

TENNESSEE BUREAU OF INVESTIGATION
Forensic Services Division



Forensic Biology STR Typing Manual

Forensic Biology
STR Typing Manual

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1. Introduction

Introduction for Forensic STR Typing Test

Short tandem repeat (STR) markers are polymorphic DNA loci that contain a repeated nucleotide sequence. The STR repeat unit ranges from 2-7 nucleotides in length. A majority of the STRs that have been evaluated by the forensic community are composed of four nucleotide repeat units. The number of repeat units within a STR locus may differ, so alleles of many different lengths are possible (polymorphic). Therefore, STR loci are useful for human identification purposes.

STR loci can be amplified using the polymerase chain reaction (PCR) process and the PCR products are then analyzed by electrophoresis to separate the alleles according to size. PCR-amplified STR alleles can be detected using various methods, such as fluorescent dye labeling, silver staining or fluorescent dye staining.

In capillary electrophoresis (CE), dye-labeled amplified fragments are separated by size as they migrate through a sieving medium contained within a narrow (50 μm) diameter capillary. The use of a narrow diameter capillary enables efficient heat dissipation during electrophoresis and allows much higher separation voltages to be used.

As the labeled amplified fragments migrate through the capillary and pass a detection window, they are illuminated by a laser. The dyes attached to the fragments are excited and emit light at a specific wavelength for each dye. This data can be then collected, analyzed and translated by the appropriate software.

This manual covers appropriate laboratory procedures for the extraction, quantification, amplification and typing of human DNA for both autosomal STR and Y-STR profiles using the GlobalFiler® and Yfiler® Plus amplification kits.



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2. Reagents and Supplies

The following general instructions are applicable in the preparation of all reagents:

- Clean disposable gloves, a mask and lab coat should be worn at all times while preparing reagents.
- Use graduated cylinders or pipettes closest in capacity to the volume being measured for preparing liquid reagents.
- Prepare all reagents in an area free of any source of DNA, including extracted DNA and amplified DNA.
- Label all reagents with name of reagent, date prepared, expiration date and preparer's initials. If applicable, label reagent with storage conditions and sterilization date.
- Complete Reagent Quality Control Sheet for each reagent prepared.
- For a commercially purchased reagent not having an expiration date, the expiration date will be five years from the date of receipt unless noted otherwise in policy.
- In-house prepared reagents will be discarded after one year unless noted otherwise in policy.

Any specific temperature mentioned in this protocol refers to the temperature at which the equipment is set given its temperature range, determined by the manufacturer and monitored by the Forensic Biology Unit's staff.

Some examples are: 5°C is considered to be refrigerated. A refrigerator set for 5°C could read between 2°C and 8°C depending on the cyclic range during the time the temperature was taken. A freezer set for -20°C could read between -15°C and -25°C depending on its range.

NOTE: All consumable products provided by Life Technologies are guaranteed to perform as specified for one year from the date received, unless otherwise indicated on the product label.

FOR A LIST OF CRITICAL REAGENTS SEE APPENDIX B.

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2.1 Reagents and Supplies for DNA Extraction

Brand name and part number after reagent / supply items is the recommended item, not necessarily the brand in use. Aliquot storage volumes are recommendations and may be adjusted.

CONICAL TUBES:

Falcon 15 mL Polypropylene Tubes

Fisher, 05-539-5

Falcon 50 mL Polypropylene Tubes

Fisher, 05-539-6

DTT:

0.39 M Dithiothreitol, 10 mL

Dissolve 0.60g dithiothreitol ($C_4H_{10}O_2S_2$, e.g. Sigma D-9779 molecular biology grade) in 10 mL sterile, deionized water in a sterile, disposable plastic 15 mL conical tube. Do not autoclave. Store 100 μ L aliquots in sterile 1.5 mL microcentrifuge tubes at $-20^{\circ}C$. Discard any unused portion of a thawed tube. The aliquots expire one year from the date of preparation.

1.0M Dithiothreitol, 10mL

Dissolve 1.545g dithiothreitol ($C_4H_{10}O_2S_2$, e.g. Sigma D-9779 molecular biology grade) in 10 mL sterile, deionized water in a sterile, disposable plastic 15 mL conical tube. Do not autoclave. Store 50 μ L aliquots in sterile 1.5 mL microcentrifuge tubes at $-20^{\circ}C$. Discard any unused portion of a thawed tube. The aliquots expire 1 year from the date of preparation.

EZ1 DNA INVESTIGATOR KIT:

Purchased commercially from Qiagen (952034).

EZ1 DNA Investigator Kit (48) for 48 preps: Reagent Cartridges, Disposable Tip Holders, Disposable Filter-Tips, Sample tubes, Elution Tubes, Buffers and Reagents; Buffer G2 (260mL) Lysis Buffer, Proteinase K (2mL), and Carrier RNA.

Buffer MTL (19112) is purchased separately for Qiagen. Store at room temperature. Buffer G2, Buffer MTL, and Proteinase K expire one year from the date of receipt if the manufacturer does not provide an expiration date for components of the EZ1 Investigator Kit.

EDTA:

Ethylenediaminetetraacetic acid, 0.5 M, pH 8.0

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Purchased commercially (Invitrogen 15575-038). Store at room temperature.

HYDROXYQUINOLINE:

Purchased Commercially.

8-hydroxyquinoline (C₉H₇NO), free base (e.g. Sigma, H687825G)

NON-STICK RNASE-FREE MICROFUGE TUBES (1.5mL):

Life Technologies, AM12450

MICROCONCENTRATOR UNITS:

Millipore, MRCF0R100, (Microcon DNA Fast Flow)

NON-SPERM EXTRACTION BUFFER:

8 mM Tris/80 mM NaCl/0.8 mM EDTA, 1% Sarkosyl, Water, 500 mL

Add 400 mL TNE and 25 mL of 20% Sarkosyl to 75 mL sterile deionized water. Store at room temperature. Aliquot amount needed for appropriate number of extractions into a sterile container. Expires one year from the date of preparation.

PHENOL:CHLOROFORM:ISOAMYL ALCOHOL:

Phenol:Chloroform:Isoamyl Alcohol, pH 7.9 (25:24:1;v/v)

Sigma P2069-100mL or Fisher Scientific 15593-049

Sigma P2069: The PCI is saturated with equilibration buffer (supplied) for a pH of 7.9. Add the entire bottle of equilibration buffer and 100 mg 8-hydroxyquinoline (an antioxidant which produces a yellow coloration) to the large bottle (100 mL) of Phenol:Chloroform:Isoamyl Alcohol. Mix gently and allow the phases to separate before use, approximately 2-4 hours. Shake vigorously and aliquot in to 15 mL conical tubes (a separatory funnel may be used to aid in aliquoting).

Fisher Scientific 15593-049: Add 100 mg 8-hydroxyquinoline to 100 mL of PCI. Mix and aliquot in to 15 mL polypropylene conical tubes. Add 1 mL of TRIS-HCl (1M, pH 8.0) to the mixture.

Store at 5°C in polypropylene tubes. PCI expires six months from the date of preparation.

CAUTION: This solution is an irritant and is toxic. Its use should be confined to a fume hood. Wear safety glasses and gloves when handling.



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PIPETTE TIPS:

Aerosol Resistant Pipette Tips (or equivalent)

10 μ L – Fisher, 21-402-482
20 μ L – Fisher, 21-236-4
100 μ L – Fisher, 21-236-8
200 μ L – Fisher, 21-236-1
1000 μ L – Fisher, 21-236-2A

Aerosol Resistant Shaft Guard Tips w/Refill Racks:

10 μ L – Ranin GP-10GF

PROTEINASE K: (10 mg/mL, 10mL)

Dissolve 100 mg of Proteinase K (Invitrogen/ABI 25530-015) in 10 mL of sterile deionized water in a sterile disposable plastic 15 mL tube. Store up to 120 μ L aliquots in sterile 1.5 mL microcentrifuge tubes at -20°C. Thaw tubes as needed for appropriate number of extractions. Discard any unused portions of thawed tubes. Expires one year from the date of preparation.

CAUTION: Powdered Proteinase K and solution of Proteinase K can be irritating to mucous membranes. Wear safety glasses and gloves when handling.

BrandTech 0.5 mL Tips for Repeat Pipetter:

Brand Tech Scientific, 702684

20% SARKOSYL:

Purchased commercially from Teknova Cat # 2S3380

SODIUM CHLORIDE:

5M NaCl, 1 L, Purchased commercially from Teknova Cat # S0250

SPERM EXTRACTION BUFFER:

(4.3 mM Tris/43 mM NaCl/0.43 mM EDTA, 2.9% Sarkosyl, Water, 350 mL).

Add 150 mL TNE and 50 mL 20% Sarkosyl to 150 mL sterile deionized water. Store at room temperature. Aliquot amount needed for appropriate number of extractions into a sterile disposable container. Expires one year from the date of preparation.

SPERM WASH BUFFER:

10 mM Tris-HCL, 10 mM EDTA, 50mM NaCl, 2% SDS, pH 7.5, 500 mL.



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Add 5 mL of 1M Tris-HCl, pH 7.5, 10 mL of 0.5M EDTA (pH 8.0), 5 mL of 5M NaCl and 100 mL of 10% SDS to 380 mL deionized water. Check pH. Autoclave. Store at room temperature. Aliquot amount needed for appropriate number of extraction into a sterile disposable plastic tube.

SPIN BUCKETS WITH DOLPHIN TUBES:

Lab Product Sales, M850002

STAIN EXTRACTION BUFFER:

10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 2% SDS, pH 8.0, 1L.

Dissolve 5.84 g NaCl in 500 mL of deionized water with stirring. To this solution add 10 mL of 1 M Tris-HCl, pH 8.0, 20 mL of 0.5 M EDTA and 200 mL of 10% SDS. Titrate to pH 8.0 with HCl. Bring to a final volume of 1 liter with deionized water. Store at room temperature. Expires one year from the date of preparation.

TE BUFFER:

10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0, 1 L

Add 10 mL of 1 M Tris-HCl, pH 8.0 and 200 μ L of 0.5 M EDTA to 990 mL of deionized water. Autoclave and aliquot into sterile, disposable plastic tubes when needed. Store at room temperature. Expires one year from the date of preparation.

TRIS/NaCl/EDTA (STE or TNE):

10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0, 1 L (Fisher Scientific BP2478-1)

TRIS-HCl:

1 M, pH 7.5
(Invitrogen 15567027). Store at 5°C.
1 M, pH 8.0
(Invitrogen 15568025). Store at 5°C.

STERILE WATER:

In-house Reverse Osmosis Water (R.O.) or In-house deionized or sterile (Fisher Scientific BP24701).

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2.2 Reagents and Supplies for Quantification

Quantifiler® Trio DNA Quantification Kit (400 reactions):

Life Technologies (Part Number 4482910)

- Quantifiler® THP PCR Reaction Mix (four tubes)
- Quantifiler® Trio Primer Mix (4 tubes)
- Quantifiler® THP DNA Dilution Buffer (2 tubes)
- DNA Control 007 (one tube)

Reagents should be stored at -15°C to -25°C upon receipt and then stored at 2°C to 8°C after initial use. The reagents should be protected from light.

96 well optical reaction plate:

P/N 4326659 (bulk)

MicroAmp optical adhesive cover

P/N 4311971

NON-STICK RNASE-FREE MICROFUGE TUBES (1.5 mL):

See Section 2.1

BrandTech 0.5 mL Tips for Repeat Pipette:

See Section 2.1

TE BUFFER:

See Section 2.1

2.3 Reagent and Supplies for Nuclear STR & Y-STR Amplification

GlobalFiler® Amplification Kit (200 reactions):

Life Technologies (P/N 4476135)

- GlobalFiler® Master Mix contains: enzyme, salts, dNTPs, bovine albumin, enzyme and sodium azide in buffer and salt.
- GlobalFiler® Primer Set contains: Forward and reverse primers to amplify human DNA targets.
- GlobalFiler® control DNA 007 contains: 0.10 ng/μg human male genomic DNA from cell line in 0.05% sodium azide and buffer.
- GlobalFiler® Allelic Ladder contains amplified alleles.



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Reagents should be stored at -15°C to -25°C upon receipt and then stored at 2°C to 8°C after initial use. The reagents should be protected from light.

MICROAMP REACTION TUBES:

0.2 mL Autoclaved MicroAmp Reaction Tubes with lids
Life Technologies (P/N N801-0612)

NON-STICK RNASE-FREE MICROFUGE TUBES (1.5 mL):

See Section 2.1

PIPETTE TIPS:

See Section 2.1

BrandTech 0.5 mL Tips for Repeat Pipette:

See Section 2.1

TE BUFFER:

See Section 2.1

TRIS-HCl:

See Section 2.1

Yfiler® Plus Amplification Kit (100 reactions):

Life Technologies (P/N 4484678)

- Yfiler® Plus Master Mix contains: enzyme, salts, dNTPs, bovine serum albumin and sodium azide in buffer.
- DNA Control 007 contains 2.0 ng/ μL human male genomic DNA in 0.05% sodium azide and buffer.
- Yfiler® Plus Primer Set contains locus-specific dye-labeled and unlabeled, forward and reverse primers to amplify human male DNA target.
- Yfiler® Plus Allelic Ladder contains amplified alleles.

Reagents should be stored at -15°C to -25°C upon receipt and then stored at 2°C to 8°C after initial use. The reagents should be protected from light.

2.4 Reagents and Supplies for Capillary Electrophoresis

ANODE BUFFER CONTAINER (3500)

Life Technologies (P/N 4393927)

CAPILLARY ARRAY (3500)

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Life Technologies (P/N 4404683)

CATHODE BUFFER CONTAINER

Life Technologies (P/N 4408256)

CONDITIONING REAGENT (3500)

Life Technologies (P/N 4393718)

CONICAL TUBES

See Section 2.1

DS-36 Matrix Standard (J6 Dye Set)

Life Technologies (P/N 4425042)

FORMAMIDE, DEIONIZED

HiDi Formamide pure grade

Life Technologies (P/N 4311320)

Allow bottle to thaw, aliquot up to 1000 μ L in each tube and store for up to 3 months at -15°C to -25°C . Use only needed amount of aliquots per set of samples. Discard any unused portion(s) of aliquoted deionized formamide.

Warning! Chemical Hazard. Formamide is a known teratogen and is harmful by inhalation, skin contact or ingestion. It can cause birth defect. Use in a well-ventilated area; use chemical-resistant gloves and safety glasses when handling. Wash thoroughly after handling formamide.

GENESCAN 600 LIZ® SIZE STANDARD VERSION 2.0

Life Technologies (P/N 4408399)

GeneScan™ 600 LIZ® Size Standard v.2.0 provides 36 single stranded labeled fragments of: 20, 40, 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, 460, 480, 500, 514, 520, 540, 560, 580 and 600bp.

NON-STICK RNASE-FREE MICROFUGE TUBES (1.5 mL):

See Section 2.1

PERFORMANCE OPTIMIZED POLYMER (POP-4) 3500:

Life Technologies (P/N 4393715)

PIPETTE TIPS:

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See Section 2.1

BrandTech 0.5 mL Tips for Repeat Pipette:

See Section 2.1

3500 SERIES SEPTA CATHODE BUFFER CONTAINER:

Life Technologies (P/N 4410715)

3500 SERIES SEPTA 96-WELL:

Life Technologies (P/N 4412614)

3500 SERIES 96-WELL STANDARD RETAINER AND BASE SET:

Life Technologies (P/N 4410228)

STERILE WATER

See Section 2.1

96 WELL OPTICAL REACTION PLATE (bulk):

Life Technologies (P/N 4326659)

96 WELL PLATE SEPTA

Life Technologies (P/N 4315933)

96 WELL REACTION PLATE

Life Technologies (P/N N801-0560)



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3. Laboratory Setup

The sensitivity of PCR amplification kits necessitates precautions to avoid contamination of samples yet to be amplified from other sources of DNA. To minimize the potential for laboratory-induced DNA contamination, time and /or space will separate DNA extraction, PCR setup and DNA typing. The following section addresses the dedicated equipment and supplies, special precautions to be taken, and controls to be used in each of these areas. In the event of cross-contamination of a sample from another DNA source during any stage of the DNA typing process, the technical leader will be notified and documentation must be placed in the affected case file(s).

3.1 DNA Extraction Work Area

This work area should be used for the extraction and isolation of DNA. Dedicated equipment and supplies should not leave the DNA extraction work area. If dedicated equipment and supplies must leave the DNA extraction work area, the items should be cleaned and/or UV treated.

Special Precautions:

1. It is important that the DNA extraction of questioned samples be performed at a separate time or separate space from the DNA extraction of known samples. Separate the DNA extractions that are set up in the same space by at least 20 minutes to allow UV irradiation of the hood. This precaution will help to prevent potential cross-contamination between evidence samples and reference samples.
2. Perform the DNA extraction from samples containing high levels of DNA (for example, whole blood) separately from samples containing a low level of DNA (cigarette butts, small bloodstains, etc.) to minimize the potential for sample-to-sample contamination.
3. Use disposable gloves and wear lab coats and masks at all times. Extraction hood shields may be used in place of a nuisance mask at analyst discretion.
4. Clean scissors thoroughly with alcohol wipes, diluted bleach or use fresh scalpel blades after cutting each evidentiary sample.
5. Use a clean cutting surface for each piece of evidence.



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6. Sterilize those solutions which can be heated in an autoclave without affecting their performance. Steam sterilization under bacterial decontamination conditions degrades DNA to a very low molecular weight, rendering it un-amplifiable.
7. Use sterile disposable pipette tips and microcentrifuge tubes.
8. Always change pipette tips between each sample, even when dispensing reagents.
9. Store reagents as small aliquots to minimize the number of times a given tube of reagent is thawed. Record the lot(s) of reagents used in each set of samples so that if contamination occurs it can be traced more readily. It is recommended that the small aliquots be retained until typing of the set of samples for which the aliquots were used is completed.
10. Centrifuge all tubes before opening them.
11. Include reagent blank control(s) with each set of DNA extractions to check for the presence of contaminating DNA in reagents.
12. Each month and as needed, clean all work surfaces thoroughly with 10% dilution of household bleach or 35% isopropyl alcohol (dilute from 70% stock). Use disposable bench paper to prevent the potential accumulation of human DNA on permanent work surfaces. Use caution when handling bleach.
13. Limit the quantity of samples handled in a single run to a manageable number. This precaution will reduce the risk of sample mix-up and the potential for sample-to-sample contamination.
14. Wear a dedicated lab coat for pre-amplification sample handling when working in the DNA extraction work area. In addition, wear a dedicated or disposable lab coat in the amplification / typing room.
15. Facemasks or a shield should be work during sample handling to prevent contamination.

3.2 Quantification Area

This work area is for the quantification of human DNA. Dedicated equipment and supplies should not leave the DNA quantification work area. If dedicated

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equipment and supplies must leave the DNA quantification work area, these items should be cleaned and / or UV treated.

NOTE: The preparation of samples for quantification is generally carried out in the DNA extraction work area.

Special Precautions:

1. Use dedicated lab coat and wear disposable gloves and mask at all times. Extraction hood shields may be used in place of a nuisance mask at analyst discretion.
2. Always change pipette tips between each sample.
3. Include all recommended controls with each quantification.
4. Do not use kit components beyond the expiration date.

3.3 PCR Setup Work Area

This work area is used for combining PCR reagents and extracted DNA in the appropriate reaction tubes. Dedicated equipment and supplies should never leave the PCR setup work area. If dedicated equipment and supplies must leave the PCR setup work area, the items should be cleaned and / or UV treated.

Special Precautions:

1. Use an adjustable pipette for adding components to the PCR Master Mix.
2. Use an adjustable pipette to add sample DNA to each tube with PCR Master Mix.
3. Always add DNA to the PCR Master Mix last. This minimizes cross-contamination by reducing the number of opportunities for inadvertent transfer of DNA between samples.
4. After the addition of each DNA sample, cap the tube before proceeding to the next sample. Have all tube capped when not being used.
5. Use disposable gloves and wear lab coat and mask at all times when handling DNA. Extraction hood shields may be used in place of a nuisance mask at analyst discretion.

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6. Avoid touching the inside surface of the tube caps.
7. Change pipette tips after addition of each sample DNA to each tube with PCR Master Mix.
8. Store the DNA amplification reagents together in the rack or the box provided, which will serve as a barrier against possible contamination by exogenous DNA. The rack or box is store in a refrigerator that does not contain DNA samples.
9. At least two microamp tube racks should be designated for specific tasks. One rack is used for holding the tubes during PCR setup and should be kept in the PCR setup work area at all times. The second rack or carrier rack should be used exclusively for carrying the PCR tubes to the thermal cycler located in the amplified DNA work area. If the carrier rack touches any surface in the amplified DNA work area, it must be irradiated with UV light.
10. Never use kit components beyond expiration date.

3.4 Amplified DNA Work Area

This work area shall be a physically separate area and used only for those activities that involve the handling of amplified DNA. This includes capillary electrophoresis of amplified DNA, handling of the allelic ladders, and waste disposal of amplified DNA solutions and frozen storage of the following: amplified DNA and the allelic ladders.

Dedicated Equipment and Supplies: (amplified DNA or equipment and supplies used to handle amplified DNA should not be taken out of the amplified DNA work area. Items that must be removed from the amplified DNA work area must be decontaminated and / or UV treated.)

Special Precautions:

1. Always remove gloves when leaving the amplified DNA work area to avoid the transfer of amplified DNA into other work areas.
2. Change glove when they may have come in contact with amplified DNA.
3. Use disposable bench paper to cover the work area used to perform the typing steps to prevent the potential accumulation of amplified DNA on

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permanent work surfaces. Diluted bleach or alcohol should be used as needed to wash exposed work surfaces. Soap and water can also be used to clean work surfaces.

4. Use the thermal cycler only for amplification and denaturation of DNA for typing.
5. Store tubes of amplified DNA in the freezer at -20°C. Do not store amplified DNA in the same area as reagents.
6. Store the allelic ladders frozen until use.
7. Wash hands after leaving amplification room.

3.5 Post Amplification Room Procedures:

The post amplification (amp) room is designed to have a one-way flow of people. There is a gowning room which is only an entrance and there is a de-gowning room that may be used as an entrance or an exit.

A person bringing DNA into the post amp room shall enter through the gowning room. Everyone working with amplified product is required to wear a disposable lab coat and gloves. If entering the room for other reasons (e.g. to check on a run), only gloves need to be worn.

A person desiring to exit the post amp room can only exit through the de-gowning room. Gloves will be placed in a trash container and lab coats will be placed on a hanger in the gowning area for re-use. All coats should be discarded when soiled or as needed.

Anyone exiting the de-gowning room should wash his/her hands in the bio-vestibule sink.

All trash in the post amp room and de-gowning area will exit through the trash door or bio-vestibule leading into the exterior hallway. No trash should exit through the de-gowning room door leading in to the laboratory.

In the event an item must leave the post amp room (e.g. pipettes for calibration), the item must be decontaminated with bleach, alcohol, or UV light exposure.

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4. Guidelines for Control Samples

It is essential that proper control samples are included when evidence samples are processed. The typing results obtained from these controls are important for the interpretation of the typing results obtained from evidence samples. Controls are described below.

4.1 Extraction Reagent Blanks

Reagent Blanks: With each set of extractions, a reagent blank must be extracted concurrently. The reagent blank consists of all reagents used in the test procedure (minus any sample) and is processed through the entire extraction, quantification, amplification and typing procedures alongside the evidence samples. If more than one type of extraction procedure is used (with different types of extraction reagents), then a reagent blank shall be set up for each type of extraction or group of extraction reagents used. Reagent blanks must be amplified using the same primers, instrument model and concentration condition as required by the forensic sample(s) containing the least amount of DNA. Reagent blanks are typed using the same instrument model, injection conditions and the most sensitive volume condition of the extraction set.

Documenting Blanks:

The reagent blanks will be documented on the extraction sheet with the samples being extracted. The reagent blanks will be identified with the date of extraction, a sequential number and the scientist's initials, e.g. "QRB080614-1BRE, QRB080614-2BRE, QRB080614-3BRE, etc."

4.2 Quantification Controls

Negative Control: With each Quantifiler® Trio run, a no template control (NTC) must be used as a negative control. The NTC consists of the amplification reagents and 2 μ L Quantifiler® THP DNA Dilution Buffer.

DNA Standards: DNA Standards 1 through 5 of known concentrations are prepared as a serial dilution from the Quantifiler® THP DNA Standard (100ng/ μ L). The standards will have concentrations ranging from 50 ng/ μ L (std. 1) to 0.005ng/ μ L (std. 5).

