the contents.
   h. Do not add anything to the NTC tube.

G. Sample Preparation

1. Remove calibrator: 1500 pg/µL from freezer and record lot number.
   a. Centrifuge the calibrator tube at full speed briefly.
   b. Make a 0.166 dilution (1/6) of the calibrator with 2 µL of the calibrator and 10 µL of irradiated water.
2. Sample dilutions:
   a. LCN samples use at neat concentration; no dilution necessary.
   b. HCN samples require a 0.1 dilution.
3. Vortex all samples including the standards, NTC, calibrator, and the dilution and/or extract tubes.
4. Centrifuge all samples briefly for 3 seconds at no greater than 3000 rpm; this will prevent the DNA from aggregating at the bottom of the tube.

5. **Witness Step: LCN samples**
   a. Arrange samples in order according to the sample sheet in a 96 well rack.
   b. Place samples in exactly the same place on the rack as they will appear vertically positioned in the rotor. (the Rotor positions A1-A8, B1- B8, C1-C8, D1-D8, E1-E8, F1-F8, G1-G8, H1-H8, and I1-I8, whereas the microcentrifuge rack positions A1-H1, A2- H2, A3-H3, A4-H4, A5-H5, A6-H6, A7-H8, and A9-H9. Therefore, A1 for the rotor is equivalent to A1 on the rack, B1=A2, C1=A3, D1=A4, E1=A5, F1=A6, G1=A7, H1=A8, and I1=A9.)
   c. Specifically, standard 1600a will reside in well A1 in the rotor and the rack. Standard 1600b will reside in A2 on the rotor, but well B1 in the rack. However, well B1 will be empty, since one will take the duplicate 1600 standard from the first tube. Rotor position A3 corresponds to rack well C1, standard 400a, and rotor position A4, represents standard 400b in rack well D1 which is empty.
   d. Only the standards 1600 pg/µL – 0.39 pg/µL are duplicated. The no template control (0 pg/µL), the calibrator, and the samples are not duplicated.
   e. In brief, the standards are vertically placed in positions A1 through B4.
   f. The no template control resides in B7, and the calibrator in B8. Samples are located in rotor wells C1-I8 and rack positions A3-H9.

H. Master Mix preparation

1. Prepare a 1/100 dilution of SYBR Green I.
   Take 2 µL of SYBR Green I in 198 µL of irradiated water, vortex, and tap the tube on the bench to prevent the reagent in the cap.
2. Mix each reagent before adding.
   a. After each reagent has thawed, vortex each reagent, with the exception of TAQ GOLD.
   b. Centrifuge reagents in the table top centrifuge at full speed.
3. Add each reagent in the order as it appears on the worksheet. Thoroughly mix each tube reagent by pipetting up and down, or vortexing briefly. If vortexing, afterwards tap the tube on the bench to prevent the reagent from being trapped in the cap.
4. For total reagent volumes above 20 µL, use a P200 even for multiple dispenses as opposed to one dispense with a P1000. To ensure accurate pipetting, aspirate and dispense the reagent as specified on the run sheet.

5. After adding each reagent, check that it has been added on the quantitation sheet, and place the reagent back in the Nalgene cooler, or for water and DMSO, in the opposite corner of the 48 well microcentrifuge rack.
6. Thoroughly mix the master mix by vortexing. Tap the tube on the bench to prevent the reagent from being trapped in the cap.
7. Add 23 µL of master mix to each tube. Fill tubes in a vertical fashion (A1 to A8, and B1-B8). After adding master mix to 16 tubes, re-vortex the master mix and tap the tube on the bench to prevent the reagent from being trapped in the cap. Use a new pipette tip.
8. In order to track additions, it may be helpful to recite each well position to be oneself.

See below for reagent concentrations, the spreadsheet will calculate amounts for n+10%n samples and will display rounded values for pipetting.

Reagent	Concentration	1 rx µL
Irradiated GIBCO™ ULTRA PURE™ distilled water		8.38
10X PCR Buffer	10mM Tris/ 50mM KCL	2.50
25 mM MgCl2	275 µM	2.75
5 mg/mL BSA	0.525µg/µL	4.00
2.5 mM dNTPs	200 µM each	2.00
DMSO	8%	1.96
1/100 dilution of 10,000X SYBR Green I	100X	0.28
20 pmol/µL Primer EB1	0.4 µM	0.50
20 pmol/µL Primer EB2	0.4 µM	0.50
5U/µL ABI Taq Gold	0.625U	0.13
Total volume		23.00

I. Sample Addition

1. In order to avoid the creation of aerosols, thoroughly mix the contents of each tube by pipetting up and down repeatedly.
2. Add 2 µL of each sample, including the standards, NTC, the calibrator dilution, and the sample dilutions and/or extracts, to each tube with master mix.
   a. It is helpful to place the sample extract tube in its cryobox or another rack directly after its addition.
   b. Every four reaction tubes, place caps on the tubes. (The caps are attached in sets of four.)
   c. Number the first cap in every set of four as they will appear in the rotor. A1 for A1, A5 for A5, B1 for B1 etc.

      IMPORTANT: **DO NOT** label the tube itself, as this may interfere with fluorescent detection.

   d. Open the machine. Add tubes to the Rotorgene. Ensure that tube A1 is in position A1 etc.
   e. Ensure that all positions on the rotor are filled. (use blanks if necessary)
   f. Add the silver ring, turn the locking ring in place, and close machine.

J. Software Operation

1. Open excel and the relevant sample sheet to the sheet with the sample names, then collapse the window.
2. Open Rotorgene 6 software on the desktop
3. Click File, New. Perform last run (highlighted), and click "new"
4. In the wizard
   a. "Rotorgene 72 well rotor" (highlighted)
   b. Check off "locking ring attached", and click next.
   c. Type initials for Operator and add any notes (extraction date/time)
   d. Reaction volume "25 µL"
   e. Sample layout "in rows 1-8"
   f. Click "next" and calibrate
      i. "Perform Calibration before 1st acquisition"
      ii. Click on "calibrate all"
      iii. "This will remove your existing setting for auto gain calibration?"
      iv. Click YES, and "close"
   g. Click next in wizard and "start run"

5. "Save as" the date and time (1129040200) nov 29, 2004 at 2:00) in Log Archive folder
6. Sample sheet window
   a. Expand the excel sample sheet window. Copy the sample names.
   b. Paste sample names in the appropriate rows in the Rotorgene sample window by right clicking and selecting paste.
   c. Settings:
      Given concentration format: 123,456.78912 unit pg/µL
      i. Type category
         1. Standards: std
         2. Zero standard: NTC
         3. Samples and calibrator: unk
      ii. In all wells with standard, calibrator or sample, select "YES"
      You may copy and paste selections by right clicking.
   d. Hit "Finish"

See below for cycling parameters that should not be changed:

95ºC	10 min	
94ºC	15 sec	35 cycles
68ºC	30 sec	
72ºC	30 sec	
72ºC	15 sec	

7. Enter run information in the Rotorgene log book.
8. The run will approximately require 1 hour and 15 minutes for completion.
9. If you are leaving for the day, the machine needs no further assistance.
10. If you will remain upon completion of the run, and no more runs will be conducted that business day, turn off the computer
11. Following the initial heating to activate the TAQ and the gain calibration, the raw data will appear on the screen. With this information, one can monitor the progress of the run. Fluorescence for the highest standard should be apparent from ~ cycle 15.
12. Previous run files may be examined while the computer is collecting data.
13. Collapse the window
14. Double click on the Rotorgene icon on the desktop.
15. The computer will prompt that another version of the software is running and ask if you want to run an analysis version only. Click yes.

K. Clean Up

1. Wipe entire work area surface and pipets with bleach followed by water and ETOH.
2. Soak and/or clean racks, pipets, cap openers, and other equipment that would not be adequately cleaned with the stratalinker with 10% bleach, 70% ETOH, and water.
3. If necessary, place fresh microcentrifuge tubes in the stratalinker and apply ultraviolet radiation for the 30 minutes.
4. Turn on the UV lamp in the hood for ten minutes. Do not forget to turn off the light.
5. Return water, dNTPs, MgCl2, 10X PCR buffer, BSA, DMSO, EBI primer, EB2 primer, and TAQ GOLD tubes with any remaining reagents to storage.
6. Dispose of all dilution tubes and the standard, calibrator, and SYBR Green I tubes. Sample dilutions may be stored until assay success is confirmed.

L. Sample Storage

1. Store extracts in a cryobox in the DNA refrigerator in the pre-amp room on the shelf labeled “samples waiting for amplification”.
2. Place the quantitation sheet on the bulletin board in the pre-amp room.

M. Analysis

1. Open Rotorgene 6 software on the desktop
2. Click “Open” and click on the run to be analyzed in the Log Archive folder
3. Click “Analysis” on the toolbar.
   a. Select “Quantitation”, “Show”.
      i. 3 windows will open with the standard curve, the samples, and fluorescence.
      ii. Ensure that “dynamic tube” and “slope correct” are selected on the tool bar.
      iii. Click “auto find threshold” in the lower right corner.
      iv. Select “calculate automatic threshold” and “okay”

b. Check if any sample curve crosses the threshold at an early cycle due to background fluorescence. The sample in question would have no value, but the normalized data would display a curve that crosses the threshold both at an early and at a later cycle (Figure 1A).

i. Dynamic tube normalization uses the second derivative of each sample trace to determine a starting point for each sample. The second derivative measures the rate of the rate of change. In other words, the increase in fluorescence from cycle to cycle is averaged from cycle 1 up to the where the fluorescence "takes off" or the starting cycle number for each sample. This method gives the most precise quantitation results.

ii. In order to avoid disabling the dynamic tube normalization setting, move the threshold to the right, ignoring the first few cycles, so that the sample does not cross the threshold. This can be achieved by the following:

1) In the normalized data windowpane, on the lower right side, under CT calculation, change the number for "Eliminate Cycles before:" from 0 to 1-5. Chose the smallest number where the threshold does not cross the data curve in question (Figure 1B).

2) Alternatively, select the grid immediately to the right of "Eliminate cycles before". This allows manually manipulation of the threshold.

3) Reanalyze the data by selecting "auto find threshold".

4. Save experiment as " QL.date.time" or "QH.date.time" in the log archive folder.

N. Report

1. Select "Report" from toolbar
   a. Select "Quantitation, cycling A FAM/SYBR
   b. Select "full report" double click
   c. Generate report
2. Print.
3. Submit reaction sheet and report paperwork to supervisor for review.

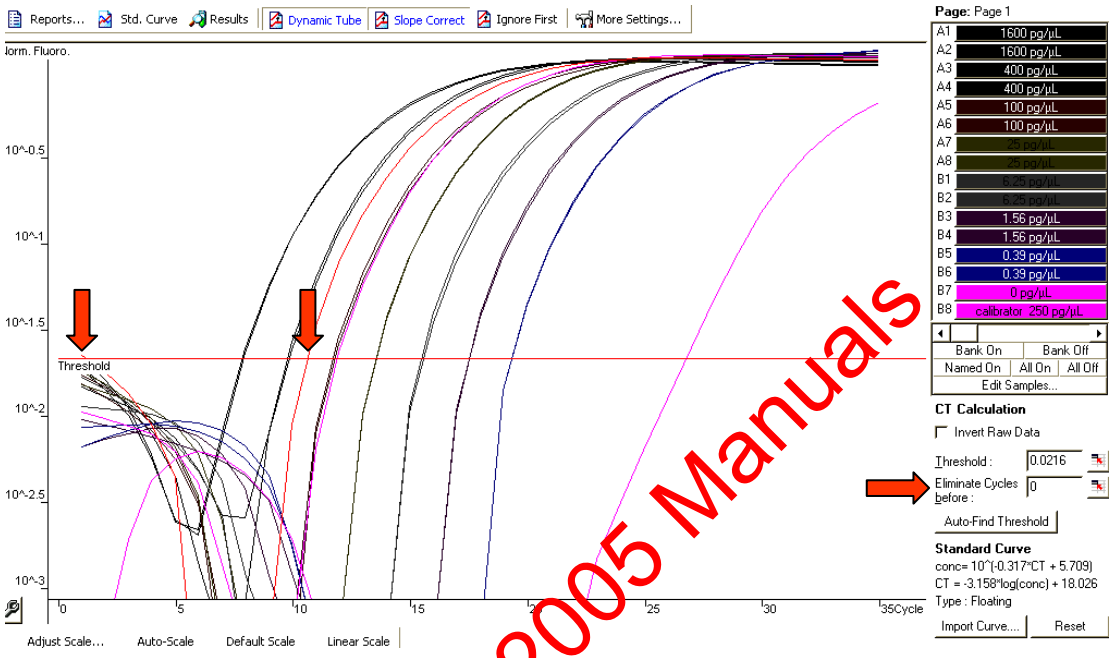


Figure 1A: Normalization with the dynamic tube method usually does not required one to eliminate early cycles. However, the sample depicted by the red curve above crosses the threshold during the first and the ninth cycle yielding a negative value.