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4.3 Amplification Controls

Amplification Blank (Negative Control): An amplification blank must be used with each set of amplifications. This sample contains all of the amplification reagents with no DNA template. For this control, 15 μ L of TE must be added for the GlobalFiler® amplification reaction and 10 μ L of TE must be added for the Yfiler® Plus amplification reaction.

Positive Control: With each set of amplifications, a sample containing DNA of known types must be amplified and carried through the remainder of the typing procedure. The DNA Control 007 is included in both the GlobalFiler® and Yfiler® Plus Amplification Kits. Up to 1ng of control DNA may be added to the amplification reaction for both GlobalFiler® and Yfiler® Plus. However, lower amounts of the positive control may be added based on instrument sensitivity, software platforms, pipetting techniques, etc.

Documenting Controls:

The positive and negative controls will be documented on the amplification calculation sheet along with the samples being amplified. The controls will be identified with the date of amplification, a sequential number and the scientist's initials, e.g. "POS080614-1BRE, POS080614-2BRE, POS080614-3BRE, etc." and "NEG080614-1BRE, NEG080614-2BRE, NEG080614-3BRE, etc."



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5. DNA Extractions

All organic extraction steps must be performed in the UV enclosure with the exception of Phenol:Chloroform:Isoamyl Alcohol (PCI) steps.

Extract known samples at a different time and/or space than questioned samples. Separate known and questioned extraction sets carried out in the same space by at least 20 minutes to allow time to UV irradiate the hood area. Use reagents and pipettes dedicated to this area.

For robotic extraction, known samples and questioned samples are not run on the same robot at the same time.

An analyst may choose to perform DNA extractions either manually or robotically using the EZ1 biorobots. Both methods provide extracted DNA of sufficient quality for DNA typing.

A differential extraction should be performed on semen stained items.

If using robotic extraction, the following protocols can be chosen from the EZ1 biorobots: trace or trace tip dance, large volume or normalization. The trace protocols should be used for small samples not requiring more than 190 μL of extraction buffer to cover the sample.

The large volume protocol should be used for larger cuttings requiring more than 190 μL of extraction buffer to adequately cover the sample.

The normalization protocol may be used for samples expected to yield a high concentration of DNA. The normalization protocol will limit the concentration of DNA to approximately 1 to 3 $\text{ng}/\mu\text{L}$ depending on the elution volume. The normalization protocol is typically used for known standards, but may be used for questioned samples as well.

The procedures for robotic extraction using the EZ1 biorobots are in sections 5.3 through 5.6 of this manual. The procedures for manual extraction are in sections 5.1 and 5.2 of this manual.

NOTE: A final extraction volume of approximately 30 μL will allow for 2 μL for quantification and up to 15 μL for GlobalFiler® and up to 10 μL for Yfiler® Plus amplification if necessary. For questioned sample extraction sets, it is recommended to have at least two reagent blanks if amplification with multiple kits may be needed. Each

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reagent blank must be quantified. The reagent blank demonstrating the highest signal, if any, is required to be amplified and typed.

5.1 Organic Extraction Procedure

1. Place the sample cutting in a sterile 1.5 mL microcentrifuge tube.
2. To the sample add 300 μ L stain extraction buffer and 7.5 μ L of Proteinase K. Vortex on low speed for one second and pulse spin in a microcentrifuge for two second to force the cutting into the extraction fluid.
3. Incubate sample at 56°C for 2-24 hours.
4. Pulse spin in a microcentrifuge for two seconds to force condensate into the bottom of the tube.
5. Remove supernatant and transfer to a fresh, sterile 1.5 mL microcentrifuge tube. Retain substrate until completion of all typing.
6. In a fume hood, add 500 μ L Phenol:Chloroform:Isoamyl Alcohol to the stain extract. Vortex on low speed briefly to attain a milky emulsion.
7. Spin then tube in a microcentrifuge at maximum speed (10,000-15,000 rpm) for three minutes.
8. To a Microcon microconcentrator unit (blue concentrator on microcentrifuge tube, supplied), add 100 μ L TE buffer. Transfer the aqueous phase (top layer) from the tube in step 7 to the concentrator unit. Avoid pipetting any of the organic phase (colored yellow) or interface from the tube into the concentrator unit. Discard tube containing organic phase and interface into a dedicated waste container.
9. Cap the microconcentrator unit and spin in a microcentrifuge at 500 X g (2300 rpm) for at least 10 minutes.
10. Carefully remove the concentrator unit from the Microcon centrifuge tube and discard the fluid from the microcentrifuge tube. Return the concentrator to the top of the microcentrifuge tube.

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11. Add 200 μ L TE buffer to the concentrator unit. Cap and spin the microconcentrator unit in a microcentrifuge at 500 X g (2300 rpm) for at least 10 minutes.
12. Add TE buffer to the microconcentrator unit to bring total volume between 20-200 μ L. Remove the concentrator from the microcentrifuge tube and carefully invert the concentrator onto a fresh, sterile, labeled microcentrifuge tube.
13. Spin the microconcentrator unit in a microcentrifuge at 500 X g (2300 rpm) for 5 minutes.
14. Discard the concentrator and cap the microcentrifuge tube.
15. Sample is now ready for quantification and amplification.
16. Store samples at 5°C or frozen. Prior to the use of samples, vortex briefly and spin in a microcentrifuge for 5 seconds.

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5.2 Semen Swab and Semen Stains (Differential Extraction)

Semen swabs and semen stains should be air-dried and stored until used.

1. Using a clean surface for each swab, dissect the swab material from the applicator stick and place it into a sterile 1.5 mL microcentrifuge tube. If the stain is on a piece of material, cut a portion approximately 3 mm by 3 mm in size and place in a tube.
2. To the sample add 500 μ L non-sperm extraction buffer and 5 μ L Proteinase K. Vortex for 1 second and pulse spin in a microcentrifuge tube for 2 seconds to force the material into the extraction fluid.
3. Incubate at 37°C for 2 hours.
4. Transfer the supernatant to a fresh, sterile 1.5 mL microcentrifuge tube. Retain the swab or material substrate until completion of typing.

NOTE: If using a spin bucket, use forceps or a sterile applicator stick to place the swab or cutting into a spin bucket. Replace the cap. Spin for 5 minutes at maximum speed (10,000-15,000 rpm). Resume at Step 6.

5. Spin the tube in a microcentrifuge at maximum speed (10,000-15,000 rpm) for 5 minutes.
6. While being very careful not to disturb any pelleted material, remove the supernatant and place it into a new, labeled 1.5 mL tube. This supernatant is the non-sperm fraction. Analysis of the non-sperm fraction resumes at Step 11. The pellet remaining in the tube is the sperm fraction pellet.
7. Wash the sperm fraction pellet by re-suspending it in 500 μ L sperm wash buffer. Vortex the suspension briefly. Spin the tube in a microcentrifuge at maximum speed for 5 minutes. Remove and discard the supernatant fluid, being careful not to disturb the sperm fraction pellet.
8. Repeat step 7 at least two additional times for a minimum of three washes of the sperm fraction pellet.

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9. To the tube containing the washed pellet, add 350 μ L sperm extraction buffer, 40 μ L 0.39 M DTT and 10 μ L Proteinase K. Close the tube caps, vortex for 1 second and pulse spin in a microcentrifuge for 2 seconds.
10. Incubate at 37 °C for 2 hours.
11. To the tube containing the sperm fraction pellet and to the tube containing the non-sperm fraction, add 400 μ L Phenol:Chloroform:Isoamyl Alcohol. Vortex the mixture briefly at low speed to attain a milky emulsion. Spin the tube in a microcentrifuge for 3 minutes at maximum speed.
12. To a Microcon microconcentrator unit (blue concentrator in microcentrifuge tube, supplied) add 100 μ L TE buffer. Transfer the aqueous phase (top layer) from the tube in Step 11 to a concentrator unit. Avoid pipetting organic phase (colored yellow) or interface from the tube into the concentrator unit. Discard tube containing organic phase and interface into a dedicated waste container.
13. Cap the microconcentrator unit and spin in a microcentrifuge at 500 X g (2300 rpm) for at least 10 minutes.
14. Carefully remove the concentrator unit from the microcentrifuge tube and discard the fluid from the microcentrifuge tube. Return the concentrator to the top of the microcentrifuge tube.
15. Add 200 μ L TE buffer to the concentrator unit. Cap and spin the microconcentrator unit in a microcentrifuge at 500 X g (2300 rpm) for at least 10 minutes.
16. Add TE buffer to the microconcentrator unit to bring total volume between 20-200 μ L. Remove the concentrator from the microcentrifuge tube and carefully invert the concentrator onto a fresh, sterile, labeled microcentrifuge tube. Discard the used microcentrifuge tube.
17. Spin the microconcentrator unit in a microcentrifuge at 500 X g (2300 rpm) for 5 minutes.
18. Discard the concentrator and cap the microcentrifuge tube.
19. Sample is now ready for quantification and amplification.

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20. Store samples at 5° C or frozen. Prior to the use of samples, vortex briefly and spin in a microcentrifuge for 5 seconds.

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5.3 Protocol: Pretreatment of forensic samples for nuclear DNA extraction using the EZ1 DNA Investigator Kit.

1. Place sample in appropriate extraction tube or 2 mL Qiagen sample tube.
2. Add Buffer G2 to the sample.
 - If sample is non-absorbent, add 190 μ L Buffer G2 to the sample.
 - If sample is absorbent, dilute Buffer G2 1:1 with ultrapure water for $n + 1$ samples (n is the number of samples to be digested). Add 190 μ L diluted Buffer G2 to the sample.
 - If the sample size is large and additional buffer is needed to properly extract the DNA, 490 μ L diluted Buffer G2 can be added to the sample. However, if this volume of buffer is added to the sample for extraction, the Large-Volume purification protocol must be used on the EZ1 or EZ1-Advanced XL BioRobots.
3. Add 10 μ L Proteinase K, vortex for 10 seconds and centrifuge briefly.
4. Incubate at 56° C for a minimum of 15 minutes. You should vortex the tube once or twice during incubation or place the sample on a thermomixer. Overnight incubation is acceptable.
5. To eliminate the risk of clogging the tip in the EZ1 biorobot, solid material must be removed from the tube unless the “Tip Dance” purification protocol is selected.
 - If a Qiagen 2 mL sample tube was used in Step 1, press the solid material against the inside of the tube to retain maximum volume and then remove the solid material.
 - If a tube other than a Qiagen 2 mL sample tube was used in Step 1, transfer the liquid from that tube to a 2 mL Qiagen sample tube.
 - Removal of solid material is optional, but highly recommended, if using the “Tip Dance” purification protocol on the EZ1 biorobots.

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6. For forensic samples thought to contain <100 ng DNA, addition of 1 μ L carrier RNA (1 μ g/ μ L) to the sample may increase the DNA recovery. This step is optional, but recommended.

7. Continue with DNA purification using the EZ1 or EZ1-Advanced XL Biorobot.

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5.4 Protocol: Pretreatment of epithelial (or other) cells mixed with sperm cells for DNA extraction using the EZ1 DNA Investigator Kit.

1. Place the sample in a Qiagen 2 mL sample tube or dolphin-nose tube (spin bucket tube).
2. Add 190 μ L Buffer G2 to the sample.
3. Add 10 μ L Proteinase K to the sample, vortex for 10 seconds and centrifuge briefly.
4. Incubate at 56° C for 15 minutes. Vortex the tube once or twice during the incubation or place on a thermomixer.
5. Centrifuge the tube briefly to remove drops from inside the lid.
6. Remove any solid material from the tube. Press the solid material against the inside of the tube to obtain maximum volume. If dolphin-nose tubes were used, place material in spin bucket with sterile wooden stick.
7. Centrifuge the tube at maximum speed for 5 minutes.
8. Transfer the supernatant to a new Qiagen 2 mL sample tube without disturbing the sperm cell pellet. This is the non-sperm fraction.
9. The non-sperm fraction is now ready for purification using the EZ1 or EZ1-Advanced XL BioRobot.
10. Wash the sperm cell pellet by re-suspending the pellet in 500 μ L Buffer G2. Centrifuge the tube at maximum speed for 5 minutes and discard the supernatant. Repeat this step at least two times.
11. Add 180 μ L Buffer G2 to the sperm cell pellet and re-suspend.
12. Add 10 μ L Proteinase K and 10 μ L 1 M DTT, vortex for 10 seconds, the centrifuge briefly.
13. Incubate at 56° C overnight. Vortex the sample several times during the incubation or incubate on a thermomixer.
14. Centrifuge briefly to remove any drops from inside the lid.

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15. For forensic samples thought to contain <100 ng DNA, addition of 1 μ L carrier RNA (1 μ g/ μ L) to the sample may increase the DNA recovery. This step is optional, but recommended.
16. Vortex and centrifuge briefly. DNA from sperm cells can now be purified from this tube.
17. Continue with DNA purification using the EZ1 or EZ1-Advanced XL BioRobot.

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5.5 Protocol: DNA Purification using the EZ1 BioRobot

1. Insert the EZ1 Investigator Card v1.0 completely into the EZ1 card slot of the EZ1 BioRobot utilizing firmware v1.1Q04.
2. Switch on the EZ1 BioRobot.
3. Press “START” to display the “Protocols” menu.
4. Press “1” for the Trace protocol, press “2” for the Trace Tip Dance protocol, press “3” for the Normalization protocol or press “4” for the Large-Volume protocol.
5. Choose TE buffer as the elution buffer by pressing “2”. Then press “1” to elute in 50 μ L, “2” to elute in 100 μ L or “3” to elute in 200 μ L.
6. Press any key to proceed through the text displayed in the LCD.
7. Open the workstation door.
8. Flick reagent cartridge(s) to mix the magnetic particles.
9. Load the reagent cartridge(s) into the cartridge rack.
10. Load 1-6 tip opened elution tube(s) into the first row of the tip rack.
11. Load 1-6 tip holder(s) containing filter-tip(s) into the second row of the tip rack.
12. Load 1-6 tip opened sample tube(s) or other extraction tube(s) containing digested sample into the back row of the tip rack. If using the Large-Volume protocol, add 400 μ L Buffer MTL to the samples prior to loading.
13. Close the workstation door.
14. Press “START” to start the purification procedure.
15. When the protocol ends, the LCD displays “Protocol finished”.

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16. Open the workstation door and cap the elution tubes containing the purified DNA. Store samples at 5° C or frozen. Prior to the use of samples, vortex briefly and spin in a microcentrifuge for 5 seconds.
17. Carry-over beads can interfere with quantification using real-time PCR. If any beads are visible in the elution tube (usually noted when using an elution volume of 100µL), remove the liquid from the beads using a magnetic rack. Alternatively, centrifuge the beads to the bottom of the tube and pipette from the upper supernatant when placing sample in the 96-well plate for quantification.
18. Close the workstation door and switch off the EZ1 BioRobot.

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5.6 Protocol: DNA Purification using the EZ1-Advanced XL BioRobot

1. Insert the EZ1 Advanced BioRobot card v1.1 completely into the EZ1-Advanced XL card slot of the EZ1-Advanced XL BioRobot utilizing firmware v1.0.1.
2. Switch on the EZ1-Advanced XL BioRobot. A maintenance reminder screen may appear. Press “1” to move to the next screen.
3. Press “START” to display the “Protocols”. A screen will first appear “Create Report File”. Press “ESC” to advance to the protocols screen.
4. Press “1” for the Trace protocol, press “2” for the Trace Tip Dance protocol, press “3” for the Large Volume protocol, press “4” for the Normalization protocol.
5. Choose TE buffer as the elution buffer by pressing “2”. Then press “1” to elute in 40 μ L, “2” to elute in 50 μ L, “3” to elute in 100 μ L, or “4” to elute in 200 μ L.
6. Press “ENT” to proceed through the text displayed on the LCD or press “ESC” to go back to the previous screen.
7. Open the workstation door.
8. Flick the reagent cartridge(s) to mix the magnetic particles.
9. Load the reagent cartridge(s) into the cartridge rack.
10. Load 1-14 opened elution tube(s) into the first row of the tip rack.
11. Load 1-14 tip holder(s) containing filter tip(s) into the second row of the tip rack.

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12. Load 1-14 opened sample tube(s) or other extraction tube(s) containing digested sample into the back row of the tip rack. If using the Large-Volume protocol, add 400 μ L of Buffer MTL to the sample(s) prior to loading.
13. Press “START” to start the purification procedure.
14. When the protocol ends, the LCD display “Protocol finished”.
15. Open the workstation door and cap the elution tubes containing the purified DNA. Store samples at 5° C or frozen. Prior to the use of samples, vortex briefly and spin in a microcentrifuge for 5 seconds.
16. Carry-over beads can interfere with quantification using real-time PCR. If any beads are visible in the elution tube, remove the liquid from the beads using a magnetic rack. Alternatively, centrifuge the beads to the bottom of the tube and pipette from the upper supernatant when placing sample in the 96-well plate for quantification.
17. Discard reagent cartridge(s), tip holder(s) containing filter-tips and sample tube(s). Clean the tip plungers and piercing units with alcohol wipes (Do not use bleach).
18. Close the workstation door. It is recommended to return to the Main Menu and Press “1” to select the UV light function. The UV light can be started by pressing “ENT” then setting the timer (the minimum time is 20 minutes). Pressing “START” will turn the UV light on and the door will be locked until the cycle completes and the UV lamp is cool.
19. Switch off the EZ-Advanced XL BioRobot.



6. DNA Quantification

6.1 Introduction

The Quantifiler® Trio Kit is designed to simultaneously quantify the total amount of amplifiable human DNA and human male DNA in a sample. The kit uses TaqMan® quantitative real-time PCR technology. The result obtained using the kit can aid in determining:

- The amount of sample to use in STR analysis applications.
- The relative quantities of human male and female DNA in a sample that can assist in the selection of the applicable STR chemistry.
- The DNA quality, with respect to both the DNA degradation level and the inhibition level.

The Quantifiler® Trio DNA Quantification Kit uses multiple-copy target loci. The human-specific target loci (Small Autosomal, Large Autosomal, and Y-chromosome targets) each consist of multiple copies dispersed on various autosomal chromosomes (Small Autosomal and Large Autosomal), or multiple copies on the Y-chromosome. The primary quantification targets (Small Autosomal and Y) consist of relatively short amplicons to improve the detection of degraded DNA samples. The small autosomal target is 80 bases and the Y target is 75 bases. In addition, the Quantifiler® Trio Kit contains a Large Autosomal target with a longer amplicon, 214 bases, to aid in determining if a DNA sample is degraded.

Quantifiler® Trio Chemistry Overview

The Quantifiler® Trio assay combines four 5' nuclease assays:

- Two separate target-specific human assays; one with a short PCR amplicon and one with a long PCR amplicon.
- A target-specific human male DNA assay.
- An internal PCR control (IPC) assay.

The Quantifiler® Trio assay targets serve the following functions:

- The Small Autosomal target (VIC® dye) is the primary quantification target for total human genomic DNA. Its smaller amplicon size (80 bp) is aligned with the sizes of typical “mini” STR loci and makes it better able to detect degraded DNA samples.
- The Large Autosomal Target (ABY® dye) is used mainly as an indicator of DNA degradation, by comparing the ratio of its quantification result with that of the Small Autosomal target. The ratio between the small and large autosomal targets, or degradation index, can be used to determine if the DNA in the sample is degraded. The degradation index (DI) is calculated by dividing the

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concentration of the small autosomal target by the concentration of the large autosomal target. A $DI \leq 1$ indicates that DNA is not likely degraded. A DI between 1 and 10 indicates moderate DNA degradation that may affect STR amplification. A $DI > 10$ indicates that the sample could be severely degraded. If the DI is blank (indicating no quantification result for the small and/or large autosomal target), the sample could be severely degraded or inhibited, or contain no DNA.

- The Y Chromosome Target (FAM® dye) allows the quantification of a sample's human male genomic DNA component.

The internal PCR control (JUN® dye) is a synthetic sequence not found in nature. The internal PCR control (IPC) present in each sample contains a synthetic DNA template and provides confirmation that all assay components are functioning as expected.

Quantifiler® Trio DNA Quantification Kit contains reagents for the amplification, detection and quantification of two human-specific DNA targets and human-male specific DNA target. The reagents are used with the Applied Biosystems® 7500 Real-Time PCR System for Human Identification with the HID Real-Time PCR Analysis Software v1.2.

The Quantifiler® THP PCR Reaction Mix, Quantifiler® Trio Primer Mix, Quantifiler® THP DNA Dilution Buffer and Quantifiler® THP DNA Standard are stored at -15 to -25° C upon receipt. After initial use, each item is stored at 2 to 8° C. Both the Primer and PCR Reaction Mix should be protected from direct exposure to light. Excessive exposure to light may affect the fluorescent probes and/or the passive reference dye.



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6.2 Standards Dilution Series

Below is an example for preparing the DNA quantification standard dilution series with the concentrations ranging from 50 ng/ μ L (std. 1) to 0.005 ng/ μ L (std. 5). When 2.0 μ L of a sample at the lowest concentration (0.005 ng/ μ L) is loaded in a reaction, the well contains approximately 1.5 diploid human genome equivalents.

Standards Dilution Series:

Standard	Concentration (ng/ μ L)	Example Volumes	Dilution Factor
Std. 1	50.000	25 μ L [100ng/ μ L stock] + 25 μ L Quantifiler® THP DNA dilution buffer	2X
Std. 2	5.000	10 μ L [Std. 1] + 90 μ L Quantifiler® THP DNA dilution buffer	10X
Std. 3	0.500	10 μ L [Std. 2] + 90 μ L Quantifiler® THP DNA dilution buffer	10X
Std. 4	0.050	10 μ L [Std. 3] + 90 μ L Quantifiler® THP DNA dilution buffer	10X
Std. 5	0.005	10 μ L [Std. 4] + 90 μ L Quantifiler® THP DNA dilution buffer	10X

6.2.1 Preparing the DNA Quantification Standards

NOTE: The diluted DNA quantification standards are stored at 2 to 8° C and used for only 2 weeks after preparation.

1. Label 5 low-bind microfuge tubes: Std. 1, Std. 2, Std. 3, etc.
2. Dispense the required amount of diluent (Quantifiler® THP DNA Dilution Buffer) into each tube.

Note: Approximately 40 μ L of the Quantifiler® THP DNA Dilution Buffer should be pipetted into a low-bind microfuge tube for use as a reagent blank / NTC (no template control) during sample setup.

3. Prepare Std. 1:
 - a. Vortex the Quantifiler® THP DNA Standard for 3 to 5 seconds.



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- b. Using a new pipette tip, add the appropriate volume of Quantifiler® THP DNA Standard to the tube for Std 1.
 - c. Vortex and centrifuge briefly.
 4. Prepare Std 2 through 5:
 - a. Using a new pipette tip, add the appropriate volume of the prepared standard to the tube for the next standard (refer to the table above for volumes).
 - b. Vortex and centrifuge briefly.
 - c. Repeat steps 4a and 4b until the dilution series is completed.

6.3 Preparing the Reaction Plate:

NOTE: During the preparation, the 96-well plate should be secured in its black base. Do not place it directly on the counter.

1. Thaw the Quantifiler® Trio Primer Mix completely, then vortex 3 to 5 seconds and centrifuge briefly before opening the tube.
2. Gently vortex the Quantifiler® THP PCR Reaction Mix and centrifuge briefly before opening the tube.
3. Pipette the required volumes of each component into an appropriately sized microcentrifuge tube. Prepare the appropriate volume of each component needed by combining the following volumes of reagents for each reaction in a 1.5 mL microcentrifuge tube:

Quantifiler® Trio Primer Mix 8.0 μ L
Quantifiler® THP PCR Reaction Mix 10.0 μ L

NOTE: Include 2 additional reactions in your calculation to provide excess volume for the loss that occurs during reagent transfer.

4. Vortex the PCR master mix 3 to 5 seconds, then centrifuge briefly.
 5. Dispense 18 μ L of the PCR master mix into each reaction well.



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6. Add 2 μ L of sample, standard or control to the appropriate wells. Always run duplicates of the standards. Include two blanks (Quantifiler® THP DNA Dilution Buffer) also referred to as NTC (no template control).
7. Seal the reaction plate with an Optical Adhesive Cover using the Optical Adhesive Cover Tool. Score each well to ensure a proper seal.
8. Centrifuge the plate at 3000 rpm for about 20 seconds in a tabletop centrifuge with plate holders to remove any bubbles from the bottom of the wells. If a tabletop centrifuge is not available, the plate can be lightly tapped to remove the bubbles in the wells or a salad spinner may be used.