Figure 1B: Eliminating the first four cycles prevented the sample depicted in red from crossing the threshold at an early cycle. Therefore, the value at which the sample crossed the threshold, cycle nine was employed to determine the quantitative value.

O. Value Calculation by Software

1. Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction.
2. The more template present at the beginning of the amplification reaction, the less cycles that will be required to obtain sufficient fluorescence signal above background. This point is defined as the Ct and will occur during the exponential phase of amplification.
3. The rotorgene software scans through all possible threshold levels until the best fit is found for the standard curve.
4. The R value is a number between -1 and 1 which defines how well a line of best fit or a least squares line describes the relationship between two variables. An R-value of zero implies no relationship, whereas an R-value of -1 or 1 indicates that the data values fall directly on the line of best fit.

5. The following are calculated :
   a. The slope of a reaction: change in Ct/change in log input, opt -3.322
   b. Reaction efficiency = $[10^{(-1/slope)}] - 1$, optimum 1
      A 100% efficient reaction results in a doubling of the amplification product each cycle.
   c. exponential amplification = $10^{(-1/slope)}$, optimum 2

P. Assay Interpretation

Standards and Controls

1. Check the raw data for cycling. If the fluorescence is below 80 RFUs, yet the reaction efficiency is acceptable (see 2b), determine if the SYBR Green I was thawed more than once. If not, notify QC in order to test stock. The assay still passes as long as conditions 2b and 3 are fulfilled.
2. Confirm that the following settings are correct:
   a. standard curve imported "no"
   b. Start normalizing from cycle "1"
   c. noise slope correction "yes"
   d. reaction efficiency threshold "disabled"
   e. normalization method "dynamic tube normalization"
   f. digital filter "light"
   g. no template control threshold "10%"
3. Slope optimum: -3.322
4. R^2 value optimum: 0.999
5. **Reaction efficiencies should range from "0.88 – 1.15"**
   a. Reaction efficiencies above "1.15" are too high and the run fails.
   b. Reaction efficiencies between 0.8 and 0.88 require consultation with a supervisor.
   c. Reaction efficiencies below "0.8" are insufficient and the run fails.
6. **Calibrator must lie between 400 pg/µL and 100 pg/µL standards.**
   a. This assay has a potential inherent error of a factor of 0.3.
   b. Therefore, the expected value for the calibrator is between 175 pg/µL and 325 pg/µL
7. **Non template controls or zero standards should be ≤ 0.1 pg/µL**
8. At least one of each duplicate standard concentration should be apparent.
9. Record the reaction efficiency, calibrator and non-template control values, and fail the assay if these values or the standards are unacceptable. The reviewer must sign and date the sheet as well.

10. Complete an Assay Resolution sheet if necessary and identify the problem, the assay name, and the samples affected, and select a resolution from the drop down menu. Post the sheet on the appropriate bulletin board in either the extraction or the pre-amp room. Save the sheet to the resolutions folder on the desktop of either the extraction or the pre-amp room's computer.
11. Initiate retesting of all samples in a failed run.

Sample Interpretation

1. Samples that are 1000 pg/µL and above should be requantitated at a 1:100 dilution.

2. If the extraction negative is ≥ 0.2 pg/µL the sample set must be re-extracted. A concentration of 0.2 pg/µL would equate to 1pg amplified, which would produce ample alleles to fail the amplification.

3. If the no template control is ≥ 0.1 pg/µL, LCN samples are amplified since there is not sufficient sample to retest. However, HCN are requantitated.

4. If a sample appears to be inhibited since, the curve initially increases and then plateaus (see Figure 2), purify the sample with a Microcon® 100 and amplify or re-quantify if necessary.
   a. Figure 2A displays samples that inherently fluoresce.
   b. For other types of inhibition and/or quenching, the curves will appear as the other curves but then plateau earlier (Figure 2B). Although purification of the samples in Figure 2B may enhance fluorescence in this assay, since sufficient DNA is present for amplification, the samples produced DNA STR profiles (data not shown). The important factor is whether the samples increase in fluorescence by at least ten fold.

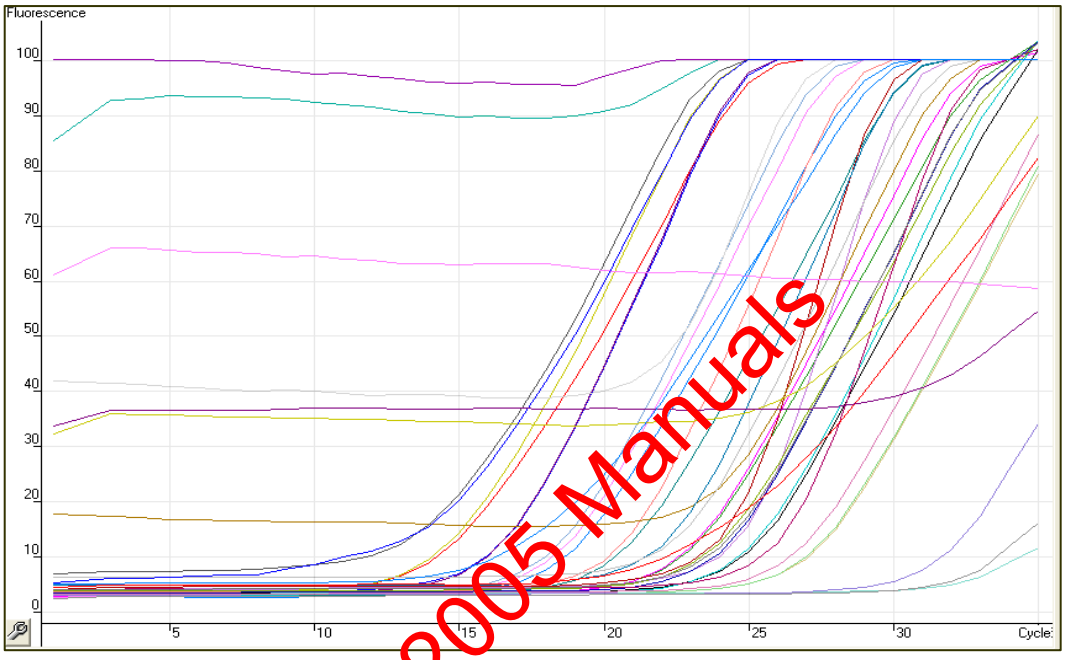


Figure 2A (above): The plateau lines are examples for inhibited samples that have inherent fluorescence. The slopes starting at cycle 15 are the expected DNA value increase.

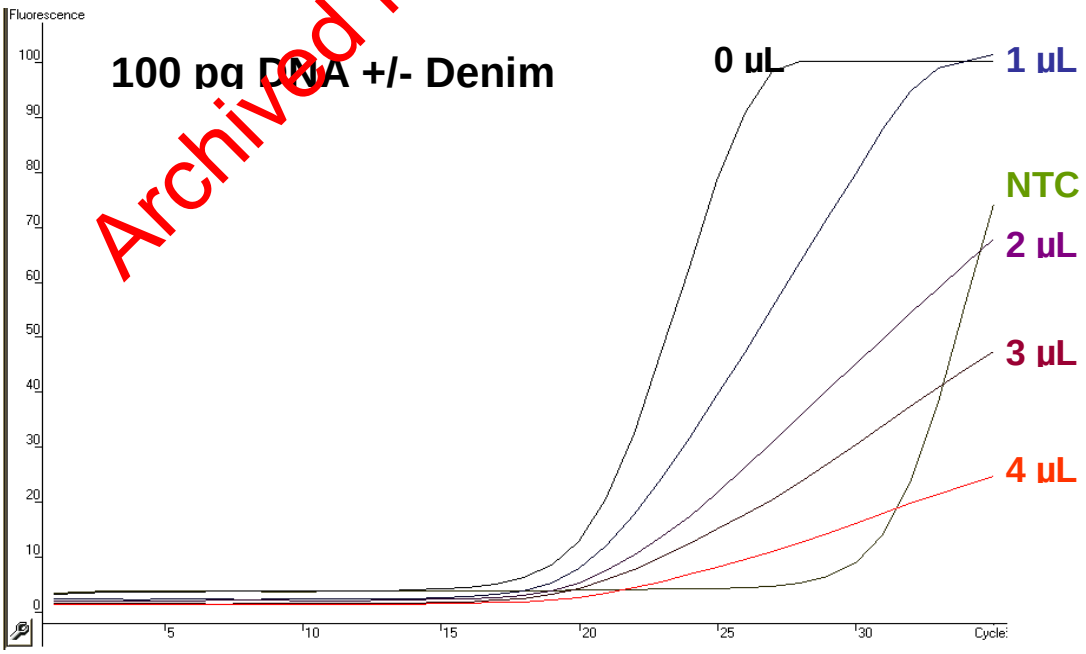


Figure 2B: 100 pg of DNA was measured with 0 to 4 µL of denim dye. The addition of denim dye to the samples reduced the amount of recorded fluorescence. However, DNA was still amplified, as evidenced by the increase in fluorescence over time.

5. Proceed to amplification for all samples with values according to table below:

System	Minimum desired DNA template amount	Template volume for amplification	Sample Conc.
Cofiler™/Profiler Plus™	150 pg	20 µL	7.5 pg/µL
Identifiler™ 28 cycles	150 pg	5 µL	30 pg/µL
Identifiler™ 31 cycles databasing samples	20 pg	5 µL	4 pg/µL
Identifiler™ 31 cycles comparison samples	10 pg	5 µL	2 pg/µL

Samples with less than 10 pg may be amplified upon referral with the supervisor.

References

Nicklas, J. A., Buel, E. Development of an *Alu*-based, Real-Time PCR Method for Quantitation of Human DNA in Forensic Samples

Nicklas, J. A., Buel, E. Development of an *Alu*-based, QSY 7-Labeled Primer PCR Method for Quantitation of Human DNA in Forensic Samples

3. DNA QUANTITATION			
DATE EFFECTIVE 01-11-2006	DATE REVISED 10-05-2006	VERSION 1.0	PAGE 1 OF 1

As of October 05, 2006, this section has been moved to the Protocols for Forensic STR Testing manual.

See Approval Form

Go directly to the new section

Amplification of Low Copy Number (LCN) DNA Samples (< 150 pg/µL) with Identifiler™

A. Paperwork preparation

The PCR strategy for LCN samples is to do three parallel amplifications.