NOTE: All bubbles must be removed from bottom of well.

6.4 Loading the reaction plate on the ABI 7500:

1. Depress the dimple on the right side of the tray and allow the tray to fully extend. The tray is located in the middle of the colored band on the front of the instrument between the power button and the “Power/Use” indicator lights.
2. With the tray open, place the 96-well plate on the tray with the notched corner facing to the right rear.
3. To close, push the tray on the dimple on the right hand side until the tray has returned into the instrument and has clicked into place.

NOTE: Push the tray back into the instrument using ONLY dimpled right side of tray.

6.5 Running the reaction plate on the ABI 7500:

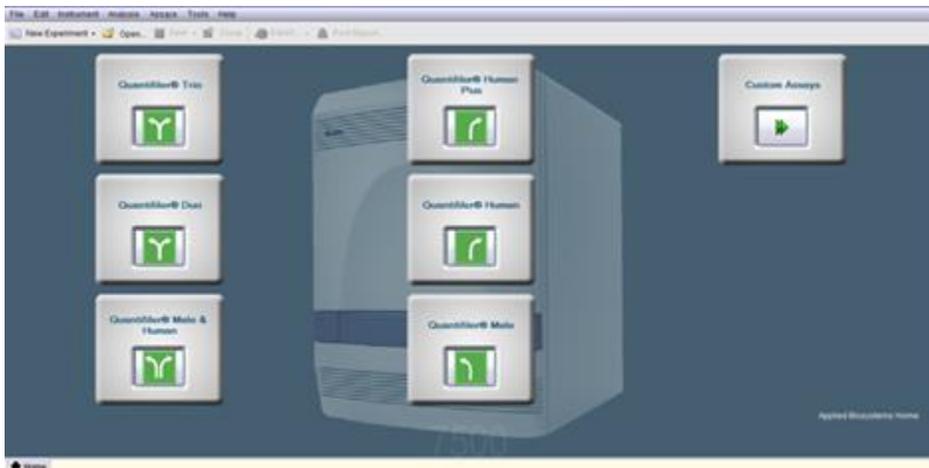
1. Turn on the computer. *NOTE: Wait for the computer to finish starting up before powering on the 7500 instrument.*
2. It is recommended to log in as user INSTR-ADMIN
3. Type the password into the Password field.

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4. Turn on the 7500 by pressing the power button on the lower right front of the 7500. When the green indicator is lit (not flashing), communication is established between the computer and instrument. If the green power-on indicator is flashing or the red indicator is lit, notify the supervisor.
5. Start the HID Real-Time PCR Analysis Software v1.2 from the desktop shortcut. The software can also be accessed by the following path:
Start>Programs>Applied Biosystems>HID Real-Time PCR Analysis Software>HID Real-Time PCR Analysis Software v1.2
6. Login with the appropriate username.
7. In the Home Screen, click the Quantifiler® Trio icon.





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- In the Experiment Properties screen, enter a name for the “Experiment Name”. The name will generally be the date and analyst initials e.g. “11-07-15 BRE”. All other settings are automatically set for the Quantifiler® Trio application.

How do you want to identify this experiment?

- Experiment Name: Quantifiler-HumanPlus
- Barcode (Optional):
- User Name (Optional):
- Comments (Optional):

Instrument

- 7500 (96 Wells)
- Set up, run, and analyze an experiment using a 4- or 5-color, 96-well system.

Experiment Type

- Quantitation - HID Standard Curve
- Use standards to determine the absolute quantity of target nucleic acid sequence in samples.

Reagents

- TaqMan® Reagents
- The PCR reactions contain primers designed to amplify the target sequence and a TaqMan® probe.

Ramp speed

- Standard (~ 1 hours to complete a run)
- For optimal results with the standard ramp speed, Applied Biosystems recommends using standard

- In the left navigational panel, click **Setup>Plate Setup**.



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10. The targets are automatically defined for the Quantifiler® Trio Application.

Defined Targets		
Target Name	Reporter	Quencher
T.Large Autosomal	ABY	QSY7
T.Small Autosomal	VIC	NFQ-MGB
T.IPC	JUN	QSY7
T.Y	FAM	NFQ-MGB

11. Define the samples: Click **Add New Samples**, then type the name for the sample. Repeat for the remaining samples.

The "Define Samples" interface shows a table with columns for Sample Name, Color, and Sample Type. It includes buttons for "Add New Sample", "Add Saved Sample", and "Save Samples". A mouse cursor is pointing at the "Add New Sample" button. The table contains entries for "Trio Standard 1" through "Trio Standard 5", "NTC", and "Sample 1".

Sample Name	Color	Sample Type
Trio Standard 1	Orange	Standard
Trio Standard 2	Purple	Standard
Trio Standard 3	Blue	Standard
Trio Standard 4	Light Green	Standard
Trio Standard 5	Yellow-Green	Standard
NTC	Pink	NTC
Sample 1	Blue	UnKnown



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12. Click **Assign Targets and Samples**. Targets are automatically assigned and the standard quantities are automatically specified.

Define Targets and Samples | **Assign Targets and Samples**

Instructions: Standards and NTC are set by default. Select wells, then assign targets if applicable.

Assign sample(s) to the selected wells.

Assign	Sample
<input checked="" type="checkbox"/>	Trio Standard 1
<input type="checkbox"/>	Trio Standard 2
<input type="checkbox"/>	Trio Standard 3
<input type="checkbox"/>	Trio Standard 4
<input type="checkbox"/>	Trio Standard 5
<input type="checkbox"/>	NTC
<input type="checkbox"/>	Sample 1

Assign target(s) to the selected wells.

Assign	Target	Task	Quantity
<input checked="" type="checkbox"/>	T.Large A...	<input type="checkbox"/> U <input checked="" type="checkbox"/> S <input type="checkbox"/> N	20
<input checked="" type="checkbox"/>	T.Small A...	<input type="checkbox"/> U <input checked="" type="checkbox"/> S <input type="checkbox"/> N	20
<input checked="" type="checkbox"/>	T.IPC	<input type="checkbox"/> U <input checked="" type="checkbox"/> S <input type="checkbox"/> N	
<input checked="" type="checkbox"/>	T.Y	<input type="checkbox"/> U <input checked="" type="checkbox"/> S <input type="checkbox"/> N	20

View Plate Layout

Show in Wells ▾

	1
A	T.IPC T.Large Autosomal T.Small Autosomal
B	T.IPC T.Large Autosomal T.Small Autosomal
C	T.IPC T.Large Autosomal T.Small Autosomal
D	T.IPC T.Large Autosomal T.Small Autosomal

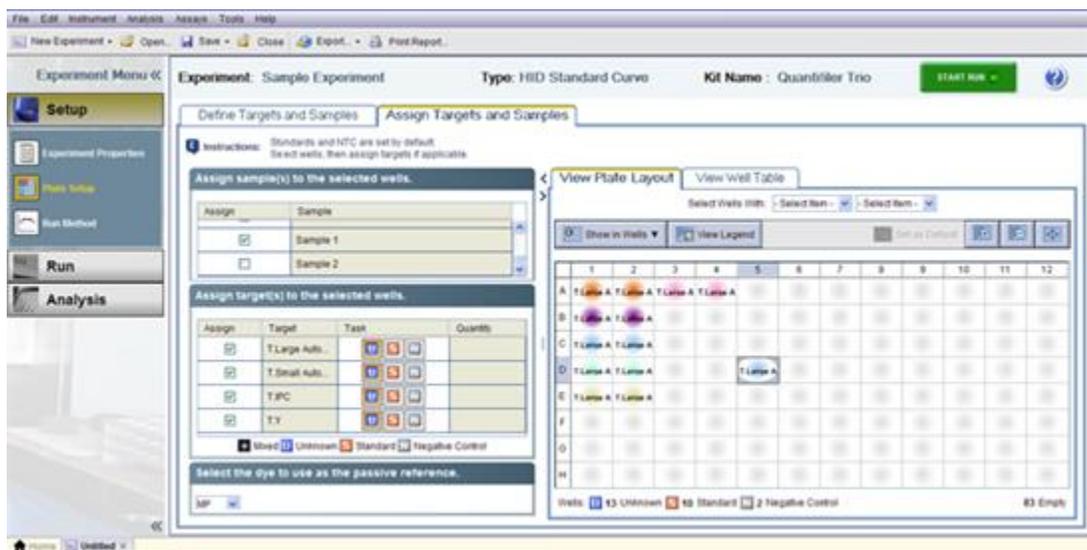


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- Click a well in the plate layout. In the Assign Sample(s) to well section to the left of the plate layout, locate the desired sample to enter in the well and select the checkbox in the Assign column next to the sample name. The target for each sample is set by default. Repeat the steps for the remaining samples. Samples can also be added by double clicking on a well in the plate layout and selecting **Add New Sample** in the box that opens.



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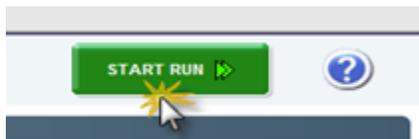
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14. In the left navigational panel, click **Setup>Run Method** to view the parameters. The parameters are automatically specified as shown in the figure below.



15. Click **Save**.

16. Click **Start Run**.



6.6 Analyzing the Run

1. To analyze a run, navigate to the folder where the run is stored and double click the run file or open the file after launching the HID Real-Time PCR Analysis software icon from the shortcut on the desktop.

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2. Verify the analysis setting by clicking **Analysis Settings>Cr Settings**. Verify that the settings are as shown in the figures below for the large, small and Y target threshold and baseline setting.

Analysis Settings for Quantifier Trio

HID Settings | **Cr Settings** | Flag Settings

Review the default settings for analysis of targets in this experiment. To edit the default settings, click "Edit Default Settings." To use different settings for a target, select the target from the table, deselect "Use Default Settings," then change the settings that are displayed.

Default Cr Settings
Default Cr settings are used to calculate the Cr for targets without custom settings. To edit the default settings, click "Edit Default Settings."
Threshold: 0.2 Baseline Start Cycle: 3 Baseline End Cycle: 15 [Edit Default Settings](#)

Select a Target

Target	Threshold	Baseline Start	Baseline End
T.IPC	0.1	3	15
T.Large Autosomal	0.2	3	15
T.Small Autosomal	0.2	3	15
T.Y	0.2	3	15

Cr Settings for T.Large Autosomal
Cr Settings to Use: Use Default Settings
 Automatic Threshold
Threshold: 0.2
 Automatic Baseline
Baseline Start Cycle: 3 End Cycle: 15

[Revert to Default Analysis Settings](#) [Apply Analysis Settings](#) [Cancel](#)

IPC target threshold and baseline settings:

Analysis Settings for Quantifier Trio

HID Settings | **Cr Settings** | Flag Settings

Review the default settings for analysis of targets in this experiment. To edit the default settings, click "Edit Default Settings." To use different settings for a target, select the target from the table, deselect "Use Default Settings," then change the settings that are displayed.

Default Cr Settings
Default Cr settings are used to calculate the Cr for targets without custom settings. To edit the default settings, click "Edit Default Settings."
Threshold: 0.2 Baseline Start Cycle: 3 Baseline End Cycle: 15 [Edit Default Settings](#)

Select a Target

Target	Threshold	Baseline Start	Baseline End
T.IPC	0.1	3	15
T.Large Autosomal	0.2	3	15
T.Small Autosomal	0.2	3	15
T.Y	0.2	3	15

Cr Settings for T.IPC
Cr Settings to Use: Use Default Settings
 Automatic Threshold
Threshold: 0.1
 Automatic Baseline
Baseline Start Cycle: 3 End Cycle: 15

[Revert to Default Analysis Settings](#) [Apply Analysis Settings](#) [Cancel](#)

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3. Click **Analyze**.

6.7 Evaluating the Run

1. Use the left navigational pane to evaluate the run data.



2. In the left navigational pane, click the **Amplification Plot** to view each sample. Evaluate the Internal Positive Control (IPC) to distinguish between true negatives and samples possibly affected by PCR inhibitors, chemistry / setup issues and/or instrument issues. The IPC for each sample should cross the threshold line between 20 and 30 C_T . If the value for any sample is outside this range, the IPC needs to be evaluated further.



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Interpreting IPC Amplification Results:

Quantifiler® Trio Sample Targets	IPC	Interpretation
No Amplification	No Amplification	Invalid Result, repeat sample. Perform sample cleanup if inhibitors are suspected.
No Amplification	Amplification	True Negative.
Amplification (Low C _T)	No Amplification	High sample concentration creating IPC inhibition. The sample should be diluted before STR amplification
Amplification (High C _T)	No Amplification	Partial PCR inhibition. Perform sample cleanup and repeat the sample.

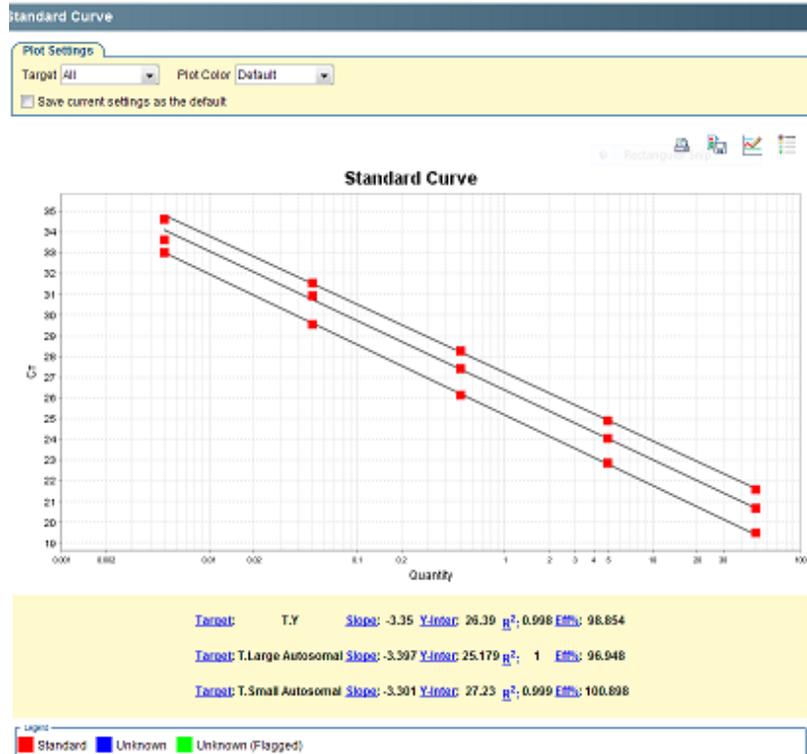
3. Click Standard Curve from the left navigational pane to examine the standard curve results. Evaluate the quality of the results for the quantification standard reactions. The R² value is a measure of the closeness of fit between the standard curve regression line and the individual C_T data points of quantification standard reaction. An R² value ≥0.99 indicates a close fit between the standard curve regression line and the individual C_T data points of quantification standard reaction. An R² value <0.98 needs further analysis of the standard curve for problems. The slope indicates the PCR amplification efficiency for the assay. A slope of -3.3 indicates 100% amplification efficiency. The slope of the standard curve should be between -3.0 and -3.6 for both the Small Autosomal and Y target. The slope of the Large Autosomal target should be between -3.1 and -3.7. No more than two data points may be omitted from the standard curve. To omit a standard, select the well in the **View Plate Layout tab**, then right click and choose **Omit**. The well may be added back by following the same steps and choosing **Include**. If after omitting two data points both the R² value is <0.98 and the slope is outside of -3.0 to -3.6 for the Small Autosomal or Y target, the procedure should be repeated with new standards. Note: If any conditions are changed (well omitted, sample name change, etc.), the run must be reanalyzed. **Print the standard curve by using the printer icon in the standard curve plot window. Select "All" in the Target drop down box to print all three standard curves.**

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4. Click the Multicomponent Plot to check for noise in the baseline. The multicomponent plot displays the fluorescence data for each target in the quantification assay plotted against cycle number. Samples in a normal multicomponent plot generally demonstrate a flat line for at least the first 15-20 cycles, before exponential growth of the PCR product can be detected. For the Quantifiler® Trio Kit, the line should be flat between cycles 3-15 to calculate the baseline for the sample. If noise occurs between cycles 3-15, it may affect the baseline calculation for the sample, which can in turn affect the C_T value calculated for the DNA targets. A noisy baseline may require repeating the run.

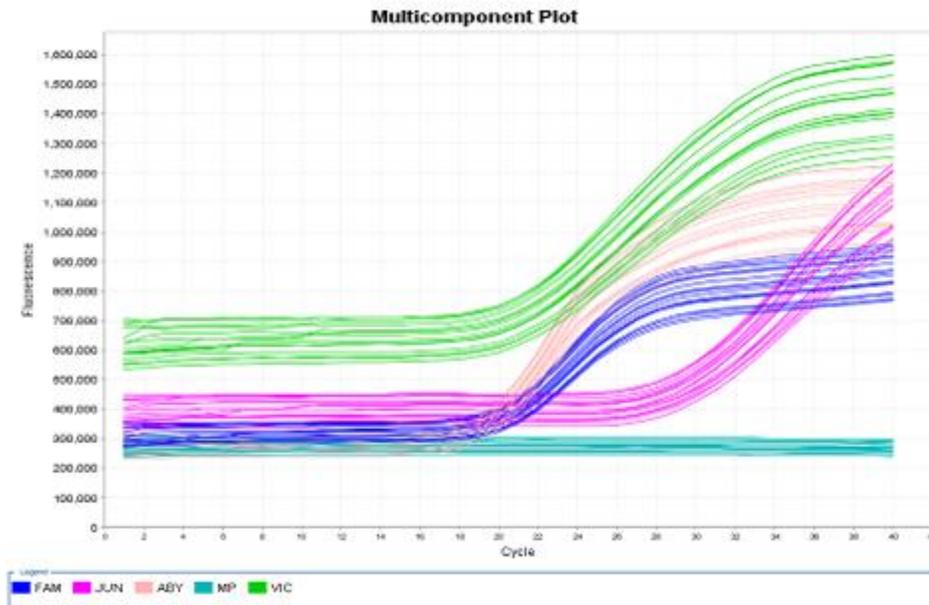


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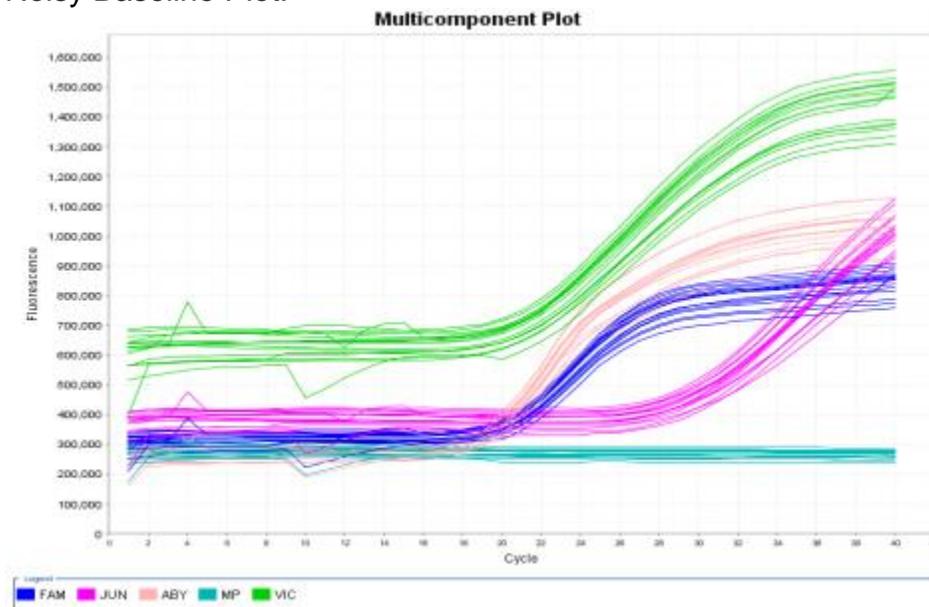
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Normal Baseline Plot:



Noisy Baseline Plot:



5. Non-Template Control (NTC): Contains PCR reagent, but no template DNA. Occasionally, a value may be given for the NTC ($C_T > 36$) due to



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background fluorescence or stochastic effects. Analysts may proceed with amplification and typing if NTC's demonstrate a $C_T > 36$ for the small autosomal or Y targets or if only one of the NTC's indicates signal. An NTC with amplification occurring only in the large autosomal target will be considered undetermined. If both NTC's indicate a $C_T < 36$, the analyst should notify the technical leader prior to amplification and typing of samples.

6. The QC Summary can be checked to provide the analyst a view of samples that may have issues. The QC Summary is **not** a replacement for analyst interpretation of each sample.

6.8 Assessing the Quantity

NOTE: The primary quantification value for autosomal typing is from the small autosomal target. Use this value for determination of input amount for the GlobalFiler® amplification kit. The Y-target will be used for determining the input amount for the Yfiler® Plus kit.

1. Select the **View Well Table** tab to view results. In the **Show in Table** tab, select the following items to view in the results table: Well, Sample Name, Target Name, C_T , Quantity, Male:Female (M:F) Ratio and Degradation index.
2. Choose the **export** function from the toolbar.
3. In the **Export Properties** tab checkmark **Results** for the data to export and provide a name and location for the export.
4. In the **Customize Export** tab, select the content to export: Well, Sample Name, Target Name, C_T , Quantity, M:F ratio, and Degradation Index.
5. Click **Start Export**.

Results may be printed in Excel.

6.9 Cleanup of Inhibited Samples

The steps below describe methods an analyst may use when a sample demonstrates the presence of an inhibitor:



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1. For samples extracted using either the organic or robotic EZ1 Method, the extract may be diluted in order to minimize the effects of the inhibitor.
2. For samples extracted using an organic method including differential extractions, the extract may be run through the EZ1 or EZ1-XL purification protocols found in section 5 of this manual. Adjust the total volume of the extract with 1:1 G2 Buffer to either 200 μ L for the trace protocol or 500 μ L for the large-volume protocol. Since the DNA has been extracted, no Proteinase K or additional incubation is required. A new reagent blank must be started for the robotic purification process unless the reagent blank and all of the associated samples are carried through the purification process or an additional reagent blank was included in the extraction set. If a new reagent blank is started, both the organic and the robotic extraction blank must be typed and both must be free of contamination.
3. Samples exhibiting inhibition after dilution and /or extraction and purification on the EZ1 may require re-extraction if the extract fails to produce a profile during typing.

6.10 Concentration of Samples

If the results from the Quantifiler® Trio run are between 0.001 ng/ μ L and 0.04 ng/ μ L, the analyst will do one of the following or a combination:

1. Perform additional extraction; repeat quantification.
2. Concentrate the extracted DNA sample and process for STR analysis.
 - It is recommended to concentrate only if the extract volume will be reduced by at least half.
 - Concentration to 30 μ L is recommended since this allows for repeat autosomal testing or additional Y-STR testing if necessary.
 - Analysts may concentrate samples to less than 30 μ L at their discretion; however, samples should not be concentrated to less than 20 μ L.
 - Samples eluted in 50 μ L or less that have a Quantifiler® Trio concentration less than 0.001 ng/ μ L for the Y or small

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autosomal target are not recommended to be concentrated even if the volume will be reduced by half. Refer to section 6.11.3 for stopping at quantification considerations.

- If two (2) extractions have been made of the sample, the analyst may combine these and concentrate. Note: When combining extracts, extracts should only be combined from the same extraction batch.
 - Samples and blanks are concentrated using a Microcon microconcentrator unit following the steps below:
 - a. Add approximately 100 μ L of TE buffer to a Microcon microconcentrator unit.
 - b. Transfer the extract(s) to the microconcentrator unit.
 - c. Cap the microconcentrator unit and spin in a microcentrifuge at 500 X g (2300 rpm) for at least 10 minutes.
 - d. Add TE buffer to the microconcentrator unit for the desired final volume (20-200 μ L).
 - e. Remove the concentrator from the microcentrifuge tube and carefully invert the concentrator onto a fresh, sterile, labeled microcentrifuge tube.
 - f. Spin the microconcentrator unit in a microcentrifuge at 500 X g (2300 rpm) for 5 minutes.
 - g. Discard the concentrator and cap the microcentrifuge tube.
 - h. Sample is ready for amplification.
3. Continue processing of the sample for possible partial probative results. When applicable, concentration of the sample should be performed prior to amplification and genotyping of the sample.