1. Open the "FBIO" folder on the G drive and click on the "HighSens" folder followed by the "preamp" folder and the "IDLCNMAC" excel file.
2. Copy and paste the samples and their concentration from the Rotorgene results sheet into the RG VALUES sheet in the column labeled "SAMPLE NAME" and "RGVALUE".
3. Hold the control key and the key "L" or Tools→Macro→ IDLCN
   a. The macro will filter and eliminate all values that are less than 4 pg/µL and greater than 30 pg/µL and will place the extraction negatives at the top of the list with all samples in the order that was specified on the Rotorgene Values sheet.
   b. The data will then be automatically populated into the amplification and 3100 run sheets.
4. Samples with ≥ 30 pg/µL samples should be considered HCN and amplified separately. Samples with less than 4pg/µL can only be amplified for sample comparisons, not routine databasing. These samples may be added to the sheet following the macros execution.
   a. Copy the sample name and RG value from the RG VALUES sheet.
   b. Paste these into the SAMPLES sheet under the Sample Name and RG Values columns at the end.
5. Select the IDLCN worksheet and type the name of the amplification in cell C1 as follows: Amonthdateyear.time for example, A011106.1000
   a. The sheet will automatically calculate the number of samples that are to be amplified. This will populate cell C2 of the IDLCN worksheet.
   b. The sheet will also calculate the amount of reagents required, and the dilution factor necessary for the samples.
6. Save the sheet and review.

TABLE 1: PCR amplification input based on Rotorgene values

RG value at 1:10 dilution pg/µL	RG value neat pg/µL	Amplification Sheet	Dilution
< 0.4 pg/µL	< 4.0 pg/µL	Amplify with 31 cycles*	Neat = 1
< 3.0 pg/µL	4.0 pg/µL to 30 pg/µL	Amplify with 31 cycles	Neat = 1
≥ 3.0 pg/µL	≥ 30 pg/µL	Amplify as HCN DNA**	As appropriate

* Samples providing less than 20 pg per amplification are suitable only for comparison, and can only be amplified with permission of a supervisor.

** Refer to the STR DNA manual for procedure

B. Personal Preparation

1. Put on a mask, lab coat, hair covering, and gloves.
   a. Ensure that the cuffs of the lab coat completely cover the arm.
   b. Lab coats may be recycled.
   c. Masks, hair coverings and gloves may be disposed after one use.
2. Do not enter room without appropriate attire. Moreover, never touch any apparatus, bench etc with bare hands.
3. Remove gloves when answering the phone and touching door handles in order to maintain a DNA free glove.
4. Do not hesitate to change gloves or at least rinse gloves with bleach followed by ETOH if contamination is suspected.

C. Work Place Preparation

1. If using a plate, turn on the heated plate seal apparatus.
2. Apply 10% bleach followed by water, and 70% ETOH to the entire work surface and pipets.
3. Retrieve clean racks and cap openers, and irradiated microcentrifuge and PCR tubes and/or plates and molecular biology grade sterile water free of DNAse from storage or the stratalinker. Autoclaving the reagents is not sufficient to remove all traces of DNA. Refer to the irradiation protocol for the specific time to apply radiation for each object or reagent.

4. If using a PCR plate, place a super pierce strong seal on top of the plate, and place the plate in the plate adapter on the ABgene heat sealer.
   a. Push the heat sealer on top of the plate for 2 seconds.
   b. Rotate the plate and reseal for 2 additional seconds.
5. Use PCR tubes compatible with the thermocycler. If using the ABI 9700, use the ABI or ABgene PCR tubes.
6. Arrange work place to minimize crossover. For example, the sharps waste for tips should be on one side whereas the fresh filter pipet tips and reagents should be on the opposing side.
7. Position gloves nearby with bleach/ETOH/water in order to facilitate frequent glove changes and cleaning.

D. Sample Preparation

1. Prepare dilutions for each sample if necessary.
   a. Centrifuge samples at full speed briefly.
   b. Line up samples behind labeled tubes for dilutions.
   c. Pipet sample up and down several times to thoroughly mix sample. Avoid vortexing sample and the possible creation of aerosols.
   d. Aliquot sample. Open one sample tube at a time with a cap opener and change tips for every sample. Clean cap opener periodically with 10% bleach followed by 70% ETOH and water.
2. **For an Identifiler™ 31 cycle amplification, make a 0.2 dilution of the ABI Positive (A9947) control at 100 pg/µL (2 µL in 8µL of water). This yields 20 pg/µL of which 5 µL will be used.**
3. Irradiated water will serve as an amplification negative control.
4. Arrange samples in a 96 well rack in precisely the positions they appear on the sheet.
5. **Witness step.** Ensure that your samples are properly positioned.

E. Master Mix Preparation

1. Retrieve **Identifiler™** primers and reaction mix from the refrigerator, and Taq Gold from freezer, and store in a Nalgene cooler on bench.
2. Centrifuge reagents at full speed briefly.
3. Record lot numbers of reagents.

4. Vortex or pipet the reagents up and down several times to thoroughly mix the reagents. After vortexing, tap reagent tube on bench to ensure that no sample is trapped in the cap. Do not vortex TAQ GOLD as it may degrade the enzyme.
5. Consult the pre-calculated excel amplification sheet for the exact amount of Identifiler™ Primers, Reaction mix, and TAQ GOLD, to add. The amount of reagents for one amplification reaction is listed in Table 2.
6. Once a reagent is added, return the reagent to the Nalgene cooler.

TABLE 2: Identifiler™ PCR amplification reagents for one sample

Reagent	Per reaction
Primer mix	2.5 µL
Reaction mix	5.0 uL
AmpliTaq Gold DNA Polymerase (5U/µL)	0.5 uL
MASTERMIX TOTAL	8.0 uL
DNA	5.0 µL

F. Reagent and sample Aliquot

1. Vortex mastermix to thoroughly mix. After vortexing, tap mastermix tube on bench to ensure that no reagent is trapped in the cap.
2. Add 8 µL of the Identifiler™ mastmix to each tube or well of the 96 well plate that will be utilized, changing pipette tips and remixing master mix every 16 samples. The seal on the plate is piercable.
3. Add 5uL of each sample and control to the appropriate PCR tube/well. Change pipette tips every sample and open only one sample tube at a time. Place a sample tube into a separate rack after its addition. Prior to immediately adding each sample or control, pipet each sample or control up and down several times to thoroughly mix.
4. Close each PCR tube after sample is added. If using a plate, use extreme care to avoid crossing over the open plate.

5. Sealing the plate
   a. If using a PCR plate, place a super pierce strong seal on top of the plate, and place the plate in the plate adapter on the ABgene heat sealer.
   b. Push the heat sealer on top of the plate for 2 seconds.
   c. Rotate the plate and reseal for 2 additional seconds.
   d. Label the plate with the A for amplification and the date and time. (A011104.1300)
6. Return DNA extracts to storage.

G. PCR

1. Place plate or sample tubes in the ABI 9700.
2. Select Users→HighSens→Accept→View→scroll to appropriate entry ID →confirm parameters

Identifiler 31 cycles:

a. *95°C for 11 min*
b. *28 or 31 cycles:*
   i. *94°C for 1 min - melting*
   ii. *59°C for 2 min - annealing*
   iii. *72°C for 1 min - extension*
c. *60°C for 30 min*
d. *4°C soak*

3. Select Start and enter correct volume of 13 uL
4. Record the thermocycler number and place amplification sheet on the bulletin board. Transfer the amplification macro to the Vilma computer in the folder entitled "samples waiting to be run".
5. If you are present when the amplification concludes, remove samples from the thermocyclers and store in the DNA refrigerator in order to conserve the thermocyclers.
   a. Place tubes in a 96 well tray with a cover.
   b. Outline the position of the samples and label the tray with the amp date and time. (Samples amplified in a plate have a label on the plate already.)
   c. Store racks/plates in the refrigerator in the samples to the rack labeled "samples to be run".

F. Clean up

1. Wipe entire work area surface and pipets with bleach followed by water and ETOH.
2. Clean or soak any racks or cap openers with bleach and ETOH.
3. If necessary, replenish the microcentrifuge, PCR, or water tube supply by irradiating the empty tubes and water tubes for 30, 10, or 45 minutes, respectively. Consult the irradiation protocol for further details.
4. Turn on the UV lamp in the hood for ten minutes.

3100 Capillary Electrophoresis

A. Personal Preparation

1. Don protective attire only designated for the post-amplification room including lab coat and gloves.
   a. Ensure that the cuffs of the lab coat completely cover arm.
   b. Lab coats may be recycled.
2. Do not enter room without appropriate attire. Moreover, never touch any apparatus, bench etc. with bare hands.
3. Remove gloves when answering the phone and touching door handles in order to maintain a DNA free glove.
4. Remember to change gloves directly prior to handling samples.

B. Paperwork

Manual Paperwork

1. Open the Sample sheet on the desktop of the 3100 computer.
2. Name the sample sheet as follows: Instrument name month day year underscore injection folders, for example HS2041604_70-74
   a. Designate the run folder numbers based on the information indicated on the screen of the status view window.
   b. One injection consists of sixteen samples located in two adjacent columns, and requires one run folder.
   c. The database is cleaned when the capillary is changed. Therefore, the number of capillary injections should equal the number of run folders, unless otherwise indicated.
3. Complete the 3100 run log with the sample sheet name, run folders utilized, and the number of capillary injections, for example.
4. Select a new sample plate to be used, or if only a few samples require injection, select a plate with an appropriate number of wells empty.
   a. Ensure that the samples are assigned to the corresponding empty wells on the sample sheet.
   b. Label the plate with the sample sheet name.

5. Complete the cells in the columns of the sample sheet as follows:
   a. "Tube label": label on the PCR tube
   b. "Case-Number-Description:" sample name
      Each sample name has a suffix describing the injection parameters as follows:
      - "L" = 1 kV 22 sec , 31 cycle samples low
      - "N" = 3 kV 20 sec, 31 cycle samples optimal
      - "H" = 6 kV 30 sec, 31 cycle samples high
   c. "SYS:" the filter to be applied for the system amplified. Identifiler™ amplifications are referred to as "I" for the G5 filter.
      i. You may copy "I" and paste special, values in the appropriate cells.
      ii. Do not just copy and paste for the "SYS" column.
   d. "INJ:" the injection parameters
      i. "L" for injections at 1 kV for 22 sec
      ii. "N" for injections at 3 kV for 20 sec
      iii. "H" for injections at 6 kV for 30 sec
   e. "RUN:" run folder
   f. "IA": Interpreting analyst who will process the case
   g. "Amplification:" The name of the sample's amplification assay, for example, A041204_1500
   h. "Re-injection:" the name of the original run in which the sample was originally injected.
6. The sample sheet automatically calculates the required amount of HIDI and size standard to be aliquotted.
7. Save excel worksheet as follows:
   File→ save as→ My Comp→ D:→ Applied Bio→3100→ Data Collection→ sample sheets→File name *i.e. HS1092904_45*→ Save
8. Print the sample sheet, pages 1-2.
9. Confirm and save the plate record
   a. Click on the plate record tab
   b. Examine the columns for apparent errors.
   c. Save Plate Record
      i. File→Save as →My computer→ D: → Applied Bio→ 3100→ Data collection → plate records
      ii. Change file type to Text tab delimited
      iii. file name is the sample sheet name followed by ".plt". For example: *HS2092904_45-46.plt* click OK.
      iv. The message "Does not support workbooks with multiple sheets may not be compatible with Tab delimited" will appear, click OK
      v. A second message "Name.plt.txt may contain features that are not compatible with Text (Tab delimited)" will appear, click YES

Automated Macro

1. Use the 3100 sheet linked to the amplification sheet.
2. Complete the sample sheet as specified above in 5a-5h.
   a. For re-injections, where some loaded samples do not require re-injection, only enter an "I" as the SYS for those samples whose data should be collected.
   b. Although the capillaries will fill for all the samples loaded on the plate for an injection, the data will only be collected for those samples with a system selected.
3. Samples may be manually added to the sample sheet. However, copy and paste special, values.
4. Confirm and save the plate record as indicated above in 9.

Import Plate

1. Delete any existing plates, then click on import button (or go to file→ import plate)
2. Select the appropriate file in the plate records folder in the data collection folder, and chose all files.
3. Confirm settings in the plate editor in the tools menu.
   a. Double click on the plate name (or go to tools→ plate editor)
   b. The specified filter for Identifiler™ (I) should be = G5
   c. Confirm that the correct run module was selected.
      <u>31 cycle amplifications:</u>
      - GeneScanPOP4_1kV22sec: low injection
      - GeneScanPOP4_3kV20sec: optimal injection
      - GeneScanPOP4_6kV30sec: high injection
   d. If necessary, tab down to select a module for each injection **or** highlight run module then press control D to make all injections to have the same module. Check all your samples then click "OK".

C. Workplace preparation

1. If using a plate, turn on the heated plate seal apparatus.
2. Turn on the heat blocks for the denature/chill step.
3. Apply 10% bleach followed by 70% ETOH and water to the entire work surface, pipets and cap openers.
4. Retrieve clean racks and cap openers, tubes and/or plates and molecular biology grade sterile water free of DNAse (the GIBCO water) from the storage cabinet below "HS1"

<u>Note:</u> Only use filter tips for pipetting.

5. Arrange work place to minimize crossover. For example, the sharps waste for tips should be on one side whereas the fresh filter pipet tips and reagents should be on the opposing side.
6. Position gloves nearby with bleach/ETOH/water in order to facilitate frequent glove changes and cleaning

D. **Sample preparation**

1. Retrieve the amplified samples from the post amp DNA refrigerator on the shelf labeled "samples waiting to be run", LIZ and Identifiler™ allelic ladder from the post amp reagent refrigerator, and HIDI from the post amp reagent freezer.
2. Prepare Pooled samples
   a. Centrifuge all tubes at full speed briefly.
   b. Label one tube for each sample set as the sample name and "abc" to represent the pooled sample injection.
   c. Take 5 µL of each sample replicate, after mixing by pipeting up and down, and place each aliquot in the same PCR tube labeled "abc".
   d. Place each pooled sample directly next to the third amplification replicate labeled "c" of each sample set.
3. Arrange sample tubes in the order in which they will be placed on the 3100 plate. Line up the PCR tubes in a 96 well fashion for ease of loading. **Witness step: Confirm the placement of the sample tubes with the names specified on the sample sheet.**
4. For 31 cycle samples:
   a. Prepare a mastermix for samples and negative controls plus two as specified in Table 1B and on the sample sheet
      i. 14.6 µL of HIDI per sample (DNA is ~10% (12%) of total vol)
      ii. 0.375 µL of LIZ per sample
      iii. Aliquot 45 µL of mastermix to each well
   b. Prepare a separate mastermix for allelic ladders and controls
      i. Add 14.6 µL of HIDI to each AL and PE.
      ii. Add 0.375 µL of LIZ to each Al and PE.
      iii. Add 15 µL of mastermix for each AL and PE.
5. Aliquot 5 µL of each sample and negative control from the PCR plate or tubes to the 3100 plate into the exact same position.
   a. You may use a multichannel pipette.
   b. If pipetting from a 96 well PCR plate, pierce the seal.
   c. **Load at least 10 µL of HIDI in any wells without a sample, but are in an injection that is being utilized.**

6. **Allelic Ladders and Positive Controls**
   a. In order to simplify re-injections at high parameters and avoid re-aliquotting of samples, load the allelic ladder and positive control at two different dilutions in each injection.
   b. For optimal and low parameter injections add the following:
      i. 1 µL of a 1/10 or 0.1 dilution of PE (2 µL in 18 µL water)
      ii. 0.5 µL of allelic ladder or 1 µL of ½ or 0.5 dilution of AL (2 µL of ladder in 2 µL of water)
   c. For high parameter injections add the following:
      i. 1 µL of a 1/20 or 0.05 dilution (2 µL PE in 38 µL water)
      ii. 0.3 µL of allelic ladder or 1µL 3/10 or 0.3 dilution of AL (1.5µL AL in 3.5 µL water)

TABLE 1: 31 CYCLE SAMPLES

Injection Parameters	Samples and negs	LIZ for samples and negs	HIDI for samples and negs	ALs	PEs 100 pg	LIZ for ALs and PEs	HIDI for ALs and PEs
1 kV 22 sec	5 µL	0.375 µL	44.6 µL	0.5µL	1µL of 0.1 dil	0.375µL	14.6 µL
3 kV 20 sec	5 µL	0.375 µL	44.6 µL	0.5µL	1µL of 0.1 dil	0.375µL	14.6 µL
6 kv 30 sec	5 µL	0.375 µL	44.6 µL	0.3µL*	1µL of 0.05 dil	0.375µL	14.6 µL

*1µL of 1.2 µL ladder in 2.8 µL water

E. Denature/Cool Samples

1. Add septa to cover plate.
2. Centrifuge plate for 1 minute at 1000 rpm.
3. Check to see if the temperatures for heat plates were recorded for the day. If they have not been recorded do so now.
4. Heat plate at 95ºC for 5 minutes in a heat block
5. Snap cool plate at 4ºC in the second block
6. Centrifuge plate for 1 min at 1000 rpm.
7. This process may also be done using a programmed thermocycler.

F. Record lot numbers of reagents

G. Sample Storage

1. PCR plate
   a. Place a super pierce strong seal on top of the plate, and place the plate in the plate adapter on the ABgene heat sealer.
   b. Push the heat sealer on top of the plate for 2 seconds.
   c. Rotate the plate and reseal for 2 additional seconds
   d. Place plate in the post amp DNA refrigerator on the section of the top shelf labeled "samples already ran" in chronological order
2. PCR tubes
   a. Place tubes in the order in which they were injected in a 96 well rack with a cover.
   b. Label the cover with the name of the injection and out line the place of where the samples are located on the cover.
   c. Place rack in the post amp DNA refrigerator on the top shelf labeled samples already ran in chronological order.

H. Preparation of 3100

1. Change water and the buffer in the machine: Do Not leave capillaries outside solution for very long.
   a. Change buffer and water daily or every 12 injections.
   b. Use only store bought DNAse free molecular biology grade water, (for example GIBCO water).
      i. Make a 1:1 dilution of the 10X buffer in a 50 ml conical tube.
      ii. For 25ml or one buffer change : mix 2.5ml of buffer with 22.5ml of Gibco water and vortex well
      iii. For 50ml or two changes: take 5ml buffer in 45ml Gibco water and vortex well.
      iv. Write 1X buffer, initial, date and Lot# on the tube and store in post-amp reagent refrigerator if extra buffer remains.
   c. Rinse each chamber, dry thoroughly, and fill buffer and water in appropriate chambers as labeled on the 3100 machine. Add buffer to the anode chamber (the jar below the lower pump block) as well.
   d. Ensure there are no drops of liquid around the septa or above the mark in the chamber. Clean with Kim wipes while preventing lint from entering the water.
   e. Change the septa every fifty runs or as needed. Septa that appear dirty or feel tacky should be changed. Record the change in log book.

2. Check POP4 expiration date and amount and refill if necessary.
   a. POP4 expires after 7 days. Follow the change polymer wizard to refresh the POP4. Record change of POP4 in the log book.
   b. Clean the block every other time the polymer is changed. Record block cleaning in the log book. Refer to the pump block cleaning procedure.
   c. 0.6 mL of POP4 fills one 96 well plate or 6 injections.
   d. Ensure that no bubbles are in the upper or lower pump block or the syringe. If bubbles are located, run the remove bubble wizard within the change polymer wizard.
3. To manually warm up the oven, open 3100 Collection software on desktop: Instrument → manual control → oven → send command → set temperature → 60° → send command
4. Loading the plate following centrifugation
   a. Place plate on black tray with the A1 well on the sample tray corresponding to the A1 well on the black tray.
   b. Place white cover over tray. Make sure it snaps into place and is on securely. Test the fit, by holding the apparatus with one hand on top while placing your other hand below the apparatus.
   c. Place the tray apparatus in the 3100 with the notch facing backwards. This is the only orientation feasible.
   d. The tray position on the left is referred to as "A" and the position on the right is "B".
5. Linking plates:
   a. Highlight your imported plate then click on the yellow plate on the screen. It should link and turn green.
      i. The message "one or more plate wells are missing information, and will be skipped..."
      ii. Click okay if indeed wells are skipped.
      iii. If necessary, unlink the plate record, double click on the plate record name, and make corrections. Link the plate again.
   b. Verify that the plate has been linked.
      i. Run instrument button in toolbar is enabled.
      ii. Plate position indicator for the linked plate becomes green.
      iii. Plate record moves from the pending plate records table to the linked plate records table.
   c. Unlinking the Plate
      i. In the plate record table in the plate view page, select the plate record.
      ii. Click unlink
   d. Before initiating run, confirm run order as specified below.

6. Run Scheduling: Run view page
   a. Select a row for any run, and the corresponding wells to be injected for that run to will be highlighted.
   b. Sample run order
      i. Order the plates are linked and then,
      ii. Alphanumeric by run module name with numerics scheduled first followed by upper case and then lower case.
      iii. Example:
         a) plate A before Plate B if plate A linked first
         b) Within plate A, low injection (1 kV), followed by optimal (3 kV) and then high (6 kV).
         c) Multiple injections within wells are run before the next set of wells
      iv. Deleted Runs may be rescheduled
         a) The run scheduler re-initializes every time you use the plate editor to link/unlink a plate, create a new plate record, or edit an existing plate.
         b) If you delete runs associated with a plate, and then use the plate editor, the deleted runs will be rescheduled. Remove any unwanted runs in the Run view window.

      Capillary use indicator

15							**1**
16							**2**

   c. Scheduled runs are displayed in table in order.
      i. Select a run by clicking on the row in the table.
      ii. By positioning the pointer over an individual cell, the name of the sample is displayed.
      iii. Plate image provides a visual representation of the physical sample layout for the selected run.
      iv. Once a run has begun, the cells representing the capillaries in use turn blue.

H	G	F	E	D	C	B	A	
								1
								2
								3
								4
								5
								6
								7
								8
								9
								10
								11
								12

d. Delete Button
   i. Select a run and then click delete to remove run from the list of scheduled runs.
   ii. The run is not deleted from the plate record.

7. Starting the Run
   a. Select the green run instrument button in the toolbar.
   b. The software checks for available space.
      i. If the database is not full, the run initiates.
      ii. If the database is full, follow the **"cleanup the database"** procedure**.**
   c. Hit the green arrow for go.
      i. Check the run view to verify if the correct samples are highlighted and are being injected, and to confirm the injection order.
      ii. Injections are ordered alphanumerically.
      iii. Check the run status view and observe the currect.
      iv. If the current fluctuates, a bubble may be present in the pop: go to change pop wizard and bubble trouble shooting.

8. Record Run(s) In Log Book

I. Monitoring the Run

In the status window, ensure that the current does not fluctuate.

1. The pH of the buffer may not be optimal causing the current to drop; change buffer with a fresh solution
2. Bubble in the polymer; check pump block for bubbles and run the remove bubble wizard within the change polymer wizard.

J. Clean up

1. Ensure that all reagents and equipment, including protective gear, utilized in the post amp laboratory remains in the post-amp laboratory. However, reagents and equipment may be transferred from the pre-amp to the post-amp area.
2. During the scheduled laboratory cleanup period, change the septa on the buffer chambers every two weeks.
3. Wipe entire work area surface and pipets with bleach followed by ETOH and water.
4. Clean work surface, pipets, racks and cap openers with bleach followed by ETOH. Replenish the microcentrifuge tube supply by irradiating the tubes for 30 minutes.
5. The ABI 3100 should only be wiped down with deionized water and a Kimwipe.