6.11 Amplification Calculation and Kit Consideration

6.11.1 Successful amplification of human DNA leading to partial or complete DNA profiles can be achieved over a wide range of

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input DNA quantified using the Quantifiler® Trio and amplified using either the GlobalFiler® or Yfiler® Plus amplification kits. The amount of DNA selected for amplification could vary over a wide range depending on the condition and type of the DNA being amplified (e.g. degraded vs. high molecular weight; single source vs. mixture; inhibited vs. not inhibited; instrument sensitivity; software platforms; pipetting techniques; etc.). Ultimately, the analyst should consider all of the above when necessary as a guideline for input DNA to perform successful casework analysis. As a general guideline, approximately 0.5 to 1.0 ng of input DNA is optimal when using the GlobalFiler® or Yfiler® Plus amplification kit. Degraded or compromised samples may require an increased input of DNA for amplification.

Note: Caution should be used when approaching inputs close to 2 ng as instrument saturation and increased artifacts may occur. Caution should be used when amplifying samples with less than 0.1 ng of input DNA due to the potential of increased stochastic effects.

- 6.11.2** If applicable, analysts should perform autosomal DNA testing prior to performing or recommending Y-STR testing. Samples demonstrating a ratio of male: female DNA greater than 1:10 may proceed directly to Y-STR testing at the analyst's discretion. Validation studies have shown that once the ratio of male to female DNA exceeds 1:10, the ability to obtain an interpretable minor male profile decreases.

Y-STR testing will generally apply to samples collected from a sexual assault of a female, but may also apply to certain samples from other violent crimes such as a female victim's fingernail scrapings. If no subject standard is available, the sample should be saved for future Y-STR testing in the event a subject standard becomes available.

Y-STR testing may not be performed if there is a probative profile developed using the GlobalFiler® amplification kit. Both GlobalFiler® and Yfiler® Plus testing may be necessary when the M:F ratio is greater than 1:10 and both the male

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and female portions of a sample may be considered probative to a case.

A case involving swabs collected for the digital penetration of a female victim by a male subject may proceed directly to Y-STR testing if requested by the District Attorney General and a subject standard has been submitted. Y-STR testing may be attempted on swabs collected from sites of penetration (e.g. penile or oral penetration swabs from the vagina and/or anus) that screen negative for the presence of semen or saliva if requested by the District Attorney General and a subject standard has been submitted.

Y-STR testing will generally not be performed or recommended for samples if less than 0.01 ng of male DNA can be targeted for amplification.

- 6.11.3** In general, blanks will be processed for STR analysis. However, for instances where multiple blanks are extracted within an extraction set, only one blank displaying the highest signal, if any, is required to be subjected to STR analysis. If no samples from the extraction set are amplified, no reagent blank is required to be amplified.

Samples eluted in 50 μ L or less having a small autosomal target value <0.001 ng/ μ L may stop at quantification. This may be reported as "Quantification results from real-time PCR testing did not indicate a sufficient amount of human DNA for analysis. No further testing was performed." If no human DNA is detected for the small autosomal target, the results may be reported as "Quantification results from real-time PCR testing did not indicate the presence of human DNA. No further testing was performed."

Samples eluted in 50 μ L or less and having a Y-target value <0.001 ng/ μ L may stop at quantification and may not be recommended for Y-STR testing. This may be reported as "Quantification results from real-time PCR testing did not indicate a sufficient amount of male DNA for analysis. No further testing was performed." If no male DNA is detected for the Y-target, the results may be reported as

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“Quantification results from real-time PCR testing did not indicate the presence of male DNA. No further testing was performed.” Prior to stopping at quantification, analysts should consider if concentration of a sample eluted in more than 50 μL would provide a concentration ≥ 0.001 $\text{ng}/\mu\text{L}$. If concentrating would produce a concentration ≥ 0.001 $\text{ng}/\mu\text{L}$, the sample should be concentrated and amplified with the appropriate kit.

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7. STR Amplification with GlobalFiler® and Yfiler® Plus

7.1 PCR Amplification using GlobalFiler®

1. Determine the number of samples to be amplified, including positive and negative controls. The Positive Control DNA (Control DNA 007 provided in kit) must be amplified with every run. The Negative Control consisting of 15 μ L of TE Buffer must also be included in every run.
2. Label the required number of PCR tubes (0.2 mL MicroAmp Reaction Tubes with caps) and place them into a rack.
3. Vortex the Master Mix and Primer Set for 3 seconds. Spin the tubes briefly in a microcentrifuge to remove any liquid from the caps.
4. Prepare the appropriate volume of each component needed by combining the following volumes of reagents for each reaction in a 1.5 mL microcentrifuge tube:

GlobalFiler® Master Mix 7.5 μ L
GlobalFiler® Primer Set 2.5 μ L

NOTE: Include at least 3 additional reactions in your calculation to provide excess volume for the loss that occurs during reagent transfer.

5. Mix thoroughly by vortexing at medium speed for 3 seconds.
6. Spin the tube briefly in a microcentrifuge to remove any liquid from the cap.
7. Dispense 10 μ L of the reaction mix into each PCR tube with a dedicated pipette.
8. Add TE followed by your sample based on the calculations previously obtained. The final volume for the reaction is 25 μ L. The optimal target for amplification is approximately 0.5-1.0 ng.
9. Vortex the Control DNA 007 tube (0.1ng/ μ L). Spin the tube briefly in a microcentrifuge to remove any liquid from the cap. Add up to 10 μ L (1.0 ng) of the control DNA to the "Positive Control" tube. If using



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10 μ L of control DNA, 5 μ L of TE must be added to bring the final volume to 25 μ L. However, lower volumes of the positive control may be added based on instrument sensitivity, software platform, pipetting technique, etc.

10. Add 15 μ L of TE buffer to the “Negative Control” tube.
NOTE: The final volume in each PCR tube is 25 μ L.
11. Carry tubes on a carrier rack into the PCR amplification room and place in thermal cycler.
12. GeneAmp® PCR System 9700 or Veriti – Close cover, turn on thermal cycler, select program to run, and press Start/Run.
13. To assure that no problems were experienced during the run, verify either the set points of the run or the history at the end of the amplification. The run history is verified from the history screen on the thermal cycler.
14. After amplification, remove tubes from thermal cycler and store the amplified products in a freezer at -15° C to -25° C. Store amplified DNA samples separate from all PCR amplification reagents, extracted DNA samples and casework samples. Amplified DNA may be discarded after the case is technically reviewed.

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7.2 Specifications for GeneAmp® PCR System 9700 or Veriti for GlobalFiler®

The following method for the GeneAmp PCR System 9700 or Veriti must be used for GlobalFiler® amplification.

Tube type: 0.2 mL MicroAmp Reaction Tube with Cap
Total Reaction Volume: 25 µL per tube
Ramp Speed: Max or 100%

Thermal Cycler PCR Parameters for GlobalFiler®:

Initial Incubation Step: Hold 95° C for 1 minute

29 cycles

Denature: 94° C for 10 seconds
Anneal / Extend: 59° C for 90 seconds

NOTE: Denature and Anneal/Extend are considered one cycle.

Final Extension: 60° C for 10 minutes

Final Step: Hold at 4° C



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7.3 PCR Amplification using Yfiler® Plus

1. Determine the number of samples to be amplified, including positive and negative controls. The Positive Control DNA (Control DNA 007 provided in kit) must be amplified with every run. The Negative Control consisting of 10 μ L of TE Buffer must also be included in every run.
2. Label the required number of PCR tubes (0.2 μ L MicroAmp Reaction Tubes with caps) and place them into a rack.
3. Vortex the Master Mix and Primer Set for 3 seconds. Spin the tubes briefly in a microcentrifuge to remove any liquid from the caps.
4. Prepare the appropriate volume of each component needed by combining the following volumes of reagents for each reaction in a 1.5 mL microcentrifuge tube:

Yfiler® Plus Master Mix 10.0 μ L

Yfiler® Plus Primer Set 5.0 μ L

NOTE: Include at least 3 additional reactions in your calculation to provide excess volume for the loss that occurs during reagent transfer.

5. Mix thoroughly by vortexing at medium speed for 3 seconds.
6. Spin the tube briefly in a microcentrifuge to remove any liquid from the cap.
7. Dispense 15 μ L of the reaction mix into each PCR tube with a dedicated pipette.
8. Add TE followed by your sample based on the calculations previously obtained. The final volume for the reaction is 25 μ L. The optimal target for amplification is approximately 0.5-1.0 ng.
9. Vortex the Control DNA 007 tube (2.0 ng/ μ L). Spin the tube briefly in a microcentrifuge to remove any liquid from the cap. Add up to 1.0 ng of the Control DNA 007 to the "Positive Control" tube. Lower volumes of the positive control may be added based on instrument sensitivity, software platform, pipetting technique, etc.

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NOTE: The Control DNA 007 supplied in the Yfiler® Plus kit has a concentration of 2.0 ng/μL.

10. Add 10 μL of TE buffer to the “Negative Control” tube.

NOTE: The final volume in each PCR tube is 25 μL.

11. Carry tubes on a carrier rack into the PCR amplification room and place in thermal cycler.
12. GeneAmp® PCR System 9700 or Veriti – Close cover, turn on thermal cycler, select program to run, press Start/Run.
13. To assure that no problems were experienced during the run, verify either the set points of the run or the history at the end of the amplification.
14. After amplification, remove tubes from thermal cycler and store the amplified products in a freezer at -15° C to -25° C. Store amplified DNA samples separate from all PCR amplification reagents, extracted DNA samples and casework samples. Amplified DNA may be discarded after the case is technically reviewed.

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7.4 Specification for GeneAmp® PCR System 9700 or Veriti for Yfiler® Plus

The following method for the GeneAmp® PCR System 9700 or Veriti must be used for Yfiler® Plus amplification.

Tube Type: 0.2 mL MicroAmp Reaction Tube with Cap
Total Reaction Volume: 25 µL per tube
Ramp Speed/Rate: 9600

Thermal Cycler PCR Parameters for Yfiler® Plus:

Initial Incubation Step: Hold 95° C for 1 minute.

30 cycles:

Denature: 94° C for 4 seconds

Anneal/Extend: 61.5° C for 1 minute

NOTE: Denature and Anneal/Extend are considered one cycle

Final Extension: 60° C for 22 minutes

Final Step: Hold at 4° C

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8. Protocol for Applied Biosystems 3500 Genetic Analyzer Using Data Collection Software v.2.0 with GlobalFiler® or Yfiler® Plus

8.1 Software Start-up

1. Turn on 3500 instrument. Wait for a solid green light before starting the computer and launching the 3500 software.
2. Turn on the PC. Enter the appropriate username (INSTR-ADMIN) and password. Wait for 3500 Daemon to fully load. A green check mark will appear on the Server Monitor Icon.



3. If the Server Monitor does not start automatically, launch the Server Monitor: **Start>Programs>Applied Biosystems>3500>>Server Monitor**. This takes 1-2 minutes to launch.
4. Launch the 3500 software.

8.2 Check Instrument Consumables

1. Check the dashboard for the status of consumables on the instrument. Click "Refresh" to verify the current status of the consumables.
2. The "Maintenance" tab or the "Maintain Instrument" button on the dashboard will allow access to the "Maintenance Wizards". The following wizards are available: Install Capillary Array, Remove Bubbles, Wash Pump and Channels, Shut Down the Instrument, Fill Array with Polymer, Replenish Polymer and Change Polymer Type.
3. Replace expired anode and cathode buffer. Refer to the Applied Biosystems 3500/3500xl Genetic Analyzer User Guide (Revision Date May 2012) page 227-229 for instructions on buffer replacement. The buffers expire 14 days after placing on the 3500 or after 240 injections.
4. The POP-4 polymer may be used up to 14 days after placing on the 3500. Replace polymer after 14 days or if there is an insufficient amount for a run. The Replenish Polymer Wizard may be used to assist in replacing polymer.



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5. The capillary array is guaranteed by Applied Biosystems for 160 injections and a certain date. The array may be used past these points at analyst discretion as long as there is no significant decrease in resolution or peak height. The Install Capillary Array Maintenance Wizard can be used to assist in replacing the capillary array.
6. Spatial Calibration. A new spatial calibration will be performed when the capillary array is removed or replaced, the detector door is opened or detection cell is moved or if the instrument is moved. Refer to the Appendix section of this manual.
7. Spectral Calibration. A new spectral calibration will be performed when the capillary array is changed, when a service engineer performs service on the optical system or if a decrease in spectral separation is observed in data. Refer to the Appendix section in this manual.

NOTE: If the capillary array is removed for any reason, a spectral calibration must be performed.

8.3 Preparing GlobalFiler® or Yfiler® Plus Samples for a 3500 Run

1. Combine the necessary amounts of HiDi Formamide and GeneScan LIZ 600 v.2.0 Internal Lane Size Standard to create a master mix for sample injection.

GlobalFiler® or Yfiler® Plus:

of samples X 9.6 µL of deionized formamide

of samples X 0.4 µL of GeneScan LIZ 600 v.2.0 size standard

*Add 4 samples to your calculation to account for master mix loss during transfers

***CAUTION:** Use only deionized formamide. Over time, formamide decomposes to formate. Formate ions are injected preferentially into the capillary, causing a loss of signal intensity.

***WARNING:** Formamide is a teratogen and is harmful by inhalation, skin contact or ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling. Wash hands thoroughly.

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2. Briefly vortex and centrifuge the master mix tube before dispensing into the wells.
3. Pipette 10.0 μ L of master mix into each well to be used on a 96-well plate.

**NOTE: Each well that will be injected to the capillary, must contain something, either sample or HiDi. If a well is empty, the capillary will inject air, which can cause damage to it.*

4. Referring to any set-up worksheet, pipette 1.0 μ L of amplified DNA sample into the designated well on the 96-well plate. Pipette 1.0 μ L of ladder into the designated wells. Place the ladders no less than every three injections.
5. Place the 96-well septum over the plate.
6. Briefly centrifuge the plate to ensure that sample is collected at the bottom of the wells and bubbles are not present in the bottom of the wells.
7. Denature the samples for three minutes at 95° C by placing the 96-well plate onto the heating block or onto a thermal cycler (use the 95° C temperature program); the plate may be snap-chilled in the freezer for 3 minutes.
8. Place the sample plate into the plate base and snap the white plate retainer onto the assembly. Check to ensure the holes on the plate retainer line up with the septa and that all wells are aligned correctly (should have a black appearance in each well). If the alignment is not correct, the capillary tips will be damaged.

8.4 Starting a Run for GlobalFiler® or Yfiler® Plus

1. Start the oven by clicking “Start Pre-heat” on the dashboard screen. The temperature is set to 60° C.

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2. On the dashboard screen, click the “Create New Plate” or “Create Plate from Template” box at the top of the screen. If the “Create Plate from Template” option is chosen, select the appropriate template (e.g. GlobalFiler® or Yfiler® Plus). Name the plate using the date and analyst initials (e.g. “6-5-13 BRE”) and fill in the remaining plate details the same as the screenshot below:

A screenshot of a software interface showing the 'Plate Details' form. The form has a title bar with buttons for 'New Plate', 'Open Plate', 'Save Plate', 'Close Plate', and 'Start Run'. The 'Plate Details' section includes the following fields:

- * Name: 6-15-13 BRE
- * Number of Wells: 96 (selected), 96-Fast, 384
- * Plate Type: HID
- * Capillary Length: 36 cm
- * Polymer: POP4

Below the 'Plate Details' section is a link for 'Secondary Analysis'.

3. Click “Assign Plate Contents”. The plate view or table view may be used to add your sample names to the appropriate wells.
4. The Sample Type for each well is defaulted to “Sample”. Wells containing allelic ladders, positive control and negative controls may be designated under Sample Type if using the Table view or from the Customize Sample Info if using the Plate view (see screen shot below). If allelic ladders are not designated at this step, a pop-up window will appear later when the run is started indicating no ladders have been assigned to the wells.

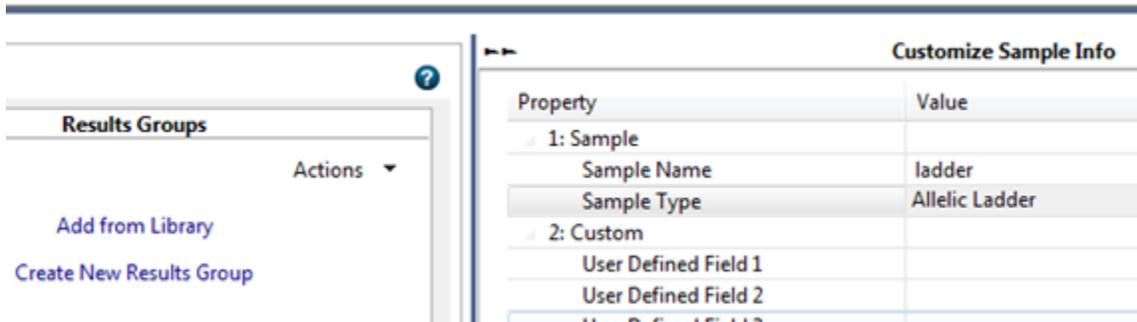


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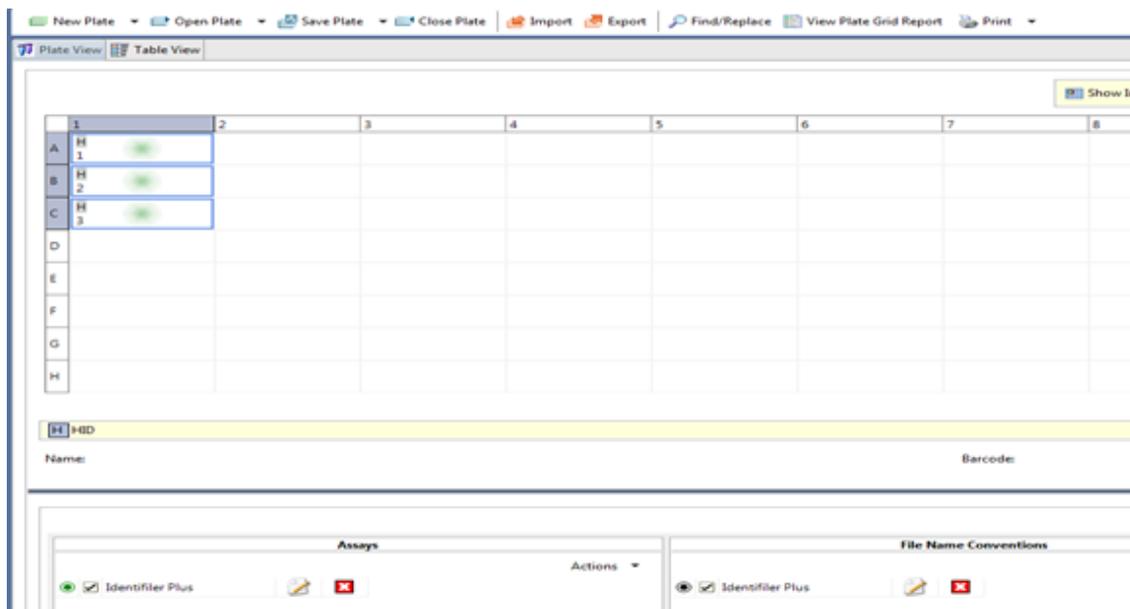
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NOTE: The Customize Sample Info tab below may be hidden at the bottom right of the screen.



5. Select the appropriate Assay, File Name Convention and Result Group from the Library (e.g. GlobalFiler® or Yfiler® Plus). Next, highlight the appropriate samples in the plate grid and enable the checkbox next to the assay name in order to assign the assay to the selected wells. Repeat this process for the File Name Conventions and Results Groups. The screenshot below shows a plate that has an assigned assay.



NOTE: Refer to sections 8.5, 8.6 and 8.7 if the Assay, File Name Convention and Results Group have not been previously set-up in the Library or if you need to verify settings and parameters. After initial set-up

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in the Library, the appropriate Assay, File Name Convention and Results Group can merely be selected from the Library for future runs.

6. Select "Save Plate".
7. Load the plate onto the 3500 instrument by pushing the Tray button on the front of the instrument panel. Ensure that the notched corner of the plate is in the notched corner of the autosampler. Close the instrument door and the autosampler will return to the home position.
8. Select "Link Plate for Run" at the bottom of the screen.
9. Select the "Create Injection List" from the bottom of the screen.
10. Before starting the run, injections may be repeated by highlighting the appropriate injection from the injection list and selecting "Duplicate". Data for the entire column samples will be collected using the same protocols as the original injection.
11. After the injection list is complete, select "Start Run" from the bottom of the screen.
12. The "Re-inject" function is now active. When the re-inject function is selected, the entire column of samples will be injected; however, data will only be collected for the samples you highlight from the plate grid or table view. Two re-injection windows will pop up when the re-inject function is selected as seen below. The first pop-up will ask if the analyst wants to select a ladder(s) for the re-injection. The analyst may choose to add a ladder(s) at their discretion.

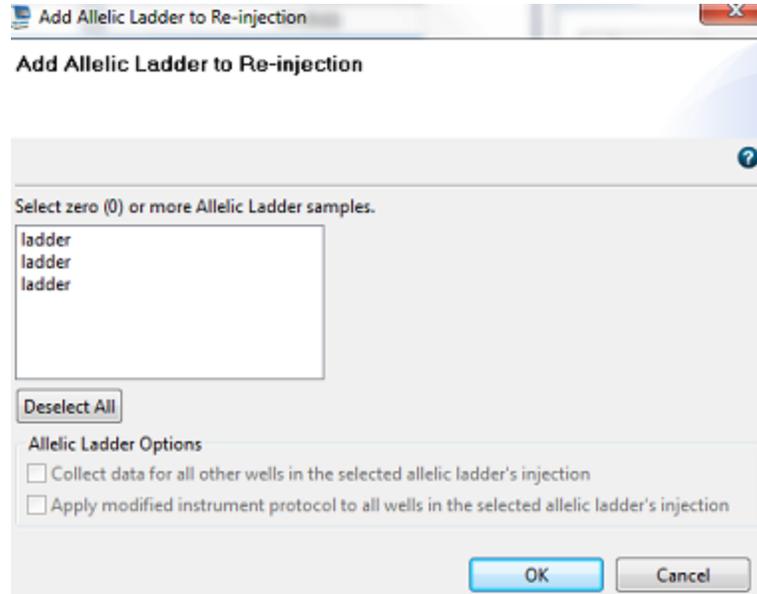
NOTE: *If the allelic ladder selected for re-injection is in a different column than the samples selected for re-injection, two re-injections will be added to the injection list. The first re-injection collects data for the selected sample. The second re-injection collects data for the allelic ladder.*



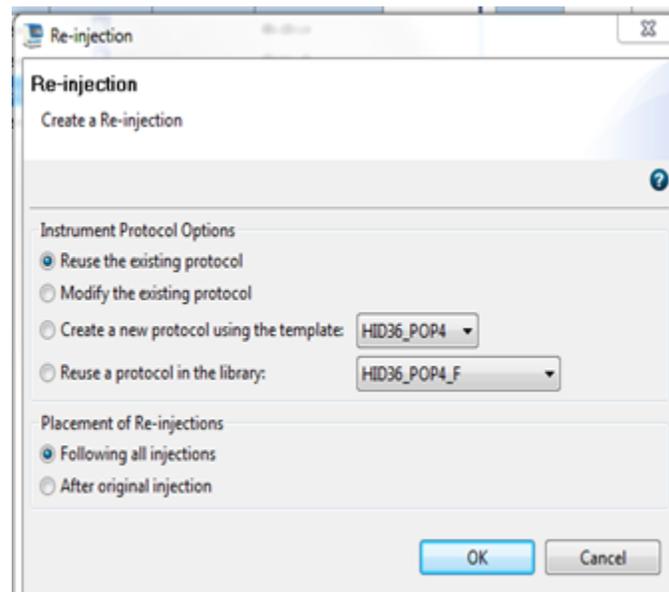
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This second re-injection pop-up window will provide Instrument Protocol and Placement of Re-injection options. Select "Reuse the existing protocol". Either selection for "Placement of Re-injections" may be chosen.