Data Access and Archive

A. **Collection of data from 3100**

1. Sample Sheets and Plate Records are located in D:\Appliedbio\3100\DataCollection and are named as follows:
   a. Sample Sheets: instrument month day year_run folder-run folder
   b. Plate Records: instrument month day year_run folder-run folder
   c. Example: **HS1041405_70-73**
2. The run folder located in D:\Appliedbio\3100\DataExtractor\Run folder is named as follows: Run_instrument_year_month_day_folder: **Run_HS1_2005_04-14_70**
3. The following files are contained within the run folder:
   a. Text document of an analysis log: Run folder name_analysis
   b. Text document: analysis log: instrument date_run folder
   c. Text document of an extraction log: Run folder name_extraction
   d. Sample files:
      i. FSA or GeneScan if analyzed
      ii. Sample Name (sample #)_well position_capillary #

4. Analysis may be conducted on the 3100s directly or on one of the analysis stations in the office area.
5. Archive the run files from the 3100 computers.
   a. Drag the relevant run folders on HS1 or HS2 as well as the sample sheets and plate records to the "Share on Vilma" folder on the desktop.
   b. On the Vilma computer, in Windows Explorer, drag the relevant files from the share folder on the desktop to a USB jump drive.
   c. After confirming that the files are on the drive, delete the files from the share folder.
6. Transport run files to working folder at High sensitivity analysis stations
   a. In Windows Explorer, drag the relevant folders from the USB drive to the working folder on the desktop.
   b. After confirming that the files are on the desktop, delete the files from the jump drive.
   c. Create a new folder in windows explorer with the name of the relevant plate record. Place the relevant, sample sheet, plate record, and run folders in the folder.
   d. For an Identifiler™ run, analyze each injection or run folder separately.
   e. In addition to the relevant run folders, sample sheet, and plate record, the major folder should contain the GeneScan projects as well as the Genotyper files.
   g. Transport the folders to the working folder on HS analysis computer one for technical review. The responsibility for this data transfer falls with the analyst who edits the run.

B. **Permanent Archive**

1. Archive all runs, sample and Genotyper files following review of the data, on High Sensitivity Analysis 1 and 2.
   a. In Windows Explorer, drag the relevant files to the archive 1 folder (HS1) and the archive 2 folder (HS2).
   b. The responsibility to archive the reviewed runs lies with the technical reviewer.
   c. Within the archive folder, the folders will appear alpha-numerically. Therefore, all HS1 folders will precede all HS2 folders.
   d. Similarly, all extraction sheets will precede the HS folders and all quantitation files will appear last.

2. Every Friday, the entire contents of HS1 is automatically archived to an external hard drive, creating three copies, HS1 hard drive, HS2 hard drive, and the external hard drive of current data.
3. Moreover, every three months, archive data that is at least three months old to CDs.
    a. Make two identical copies of each CD and label with first-last date.
    b. One CD is stored at Bellevue and the other at 520.
    c. The responsibility of the CD archive is a QC function assigned with the rotational duties.
    d. Transfer of files to CD
        i. When available, always utilize a CD-R, not a CD-RW for data archive
        ii. To format a CD-R or CD-RW in DirectCD:
        iii. Insert a blank CD-R or CD-RW disc into the CD-RW drive.
        iv. Wait for the program **Easy CD Creator 4** to launch itself.
        v. 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**.
        vi. Click **DATA**.
        vii. Click **DIRECTCD**. The **Adaptec DirectCD Wizard** will open.
        viii. Click Next. The Drive Information window will open.
        ix. Click **Next**. The **Format Disc** window will open.
        x. Click **Next**. The **Name Your Disc** window will open. Name the disk as follows: 1st date-last date: 041405-07-15-05
        xi. Click **Finish**. You will receive the message, "Disc in Drive _: has been successfully formatted."
        xii. 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).
        xiii. 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.

C. Editing of stored runs

1. Refer to the analysis section for editing guidelines.
2. Edits may be applied to runs in the archive folder. However, the run must be saved onto HS1 archive, HS2 archive, as well as the external hard drive.
3. If editing must be performed on runs already burned to the CD-R, do the following:
   a. Find the desired CD-R disk at the analysis network area and copy all of its contents to a folder entitled "Temp Archive" on the desktop of one of the analysis workstations.
   b. Following editing, re-burn two new CD-R disks including all of the contents of the temporary working file.
   c. Store one CD-Rs will replace the one at the analysis network area and the other will replace the backup located at 520.
   d. 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.
   e. After new copies of disks have been burned, delete all of the files from the Temp Archive folder on the desktop.

Cleaning the Pump Block and Changing POP

A. Removing the Upper Block

1. Set Plungers into the "Home Position"
2. Remove the syringe support from the upper polymer block
3. Remove the syringes from the upper polymer block
4. Remove the array from the upper polymer block

   NOTE: Be Very CAREFUL removing the array!

   In order to remove the array you must first unscrew the laser cover and unscrew the fitting attached to the polymer block. Gently pull the array from the block making sure not to lose the plastic ferrule fitted over the array connection.
5. Disconnect the peak tubing from the lower polymer block
6. Remove the upper polymer block from the instrument

B. Cleaning the Upper Polymer Block

1. Rinse all the fittings with the deionized bottled water
2. Thread the 6-mm plastic syringe into the reserve syringe fitting and flush with room temp. dH2O. Force the dH2O out each opening by sealing them with your fingers.
3. Visually inspect the block for dried POP4 (white residue). Continue to flush with dH2O till no more POP4 can be seen. Channel inside the block should be clear not hazy.
4. Rinse the outside of the block and all the fittings with Gibco dH2O.
5. Wipe the outside of the block dry with a Kimwipe and use the vacuum pump to force air through the channel inside the block. *When the block has been thoroughly dried, re-examine it for dried POP4 (white residue or haze)*

NOTE: BLOCK MUST BE DRY BEFORE REPLACING IT INTO THE INSTRUMENT

C. Reinstalling the Upper Polymer Block

1. Reconnect the peek tubing to the lower polymer block
2. Reinstall the cleaned reserve and array-fill syringes. The reserve syringe should be filled to ~2.5mL with POP4 and the array-fill should be filled all the way.
3. Reinstall the capillary array (see below)
4. Reconnect the syringe support

D. Cleaning the Lower Polymer Block

1. Make sure that the buffer valve is open (in up position)
2. Remove the peek tube and fitting from the upper polymer block
3. Remove the lower block by pulling straight out
4. Rinse all the fittings with room temp. Gibco dH20
5. Rinse the buffer valve with the Gibco dH20 while moving it in and out
6. Thread the 6mm plastic syringe into the peeking tube fitting and flush with dH2O
7. Wipe the outside of the block dry with a Kimwipe and use the vacuum pump to force air through the channel inside the block. *When the block has been thoroughly dried, re-examine it for dried POP4 (white residue or haze)*

NOTE: BLOCK MUST BE DRY BEFORE REPLACING IT INTO THE INSTRUMENT

D. Reinstalling the Lower Polymer Block

1. Buffer valve must be open (the up position) before reinstalling
2. Reconnect the peek tubing. *Be sure to butt the tube against the stop before tightening the fitting.*

E. Reconnecting the Capillary Array

1. With the polymer block pulled out about an inch reinsert the capillary array with plastic ferrule into the block.
2. Push the block all the way back into the instrument and align the laser window before screwing the tubing into the block.
3. Once the laser window is in place, tighten the screw into the polymer block.

NOTE: MAKE SURE ALL TUBINGS (INCLUDING THE ARRAY) ARE SECURELY ATTACHED BEFORE PROCEEDING*

F. Changing the POP4

1. Set the syringes to the "Home Position" using Change POP4 Wizard.
2. The reserve syringe and array-fill syringe (250-uL) must be cleaned with Gibco dH2O before new POP4 can be added.
3. *Slowly* push out the old POP4 in both syringes
4. Rinse the outside of the syringes with the Gibco water and dry off. Be careful with the tip. If it comes loose **do not** put it back into the instrument.
5. Suck up the Gibco water with the syringes and slowly push the water out. This will flush out any old POP4. **NEVER PULL THE PLUNGERS OUT OF THE GLASS SYRINGES.** If you see dried POP4 (white residue or haze) within the syringe continue to flush with Gibco water till it is gone.
6. For each syringe suck up a small amount (a few drops) of the new POP4 and invert the syringe. Use this little amount a POP4 to coat the entire inside of the syringe. Do this slowly and push it back out. This will lubricate the inside of the syringe while removing any residual water that could dilute the POP4.
7. Fill the reserve syringe to 2.5mL and fill the array-fill syringe.

Once the instrument is cleaned, reassembled, and POP4 replaced perform a Spatial Calibration according to the casework manual.

Cleanup Database Utility

The Cleanup Database utility deletes the processed frame data and all associated run information, such as plate records and run data stored in the 3100 Data Collection software database. It makes room for new run data. **Before running the utility, be sure that all runs have been extracted from the database.**

1. Make sure that the OrbixWeb Daemon and AE server are running.
2. Quit the 3100 Data Collection Software.
3. Using Window NT Explorer, navigate to the following directory: D: Applied Bio/ 3100/ Bin.
4. Locate and double-click *CleanUpDB.bat*. The utility takes a few seconds to complete.
5. Shut down and then relaunch OrbixWeb Daemon. If you do not perform this step, any new run data will not be saved to the database.
6. Following cleanup of the database, the subsequent run folder is designated as run folder number 1.

3100 Troubleshooting

A. Data Problems

1. Many artifacts in sample
   a. Secondary structure present. Sample not denatured properly.
   b. Clean pump block and change polymer to refresh the urea environment.
   c. Denature chill samples.

2. Decreasing peak heights in all samples.
   a. Poor quality formamide or sample environment very ionic.
   b. Realiquot samples with fresh HIDI

3. Individual injections run at varying speeds. For example, the scan number where the 100 bp size standard appears significantly differs from one injection to another, usually appearing earlier. When laboratory temperatures are warm, the run will occur much faster affecting sizing.
   a. Redefine size standard.
   b. If this fails, reinject.

4. Loss of resolution of peaks
   a. Capillary failure.
   b. Change Capillary. Refer to capillary change wizard and record change in log book

5. An off ladder peak appears to be a pull up, but it is not exactly the same basepair as the true peak.
   a. Matrix over-subtraction. Usually in the green channel, the true peak is overblown and is split.
   b. Pull up peaks appear in the blue and the red channels.
   c. In the yellow channel, there is a negative peak at the base pairs of the true peak, however immediately to the right and to the left are off ladder peaks.
   d. Remove off ladder peaks as matrix over-subtraction

6. Peaks overblown and running as off ladder alleles
   a. More than 100 pg of sample amplified.
   b. Rerun samples at lower injection parameters
   c. Or rerun samples with 1 or 3 µL

7. Pull up peaks
   a. Colors bleeding into other colors
   b. Re-run matrix.

8. Spikes in the electropherogram
   a. Crystals in the polymer solution due to the polymer warming and congealing from fluctuations in the room temperature.
   b. Change the polymer.

9. Spikes in electropherogram and artifacts
   a. Arcing: water around the buffer chambers
   b. Clean chambers
   c. Beware of drops accumulating around the septa.

10. Split peaks
   a. Lower pump block is in the process of burning out due to the formation of a bubble.
   b. Clean the block.

11. Increasing number of spurious alleles
   a. Stop laboratory work under advisement of technical leader.
   b. Implement a major laboratory clean-up.

B. **General Problems**

1. Fatal Errors
   a. Close collection software.
   b. Restart collection software
2. Do not open the 3100 door before the tray if stopped.
3. 3100 not cooperating
   Have patience. Machine will perform functions slowly.

C. Recovering Data if Autoextraction Fails

Observation:
No folder in extracted data folder.

Cause:
The data was not extracted.

Recommended actions:

1. Ensure that the Orbix Web Daemon and AEServer are running.
2. Quit the data collection software.
3. Select Start>Applied Biosystems?>3100 Utilities>**Extractor Utility.**
   a. Select a run or runs to extract.
   b. Runs that may be extracted will be listed as “extractable”.
4. Do not extract runs with NOT extractable status.
5. Click extract.
6. The data will be extracted to the location defined, for example:
   D:/AppliedBio/3100/Data Extractor/Extracted Runs/

D. Shutter Problems

Observation:
First peak big, last small and in between a flat line for the spatial calibration or noisy line with very low intensity, which equals to no signal from the laser. No clicking shutter noise during spatial calibration or when the shutter is manually opened and closed from the manual control window.

Cause:
Stuck shutter, due to heat.