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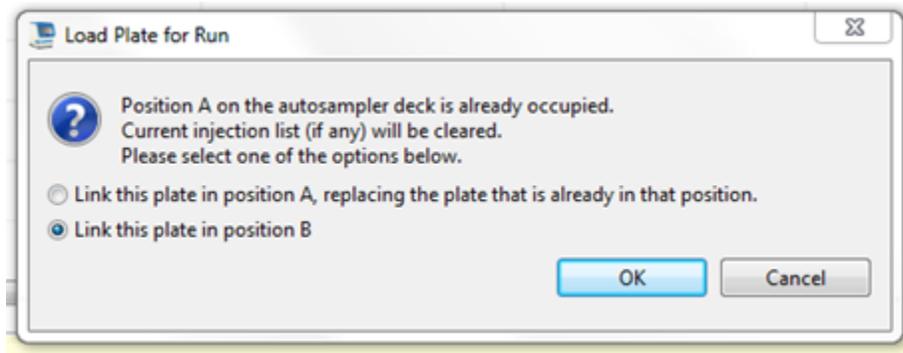
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13. A second plate can be set up and run on the 3500 by returning to the Dashboard and following the above steps beginning at “Create a New Plate”.

NOTE: The plate must be loaded onto the open plate deck on the 3500 before selecting “Link Plate for Run”. The correct plate position will need to be selected after selecting link plate so as not to delete and replace the first plate.



14. After completion of an injection, the “Review Results” function may be selected to view data from each sample. To return back to the injection list screen, select “Monitor Run” from the Main Workflow.

8.5 Setting up Assays in the Library

1. The Assay is composed of the Instrument Protocols and the QC Protocol. The name of an assay may vary, but the parameters shown in the screenshots below for the Instrument Protocols will be used for data collection of GlobalFiler® and Yfiler® Plus. If necessary, the run time may be adjusted.



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GlobalFiler® Instrument Protocol:

Edit Instrument Protocol GlobalFiler 28

Setup an Instrument Protocol

Application Type: Capillary Length: cm Polymer:

Dye Set:

Instrument Protocol Properties

* Run Module:

* Protocol Name:

Description:

Oven Temperature (°C): Run Voltage (kVolts): PreRun Voltage (kVolts): Injection Voltage (kVolts):

Run Time (sec.): PreRun Time (sec.): Injection Time (sec.): Data Delay (sec.):

Advanced Options

Following values are not recommended to be changed.

Voltage Tolerance (kVolts): Voltage # of Steps (nk): Voltage Step Interval (sec.):

First Read Out Time (ms): Second Read Out Time (ms):

Normalization Target: Normalization Factor Threshold Min: Normalization Factor Threshold Max:



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Yfiler® Plus Instrument Protocol:

Edit Instrument Protocol Yfiler Plus

Setup an Instrument Protocol

Application Type: Capillary Length: cm Polymer:

Dye Set:

Instrument Protocol Properties

* Run Module:

* Protocol Name:

Description:

Oven Temperature (°C): Run Voltage (kVolts): PreRun Voltage (kVolts): Injection Voltage (kVolts):

Run Time (sec.): PreRun Time (sec.): Injection Time (sec.): Data Delay (sec.):

Advanced Options

Following values are not recommended to be changed.

Voltage Tolerance (kVolts): Voltage # of Steps (nk): Voltage Step Interval (sec.):

First Read Out Time (ms): Second Read Out Time (ms):

Normalization Target: Normalization Factor Threshold Min: Normalization Factor Threshold Max:



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- The screenshot below provides the recommended setting in the QC protocols for GlobalFiler® and Yfiler® Plus. These settings may be adjusted if needed.

GlobalFiler® QC Protocol:

The screenshot shows the 'Edit QC Protocol Globalfiler' window with the following settings:

- Protocol Name: Globalfiler
- Description: GS600LIZ (60-460)
- Size Standard: GS600_LIZ_(60-460)
- Sizecaller: SizeCaller v1.1.0

Analysis Settings

- Analysis Range: Full
- Analysis Start Point: 0
- Analysis Stop Point: 1000000
- Sizing Range: Partial
- Sizing Start Size: 60
- Sizing Stop Size: 460
- Size Calling Method: Local Southern

Peak Amplitude Threshold

Color	Threshold
Blue	150
Green	150
Yellow	150
Red	150
Purple	150
Orange	700

Common Settings

- Use Smoothing: Light
- Use Baseline (Baseline Window (Pts)): 33
- Minimum Peak Half Width: 2
- Peak Window Size: 13
- Polynomial Degree: 3
- Slope Threshold Peak Start: 0.0
- Slope Threshold Peak End: 0.0

Buttons: Close, Save



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Yfiler® Plus QC Protocol:

- The following question will be asked within the Assay. “Do you wish to assign multiple instrument protocols to this assay?” Check “No”.



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8.6 Setting up the File Name Convention in the Library

1. The “File Name Convention” must include at least the “Sample Name”. It is recommended to also include the Capillary Number, Well Position, Injection Number and Date of Run. It is recommended to use the default file location: D:\Applied Biosystems\3500\Data for saving files. The screen shot below provides guidance in the setup of the File Name Convention setting for either GlobalFiler® or Yfiler® Plus.



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8.7 Setting up the Results Group in the Library

1. The “Results Group” naming may vary, but the “Results Group Attributes” must at least contain the “Plate Name”. It is recommended to follow the setup shown in the screenshot below for storing folders for either GlobalFiler® or Yfiler® Plus.



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9. Procedure for using GeneMapper ID-X Analysis Software Version 1.4 for Analysis of AB 3500 Data for GlobalFiler® or Yfiler® Plus

SCOPE

This procedure describes the methods by which the individual fragments separated by size using the AB 3500 are genotyped using the Applied Biosystems GeneMapper™ ID-X Software (GMID-X). This procedure describes the steps that are common to using this software.

The following analysis variables used by GMID-X are set when the plate record is established prior to CE on the 3500: Sample name and Run name.

9.1 Starting the GeneMapper ID-X Software

1. Click on the GeneMapper ID-X Icon located on the desktop or select **Start>All Programs>Applied Biosystems>GeneMapper ID-X**.
2. Login using the appropriate username and password.

9.2 Creating a Project

1. Add samples to the project either by going to **Edit>Add Samples to Project** or by selecting Ctrl + K.
2. Select the location of your data runs from the 3500. Highlight plate name and then click **Add to List>**. The plate will then be added to the right hand side. Next, click **ADD** on the bottom right of the screen.
3. Once the samples have been imported, edit and fill down the following columns for either GlobalFiler® or Yfiler® Plus as follows:

GlobalFiler®:

Analysis Method: GlobalFiler Analytical or GlobalFiler Stochastic

Panel: GlobalFiler_Panel_v1

Size Standard: GS600_LIZ (60-460)

Sample Type: Label Allelic Ladder, Positive and Negative Control appropriately.



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Yfiler® Plus:

Analysis Method: Yfiler Plus

Panel: Yfiler_Plus_Panel_v4

Size Standard: GS600_LIZ (60-460)

Sample Type: Label Allelic Ladder, Positive and Negative Control appropriately

The columns may be filled down by highlighting the column name, then clicking **Edit>Fill Down** or Ctrl + D to fill in the information for each sample.

For GlobalFiler®, all questioned samples, blanks and controls shall be analyzed at the analytical threshold. Allelic ladders should be analyzed at the stochastic threshold, but may be analyzed at analytical threshold if necessary. The reference standards may be analyzed at either threshold.

For Yfiler® Plus, all questioned samples, reference standards, controls, and ladders will be analyzed at the analytical threshold.

NOTE: The “Analysis Method” naming may vary, but must use the HID version with the “Advanced” peak detection algorithm.

The following thresholds apply to all dye channels. The orange (LIZ) dye channel is recommended to be analyzed at the stochastic threshold level for GlobalFiler® (700 RFU) and Yfiler® Plus (450 RFU), but may be analyzed at analytical threshold (150 RFU) if necessary. LIZ peaks not meeting stochastic threshold may indicate an injection or instrument issue.

Thresholds for either GlobalFiler® and Yfiler® Plus

	Analytical Threshold	Stochastic Threshold
GlobalFiler®	150 RFU	700 RFU
Yfiler® Plus	150 RFU	450 RFU (DYS385 and DYF387S1)



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The screenshot below provides the appropriate settings for the analytical threshold analysis method for the GlobalFiler® amplification kit:

The screenshot shows the 'Analysis Method Editor' window with the following settings:

- General** | **Allele** | **Peak Detector** | **Peak Quality** | **SQ & GQ Settings**
- Peak Detection Algorithm:** Advanced
- Ranges**
 - Analysis:** Full Range
 - Sizing:** Partial Sizes
 - Start Pt:** 0
 - Start Size:** 60
 - Stop Pt:** 10000
 - Stop Size:** 460
- Smoothing and Baseline**
 - Smoothing:** None, Light, Heavy
 - Baseline Window:** 33 pts
- Size Calling Method**
 - 2nd Order Least Squares
 - 3rd Order Least Squares
 - Cubic Spline Interpolation
 - Local Southern Method
 - Global Southern Method
- Peak Detection**
 - Peak Amplitude Thresholds:**
 - B:** 150
 - R:** 150
 - G:** 150
 - P:** 150
 - Y:** 150
 - O:** 700
 - Min. Peak Half Width:** 2 pts
 - Polynomial Degree:** 3
 - Peak Window Size:** 13 pts
 - Slope Threshold**
 - Peak Start:** 0.0
 - Peak End:** 0.0
 - Normalization:** Use Normalization, if applicable

Buttons: Save As, Save, Cancel, Help, Factory Defaults

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The screenshot below provides the appropriate settings for the stochastic threshold analysis method for GlobalFiler®:

The screenshot shows the "Analysis Method Editor" window with the "Peak Detector" tab selected. The settings are as follows:

- Peak Detection Algorithm:** Advanced
- Ranges:**
 - Analysis: Full Range
 - Sizing: Partial Sizes
 - Start Pt: 0
 - Start Size: 60
 - Stop Pt: 10000
 - Stop Size: 460
- Smoothing and Baseline:**
 - Smoothing: Light
 - Baseline Window: 33 pts
- Size Calling Method:**
 - Local Southern Method
- Peak Detection:**
 - Peak Amplitude Thresholds: B: 700, R: 700, G: 700, P: 700, Y: 700, O: 700
 - Min. Peak Half Width: 2 pts
 - Polynomial Degree: 3
 - Peak Window Size: 13 pts
 - Slope Threshold: Peak Start: 0.0, Peak End: 0.0
- Normalization:**
 - Use Normalization, if applicable

Buttons at the bottom: Save As, Save, Cancel, Help, and a Factory Defaults button.



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The screenshot below provides the appropriate settings for the analytical threshold for Yfiler® Plus Amplification kit:

The screenshot shows the 'Analysis Method Editor' window with the 'Peak Detector' tab selected. The 'Peak Detection Algorithm' is set to 'Advanced'. The 'Ranges' section shows 'Analysis' set to 'Full Range' and 'Sizing' set to 'Partial Sizes'. The 'Start Pt' is 0 and 'Stop Pt' is 10000. The 'Start Size' is 60 and 'Stop Size' is 460. The 'Smoothing and Baseline' section has 'Smoothing' set to 'Light' and 'Baseline Window' set to 33 pts. The 'Size Calling Method' section has 'Local Southern Method' selected. The 'Peak Detection' section has 'Peak Amplitude Thresholds' set to B: 150, R: 150, G: 150, P: 150, Y: 150, and O: 450. The 'Min. Peak Half Width' is 2 pts, 'Polynomial Degree' is 3, and 'Peak Window Size' is 13 pts. The 'Slope Threshold' section has 'Peak Start' and 'Peak End' both set to 0.0. The 'Normalization' section has 'Use Normalization, if applicable' unchecked. A 'Factory Defaults' button is located at the bottom right of the settings area. At the bottom of the window are 'Save As', 'Save', 'Cancel', and 'Help' buttons.

NOTE: A stochastic threshold of 450 RFU's applies only to the multi-copy *DYS385* and *DYF387S1* loci.

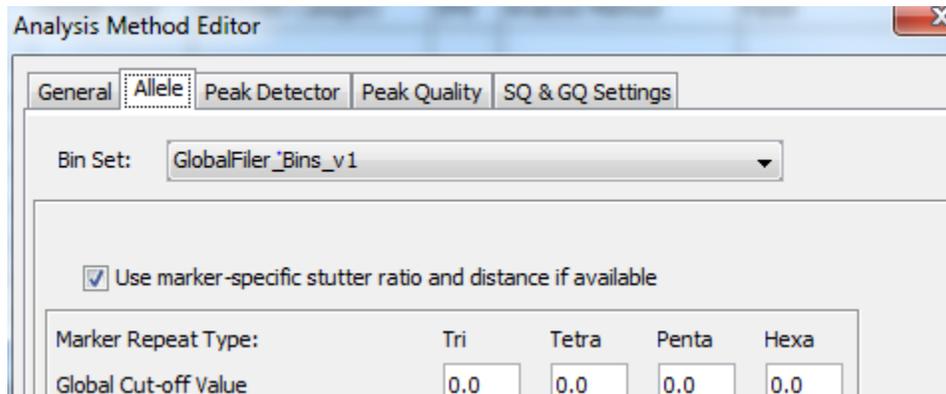


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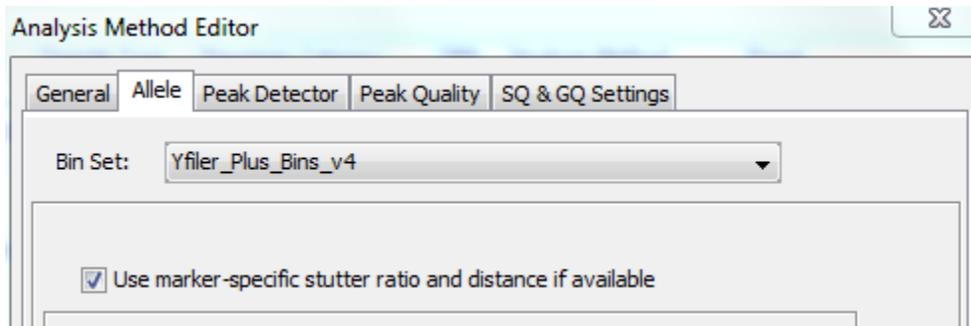
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The marker specific stutter filter for GlobalFiler® will be enabled as shown below.



The marker specific stutter filter for Yfiler® Plus will be enabled as shown below:



4. On the toolbar, press the green arrow key  to analyze the samples. A new window will open requesting the GeneMapper ID-X project to be named and saved. Analysis automatically begins after saving the project.

NOTE: The name given at this step will be displayed on all printouts. The project name should incorporate the analyst's initials, but is not required.

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5. The quality value flags may be check to provide the analyst a view of samples that may have issues. The quality flags are **not** a replacement for analyst interpretation of each sample, ladder, control or standard. The following items are assessed by the quality flags: SQ (Size Quality), SOS (Sample Off-Scale), SSPK (Sample Spike), MIX (Mixed Source), OMR (Outside Marker Range), CGQ (Composite GQ).



Green box-good quality

Yellow Triangle-check quality

Red Stop Sign-low quality

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The following screenshot provides the recommended Peak Quality settings for the GlobalFiler® Amplification kit:

The screenshot shows the "Analysis Method Editor" window with the "Peak Quality" tab selected. The settings are as follows:

Setting	Value
Min/Max Peak Height (LPH/MPH)	
Homozygous min peak height	700.0
Heterozygous min peak height	700.0
Max Peak Height (MPH)	20000.0
Peak Height Ratio (PHR)	
Min peak height ratio	0.6
Broad Peak (BD)	
Max peak width (basepairs)	1.5
Allele Number (AN)	
Max expected alleles:	
For autosomal markers & AMEL	2
For Y markers	1
Allelic Ladder Spike	
Spike Detection	Enable
Cut-off Value	0.2
Sample Spike Detection	
Spike Detection	Disable

Buttons at the bottom: Save As, Save, Cancel, Help, Factory Defaults.



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The following screen shot provides the recommended Peak Quality for the Yfiler® Plus Amplification kit:

Analysis Method Editor

General | Allele | Peak Detector | **Peak Quality** | SQ & GQ Settings

Min/Max Peak Height (LPH/MPH)

Homozygous min peak height: 450.0

Heterozygous min peak height: 450.0

Max Peak Height (MPH): 20000.0

Peak Height Ratio (PHR)

Min peak height ratio: 0.6

Broad Peak (BD)

Max peak width (basepairs): 1.5

Allele Number (AN)

Max expected alleles:

For autosomal markers & AMEL: 2

For Y markers: 1

Allelic Ladder Spike

Spike Detection: Enable

Cut-off Value: 0.2

Sample Spike Detection

Spike Detection: Disable

Factory Defaults

Save As | Save | Cancel | Help

NOTE: The SQ and GQ Settings are recommended to remain at the default settings.



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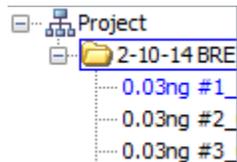
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6. Verify that the peaks for the allelic ladders are labeled with the correct allele designations. To view the ladder, see “Viewing Samples in the Project”.
7. Verify that the positive control is the known profile from the kit and that the negative control/reagent blanks are free of a profile or contamination by viewing the data from the analytical threshold and above to check for any extraneous peaks not attributable to artifact.
NOTE: To view positive and negative control and reagent blanks, see “Viewing Samples in the Project”.

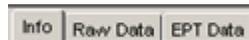
9.3 Viewing Raw Data in the Project

NOTE: Raw data may be viewed at any time before or after analyzing the samples.

1. In the left hand window, double click on the run folder (or click the plus sign next to the folder name) listed under project folder icon to show a list of all the samples in the project.



2. Single click to highlight any sample name from the list to view raw data.
3. With any sample name highlighted, the *Samples/Genotypes* tab in the right hand window will change to the *Info/Raw Data/ EPT Data* tab.



4. From here, one may view the general information, raw data or the run conditions of the selected sample.
5. Use the up and down arrow keys to scroll through the list of samples or select files individually with the mouse.
6. Clicking on the run folder or the project icons will return the project to the *Samples/Genotypes* tab screen.



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9.4 Viewing Samples in the Project

1. From the Samples Tab, to view a sample individually, highlight it and click on the icon with multiple color peaks (🌈 - Display Plots).
2. To zoom in, click above the area (a magnifying glass will appear), click and drag to desired area. To zoom back out, double click when the magnifying glass is present.

9.5 Labeling Artifacts

1. From the Samples Plot Screen, display the sample plot to be edited.
2. Select the peak that requires editing by left clicking the peak. Then, right click.
3. Select the appropriate editing option (i.e. Rename Allele Label).
4. If renaming an allele, click on the **Rename Allele** option. A **Custom Artifact Label** will appear, fill in the appropriate label name, and click ok. Next a **Reason(s) for Change** box will appear. Fill in the appropriate comment and click "ok".

Custom Artifact Label

Custom Artifact Label:

Add to predefined list:

OK Cancel Help



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Reason(s) for Change

Attribute	RUN.Inst1_Plate4_GF_Mix_NP.SAMPLE.GF-MX_1ng_MF-1-0_29c_15s_02_B01_01.hid.ALLELE.17.modified
Old Value	17 [KitT.GlobalFiler_v1.PANEL.GlobalFiler_Panel_v1.MARKER.D3S1358.BASEPAIR.129.56.SAMPLE.GF-MX_1
New Value	pull-up [KitT.GlobalFiler_v1.PANEL.GlobalFiler_Panel_v1.MARKER.D3S1358.BASEPAIR.129.56.SAMPLE.GF-M

Enter the Reason(s) for Change:

Pull-up

OK

5. To undo any changes to an allele, click “Edit” on the toolbar and select “Undo”.

NOTE: Artifacts may also be labeled by hand on the printed electropherogram.

6. To save changes, click on the “Save Project” button () on the toolbar or **File>Save Project**.

9.6 Deleting Projects

1. Once a project has been named, analyzed, and saved, the same project name cannot be used again. To delete a project, click on the **Tools>GeneMapper ID-X Manager** or CTRL + M to open the GeneMapper ID-X Manager screen.
2. Click on the project name to highlight it.
3. Click on the delete button. A GeneMapper Deletion Alert screen will pop up.

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NOTE: Once a project is deleted, the object is removed from the database and the action cannot be undone.

4. Click on the “Yes” button to permanently delete the project.
5. Projects should be removed from the database as needed.

9.7 Printing Samples

1. From the “Samples” tab, highlight all samples to print. Click the  icon.
2. Zoom to the desired region.
3. Click **File>Print**.

NOTE:

GlobalFiler® and Yfiler® Plus: All electropherograms will be printed with the allele calls. The electropherograms must also include peak heights except for the allelic ladders. Peak sizes may also be included at the analyst’s discretion.

For GlobalFiler®, all question samples will be printed at both the stochastic and analytical threshold. The blanks and controls shall be analyzed and printed at the analytical threshold. Allelic ladders and the internal lane size standard (LIZ 600) should be analyzed and printed at the stochastic threshold (700 RFU), but may be analyzed at analytical threshold if necessary. Reference standards may be analyzed and printed at either the stochastic or analytical threshold. Reference standards not providing a complete profile at the stochastic threshold should be printed at the analytical threshold.

For Yfiler® Plus, all question samples, reference samples, blanks, controls and ladders for Yfiler® Plus will be printed at the analytical threshold. The internal lane size standard (LIZ 600) should be analyzed and printed at stochastic threshold (450 RFU), but may be analyzed at analytical threshold if necessary.

Analysts must check that DYS385 and DYF387S1 meet stochastic threshold requirements for interpretation.



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NOTE: The following two screenshots provide the recommended plot settings:

Plot Settings Editor

General Sample Header Genotype Header Sizing Table Labels Display Settings

Show Labels on Samples and Genotypes Plot:

Labels

	Assigned Allele	Custom Allele	Allelic Ladder	Artifact
Label 1:	Allele Call	Original Label	Allele Call	Original Label
Label 2:	Height	Height	NONE	Height
Label 3:	NONE	NONE	NONE	Size
Label 4:	NONE	NONE	NONE	Artifact Label



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Plot Settings Editor

General | Sample Header | Genotype Header | Sizing Table | Labels | Display Settings

When opening the Plot Window:

Use the display settings last used for this plot

Use these display settings:

For both Sample and Genotype plots:

Panes: 6

Labels

No Labels

Horizontal Labels

Vertical Labels

Show

Plot Header

Marker Range

Marker Indicators

Bins

Toolbar

Peak Positions

Bring Ctrls to Top

Bring Ladders to Top

Allele Changes

Off-scale

Axes

Y-Axis: Scale individually

X-Axis: Basepairs

For Sample plot only:

Select Dyes

Blue

Green

Yellow

Red

Purple

Orange

All Dyes

All-Dye Range (bp): *

Start Range: 50.0

End Range: 470.0

Labels

Size Std Labels

Tables

No Table

Sizing Table

Genotypes Table

Label Edit Viewer

Dye Layout

Combine Dyes

Separate Dyes

Overlay All

Custom Colors

For Genotype plot only:

Marker Margin: 5 bp

* Will be overridden if Retain X-axis Zoom Range is enabled on Plots ->Zoom menu

OK Cancel Help

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10. Assessment and Reporting of GlobalFiler® Data

STR fragments are separated according to size by capillary electrophoresis. During this process, each dye-labeled DNA fragment travels through a polymer-filled capillary past a solid state laser. The fluorescent dyes are excited by the laser and emit light at a specific wavelength for each dye. It is collected onto a charge-coupled device (CCD) camera, so all of the fluorescent emissions can be detected simultaneously. The spectra of these dyes and signal are displayed as peaks. The peak height is a measure of the amount of fluorescence detected and quantity of DNA present. The electropherogram is a graphical display of colored peaks measured by height in relative fluorescence units (RFU) and time. The size of each fragment is determined by GeneMapper ID-X program that compares fragment size to the internal size standard GS LIZ 600 v.2.0 using the Local Southern method of analysis. GS LIZ 600 v.2.0 size standard is displayed as orange peaks. The software is also used to assign allelic designations from the sized data by comparison to the allelic ladders. The 3500 instrument uses a solid-state laser and data collection algorithms, resulting in an optimal dynamic range of 3000 to 12,000 RFU. The data collected from an AB 3500 will be analyzed with GeneMapper ID-X v1.4

The GlobalFiler® PCR Amplification kit is a 6-dye, short tandem repeat (STR) multiplex assay that amplifies 21 autosomal STR loci, one Y-STR (DYS391), one insertion/deletion polymorphic marker on the Y chromosome (Y-indel) and the sex determining marker, Amelogenin. The combination of a 6-dye fluorescent system and the inclusion of non-nucleotide linkers allow for simultaneous amplification and efficient separation of all 24 markers during automated DNA fragment analysis.