Recommended action:
1. Close the 3100 Collection Software.
2. Go to *Start* menu.
3. Go onto *Applied Biosystems*.
4. Go onto *3100 Service*.
5. Click on *3100 Diagnostic.*
6. Click on *Calibration Discovery.*
7. Click on *CCD Calibration*.
8. Click on *Run Calibration Module* from D: Firmware/ Diagnostics/ Service Tools/ Module 16 Cap/ Shutter Test 960.mod; click *Open* if you have to put in the test's directory on the D drive.
9. Wait and listen for the clicking shutter noise while the green light on the instrument is flashing.
10. If you cannot hear the sound repeat steps 7-8.
11. Click on *Return*.
12. Click on *Return.*
13. Click on *Exit*.
14. Open 3100 Collection Software.
15. Perform Spatial Calibration.

GeneScan Analysis

For a regular 3100 run all collected data will have been auto analyzed based on the default settings (see below). Use manual analysis if the auto analysis did not take place.

A. Access to GeneScan

On the desktop open the GeneScan Program

File→ new→ project button. Project→add sample files→ data extractor→ look for your run folder.

Double click on each folder and click the "Add All" button until all files have been added. Click the finish button.

Click on "parameter button" LIZAnalysisParameters.gsp

Click on "size standards" LIZ-250-340.szs

B. Analysis Settings

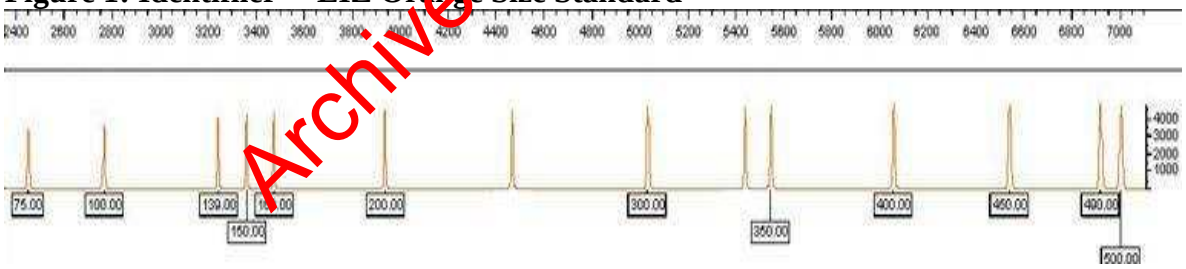
1. Analysis parameter range:
   a. Double click on the first sample such as the allelic ladder. This will open a separate window with the sample's raw data. Alternatively, select the samples and View→Raw data.
   b. Scroll to the bottom of this window where the orange peaks will be visible as well as the minutes, size, peak area, and datapoints.
   c. Locate the 75 bp LIZ standard, and set the analysis parameters to initiate approximately 25 data points prior to this 75 bp LIZ standard datapoint.
   d. Locate the 500 bp LIZ standard and set the analysis parameters to end approximately 25 data points beyond the 500 bp size standard datapoint.
   e. Repeat this process for the middle and the last sample files to ensure that this analysis range includes all of the 75 and the 500 LIZ standards for all samples in that injection. (This step is a preview to step C below.)
2. Size Call Range:
   Min: 75
   Max: 500
3. Data Processing: Light Smoothing
4. Size Calling Method: Local Southern Method

5. Peak Detection
   a. Peak Amplitude Thresholds:
      i. Blue, Green, Red, Yellow, Orange: 75 RFUs. Thus, only peaks that are at least 75 RFUs are considered alleles.
      ii. Orange may be lowered to 25 RFUs
   b. Minimum Peak half width: 2 pts
   c. Peak window size: 15
   d. Polynomial degree: 3
   e. Slope threshold for peak start 0.0
   f. Slope threshold for peak end: 0.0
6. Baselining window size: 251 pts
7. Auto Analysis only: Size standard LIZ-250-340

C. Analysis

1. Click upper left corner to highlight all colors then click the analyze button.
2. Check the size standard for correct peak calls:
   i. Windows→ results control→ # of panels 8 →Quick tile "on"
   ii. Highlight the first 8 orange size standards and click the display button.
   iii. See Figure 1 for correct labels. Do not assign values to the 250bp or the 340 bp peaks.
3. File→ save project as→ then close the program. (it will save as a prj file)

Figure 1: Identifiler™ LIZ Orange Size Standard



D. If a sample fails to be analyzed

1. Select sample and View→Raw
2. If there is no evidence of peaks, the injection failed, re-inject.
3. If peaks are present continue
   a. Check the height of the orange size standard. Lower the threshold to 25 RFU is necessary.
   b. Similar to B1a and B1b, observe where the first orange size standard is located in the sample, not the ladder, and start the analysis parameters approximately 25 bp less than that datapoint.
   c. If the baseline of the orange size standard is noisy, raise the RFU threshold of the orange to above the noise level.
   d. Alternatively, redefine the size standard. Select the size standard column in the sample and Define new.
      i. The orange peaks will appear and at the appropriate peak, type the label in the column (see above for correct values).
      ii. Save the standard as the name of the sample for example.
      iii. If the software crashes, close the project and open the size standard project and define the standard according to the relevant sample and save the redefined standard in the size standard folder.

E. Save the project

1. Save the project as the plate record name-run folder.
2. For example, if the plate record is HS1041405_70-75. The GeneScan project for one injection would be HS1041405_70.

Genotyper

The Genotyper step will assign allele calls to the labeled peaks based on the category list and the allelic ladder off-sets.

A. Importing data and allele call assignment

1. Open Genotyping program on the Desktop.
2. File → Import from GeneScan file→ choose your file→Finish
3. Double click on the appropriate macro (HS Identifiler 10%)
4. In order for the macro to run more quickly, if the category window is not collapsed such that each offset is visible, adjust the following. While in the main window view in the category window, click on a category and control A to select all the categories. Then under view select collapse categories.
5. **Save file as the plate record and the run folder and the injection parameter as follows: HS1041405_70N or H or L as appropriate.**

B. Modifying the allelic ladder category

If you receive the error message "Could not complete your request because the labeled peak could not be found," it means that the allelic ladder failed. Before the macro is rerun, the following two parameters of the ladder categories can be modified:

1. Open the categories window to see where the ladder failed.
2. Confirm this with the plot window.
3. Expand the offset of the first allele in the ladder to include the peak in the ladder.
   a. In the category window, highlight the category.
   b. Category→Edit category→type in expanded offset→Replace
   c. View→show main window→select the calculate offset for the relevant locus to confirm that expanding the offsets was beneficial.
4. Alternatively, if the baseline is noisy the RFU for the ladder must be raised in order to assign the correct allele.
   a. Category→Edit category→select the box with height of at least and type in appropriate height→Replace
   b. Rerun the macro.

C. Viewing samples

1. View→ show main window, highlight all samples
2. View→ show plot window, analyze results and fill out editing sheet.
3. Plot scan range for ID is approximately 2800 – 7000 or 90 bp to 370 bp.

D. Sample sorting

View→Dye lane sorting→file name and dye color in ascending order.

E. To closely examine peaks

For many loci it is necessary to zoom in as described to evaluate a low peak or double check on an editing step.

1. Point the mouse over the relevant area, and select view→zoom in selected range.
2. View→zoom to the plot settings.

F. Detection of rare alleles

A peak will be labeled "OL allele?" if the peak is outside the defined allele range or is not present in the allelic ladder. This peak might be a "new," previously unreported allele. OL alleles should be OL in all PCR aliquots if it is really a new allele and not an electrophoresis artifact.

G. Click History

This command will display all peaks that were manually removed.

View→Plot Options→Label options→show labels if they were manually removed→OK

H. Save file as the plate record and the run folder and the injection parameter as follows: HS1041405_70L, N, H or E as appropriate.

1. L = 1 kV 22 sec on HS1 and HS2 and 3 kV 10 sec on Stars, Stripes, and Rudy
2. N = 3 kV 20 sec
3. H = 6 kV 30 sec.

I. Editing of Genotyper files

Electrophoresis and PCR artifacts can be manually removed in order to simplify the allele call review. Make sure not to remove any labels for potential DNA alleles. All edits must have a reference point on the editing sheet. When in doubt leave the peak labeled for review. Mixture samples must be edited conservatively and only electrophoresis artifacts can be eliminated. Peaks in stutter positions cannot be edited for mixtures.

1. Stutter (1-4bp smaller than the main allele)
   a. The Identifiler™ Macro has an automatic stutter filter for each locus (see appendix for stutter values)
   b. Low Copy Number DNA samples have been shown to display increased stutter (Whitaker et al 2001). For single source samples, peaks below 20% of the main allele can be removed as stutter.

c. Peaks 1-4bp bigger than the main allele might be plus stutter but this is very rare.

d. If the main allele has an additional label prior to the main allele label (e.g. a shoulder peak, 1bp less in size) this peak will be used for stutter percentage calculation and the stutter might not have been automatically removed. In this case, the stutter peak can also be removed for mixtures.

e. Peaks that are overblown with RFUs above 6000, and thus their peak height has plateaued, will often have a stutter peak that will be more than 20% of the main peak.

i. If the sample is robust for all loci, re-inject the sample with low injection parameters or dilute the sample accordingly, refer to step "L" below.

ii. For LCN DNA samples, however, if the sample is degraded and the longer amplicons are not apparent or have very low RFUs, the sample should not be re-injected low as the long amplificons will no longer be visible. Alternatively, the stutter peaks for the alleles above 6000 RFUs may be removed, but only if the sample is not a mixture.

2. Split peaks

a. N bands due to incomplete non nucleotide template A addition should not occur for samples with low amounts of DNA

b. However, for 100 pg samples amplified for 31 cycles especially the low molecular weight loci could have incomplete A addition and should be edited as such.

c. Split peaks can also be an electrophoresis artifact and attributed to an overblown allele. Additional labels can be edited out.

Split peaks may occur in overblown samples or amplicons due to matrix over-subtraction. For example, an overblown green peak may dip at the top where a pull up peak is present in blue and in red. The yellow peak will also display over-subtraction with a dip at the peak's crest.

3. Non-specific Artifacts

a. Dye artifacts commonly occur in the beginning of the green, blue, and the yellow loci right after the primer peaks (Applied Biosystems 2004 a and b).

b. These artifacts may or may not appear in all samples, but are particularly apparent in samples with little or no DNA such as the negative controls.

4. Spikes and pull up
   a. Generally, a spike is an electrophoresis artifact that is present in all colors. For Identifiler™ a spike may appear in the red or green, but not be readily apparent in the other colors. However, zoom in and confirm the spike.
   b. Pull up is a matrix or "spectral" artifact that is caused by insufficient color overlap subtraction if a peak is very high. The label in the other color will have a basepair size very close to the real allele in the other color. The peak that is considered an artifact or "pull up" will always be shorter than the original, true peak. With Identifiler™, it is possible to for a particularly high stutter peak in for example blue or green, to create pull up in red or orange.
   c. Matrix artifacts could also be manifested as a raised baseline between two high peaks or an indentation of a large peak over another large peak. Labels placed on such artifacts can be removed.

5. Spurious alleles or drop ins
   a. Drop-ins are likely due to contamination either present in the sample before it reaches the laboratory or due to the lab processes.
   b. Drop-ins very often are not labeled as an "off ladder" allele.
   c. Spurious alleles cannot be edited during the Genotyper procedure but must be dealt with at the interpretation level.

All manual removals of peak labels must be documented on the editing sheet. This sheet also serves as documentation for the technical review. Check the appendix for the correct peak assignments to each allelic ladder and the expected genotype of the positive control.

J. Printing samples

1. Display all samples and the positive and negative controls with basepairs, peak heights, and category names, and the relevant allelic ladder with the basepairs and category names only.
   a. Highlight all samples except Ladder and go to Analysis→ change labels → select peak heights, basepairs, and category names.
   b. Highlight the relevant allelic Ladder and go to Analysis→ change labels → select basepairs and category names.
2. Display only the allelic ladder used for the macro if more than one allelic ladder is apparent. Highlight the extra allelic ladder and select Edit→Cut.