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Table 1 below shows the loci amplified, their corresponding fluorescent marker dyes, the GlobalFiler® Allelic Ladder used to genotype the samples and the genotype of GlobalFiler® Control DNA 007.

Table 1:

Locus	Alleles included in Ladder	Dye Label	DNA Control 007
D3S1358	9-20	6-FAM™	15, 16
vWA	11-24	6-FAM™	14, 16
D16S539	5, 8-15	6-FAM™	9, 10
CSF1PO	6-15	6-FAM™	11, 12
TPOX	5-15	6-FAM™	8, 8
Y indel	1, 2	VIC®	2
Amelogenin	X, Y	VIC®	X, Y
D8S1179	5-19	VIC®	12, 13
D21S11	24, 24.2, 25-28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36-38	VIC®	28, 31
D18S51	7, 9, 10, 10.2, 11-13, 13.2, 14, 14.2, 15-27	VIC®	12, 15
DYS391	7-13	VIC®	11
D2S441	8-11, 11.3, 12-17	NED™	14, 15
D19S433	6-12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2, 18.2, 19.2	NED™	14, 15
TH01	4-9, 9.3, 10, 11, 13.3	NED™	7, 9.3
FGA	13-26, 26.2, 27-30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2	NED™	24, 26
D22S1045	8-19	TAZ™	11, 16
D5S818	7-18	TAZ™	11, 11
D13S317	5-16	TAZ™	11, 11
D7S820	6-15	TAZ™	7, 12
SE33	4.2, 6.3, 8, 9, 11-20, 20.2, 21, 21.2, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 31.2, 32.2, 33.2, 34.2, 35, 35.2, 36, 37	TAZ™	17, 25.2
D10S1248	8-19	SID™	12, 15
D1S1656	9-14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18.3, 19.3, 20.3	SID™	13, 16
D12S391	14-19, 19.3, 20-27	SID™	18, 19
D2S1338	11-28	SID™	20, 23



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10.1 Sizing Standard

The internal sizing standard will be examined for each sample and control to ensure that all peaks are sized correctly [within the calling range of 60-460 base pairs (BP)].

NOTE: *GeneMapper has an internal regulator that will flag a sample with a red stop sign if there is sizing quality issue. A red stop sign indicates a failure in the internal size standard. If a yellow triangle is displayed the sample requires further interpretation. A green box indicates that the sizing quality passes. The quality flags are used as an aid to the analyst, but interpretation and assessment of the data will be performed by the analyst through examination of the raw data and/or electropherograms.*

10.2 Allelic Ladder

The allelic ladder(s) used for genotyping will be examined to determine that GeneMapper ID-X has assigned all allele designations correctly. When interpreting GlobalFiler® results, genotypes are assigned to sample alleles by comparison of their sizes to those obtained from the known alleles in the allelic ladders. Genotypes, not sizes, are used for comparison of the data between runs, instruments and laboratories.

NOTE: *GeneMapper ID-X has Sample Type menu where “Allelic Ladder” can be selected. At least one ladder must be identified before the plate can be analyzed. If multiple ladders are used for analysis, all ladders shall be printed and placed in the case file for technical review.*

10.3 Reagent Blanks and Negative Controls:

The reagent blanks and negative controls are a test for the presence of contamination occurring during extraction and amplification set-up, respectively. Both should only consist of the GS LIZ 600 v.2.0 peaks. If a negative control is determined to have non-artifact peaks at or above the analytical threshold in the 60 bp to 460 bp range, the samples amplified at the same times as the negative control must be re-amplified using the same amounts of DNA template and TE buffer previously amplified if sample size permits. If a reagent blank is determined to have non-artifact peaks at or above the analytical threshold in the 60 bp to 460 bp range, the reagent blank must be re-amplified with a negative



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and positive control. If a reagent blank contains any peaks that cannot be attributed to artifacts after re-amplification, the samples extracted at the same time as the reagent blank must be re-extracted if sample size permits.

NOTE: GeneMapper has Sample Type menu where "Negative Control" can be selected. The Reagent Blank and Negative control should be selected prior to being analyzed.

10.4 Casework Sample

A casework sample determined to have contamination must be re-amplified if sample size permits. If the contamination is still present after re-amplification, then the sample must be re-extracted; sample size permitting. In instances where sample size does not permit re-extraction or re-amplification of potentially contaminated samples, the sample data may possibly be interpreted with caution under the direction of the DNA Technical Leader. This will be documented and retained in the case file.

10.5 Positive Control:

The positive control provided in the kit must be typed correctly at all loci (see Table 1 at the beginning of this chapter for positive control types) and not contain any peaks that are not attributed to an artifact. If the positive control does not type correctly, all samples amplified at the same time as the positive control must be re-amplified.

10.6 GlobalFiler® Thresholds:

The analytical threshold (AT) is set at 150 RFU for all dye channels on a 3500.

The stochastic threshold (ST) is set at 700 RFU for all dye channels on a 3500.

Analytical threshold:

The analytical threshold is the minimum peak height requirement at and above which detected peaks can be reliably distinguished from background noise. Peaks at or above the analytical threshold are generally considered true alleles or artifacts. Peaks at or above the analytical threshold may be examined for



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comparison purposes. Peak at or above the analytical threshold may be examined to assist in the determination of the minimum number of contributors to a sample. The presence of male DNA may be established based on the presence of an allele at or above the analytical threshold for amelogenin, DYS391 or the Y-indel.

Stochastic threshold:

The stochastic threshold is the peak height value below which it is reasonable to assume that, at a given locus, allelic dropout of a sister allele in a heterozygous pair may have occurred. The stochastic threshold for GlobalFiler® is set at 700 RFU; however, caution should still be exercised when determining true homozygosity for an allele near the stochastic threshold (i.e. 700-1000 RFU) in low level profiles amplified with less than 100 pg of DNA. Peaks above 20,000 RFU on the 3500 should be interpreted with caution. Each sample will be examined for any extraneous peaks.

For known reference standards, data between the analytical and stochastic thresholds may be used for both inclusionary and exclusionary comparisons. When comparing a questioned sample to a known reference standard, loci with alleles falling between the analytical and stochastic thresholds in the known reference standard should be heterozygous before an inclusion can be declared and statistics generated for the questioned samples. Homozygous loci falling between the analytical and stochastic thresholds in the known reference standard should only be used for exclusionary purposes.

NOTE: Statistics are calculated for the questioned sample and not the known reference standard.

10.7 Artifacts

1. Each sample will be examined for fluorescent pull-up and complex pull-up peaks. These are peaks present at a given base pair size in one or more dyes which echo the presence of a relatively large peak at the same base pair size in another one of the dyes. This may result in the “pull-up” peak being given an allelic designation. Electropherograms exhibiting pull-up may be used for interpretative purposes or the sample may be re-injected with less DNA.
2. Spikes may be present due to the capillary electrophoresis process. Generally, spikes are peaks with large peak heights, present in two or



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more colors and have the same base pair number. Electropherograms exhibiting spikes may be used for interpretative purposes or the sample may be re-injected.

3. Split peaks result from incomplete A-nucleotide addition in the final extension step. Electropherograms exhibiting split peaks may be used for interpretative purposes.
4. Shoulder is an elongated or raised baseline preceding or following a true allele.
5. Dye artifacts are slightly broadened peaks that are the result of dye-related materials such as free dye.
6. TPOX artifact may occur after plate set-up for the genetic analyzer. The artifact usually requires at least 24 hours after plate set-up before being observed. The manufacture of GeneScan 600 LIZ v.2.0 Size Standard contains a by-product that has been found to have a complimentary sequence with the TPOX locus. The binding to the TPOX fragment causes a shift in electrophoretic mobility. Over time the parent allele height decreases and the artifact height increases. Electropherograms exhibiting a TPOX artifact may be used for interpretative purposes or a fresh plate may be prepared and re-injected.
7. Stutter is the result of slippage of the Taq Polymerase. Stutter is a minor peak usually four base pairs ($N - 4$) shorter than the allele peak (N). The trinucleotide repeat unit in the D22S1045 locus demonstrates n-3 stutter. SE33 and D1S1656 exhibit an n-2 stutter pattern in addition to the n-4 stutter due to complex repeat motifs. The proportion of stutter product relative to the main allele peak is calculated by dividing the height of the stutter peak by the height of the main allele peak. Peaks in these positions must be evaluated as to whether they represent stutter peaks or true alleles. For purposes of mixture interpretation, peaks in a stutter position that exceed the expected percent stutter at a particular locus may be designated as a true allele. However, if a peak in a stutter position falls below the expected percent stutter, it should be considered as a possible true allele if the peak height is consistent with or greater than the height of peaks in non-stutter positions for the minor contributor(s). The following values should be used as a guide for expected levels of stutter in the GlobalFiler® kit:

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Locus	Stutter	% Stutter
D3S1358	n-4	10.98
vWA	n-4	10.73
D16S539	n-4	9.48
CSF1PO	n-4	8.77
TPOX	n-4	5.55
D8S1179	n-4	9.60
D21S11	n-4	10.45
D18S51	n-4	12.42
DYS391	n-4	7.43
D2S441	n-4	8.10
D19S433	n-4	9.97
TH01	n-4	4.45
FGA	n-4	11.55
D22S1045	n-3 / n+3	16.26 / 6.69
D5S818	n-4	9.16
D13S317	n-4	9.19
D7S820	n-4	8.32
SE33	n-4 / n-2	14.49 / 3.97
D10S1248	n-4	11.46
D1S1656	n-4 / n-2	12.21 / 2.45
D12S391	n-4	13.66
D2S1338	n-4	11.73

Peaks in the stutter position greater than the listed values may indicate the presence of DNA from more than one individual. However, elevated stutter is also associated with both excess template DNA and very low amounts of template DNA. Elevated stutter is generally within 3% of the stutter filter values.

Except for the n+3 stutter at locus D22S1045, plus / forward stutter products are not as common as minus stutter products. These artifacts are generally less than 3% of the true allele.

8. Off-Scale Data:

Samples with off scale data may be diluted, re-amplified with less template DNA and re-injected. Off-Scale data should not be used for quantitative assessments such as determination of stutter artifacts.

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9. Off-Ladders (OL)

Off-Ladder alleles falling within a ladder range will be designated as “OL” on the data sheets. Off-Ladder alleles falling above the largest or below the smallest allele may be designated as either greater than (>) or less than (<) the respective ladder allele and may be used for interpretative purposes and statistical calculations.

Peaks that result from artifacts that are given allelic designations (including “OL” alleles) must be interpreted with caution.

10. Secondary structure within the DNA as well as amplification with non-human DNA may lead to artifacts. The table below list artifacts that have been observed by the manufacturer with the GlobalFiler® kit.

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Artifact Location	Base Pair Size Where Found	Designation & Cause
D3S1358	~132	Calls as "OL" (Not observed in negative controls) Cause: Non-STR amplification by-product
vWA	~204	Calls as "OL" (Not observed in positive or negative controls) Cause: Non-human DNA: Yeast & fungi (in plants, soil, & organic matter)
TPOX	~325	N-24 from true peak Cause: Secondary structure
Y-indel	~84	May call as an "OL" Cause: Non-STR amplification by-product
<i>between</i> Amelogenin & Y-indel	~94.5	Outside BIN "OMR" (Proportional to X peak height) Cause: Secondary structure
D8S1179	~114-121	May call as an "OL" Cause: Dye derivative
D21S11	~207	Calls as "29.3" or "OL" (Not observed in positive or negative controls) Cause: Non-human DNA: Yeast & fungi (in plants, soil, & organic matter)
<i>before</i> D2S441	~63	Cause: Primer dimer
D2S441	~92-100	Cause: Dye derivative
D2S441	~N-2 to N-2.5	Cause: N-2 stutter or secondary structure
TH01	~178-181	N-12 from true peak Cause: Secondary structure
<i>between</i> D22S1045 & D5S818	~131	Cause: Unknown



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D5S818	~140-142	Calls as "OL" Cause: Fecal or gastrointestinal bacteria
D5S818	~152-154	Calls as "OL" Cause: Secondary structure (associated with true allele "11", generally less than 1% of the 11)
D5S818	~180	Calls as "OL" (Not observed in negative controls) Cause: Non-STR amplification by-product
D13S317 & D7S820	~249-250	Outside BIN "OMR" Cause: Unknown
<i>between</i> D13S317 & SE33	~N-90	N-90 peak before parent SE33 peak Cause: SNP found in certain populations
SE33	~306	Cause: Unknown
SE33	~352	Cause: Unknown
D2S1338	~345	Cause: Unknown

11. Designating Artifacts:

Analysts should try to designate the type of artifact on the electropherogram; however, ambiguous artifacts that are deemed not to be true alleles may be labeled "artifact" or "ART" on the electropherogram.

10.8 Interpretation and Reporting of GlobalFiler® Sample Data

1. Single Source sample:

A sample may be considered to be from a single source when the observed number of alleles at each locus and the peak height ratios of alleles at a locus are consistent with a profile from a single contributor. All loci should be evaluated in making this determination. Heterozygote peak height ratios are determined by dividing the peak height of the allele with the lowest RFU value by the peak height of the

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allele with the highest RFU value and multiplying this value by 100 to obtain a percentage. Generally, if the peak height ratio of the two alleles is 60% or more, it may be considered a heterozygous pair. In a single source sample, one would expect to find no other locus in the profile exhibiting more than two alleles with the exception of a possible tri-allele.

2. Mixture sample:

A sample may be considered to have originated from two or more sources if more than two alleles are present at two or more loci (discounting three peak patterns) and/or peak height ratios for heterozygotes fall outside the expected values when all loci are considered. Generally, if the peak height ratio of the smaller allele is less than 60% of the major peak, the allele may represent a mixture from separate sources. Such a mixture may be confirmed by at least one locus with three or more alleles present in the profile.

3. Deduced Profiles and Assumed Contributors:

In some cases, when one of the contributors to a mixture profile (e.g. the victim) is known, the genetic profile of the unknown contributor may be deduced by subtracting the contribution of an assumed donor to the mixed profile. A deduced profile is defined as one needing a known DNA profile to deduce or infer the probative profile from a mixture that does not exhibit an obvious major and minor contributor. The inclusion of an assumed contributor can be reported without providing statistical weight; however, the report should clearly state when a contributor is being assumed. An assumed contributor to a profile should be limited to situations in which the presence of an individual's DNA is reasonably expected on an item such as intimate swabs (including sticks, nail clippers, and other collection devices), nail clippings, and clothing/items taken directly from an individual's body. A consensual partner profile may also be assumed on items intimate to a victim (i.e. intimate swabs, nail clippings, clothing, and items taken directly from the victim). The standard should be clearly identifiable as an elimination or consensual partner standard based on the information provided by the submitting agency. Assumption(s) regarding elimination or consensual partner standards should be clearly reported. A deduced profile can be used for comparison, interpretation and statistics. The profile should be reported as a deduced profile along with any key assumptions or interpretations.

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4. Composite Profile:

Typing results from multiple amplifications and injections of the same extract may be combined into a composite profile for determining inclusions/exclusions as well as for statistical calculations. Generally, amplification of separate extractions from different locations on a piece of evidence should not be combined into a composite profile unless there is a reasonable expectation of the sample(s) originating from a common source. Composite profiles should be limited to single source profiles.

10.9 Interpretation Procedure:

The following procedure is a general guideline for the interpretation of DNA profiles generated with the GlobalFiler® amplification kit. The procedure provides guidance based on validation studies, but the procedure may not be applicable for every profile encountered in casework.

Step 1: Determine if the profile is a mixture or single source.

Does the profile have more than two alleles at two or more loci?

Yes: Report as a mixture, unless a tri-allele pattern is determined.

NOTE: If only one locus has three alleles, this may be reported as an additional allele to account for a possible tri-allele.

No: Inspect the peak height ratios of the profile. Are the peak height ratios for heterozygous pairs generally 60% or higher for alleles above stochastic threshold?

NOTE: Peaks below stochastic threshold should not be used for determining peak height ratios.

Yes: Report as single source if peak height ratios are approximately 60% or higher for heterozygous pairs above stochastic threshold.

NOTE: For profiles interpreted to be single source, alleles below the stochastic threshold may be used for statistics if at least four loci have all alleles above the stochastic threshold. Single alleles below the stochastic threshold will be treated as obligates rather than homozygotes for statistical purposes.

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If there are less than four loci having all alleles above the stochastic threshold, no determinations regarding the number of contributors should be made unless the profile can reasonably be considered a single source profile. Profiles with all peaks below stochastic threshold should only be used for exclusions, except for the inclusion of an assumed contributor. Analysts should exercise caution before considering a profile with all alleles below stochastic threshold as originating from a single source.

Proceed to Step 6.

No: Report as a mixture after reviewing the entire profile. Proceed to Step 2.

Step 2: Determine the number of contributors.

Criteria:

Use the locus in the profile with the maximum number of alleles to determine the number of contributors e.g. maximum three or four alleles at any locus indicates two contributors or at least two contributors. Peak heights and peak height ratios may also be considered in determining the number of contributors. Profiles with two or more contributors may be reported as "at least two individuals". Profiles with three or more contributors will generally be reported as "at least three individuals". An unresolved mixture may be reported as being consistent with a certain number of contributors if there are no drop out concerns and the peak height ratios support a certain number of contributors. A resolvable profile may also be reported as being consistent with a certain number of contributors at the analyst discretion e.g. a sperm fraction profile containing a major male profile above stochastic threshold and alleles from an assumed contributor below stochastic threshold can be reported as a mixture consistent with two individuals. A resolvable profile is defined as a mixture profile that can be deconvoluted into a deduced profile based on an assumed contributor(s) or can be deconvoluted into major/minor components. If interpretation of a mixture is based on an assumed number of contributors, it will be reported as being 'consistent with' that number, and all further interpretations will be conditioned on this assumption. An assumed number of contributors allows for the use of obligate alleles



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when the true genotype or allele zygosity is in question. An obligate allele is defined as an allele in a mixture not attributed to a known donor or a major contributor and therefore must have originated from the unknown contributor or a minor contributor. Obligate alleles below stochastic threshold are not subject to peak height ratio expectations. Proceed to Step 3 or 4 for more information on assumed contributors and major/minor components.

Step 3: Can the profile be resolved into a major and minor profile?

Criteria:

A profile may be considered to consist of a mixture of major and minor contributors if there is a distinct contrast in signal intensities among the alleles. The difference is evaluated on a case-by-case basis and all loci should be evaluated in making this determination. The number of potential contributors of DNA and intra-locus peak height ratios should be considered when making this determination. For mixtures consistent with two or more contributors, the highest minor peak should generally be less than 40% of the lowest major peak and the major peak(s) should account for approximately 70% of the total RFU's of a locus. Heterozygous pairs should have a peak height ratio of approximately 60% or higher.

Example: a locus has four alleles 12 (4000 RFU), 13 (3200 RFU), 14 (1200 RFU), 15 (1000 RFU).

The highest minor allele divided by the lowest major allele should be less than 40% i.e. $14 (1200 \text{ RFU}) / 13 (3200 \text{ RFU}) \times 100 = 37.5\%$.

The peak height ratio for a locus should generally be within 60% for the resolved genotype i.e. $13 (3200 \text{ RFU}) / 12 (4000 \text{ RFU}) \times 100 = 80\%$.

The major component for the locus should also account for approximately 70% of the total RFU's of the locus i.e. $12 (4000 \text{ RFU}) + 13 (3200 \text{ RFU}) / 12 (4000 \text{ RFU}) + 13 (3200 \text{ RFU}) + 14 (1200 \text{ RFU}) + 15 (1000 \text{ RFU}) \times 100 = 76.5\%$.

Before considering a major mixture from a mixture profile of at least three individuals, the highest minor allele should be less than 20% of the lowest major allele and the major mixture should account for approximately 70%



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of the total RFU's of a locus. A resolved major mixture should not consist of three or more individuals.

Caution should be exercised when determining major and minor contributors based on peak height ratios for alleles falling between 700 and 1400 RFU. Alleles in this range, while not likely to exhibit dropout, are subject to increased stochastic effects resulting in increased peak height imbalances.

The major or minor contributor should be resolvable at approximately half of the loci detected and should be resolvable at no less than 4 loci if performing statistical calculation on the individual components. Major or minor contributor profiles resolvable at a limited number of loci, i.e. 4 to 6 loci, will generally be restricted to degraded profiles exhibiting preferential amplification at the smaller loci. It may not be possible to determine a major or minor contributor at all loci for some two-person mixtures (e.g. a degraded profile). In these cases, it is acceptable to proceed to Step 4 and deduce more of the profile if an assumed contributor is applicable.

Yes: Resolve the major contributor and proceed to Step 6. The major profile should be indicated on the allele call sheet or the appropriate electropherogram. Resolve the minor contributor if possible and proceed to Step 6. The resolved minor profile to be used for inclusionary comparisons should be indicated on the allele call sheet or the appropriate electropherogram. If the minor contributor cannot be resolved, proceed to Step 4.

Note: If minor alleles below the stochastic threshold are used for inclusions and statistics, the minor profile should have at least four loci with an obligate minor allele(s) above the stochastic threshold. The four loci above stochastic threshold refer to the minor profile, not the major contributor profile. For mixture profiles, use of minor alleles below the stochastic threshold for inclusions and statistics should be limited to two person mixtures and should be reported as a mixture of two individuals e.g. "The DNA profile is consistent with a mixture of two individuals."

No: Proceed to Step 4.



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Step 4: Can any assumptions be made regarding known contributors to the mixture profile based on the item tested e.g. swabs taken from an individual's body?

Criteria:

Some mixtures contain DNA from two individuals in proportions that cannot readily be separated into major and minor contributors due to the overall balance between the contributors. In these cases, it may be possible to use a known (assumed) contributor profile to deduce the profile of an unknown contributor. The ratio of DNA between the assumed and unknown contributor should be approximated by examining loci with four alleles above stochastic threshold or loci with three alleles above threshold where the assumed contributor is homozygous. If the ratio of contributors varies significantly across the profile, use caution when relying on the ratio to determine allele sharing. Degraded, inhibited, and low level profiles can exhibit a high variation in the estimated ratio of contributors. SE33 is not recommended for use in determining the ratio of contributors.

Peak height ratios may be used to compare different genotype possibilities. After subtraction of an assumed profile, the unknown profile peak height ratio should be approximately 60% or higher. Caution should be exercised when deducing a profile based on peak height ratios for alleles falling between 700 and 1400 RFU. Alleles in this range, while not likely to exhibit dropout, are subject to increased stochastic effects resulting in increased peak height imbalance.

Analysts can use the expected ratio between the known and unknown contributors to aid in determining if alleles are being shared. A ratio approaching 1:3 for the unknown to known/assumed contributors makes it more difficult to distinguish if peak height imbalance is normal variation within a heterozygous pair or is due to allele sharing.

Yes: Can a profile be deduced by subtracting out the assumed contributor's contribution to the mixture profile?

Yes: If a major/minor profile cannot be deconvoluted (See Step 3), report the assumed contributor and determine the deduced profile. If alleles below the stochastic threshold are used for inclusions and statistics, the deduced profile should have at least four loci with the



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obligate allele(s) above the stochastic threshold. The four loci above stochastic threshold refer to the deduced profile, not the assumed contributor profile. The deduced profile should be indicated on the allele call sheet or appropriate electropherogram. Refer to section 10.10 of this manual for further guidance in deducing a profile from a mixture with an assumed contributor. Proceed to Step 6.

NOTE: Using alleles below the stochastic threshold for statistics requires an interpretation of the number of contributors. This should be limited to two person resolvable mixtures i.e. an assumed contributor and a deduced single source profile or a major contributor and a single source minor contributor. The number of contributors and assumed contributors will be communicated in the report e.g. "The DNA profile is consistent with a mixture of two individuals. Assuming exhibit 002-a (Jane Smith) is a contributor to this mixture profile, the deduced profile is from an unknown male (male 1)."

No: Proceed to Step 5.

Step 5: Does the unresolved mixture meet any of the following criteria?

- A. The unresolved mixture is consistent with at least two individuals and has ten or more loci with alleles below stochastic threshold and the target quantity was approximately 0.1 ng or less. A target quantity of 0.1 ng would correlate to a Quantifiler® Trio value of approximately 0.007 ng/μL or less.
- B. The unresolved mixture is consistent with at least three individuals and has ten or more loci with alleles below stochastic threshold and the target quantity was approximately 0.25 ng or less. A target quantity of 0.25 ng would correlate to a Quantifiler® Trio value of approximately 0.016 ng/μL or less.
- C. The unresolved mixture is from at least four individuals.
- D. The unresolved minor contributor has ten or more loci below stochastic threshold and is consistent with being from at least two minor contributors.