3. Print the electropherogram for the controls as follows:
   a. In the main view window, highlight the ladder, and all the controls. Highlight all colors including orange.
   b. View the plot window and ensure that all alleles in the ladder are apparent refer to step C3a-b.
   c. File→ page set up→Orientation→Portrait→properties button→ multipage printing→click on "2up"→click "more" button→scaling → 105, click OK→OK
   d. File→ print→ OK→OK
4. Once printed, ensure that all alleles in the ladder are labeled. Manually enter the basepair size if necessary and initial and date.
5. For LCN samples only, sort samples by dye color first and sample info, and select samples from one case at a time or all cases together, and print as a multipage document at 80% in order to examine repeats of a sample concurrently. Print the basepairs, peak heights, and the category names. In order to fit all colors of the profile on two pages, print parameters may need to be set as low as 55%.

K. Printing the Table

1. Select all samples including the ladder
2. Table→ append table→views→show table window
3. File→ page set up →Orientation→Landscape→properties button→Multipage Printing→select 4 up
4. Click on More button→ scaling 58 click OK→OK→OK
5. File→ print→ OK→OK
6. The table can be cleared under Analysis→clear table
7. The table can be recreated under Table→Append to table

L. Assay finalization and/or sample reruns

Enter the 3100 run date and time into the database.
Complete an assay resolution sheet for future action.

1. Place the resolution sheet on the bulletin board in the post amp room.
2. If all the samples are very overblown or very low, do not edit the electropherogram, but simply complete an resolution sheet and indicate that all samples will be rerun with modified loading and/or injection conditions.
3. All samples must be rerun if no allelic ladder is of sufficient quality to run the macro.

4. If the positive or negative controls fail and an electrophoresis problem is suspected as the cause for the failure, the controls can be rerun to test them again. A sample set can pass, if the negative controls pass the rerun. A sample set must have a valid positive control as an electrophoresis control. If the positive control passes the rerun all samples must be rerun together with the control, unless for unrelated reasons a second positive control happened to be present in the first run.
5. If only some samples are overblown, edit the run, but remove all peaks for the samples that must be re-injected.

M. Re-injection guidelines

1. All controls must be re-injected for all rerun conditions that are at a higher parameter. For reruns that are lower than the original injection, only a positive control must be re-injected.
2. If peaks are evident, but are below the threshold of detection, 75 RFUs, re-inject the samples at 6 kV and 30 seconds for LCN DNA samples.
3. For LCN DNA samples, if the peaks are over blown at some but not all loci, re-inject samples at 1 kV and 22 seconds.
4. For LCN DNA samples, if the sample is very overblown with peak heights above 6000 RFUs for the blue or green loci and at least above 2000 RFU for the yellow or red loci, re-aliquot the sample at a 0.1 dilution and inject at 1 kV 22 seconds.
5. If the sample is overblown at a homozygote loci in blue or green causing it to run off ladder, re-aliquot 2 uL (LCN DNA) of the sample and inject at 1 kV and 22 seconds.

Profile Production

A. For each amplification set create a spreadsheet using a macro to compile the composite profiles.
   1. This may include injections from different runs if a replicate sample had required re-injection due to a failed size standard for example.
   2. Having all the data from an amplification in one spreadsheet facilitates detection of contamination.

B. Composite Profile Macro
   1. Create a Genotyper table with six Alleles per Locus
      a. Ensure that all relevant samples are selected in the main window
      b. Select Analysis→Clear table
      c. Select Table→Set up table→Labels Options
      d. Set the number of peaks per category to 6
      e. OK→OK→Table→Append to table
      f. Views→Show Table Window
      g. Edit→Select All→Edit→Copy
   2. Profile Generation spreadsheet macro (HIGHSENS\SHEETS\MACROS\PROCODIS)
   3. Paste into cell A12 of "extra sheet" and delete rows containing the PE and the Allelic Ladders.
      a. Ensure that samples are in the following order:
         i. row 12: Sample info and Loci names
         ii. rows 13-14: Amp Negatives
         iii. Rows 15-18 (triple amp): Extraction negatives
         iv. Samples being in row 18 (triple amp plus pooled).
         v. Sample triplicates and pooled samples should be consecutive.
      b. Make appropriate adjustments by selecting Insert or delete row(s). Ensure that two rows are skipped between each sample such that the first sample is in row 15-18, rows 19 and 20 are skipped, and the second sample is in rows 21-24.
      c. The workbook contains 29 sample sheets.
         i. The first two sheets accommodate the amp and ext negatives.
         ii. Sheets 3-29 are sample sheets for up to 27 samples. Due to the inclusion of the pooled, and the required adjustments to the current macro, one macro can accommodate only 14 samples. Therefore, use two macros for a sample set of 27. For the second macro, leave the first six row blanks where the negatives would usually be placed.

4. Compilation of triple amplifications
   a. On the "extra sheet", Edit→select all→copy
   b. Paste into cell A1 of the copy geno triple sheet. (The geno db sheet is for double amplifications that would not be utilized for casework.)
5. "NIKE" macros to filter and sort
   a. Macro 4: Select the control and the "n" keys to filter sample sheets 1-14.
   b. Marcro 4b: Select the control and the "i" keys to filter sample sheets 15-29.
   c. Profiles macro:Select the control and the "k" keys to sort sample sheets 1-14.
   d. ProfilesB macro: Select the control and the "e" keys to sort sample sheets 15-29.
6. Arrow to the right to the triple chart.
   a. Each amplification replicate is shown in the white rows, and the composite profile containing alleles that repeat in two of the three amplifications is in the grey row below the 3 amplifications. Refer to the section of the manual entitled "Allele Confirmation and Profile Determination" for detailed instructions regarding profile generation.
   b. The pooled injection is located in the dark gray row beneath the composite profile.
   c. In the pink row beneath the pooled row labeled "CODIS deduced 3 amp", the major component of the composite profile should be deduced. The macro cannot generate the deduced major component; this must be calculated manually. Refer the section in the manual entitled "Allele Confirmation and Profile Determination."
   d. In the purple row labeled "CODIS pooled" deduce the major component from the pooled injection. Only alleles that are confirmed in the composite profile may be assigned. Refer to the section in the manual entitled "Allele Confirmation and Profile Determination" for further details.
   e. The macro will automatically calculate the number of inconclusive, total, clean, mixed, and partial loci for both CODIS loci and all amplified loci.
7. Copy the chart sheet to a new file.
   a. Right Click on the triple chart sheet
   b. Select Move or Copy
   c. Select create a copy and under "To book" select "newbook"
   d. Save the Newbook with the name of the amplification set.
8. Add this sheet to the sample's casefile.

7. ALLELE CONFIRMATION AND PROFILE DETERMINATION

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All allele calls are confirmed by evaluating both or all three amplifications. An allele is only considered a duplicated part of the DNA profile if it occurred in at least two of the three amplifications ("**repeating peaks**"). For duplicate amplifications, an allele must occur in two of the two amplifications. Peaks that are seen only in one of the amplifications can be real alleles, low-level mixture peaks, or PCR or electrophoresis artifacts. These peaks are called spurious alleles or **drop-ins** (Gill et al 2000). An allele that is known to be present at a heterozygote locus but is not detected is called a dropout.

A. Controls

1. The positive controls must give the expected result for an amplification to be valid. See Appendix for correct genotype for the positive control. The positive control can be tested with a dilution and with lower injection conditions than the samples, and the amplification will pass. The amplification fails if the positive control is compromised, and would require concentration steps or a higher injection than the samples to yield the correct result.
2. Negative controls can display spurious allele peaks and still pass, unless:
   a. The allele occurs in two of the two or three amplifications, which indicates potential contamination instead of drop-in. If this happens for only one or two loci, the affected loci must be evaluated for all samples. The locus is inconclusive for samples that display the same allele, which is present in the negative control, at this locus.
   b. If more than two repeating peaks are present in a negative control, the amplification or extraction fails.
   c. Even if none of the spurious allele peaks repeat in two amplifications, a control fails if too many spurious alleles are present. The cut off is ≥ 6 drop-in peaks distributed over at least two of the three amplification aliquots for three amplifications.
   d. If a negative control fails following injection with "high" parameters but passes with injections at "optimal" or "low" parameters, data from samples in the amplification set injected with "high" parameters fails accordingly, whereas data from samples injected with "optimal" or "low" parameters passes.

B. Profile interpretation

An Identifiler™ allele is characterized by the labeling color of the locus specific primers and the length of the amplified fragment. Alleles in unknown samples are compared to an allelic ladder containing the most common alleles (see Appendix). Due to stochastic effects, the 31 cycle Identifiler™ data for 100 pg or less have different characteristics than STR data for higher copy numbers. The heterozygote peak imbalance was determined to be 15% for 100 pg and can go up to 40% for 25 pg. The higher injection conditions cause high RFU values for lower DNA amounts and therefore stochastic effects and potential allelic drop out have to be considered for RFU values as high as 2000. Therefore, the procedures outlined in the Forensic Biology Protocols for Forensic STR typing were slightly modified as described below.

1. The first step is the decision whether the sample is a mixture or can be treated as a clean type.
   a. Samples with 3 or more repeating minor peaks must be interpreted as mixtures.
   b. Samples with <3 repeating minor peaks are interpreted as clean profiles.

2. For mixed samples, only samples with a clear major component can be interpreted, unless the stain is from an intimate sample and one of the DNA sources is known.

3. The following samples will not be interpreted or used for comparison:
   - Partial profiles and/or complex mixtures with several loci with more than 6 repeating peaks or an apparent ratio of 1:1 to 1:2.

4. A locus may be assigned a “Z” to indicate that another allele may be present, particularly for potential false homozygotes.

5. Non-mixtures are treated as follows:
   a. The heterozygote type for a locus with two repeating alleles is determined based on the two highest peaks in two amplifications. The heterozygote peaks do not have to show a specific peak balance with the following exceptions:
      i. When a repeating allele is in the plus or minus 4 bp stutter position, and is less than 30% of the major peak in two out of three amplifications, the possibility of a homozygote must be considered, and a Z is assigned. The potential stutter peak should not be more than 50% of the main allele in the third amplification.
      ii. If two repeating peaks are clearly major peaks, any additional repeating alleles, which are consistently minor, are not considered in the profile.
      iii. If only two repeating peaks are present, and one of the two peaks is <30% of the major peak in all three amplifications, the possibility of a homozygote must be considered.
   b. However, homozygote types have to be interpreted carefully.
      i. An allele must appear in all three amplifications to be considered a homozygote.
      ii. The presence of an additional allele in one of the three amplifications can be indicative of allelic dropout.
      iii. But if one peak if clearly the major peak and the minor peaks (even if they repeat) are less than 30% of the major peak in all three amplifications, an allele can be considered a true homozygote.
      iv. Alternatively, if the non-repeating minor drop-in is >30% of the repeating peak, allelic drop out should be suspected and the locus is marked with a Z, to indicate the possible presence of a second allele.
      v. Similarly, if the non-repeating minor drop-in is in the stutter position and is >50% of the repeating peak, allelic dropout should be suspected, and the locus is marked with a Z to indicate the presence of a second allele.
      vi. Based on validation data, allelic dropout and a Z are always considered for the following:
         1) High molecular weight or less efficient loci: CSF1PO, THO1, D16S539, D2S1338, D18S51, and FGA if only one peak could be called.
         2) The largest loci apparent in each color in two of three amplifications in a degraded sample.

c. If alleles in one of three amplifications are completely different from the 1st two amplifications, the locus is inconclusive. For example,
   i. locus is INC when
      1st amp = 8, 8
      2nd amp = 8, 8;
      3rd amp = 11, 12
   ii. BUT locus is 8, Z if
      1st amp = 8, 8
      2nd amp = 8, 8
      3rd amp = 11

6. Mixture deduction rules are as follows:
   a. Major alleles can be assigned to a major component if they appear **in all three amplifications** and if they are the major peaks in **two out of the three.** A heterozygote pair can be called if two out of the three amplifications show a peak balance ≥0.5.
   b. Homozygote types have to be deduced carefully. If one peak is clearly the major peak and the minor peaks (even if they repeat) are less than 30% of the major RFU in all three amplifications, an allele can assigned as a true homozygote.
   c. In between cases, where the second allele is between the 30 and 50% heterozygous peak balance, it cannot be concluded if the major component is heterozygote or homozygote. In this case, a major peak can be assigned to the major component with a Z.
   d. If only one peak could be called, based on validation data, allelic dropout and a Z are always considered for the following:
      i. High molecular weight or less efficient loci such as CSF1PO, THO1, D16S539, D2S1338, D18S51 and FGA.
      ii. The last evident locus of a particular color in two of three amplifications in a degraded sample
   e. When none of the alleles can be assigned to the major component, the locus is not deduced and made inconclusive.

7. Samples amplified with less than 20 pg have been shown to have more allelic dropout and all loci with only a single repeating peak are deemed potentially false homozygotes and are assigned a Z.

8. Partial profiles can be compared to known samples but might not be database eligible.

9. Pooled samples are only interpretable in conjunction with the triplicate amplifications; alleles can only be assigned if they are confirmed by the composite profile.

C. Alleles reported to CODIS

1. A profile is considered CODIS NDIS eligible if it contains 6 or more "clean" loci and not more than 4 "mixed loci with 3 alleles or more. Refer to the Forensic Biology CODIS Manual for more detail.
2. A "clean" locus is defined as a locus where the most probable true heterozygote or homozygote genotype could be determined, and does not include loci where a Z was assigned.
3. Identifiler™ loci D2S1338 and D19S433 are not CODIS eligible and are neither reported nor counted towards the number of CODIS loci.
4. Single source samples are treated as follows:
   a. All deduced alleles are entered.
   b. Loci with an assigned Z (a second allele may be present) are entered as homozygotes. These loci will be marked on the CODIS sheets.
   c. If the profile contains less than 9 loci only the "clean" loci are reported, otherwise enter all results.
5. Mixture samples are treated as follows:
   a. Only samples with a deduced major component are entered. Some loci will be clean, whereas others are entered as mixtures.
   b. Loci with an assigned Z (a second alleles might be present) are entered together with all repeating minor alleles, with the major allele marked as obligate.
   c. If the profile contains less than 9 loci, only the deduced "clean" loci are reported, otherwise enter all results.
   d. Profiles with more than 4 loci mixed (>3 alleles) loci are not CODIS eligible (see above).
6. Identifiler™ samples will not be entered in linkage but can be compared manually.

D. Profile Comparison and Statistical evaluation

1. Based on the triple amplifications, only repeating alleles will be reported and used for comparisons or database entry. All allele calls are part of the most likely DNA type for the DNA source.
2. Consider all possible evidence and exemplar pairs and all possible evidence pairs to determine inclusions and exclusions.
3. For contact DNA, the absence of an individual's alleles does not mean this individual did not touch the object; the DNA might not have been detectable.
4. The detection of an individual's alleles indicates that their DNA is present. No inferences about the type of body fluid or the means of deposition should be made.
5. Allelic dropout caused by stochastic effects is a common occurrence for low copy number DNA samples and a mismatch between a heterozygote exemplar and an apparent homozygote locus is not necessarily an exclusion, even if no Z was assigned (a second allele may be present).
6. Refer to the Forensic Biology Protocols for Forensic STR typing (Chapter 10) and Forensic Biology Case Management Manual (Chapter 5) for further instructions on sample matching and reporting.
7. Only non-mixtures or deduced major components will used for a statistical evaluation. For loci assigned a Z, only one allele is entered in the calculation spreadsheet. In this manner, the locus is not treated as a true homozygote whose statistical values are determined by multiplying the frequency of the allele in the database by itself (p^2). Rather Z loci utilize the probability only of the one assigned allele (2p).
8. For previously, undetected rare alleles (OFF LADDER) refer to the Forensic Biology Protocols for Forensic STR typing (Chapter 11).

E. References

Applied Biosystems (2001) AmpFlSTR Identifiler PCR Amplification Kit User's Manual. Foster City, CA.

Applied Biosystems (2004a) AmpFlSTR Identifiler PCR Amplification kit. Product Bulletin. Human Identification Group, Foster City, CA.

Applied Biosystems (2004b) AmpFlSTR Identifiler PCR Amplification kit. Application Note. Human Identification Group, Foster City, CA.

Gill P, Whitaker JP, Flaxman C, Brown N, Buckleton J (2000) An investigation of the rigor of interpretation rules for STRs derived from less than 100pg of DNA. Forensic Sci Int 112:17-40

Whitaker JP, Cotton EA, Gill P. (2001) A comparison of the characteristics of profiles produced with the AmpflSTR SGM Plus multiplex system for both standard and low copy number (LCN) STR DNA analysis. Forensic Sci Int 123:215-223.

F. Appendix

Figure 1 Allelic Ladder for Identifiler™

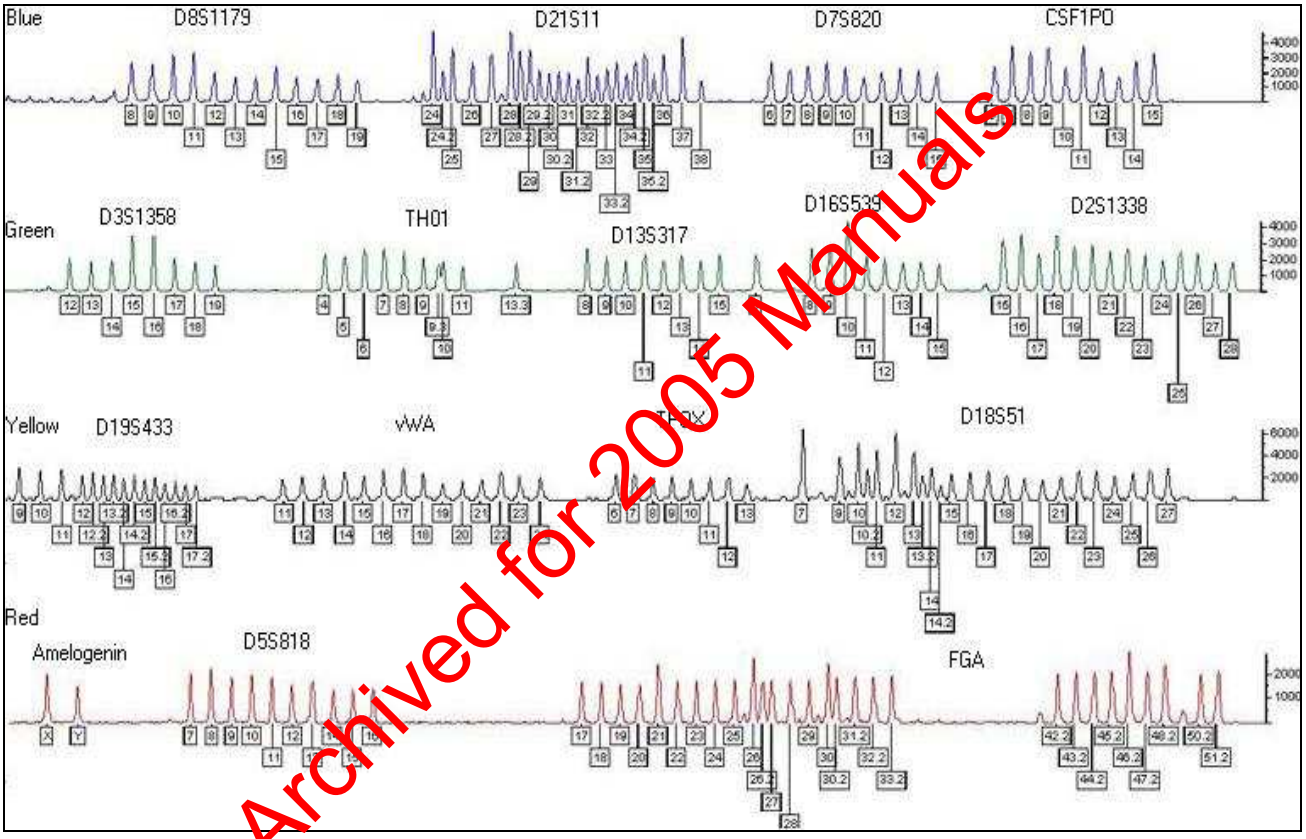

Figure 2 Positive Control Type for Identifiler™

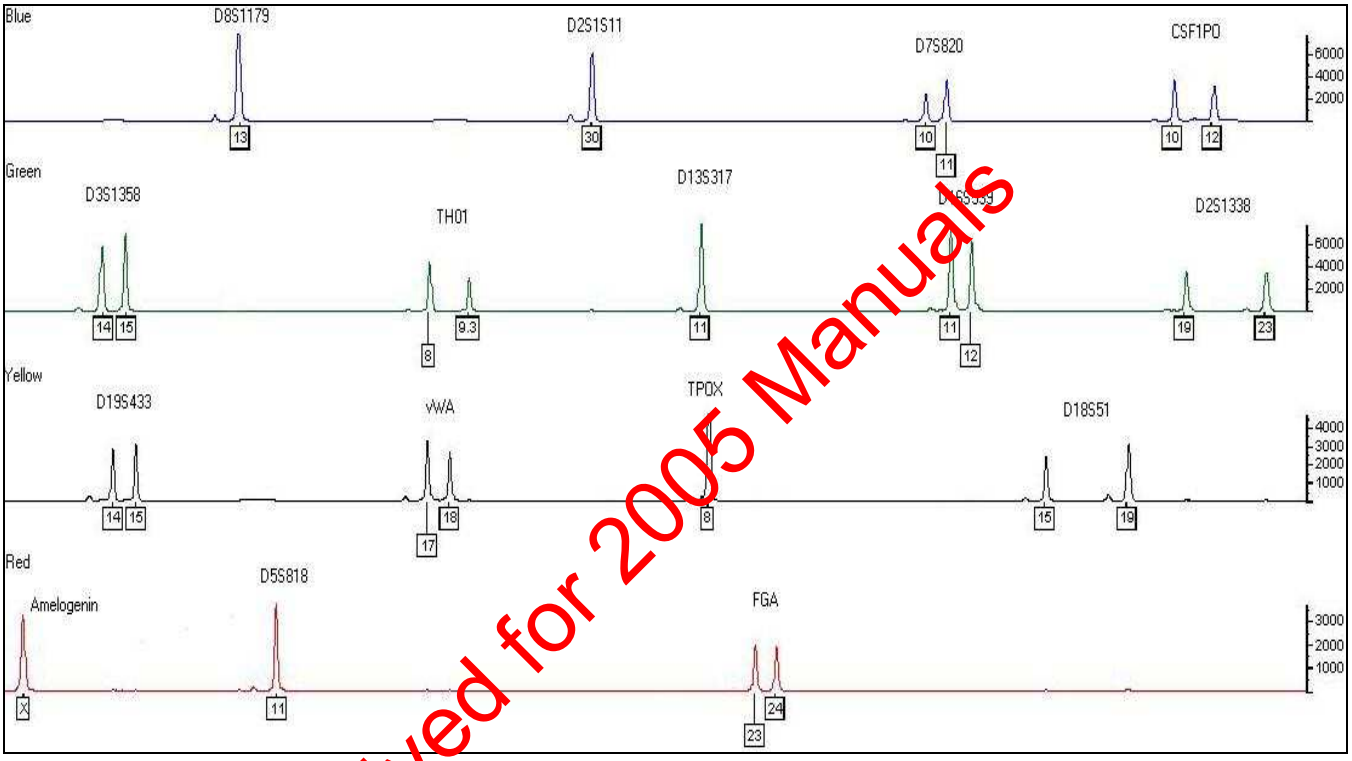

Table with ABI default stutter filter values for the Identifiler™ Genotyper Macro.

Locus	Stutter Filter
D8S1179	12%
D21S11	13%
D7S820	9%
CSF1PO	9%
D3S1358	11%
THO1	6%
D13S317	10%
D16S539	13%
D2S1338	15%
D19S433	17%
vWA	11%
TPOX	6%
D18S51	16%
Amelogenin	none
D5S818	10%
FGA	11%