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NOTE: Analysts should consider if filtered peaks in stutter position may be true alleles. Peaks in stutter positions with peak heights similar to true alleles in non-stutter positions may be potential alleles. This consideration should be given when considering the possibility of drop out or additional alleles for interpretation at a given locus.

Yes: The profile will generally be considered inconclusive for inclusionary purposes; however, the profile may be acceptable for exclusionary purposes. Caution should be exercised if considering a low level / complex mixture profile acceptable for inclusionary or exclusionary comparison due to stochastic effects and high levels of drop out associated with these types of profiles. When possible, the report should reflect the value of the profile, e.g. the profile is either inconclusive or is of value for exclusionary purposes only.

No: Proceed to Step 6.

Step 6: Can an individual be included or excluded as a contributor to the profile?

Single Source Profiles (includes single source major, minor and deduced profiles).

- A. Exclusion should be reported when the reference sample alleles are not observed in the question sample profile and no scientific explanation can be given for their absence (i.e. drop out, degradation, inhibition, etc.)
- B. Inclusion may be reported when the reference sample alleles are also observed in the question sample profile and no scientific explanation can be given for their absence (i.e. drop out, degradation, inhibition, etc.). Except for an assumed contributor, statistical weight must be provided for inclusions. Inclusions based on assumptions should be clearly defined in the report.
- C. Inconclusive may be declared when an individual can neither be included nor excluded from the profile.

Mixture Profiles

NOTE:

Before making comparisons to known standards, the analyst will interpret and determine the value of the unknown profile. The analyst



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should consider the target quantity and potential for allele stacking when determining which loci should be used for inclusionary statistics. Validation studies have shown that as the target quantity nears 0.25 ng or less, the potential for allele dropout increases.

- D. Exclusion should be reported when reference sample alleles are missing from the question sample profile and no scientific explanation can be given for their absence (i.e. drop out, degradation, inhibition, etc.) Determinations should be made based on loci not demonstrating allele drop out when considering missing alleles as the basis for exclusion. While allele sharing will generally not provide a balanced peak height ratio, the analyst should consider whether genotype pairings are reasonable.
- E. Inclusion may be reported when the reference sample alleles are also observed in the question sample profile and there is a scientific explanation for any inconsistency (i.e. drop out, degradation sample, inhibition, etc.) While allele sharing will generally not provide a balance peak height ratio, the analyst should consider whether genotype pairings are reasonable. Except for an assumed contributor, statistical weight must be provided for inclusions. Loci suitable for mixture statistics should be documented on the electropherogram or on a worksheet prior to comparison to probative standards. Inclusions based on assumptions should be clearly defined in the report.
- F. Inconclusive may be declared when an individual can neither be included nor excluded from the mixture profile.

NOTE 1: Questioned samples must be interpreted prior to comparison to a known standard with the exception of an assumed contributor. Alleles suitable for comparison and, if applicable, statistics must be determined prior to comparison to a probative known standard. A worksheet may be used to document the interpretation procedure or calculations for mixture profiles. This sheet should be placed in front of or behind the electropherogram being interpreted. Documentation of alleles suitable for comparison and, if applicable, statistics may be documented on the electropherogram, mixture worksheet, and/or allele call sheet. Single source profiles will generally be documented on the allele call sheet only.

NOTE 2: For loci utilizing an obligate allele for CODIS and/or statistics, inclusions and exclusions should be made based on reasonable genotype



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pairings when drop out is not expected (i.e. the obligate allele is above stochastic threshold).

NOTE 3: Interpretation of unknown profiles is performed prior to comparison to probative known standards. Occasionally, unexpected typing results due to genetic or amplification anomalies may occur (e.g. unexpected peak height imbalances, artifacts, drop out, etc.). This may result in a non-concordance at a locus during comparison to a reference profile that may not result in an overall exclusion if there is an explanation for the occurrence. If this occurs, the non-concordance should be noted in the case file and the locus left out of statistical calculations.

10.10 Guidelines for Deducing Profiles

The guidelines below apply when the ratio of the unknown and known contributors is approximately equal across the profile (e.g. 1:1 – 1:2) or if the known (assumed) contributor is the lesser contributor. As the ratio of contributors approaches 1:3 or 3:1, the profile may be more suitable for major/minor deductions.

NOTE: Heterozygous pairs between 700 and 1400 RFU are susceptible to increased peak height ratio (PHR) imbalance. Use caution when relying on PHR's of a known contributor below 1400 RFU when determining allele sharing between the known and unknown contributors.

Peak patterns:

A. 1- peak pattern with the known contributor being homozygous:

1. The unknown contributor may be sharing the known allele and may be designated as homozygous if the allele is above stochastic threshold and drop out of an unknown allele is unlikely at the locus in question based on the assessment of drop out for the entire profile.
2. If drop out of an unknown allele is possible, the unknown contributor should not be determined to share with the known allele or be homozygous. The locus will be designated as not determined (ND).

B. 2-peak pattern with the known contributor being homozygous:



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1. The unknown contributor may be designated as homozygous for the unknown allele if the following criteria are met:
 - The unknown allele is above stochastic threshold and approximately equal to or higher than the known allele and/or the ratio between the two alleles is consistent with the overall ratio between the unknown and known. For example, if the ratio for the unknown and known is approximately 1:1 at loci with four peaks, the ratio between the homozygous known allele and the homozygous unknown allele should be approximately 1:1.
2. If the ratio between the alleles does not support a homozygous unknown contributor, the unknown contributor may be heterozygous. In this case, it may be determined that the unknown contributor is sharing with the known contributor's allele if the following criteria are met:
 - The unknown allele is above stochastic threshold.
 - The known allele should be taller than the unknown allele with the known contributor being an approximately equal or lesser contributor to the profile.
 - The ratio of the unknown contributor to the known contributor should be consistent with the overall ratio in the profile before designating the unknown contributor as heterozygous.

Example: A locus has the following peaks 15 (7500 RFU) and 17 (2500). The unknown to known ratio based on four peak patterns across the profile is approximately 1:1 and the known contributor genotype is 15,15. If the unknown contributor is designated as a homozygous 17,17, the ratio for the unknown to known is 1:3; however, if the unknown contributor is designated a 15,17, the ratio of the unknown to known is 1:1 and is in agreement with the overall profile ratio of 1:1.

If the ratio between the alleles is ambiguous a heterozygous or homozygous designation may not be possible to determine. In this case, the unknown allele should be designated as an obligate allele e.g. 17, P or 17P. The P designates the allele as an obligate allele with an unknown sister allele. While the P designation allows the obligate to be paired with any other allele for statistical purposes, the analyst should attempt to limit the acceptable genotypes available for inclusionary/exclusionary



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comparisons when possible. Narrowing down the genotypes allowed for inclusionary/exclusionary comparisons should be reserved for loci where drop out of alleles is unlikely.

3. If the unknown allele is below stochastic threshold, the allele should be designated as an obligate allele. Due to the possibility of drop out, single obligate alleles below stochastic threshold generally cannot be used to further limit the genotypes available for inclusionary/exclusionary comparisons.

C. 2-peak pattern with the known contributor being heterozygous:

1. The unknown contributor may be determined to be heterozygous by sharing both known alleles if the following criteria are met:
 - Both alleles should be above stochastic threshold with drop out of an unknown contributor allele unlikely at the locus in question.
 - The PHR between the alleles should be $\geq 60\%$ and the known contributor should be approximately an equal or lesser contributor to the profile.

Note: If the known contributor is a significantly higher contributor to the profile than the unknown contributor is, it may not be possible to determine the exact genotype of the unknown contributor based on the PHR. If no dropout is expected, the possible genotypes available for comparison may still be determined even though no statistics may be performed at the locus.

2. The unknown contributor may be designated as homozygous for the taller allele if the following criteria are met:
 - The PHR is $< 60\%$ for the known contributor alleles with both alleles being above stochastic threshold.
 - Drop out of an unknown contributor allele is unlikely at the locus in question.

Note: Caution should be used when relying on a PHR for alleles between 700 - 1400 RFU since alleles in this range are more susceptible to peak height imbalances.

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3. No determination for the unknown contributor may be possible if the unknown contributor is a lesser contributor to the profile or drop out is expected.

D. 3-peak pattern with the known contributor being homozygous:

1. The two unknown alleles can be assumed as a heterozygous pair for the unknown contributor. This peak pattern can be used to help determine the overall ratio between the unknown and known contributors. Occasionally, heterozygous alleles can be susceptible to extreme peak height imbalance due to a low level or degraded profile. Analysts may decide not to determine (ND) an “assumed” heterozygous unknown due to an extreme peak height imbalance or an indistinguishable artifact. If more than a few loci fall into this category, the analyst may need to reconsider if the profile is only a two-person mixture.

E. 3-peak pattern with the known contributor being heterozygous:

1. The unknown contributor may be designated as homozygous if the following criteria are met.
 - The unknown allele is above stochastic threshold and approximately equal to or higher than the known alleles.
 - The known contributor is approximately an equal or lesser contributor to the profile.
 - The PHR between the known alleles should be $\geq 60\%$.
 - The ratio between the known alleles and the unknown allele should be consistent with the overall ratio of the unknown and known contributors. For example, if the unknown to known ratio is approximately 1:1 at loci with four peaks, the ratio between the heterozygous known alleles and the homozygous unknown allele should be approximately 1:1.
2. If the PHR of the known contributor alleles is $< 60\%$ and the ratio between the unknown and known alleles does not support a homozygous unknown contributor, the unknown contributor may be heterozygous and sharing one of the known alleles. The unknown contributor may be sharing with one of the known contributor's alleles if the following criteria are met:

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- The unknown allele should be above stochastic threshold before determining if allele sharing with the known alleles is possible.
 - The known contributor should be approximately an equal or lesser contributor to the profile.
 - The ratio of the unknown and known contributors should be consistent with the overall ratio in the profile before designating the unknown contributor as heterozygous.
3. When the PHR of the known alleles is $\geq 60\%$, but the ratio of contributors is ambiguous or does not support a homozygous unknown contributor, the unknown allele may be designated as an obligate allele e.g. 17, P or 17P. The P designates the allele as an obligate allele with an unknown sister allele. While the P designation allows the obligate to be paired with any other allele for statistical purposes, the analyst should limit the acceptable genotypes for inclusionary/exclusionary comparisons when possible. Narrowing down the genotypes is generally reserved for loci where drop out of alleles is unlikely.

Example: A locus has alleles 15,16,17 which are all above stochastic threshold. Alleles 15,16 are known alleles for the assumed contributor. This leaves the only unknown allele as 17. The 17 allele can be designated as an obligate (17,P) for statistical purposes, however, the genotypes to be used for inclusionary/exclusionary comparisons can be limited to 15,17; 16,17; and 17,17. The ratio of contributors may also be used to further narrow down the genotype possibilities. If the ratio of the contributors across the profile is approximately 1:1 and a homozygote 17,17 provides a ratio of 1:3, the 17,17 genotype may be eliminated as a possibility for inclusionary/exclusionary comparisons.

4. In the example above, if the unknown 17 allele were below stochastic threshold, the only designation should be 17,P with no further limiting of genotypes due to the possibility of allele drop out.

F. 4-peak pattern with a heterozygous known:

1. The unknown contributor can be designated as heterozygous for the two alleles not associated with the known contributor. This peak pattern can be used to determine the overall ratio between the unknown and known



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contributors. Occasionally, heterozygous alleles can be susceptible to extreme peak height imbalance due to a low level or degraded profile. Analysts may decide not to determine (ND) an “assumed” heterozygous unknown due to an extreme peak height imbalance or an indistinguishable artifact. If more than a few loci fall into this category, the analyst may need to reconsider if the profile is only a two-person mixture.

10.11 Calculating and Reporting Statistics for GlobalFiler®

To interpret the significance of an inclusion, it is necessary to know the population distribution of alleles at each locus in question. If an individual is excluded as the donor of the biological evidence tested, no statistical calculations are necessary.

If the individual’s reference sample and the evidence sample have the same genotype, then the suspect is “included” as a possible source of the evidence sample. The probability that another unrelated individual would also match the evidence sample is equal to the inverse frequency (1/f) of that genotype in a relevant population database.

Statistical analyses will be calculated using the U.S. Department of Justice, FBI CODIS Popstats program, using either the Random Match Probability Formula for single source profiles, the Mixture Formula for mixed profiles, or the Modified Random Match Probability for single source and resolved single source profiles where the zygosity is in question. The resultant values may be truncated for reporting, but shall not be rounded up. Except for amelogenin, Y-indel and DYS391 which are not used for statistical calculations, all loci in the GlobalFiler® kit are in Hardy-Weinberg equilibrium and are acceptable for use in statistical calculations using the product rule. The FBI Popstats program is accessed through the CODIS software. The expanded 2015 FBI population databases as referenced below will be utilized for statistical calculations.

Moretti T, Moreno L, Smerick J, Pignone M, Hizon R, Buckleton J, Bright J, Onorato A. Population data on the expanded CODIS core STR loci for eleven populations of significance for forensic DNA analyses in the United States. Data developed by the FBI Laboratory.

1. Random Match Probability (RMP):



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RMP is the probability that the DNA of a randomly chosen person has the same profile as the DNA of an evidentiary sample. To calculate the frequency of a locus genotype for a single source sample, the following formulae are used:

Heterozygote- $f = 2pq$

Homozygote- $f = p^2 + p(1-p)\theta$, where typically $\theta = 0.01$

A single allele having its zygosity in question e.g. the allele is below the stochastic threshold- $f = 2p$

When it is unclear if an allele is a true homozygote (e.g. the allele is below the stochastic threshold), the formula for heterozygotes can be modified so that the unknown sister allele frequency is equal to 1. The formula $2pq$ becomes $2p(1)$ or $2p$. If the obligate allele is a true homozygote, the $2p$ calculation is still acceptable since the statistic produced is less rare than the RMP calculation for a homozygote. Obligate alleles requiring the $2p$ calculation will be designated with a "P" either on the allele call sheet or the electropherogram. The "Rec 4.1" box must be checked at each obligate allele in Popstats in order to utilize the $2p$ calculation.

The multi-locus genotype frequency is estimated by multiplying together the genotype frequencies from the different loci.

2. Combined Probability of Inclusion (CPI; Mixture Formula):

To calculate the probability of a randomly selected individual being included as a contributor to a mixture for a particular locus, the following formula is used:

Mixture- $f = (p_1 + p_2 + \dots + p_k)^2$, where p_k = total number of alleles of the profile in locus.

The combined mixture profile probability is calculated by taking the product of the individual locus probabilities. CPI can only be performed on loci that do not show signs of drop-out or contain peaks below the stochastic threshold. This does not preclude using the CPI for multiple major contributors when minor peaks may fall below the stochastic threshold.



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3. Modified Random Match Probability (mRMP)

While the Random Match Probability (RMP) is thought of in terms of single source profiles, the formula also applies to mixture calculations where the number of contributors is assumed unlike the CPI calculation which assumes no definite number of contributors. When applied to mixture profiles, the method is referred to as modified random match probability (mRMP). This method will be limited to obligate alleles in resolved two person mixtures i.e. deduced or minor profiles. When a genotype can be completely determined either by major/minor disparity or the use of an assumed contributor, the calculations are the same as RMP. When the zygosity of a single obligate allele is in question for the deduced or minor profile, an alternative form of the RMP can be used i.e. 2p. When it is unclear whether the obligate allele is a homozygote or a heterozygote, the RMP formula for heterozygotes can be modified so that the unknown sister allele frequency is equal to 1. The formula $2pq$ becomes $2p(1)$ or $2p$. If the obligate allele is a true homozygote, the $2p$ calculation is still acceptable since the statistic produced is less rare than the RMP calculation for a homozygote. Obligate alleles requiring the $2p$ calculation will be designated with a "P" either on the allele call sheet or the electropherogram. The "Rec 4.1" box must be checked at each obligate allele in Popstats in order to utilize the $2p$ calculation.

4. Amelogenin, DYS391 and the Y-indel are not used in the frequency calculations. These loci may be documented on worksheets or allele call sheets for major, minor, and deduced profiles, but are not used for calculation of statistics.

5. Calculating Statistics for Probative Inclusions

In general, when reporting statistics on sexual assault cases, statistics are calculated on the sperm fraction. The non-sperm fraction may be assessed on a case-by-case basis. Except for assumed contributors, statistics shall be calculated and reported for all inclusions. Inconclusive or uninterpretable data will not be used for statistics supporting an inclusion. Statistical calculations are not required to support the inclusion of an assumed contributor. Inclusion of an assumed contributor(s) should be supported in the report with a



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qualitative statement indicating the included individual is an assumed contributor.

Statistics will generally be reported for the Caucasian, African-American, and Southwestern Hispanic populations.

Alleles that are less than or greater than the allowed alleles in Popstats may be entered as < or > than the allowable allele. For example, the smallest allele allowed at CSF1PO is 5. An allele less than 5 may be entered as <5. The minimum allele frequency will be used for this situation and other rare allele events where the allele has been observed less than five times in the population database. The formula for this frequency is $5/2N$ ($2N$ = number of alleles in the population database at that locus).

Any genotype frequency which exceeds 1 in 8 billion may be reported in casework as "the probability of randomly selecting an unrelated individual having the same DNA profile is 1 in a number greater than the current world population for the African- American, Caucasian, and Southwestern Hispanic populations." If one or more of the populations fall below 1 in 8 billion, then the calculated frequency will be reported for each population.

Requests for recalculation of previous statistics generated for Profiler Plus, Cofiler, and Identifiler Plus data should use the amended 2015 FBI population databases as referenced below.

Budowle B, Moretti TR, Baumstark AL, Defenbaugh, DA, Keys KM. Population data on the thirteen CODIS core short tandem repeat loci in African Americans, U.S. Caucasians, Hispanics, Bahamians, Jamaicans, and Trinidadians. *J Forensic Sci* 1999;44(6):1277–1286.

Bruce Budowle ; Patrick J. Collins ; Pero Dimsoski ; Constance K. Ganong; Lori K. Hennessy ; Craig S. Liebelt ; Sulekha Rao-Coticone ; Farideh Shadravan ; Dennis J. Reeder. Population Data on the STR Loci D2S1338 and D19S433. *Forensic Science Communications*, V 3, N 3, July 2001.

Moretti, T. R., Budowle, B. and Buckleton, J. S. (2015), Erratum. *Journal of Forensic Sciences*. DOI: 10.1111/1556-4029.12806



11. Assessment and Reporting of Yfiler® Plus Data

Scope

Y-STR analysis may be performed in situations where male DNA needs to be differentiated from female DNA.

11.1 Considerations for Performing Y-STR Testing

- Y-STR testing may not be performed without a request from one of the following: the District Attorney General or TBI Special Agent in Charge. The request may be made via a phone call or in writing.
- Limited samples with a male: female ratio greater than 1:10 should proceed directly to Y-STR testing if there is insufficient sample to perform both autosomal and Y-STR testing. Analysts should consider if determining a female autosomal profile could be probative or meaningful prior to performing only Y-STR analysis.
- Standards from any male individuals involved in a case should be submitted before Y-STR analysis is performed. A Y-STR profile is not searchable against arrestees and convicted offenders in the CODIS database. Y-STR testing should not be performed unless a subject standard is submitted.
- Sexual assault cases that have not provided meaningful autosomal results should be considered for Y-STR analysis when interpretation of a potentially probative male profile is masked by excess female DNA.
- Homicide and violent crime cases may occasionally have items to consider for Y-STR testing if no meaningful results are obtained via autosomal DNA testing (e.g. female victim's fingernail scrapings).
- Property crimes will generally not be considered for Y-STR analysis.
- Paternal relatives are expected to have the same Y-STR profile, barring a potential mutation.
- Y-STR testing results should be issued in a separate report from the autosomal testing results, if applicable.

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11.2 Introduction: Yfiler® Plus

Amplification of Y chromosome-specific short tandem repeats, Y-STRs, allows haplotyping of human male DNA. The Y-STR loci are located on the non-recombining region of the Y chromosome, producing a haploid profile. A male's Y-STR profile is inherited from his father, thus characteristic of his paternal lineage and not unique to a single individual. The Yfiler® Plus PCR Amplification Kit is a 6-dye, short tandem repeat (STR) multiplex assay optimized to allow amplification from multiple male-specific sample types such as male-female mixtures. The Yfiler® Plus System contains 27 Y-STR loci, including the following 17 Yfiler® loci: DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385a/b, DYS393, DYS391, DYS439, DYS635, DYS392, YGATAH4, DYS437, DYS438, and DYS448 plus 10 additional highly polymorphic Y-STR markers (DYS576, DYS627, DYS460, DYS518, DYS570, DYS449, DYS481, DYF387S1a/b and DYS533). These ten loci include the following 7 rapidly mutating Y-STR loci which allow for improved discrimination of related individuals: DYS449, DYS518, DYS570, DYS576, DYS627 and DYF387S1a/b. The combination of a six-dye fluorescent system and the inclusion of non-nucleotide linkers allows for simultaneous amplification and separation of the 27 Y-STR loci during automated DNA fragment analysis. Multicomponent analysis is the process that separates the six different fluorescent dye colors into distinct spectral components. The five dyes used in the Yfiler® Plus Kit to label samples are 6-FAM™, VIC®, NED™, TAZ, and SID dyes. The sixth dye, LIZ® dye is used to label the GeneScan™ 600 LIZ® Size Standard v.2.0. Each of these fluorescent dyes emits its maximum fluorescence at a different wavelength. During data collection, the fluorescence signals are separated by diffraction grating according to their wavelengths and projected onto a charge-coupled device (CCD) camera in a predictably spaced pattern. The 6-FAM™ dye emits at the shortest wavelength and it is displayed as blue, followed by the VIC® dye (green), NED™ dye (yellow), TAZ dye (red), SID dye (purple) and LIZ® dye (orange).

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Table 2 shows the loci amplified and the corresponding fluorescent marker dyes. The Yfiler® Plus Allelic Ladder is used to type the analyzed samples. The alleles contained in the allelic ladder and the haplotype of the DNA Control 007 are also listed in the table.

Table 2

Locus	Alleles included in Yfiler® Plus Ladder	Dye Label	DNA Control 007
DYS576	10-25	6-FAM™	19
DYS389I	9-17	6-FAM™	13
DYS635	15-30	6-FAM™	24
DYS389II	24-35	6-FAM™	29
DYS627	11-27	6-FAM™	21
DYS460	7-14	VIC®	11
DYS458	11-24	VIC®	17
DYS19	9-19	VIC®	15
YGATAH4	8-15	VIC®	13
DYS448	14-24	VIC®	19
DYS391	5-16	VIC®	11
DYS456	10-24	NED™	15
DYS390	17-29	NED™	24
DYS438	6-16	NED™	12
DYS392	4-20	NED™	13
DYS518	32-49	NED™	37
DYS570	10-26	NED™	17
DYS437	10-18	TAZ™	15
DYS385	6-28	TAZ™	11, 14
DYS449	22-40	TAZ™	30
DYS393	7-18	SID™	13
DYS439	6-17	SID™	12
DYS481	17-32	SID™	22
DYF387S1	30-44	SID™	35, 37
DYS533	7-17	SID™	13

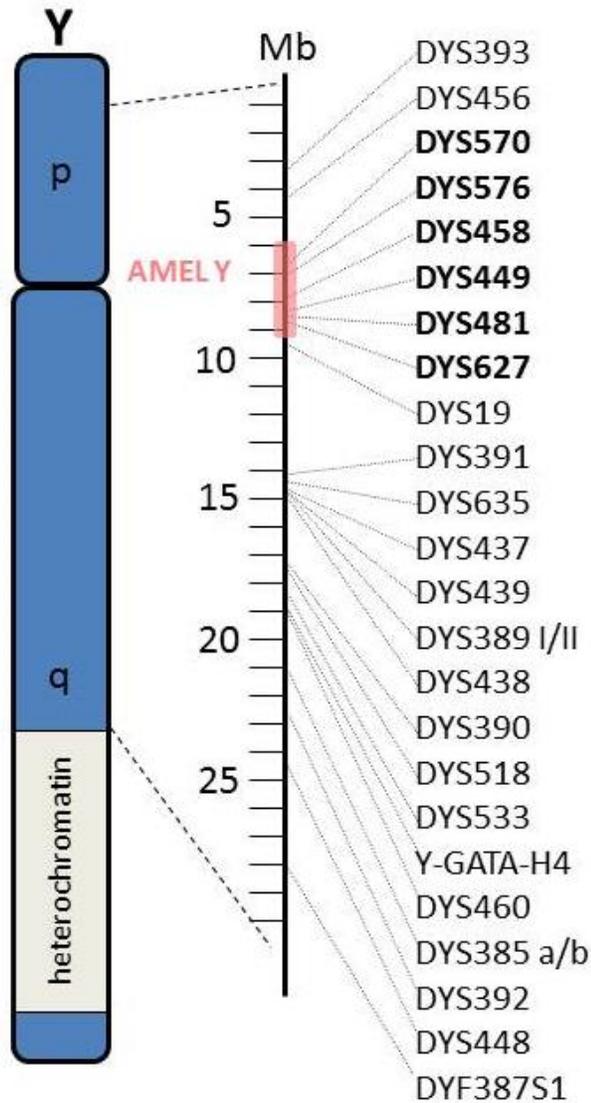


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Positions of the Yfiler® Plus markers on the Y-Chromosome:



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11.3 Sizing Standard

The internal sizing standard will be examined for each sample and control to ensure that all peaks are sized correctly [within the calling range of 60-460 base pairs (bp)].

NOTE: GeneMapper has an internal regulator that will flag a sample with a red stop sign if there is sizing quality issue. A red stop sign indicates a failure in the internal size standard. If a yellow triangle is displayed, the sample requires further interpretation. A green box indicates that the sizing quality passes. The quality flags are used as an aid to the analyst, but interpretation and assessment of the data will be performed by the analyst through examination of the raw data and/or electropherograms.

11.4 Allelic Ladder

The allelic ladder(s) used for genotyping will be examined to determine that GeneMapper ID-X has assigned all allele designations correctly. When interpreting Yfiler® Plus results, designations are assigned to sample alleles by comparison of their sizes to those obtained from the known alleles in the allelic ladders. Haplotypes, not sizes, are used for comparison of the data between runs, instruments and laboratories.

NOTE: GeneMapper ID-X has a Sample type menu where "Allelic Ladder" can be selected. At least one ladder must be identified before the plate can be analyzed. If multiple ladders are used for analysis, all ladders shall be printed and placed in the case file for technical review.

11.5 Reagent Blanks and Negative Controls:

The reagent blanks and negative controls are a test for the presence of contamination occurring during extraction and amplification set-up, respectively. Both should only consist of the GS LIZ 600 v.2.0 peaks. If a negative control is determined to have non-artifact peaks at or above the analytical threshold in the 60 bp to 460 bp range, the samples amplified at the same time as the negative control must be re-amplified using the sample amounts of DNA template and TE buffer previously amplified if sample size permits. If a reagent blank is determined to have non-artifact peaks at or above the analytical threshold in the 60 bp to 460 bp range, the reagent blank must be re-amplified with a negative and positive control. If a reagent blank contains any peaks that cannot be attributed to artifacts after re-amplification, the samples extracted at the same time as the reagent blank must be re-extracted if sample size permits.

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NOTE: GeneMapper has a Sample Type menu where “Negative Control” can be selected. The Reagent Blank and Negative Control should be selected prior to being analyzed.

11.6 Casework Sample:

A casework sample determined to have contamination must be re-amplified if sample size permits. If the contamination is still present after re-amplification, then the sample must be re-extracted; sample size permitting. In instances where sample size does not permit re-extraction or re-amplification of potentially contaminated samples, the sample data may possibly be interpreted with caution under the direction of the DNA Technical Leader. This will be documented and retained in the case file.

11.7 Positive Control:

The positive control provided in the kit must be typed correctly at all loci (see Table 2 in the introduction section of this chapter for positive control types) and not contain any peaks that are not attributed to an artifact. If the positive control does not type correctly, all samples amplified at the same time as the positive control must be re-amplified.

NOTE: The analyst must visually verify from the electropherogram that the positive control has typed correctly.

11.8 Thresholds

The analytical threshold (AT) for Yfiler® Plus is set at 150 RFU for all dye channels on a 3500.

The stochastic threshold (ST) for Yfiler® Plus is set at 450 RFU for the DYS385 and DYF387S1 loci on a 3500.

Analytical Threshold:

The analytical threshold for data interpretation is 150 RFU. The analytical threshold is the minimum peak height requirement at and above which detected peaks can be reliably distinguished from background noise. Peaks at or above the analytical threshold are generally considered true alleles or artifacts.

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Stochastic Threshold:

The stochastic threshold for data interpretation applies only to the multi-copy *DYS385* and *DYF387S1* loci and is set at 450 RFU. The stochastic threshold is the peak height requirement at or above which it is reasonable to assume that allelic dropout has not occurred within a heterozygous loci. If the *DYS385* or *DYF387S1* locus demonstrates a single allele that is at or above 150 RFU but below 450 RFU, true homozygosity cannot be determined.

11.9 Determination of a Single Source Y-STR Profile

A Yfiler® Plus haplotype result can be considered to be from a single contributor if there is no more than one allele that is greater than or equal to the analytical threshold at each locus. The exception is the duplicated loci *DYS385* and *DYF387S1* which will often exhibit the presence of two balanced alleles for a single individual. Both loci in the gene duplications can be amplified using the same primer set due to shared sequence information. In rare instances, gene duplications can occur at other loci and this should be considered when making determinations if the sample is from a single individual. If the Yfiler® Plus typing result is determined to be from a single contributor, alleles from the “single” copy loci that meet or exceed the analytical threshold can be used for comparison purposes. For *DYS385* and *DYF387S1* which exhibit duplications, a single allele below the stochastic threshold may indicate potential dropout. Alleles below stochastic threshold at *DYS385* and *DYF387S1* may be used for inclusionary/exclusionary comparisons.

11.10 Interpreting Mixed Y-STR Profiles

The Yfiler® Plus haplotype result is considered to be from a mixed source if there are two or more alleles present above the analytical threshold at two or more single copy loci. More than 2 alleles present at *DYS385* or *DYF387S1* would indicate a mixture. The analyst should also consider the presence of multiple stutter peaks that are greater in percentage than what has been established in the validation. It should be taken into consideration that loci other than *DYS385* and *DYF387S1* can experience duplications that will produce more than one allele, but this occurrence is rare.

1. Considerations for the Occurrence of Duplications



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Typically, a Y-STR locus, with the exception of DYS385 and DYF387S1, will exhibit a single PCR product. There are instances of duplications of Y-STR loci where more than one peak will appear (or more than two peaks will occur for DYS385 and DYF387S1). The possibility of duplications should be considered for determining if a Y-STR profile is a single source contributor or a mixed sample. Generally, duplicated loci will possess alleles that differ by a single repeat and possess similar peak heights. The greater the number of loci with multiple alleles, the more likely it is a mixture. If there is more than one locus with multiple alleles, the further apart these loci are physically located on the Y chromosome, the more likely it is mixture. The alleles at duplicated and multi-copy loci, such as DYS385 and DYF387S1, should be documented as genotypes (e.g. 11, 14, or 15,15).

2. Y-STR Mixtures with Major / Minor Components

When a major component can be distinguished from the mixture, inclusions and exclusions can be made between known reference sample(s) and the major contributor profile. A population database search to generate statistics can be performed on the major profile. A major contributor should be deduced locus by locus. Consideration should be given to the possibility of the flipping of the major and minor contributors from locus to locus for low level and degraded samples. A major contributor should not be deduced for alleles less than 450 RFU due to increased stochastic effects. Analysts should only deduce a major contributor when the minor allele peak height(s) is less than 40% of the major allele peak height(s). A major contributor should be interpretable at 8 or more loci. A locus should only be included in the statistics when these conditions are met. The minor contributor may be used for inclusions when the minor profile is visible at 15 or more loci and the minor profile is determined to be from a single contributor (DYS385 and DYF387S1 will be counted as one locus each for this purpose). Minor profiles deemed to be mixtures may be used for exclusionary purposes only. No inclusions will be made or statistics generated for a mixed minor component of a Y-STR genotype. The major and minor profiles may be listed separately on the allele call sheet or the electropherogram.

3. Determining Major / Minor Components at Duplicated Loci

Caution should be used when interpreting DYS385 and DYF387S1 where each contributor may have up to 2 alleles. At these loci, alleles



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should only be attributed to the major contributor under the following circumstances:

- The major allele(s) must be equal to or greater than 450 RFU and the highest minor allele should be less than 40% of the lowest major allele. The major allele(s) should account for approximately 70% of the total RFU for the locus.
- If only 1 allele is present and it is greater than or equal to 450 RFU, this allele can be attributed to the major contributor.

4. Mixtures with a Known Contributor

In instances where a contributor is known or expected to be present (such as intimate swabs, consensual partners, etc.), subtracting out the known contributor's alleles can allow for determination of the remaining deduced probative profile. The deduced profile may be used for both inclusionary and exclusionary comparisons. Caution should be used when deducing profiles for low level alleles less than 450 RFU due to increased stochastic effects. At those loci where only one allele has been identified, it can be assumed that both the known and deduced contributors have donated the same allele if it can be reasonably determined that the contribution of the deduced contributor (as determined from all loci where the known and deduced contributors have separate alleles), would result in an allele that is above the analytical threshold. The deduced profile may be listed on the allele call sheet or the electropherogram.

5. Mixtures with Indistinguishable Components

No inclusions will be made to mixtures where a single source component (e.g. major/minor contributor or deduced profile) cannot be distinguished. This is due to the inability to provide an evidentiary statistical weight to indistinguishable Y-STR mixtures. Exclusionary comparisons may still be made to indistinguishable mixtures.

11.11 Artifacts

1. Anticipated Artifacts

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Y-STR testing exhibits the normal artifacts (spikes, dye blobs, pull-up, and –A shoulders) that occur in autosomal STR's, as well as other Y-STR and kit dependent artifacts.

a. **Stutter**

Stutter peaks can occur as $n-3/n+3$, $n-4/n+4$, $n-5/n+5$ and $n-6/n+6$ in the Yfiler® Plus kit. The DYS448 locus having a hexanucleotide repeat unit exhibits N-6 and N+6 stutter. The DYS438 locus having a pentanucleotide repeat unit exhibits $n-5$ and $n+5$ stutter. The DYS392 and DYS481 loci have trinucleotide repeat units exhibiting $n-3$ and $n+3$ stutter. The remaining loci Yfiler® Plus loci exhibit $n-4$ and $n+4$ stutter. In addition to the $n-3$ or $n-4$ stutter, a non-standard (minus 2-nt) stutter has been observed in the DYS19, DYS481, DYS533 and DYS627 loci that include more complex nucleotide sequences including regions of dinucleotide repeats. The GeneMapper ID-X analysis software contains a minus 2-nt stutter filter.

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The following values should be used as a guide for the expected levels of stutter in the Yfiler® Plus Kit:

Locus	Stutter	% Minus Stutter	% Plus Stutter	% Minus 2 / % Plus 2 Stutter
DYS576	n-4 / n+4	15.15	3.38	NA
DYS389I	n-4 / n+4	9.16	3.45	NA
DYS635	n-4 / n+4	13.38	3.30	NA
DYS389II	n-4 / n+4	18.79	3.73	NA
DYS627	n-4 / n+4	15.18	2.62	2.71
DYS460	n-4 / n+4	11.65	4.27	NA
DYS458	n-4 / n+4	15.31	2.52	NA
DYS19	n-4 / n+4	12.68	3.72	10.10 / 3.42
YGATAH4	n-4 / n+4	11.53	2.27	NA
DYS448	n-6 / n+6	4.68	2.29	NA
DYS391	n-4 / n+4	9.99	3.41	NA
DYS456	n-4 / n+4	15.36	3.74	NA
DYS390	n-4 / n+4	13.58	3.51	NA
DYS438	n-5 / n+5	5.86	2.76	NA
DYS392	n-3 / n+3	16.94	11.0	NA
DYS518	n-4 / n+4	25.50	4.85	NA
DYS570	n-4 / n+4	15.65	2.88	NA
DYS437	n-4 / n+4	8.13	1.65	NA
DYS385	n-4 / n+4	18.32	3.70	NA
DYS449	n-4 / n+4	23.24	4.20	NA
DYS393	n-4 / n+4	14.07	4.95	NA
DYS439	n-4 / n+4	9.89	3.39	NA
DYS481	n-3 / n+3	28.55	5.59	9.55
DYF387S1	n-4 / n+4	15.71	2.00	NA
DYS533	n-4 / n+4	12.0	4.60	1.88

NOTE: The level of elevated stutter will generally not be greater than ~3% of the listed values for minus and plus stutter. Elevated stutter is possible due to saturation, low level template and additive affects.



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b. Additional Stutter Products

The following stutter products have also been observed with Yfiler® Plus, but are not part of the Genemapper ID-X stutter filter:

Locus	Stutter Product	% Stutter
DYS456	-2 / -6	0.70 / 0.70
DYS392	+6	0.85
DYF387S1	+4	2.0
DYS437	-5 / -12 / -16	2.0 / 2.0 / 2.0



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c. Other DNA Dependent Artifacts

Additional reproducible DNA-dependent artifacts have been characterized. Low level artifacts with female DNA may occasionally be seen. The intensity of these artifacts may depend on the individual female and the quantity of female DNA amplified. Normally, these artifacts will not affect the interpretation of the Y-STR profile. The table below provides a list of documented DNA dependent artifacts.

Artifact	Dye	Approximate Size	Comment
FAM270	Blue	270-271	Minor cross-reactive product observed with female DNA in excess of 2 µg.
FAM280	Blue	280-281	Minor cross reactive product observed with female in excess of 2 µg.
FAM348	Blue	348-349	Specific to control DNA 007.
Y391 (n-10)	Green	n-10	Specific to DYS391. Minor cross reactive product observed with male DNA in excess of 1.0 ng.
TAZ140	Red	139-140	Minor cross-reactive product observed with female DNA in excess of 2 µg.
TAZ144	Red	144-145	Minor cross-reactive product observed with female DNA in excess of 2 µg.
TAZ225-260	Red	225-260	Multiple minor cross-reactive product observed with female DNA in excess of 2 µg.
TAZ412	Red	412-413	Cross-reactive product observed with female DNA in excess of 100ng. Occurs outside of the read region. Does not impact interpretation.
VIC70	Green	70	Sporadic PCR product. Occurs outside of the VIC read region. Does not impact interpretation.

11.12 Documentation of Artifacts

Analysts should try to designate the type of artifact on the electropherogram; however, ambiguous artifacts that are deemed not to be true alleles may be labeled “artifact” or “ART” on the electropherogram.

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11.13 Results of Y-STR Analysis

1. No DNA profile obtained: No alleles were detected on the electropherogram.
2. Inconclusive: Alleles were observed at one or more loci; however no conclusive results can be drawn from them.
3. Inclusion may be reported when the reference sample alleles are also observed in the question sample profile and there is a scientific explanation for any inconsistency (i.e. drop out, degradation, inhibition, etc.). Except for an assumed contributor, statistical weight must be provided for inclusions. Inclusions based on assumptions should be clearly defined in the report.
4. Exclusion should be reported when reference sample alleles are missing from the question sample profile and no scientific explanation can be given for their absence (i.e. drop out, degradation, inhibition, etc.).

11.14 Y-STR Statistical Calculations

Y-STR loci are located on the non-recombining region of the Y chromosome and are considered linked. Statistics for Y-STR analysis are calculated using the counting method to determine the number of times a particular haplotype has been observed in a given population database. The current version of the Y-STR Haplotype Reference Database (YHRD) will be used for this statistic. The most current version of YHRD can be found online at <https://yhrd.org>.

Haplotypes can be searched by accessing the database at <https://yhrd.org> and selecting "Search the database". The haplotypes can be manually entered by selecting "Manually enter the haplotype/haplotypes to search for". Select either Yfiler Plus or Yfiler to organize the loci for entry. After entry of haplotypes, select "Search". The YHRD will automatically search against the Worldwide database. To search against the U.S. database, select "Add feature to this report" to view a dropdown menu. Select the "National Database (with subpopulations, 2014 SWGDAM-compliant)" from the dropdown menu. The total number of overall matches and the 95% upper confidence interval profile probabilities for each subpopulation may be reported from the "Observed" results box.

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Single source profiles including major / minor components and single source deduced profiles can be searched in the population database using the directions outlined on the YHRD web site in the “2014 SWGDAM Compliant YHRD User’s Guide”.

Statistics for the typing results that provide the most genetic information and/or the highest discrimination potential may be reported.

At their discretion, analysts may perform searches using only the Yfiler® loci (DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385, DYS393, DYS391, DYS439, DYS635, DYS392, YGATAH4, DYS437, DYS438, and DYS448). This may provide a more discriminating search due to the increased number of available Yfiler® profiles in the database. If matching haplotypes are obtained with a reduced locus search, the search results utilizing the most genetic information should be reported.

The minimum number of loci required to determine an inclusion and perform a statistical calculation is 8. DYS385 and DYS387S1 will each count as one locus for this purpose.

For single source or deduced single source profiles, alleles below stochastic threshold for DYS385 and DYS387S1 may be included in statistics if the locus is heterozygous. If either locus has only a single allele below stochastic threshold, the allele cannot be entered for statistics.

Loci exhibiting duplications other than DYS385 and DYS387S1 should not be included in statistics.

In cases where less information is obtained from the known sample, only those loci for which results were obtained from both the known and evidentiary sample should be used in the population database search.

Profile probability is estimated by applying a 95% confidence upper bound to the haplotype frequency. The application of a confidence interval corrects for database size and sampling variation.

- If the haplotype has not been previously observed in the database, the formula used for calculating the upper 95% confidence limit is:



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$$1-(0.05)^{1/n}$$

n = database size

- If the haplotype has been observed in the database, the formula below used for calculating the 95% confidence limit can be found on the YHRD website. The number of samples in the database is n and the number of matches is x . This gives a sample proportion p .

The formula used seeks a value P such that:

$$\sum_{k=0}^x \binom{n}{k} p_0^k (1-p_0)^{n-k} = 0.05$$

The number of overall observed matches in the database will be reported for the overall total # of haplotypes in the database. The 95% Upper Confidence Interval (UCI) from the “National Database (with subpopulations) - United States” in the YHRD will be reported for the African American, Asian, Caucasian, Hispanic, and Native American populations.

Reports will provide either a statistical result or a qualitative statement for each association. A qualitative statement may be used when reporting an inclusion of an assumed contributor to a profile.



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APPENDIX A

Reporting Statements

The following statements or combination of statements may be used as guidelines in report writing. It is understood that all cases are different; therefore the list below is not intended to cover all possible reporting scenarios. Analysts may edit/change the statements below in order to clearly communicate the results of testing.

Quantification

1. Quantification results from real-time PCR testing did not indicate a sufficient amount of human DNA for analysis. No further testing was performed.
2. Quantification results from real-time PCR testing did not indicate the presence of human DNA. No further testing was performed.
3. Quantification results from real-time PCR testing did not indicate the presence of male DNA. No further testing was performed.
4. Quantification results from real-time PCR testing did not indicate a sufficient amount of male DNA for analysis. No further testing was performed.
5. Quantification results from real-time PCR testing did not indicate a sufficient amount of male DNA for autosomal STR analysis; however, this exhibit may be suitable for Y-STR analysis. Testing may be performed upon receipt of a subject standard and a request for Y-STR analysis from the District Attorney General. For additional information, contact the undersigned analyst.

Profiles Generated with GlobalFiler®

DNA testing was performed using the polymerase chain reaction (PCR) technique for Amelogenin, Y-indel marker, and the following short tandem repeat (STR) loci: D3S1358, vWA, D16S539, CSF1PO, TPOX, D8S1179, D21S11, D18S51, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, SE33, D10S1248, D1S1656, D12S391, D2S1338, and DYS391.

GlobalFiler® Single Source Profiles

1. The DNA profile matched exhibit # _____.

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2. The DNA profile obtained is that of an unknown individual.
3. The DNA profile matched the unknown male profile obtained from exhibit # _____.
4. The DNA profile obtained is from an unknown male; therefore exhibit # _____ is excluded as a contributor.
5. The DNA profile did not match exhibit # _____.

GlobalFiler® Partial Profiles

Note: A questioned sample or known reference standard may be reported as a partial profile if one or more loci have no alleles detected at or above the analytical threshold. A questioned sample may be reported as limited if less than seven loci qualify for statistical calculation. A known reference standard with less than seven complete loci may be reported as limited.

6. A partial DNA profile was obtained which was consistent with exhibit # _____ at the following STR loci: _____. The remaining STR loci were inconclusive due to insufficient and/or degraded DNA.
7. A partial DNA profile was obtained at the following STR loci: _____. The remaining loci were inconclusive due to insufficient and/or degraded DNA. Exhibit # _____ is excluded as a contributor.
8. A partial DNA profile was obtained from an unknown male.
9. A partial DNA profile was obtained. The following loci were inconclusive due to insufficient and/or degraded DNA: _____.
10. The DNA profile obtained is only of value for exclusionary purposes. This DNA profile is not eligible for CODIS entry.
11. Due to the limited DNA profile obtained, the profile is deemed to be inconclusive.

GlobalFiler® Mixture Profiles

12. The DNA profile obtained is consistent with a mixture of at least _____ individuals.



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13. Due to the limited profile obtained and the unknown number of potential contributors to the profile, interpretation of this profile is deemed to be inconclusive.
14. The DNA profile obtained is only of value for exclusionary purposes. Due to the limited profile obtained, comparison to exhibit # _____ is inconclusive.
15. The major contributor profile matched exhibit # _____.
16. The major contributor profile is from an unknown male.
17. Due to the limited minor contributor profile obtained, interpretation of the minor contributor profile is deemed to be inconclusive.
18. The minor contributor profile is consistent with exhibit # _____.
19. The DNA profile is consistent with a mixture of _____ individuals. Assuming exhibit # _____ is a contributor to the mixture profile, the deduced male profile matches exhibit # _____.
20. The DNA profile is consistent with a mixture of _____ individuals. Assuming exhibit # _____ is a contributor to the mixture profile, the deduced profile is from an unknown male. Therefore, exhibit # _____ is excluded as a contributor to the profile.

Assumed Contributors

21. The major contributor profile is consistent with exhibit # _____ (Name), who is an assumed contributor to the profile.
22. The minor contributor profile is consistent with exhibit # _____ (Name), who is an assumed contributor to the profile.
23. The DNA profile is consistent with exhibit # _____ (Name), who is an assumed contributor to the profile.
24. No DNA profile was obtained other than exhibit # _____ (Name), who is an assumed contributor to the profile.



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25. No DNA profile other than the assumed contributor _____ (Name) was obtained.
26. The DNA profile is consistent with a mixture of _____ individuals. Assuming exhibit # _____ is a contributor to the mixture profile, the deduced profile is from an unknown male. Therefore, exhibit # _____ is excluded as a contributor to the profile.
27. The DNA profile is consistent with a mixture of _____ individuals. Assuming exhibit # _____ is a contributor to the mixture profile, the deduced male profile matches exhibit # _____.

GlobalFiler® Statistics

28. The probability of randomly selecting an unrelated individual who would be included as a contributor of the DNA profile is 1 in a number greater than the current world population for the African-American, Caucasian, and Southwestern Hispanic populations. The statistics were calculated using allele frequencies from the 2015 Expanded FBI STR Population Data.
29. The probability of randomly selecting an unrelated individual who would be included as a contributor of the DNA profile is approximately 1 in ____ for the African-American population, 1 in ____ for the Caucasian population, and 1 in ____ for the Southwestern Hispanic population. The statistics were calculated using allele frequencies from the 2015 Expanded FBI STR Population Data.
30. The probability of randomly selecting an unrelated individual who would be included as a contributor to this DNA mixture profile is approximately 1 in ____ for the African-American population, 1 in ____ for the Caucasian population, and 1 in ____ for the Southwestern Hispanic population. The statistics were calculated using allele frequencies from the 2015 Expanded FBI STR Population Data.

CODIS

31. This unknown DNA profile will be added to the State and National CODIS databases and the search results will be issued in a separate report.

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32. This DNA profile will be added to the State and National CODIS databases. This profile will remain in the databases and searched weekly. You will be notified in the event of a future investigative lead.
33. This unknown, partial DNA profile will be added to the State CODIS database. It is not eligible for addition to the National database. The search results will be issued in a separate report.
34. This partial DNA profile will be added to the State CODIS database and will remain in the State database and searched weekly. This partial DNA profile is not eligible for addition to the National database. You will be notified in the event of a future investigative lead.
35. This unknown DNA profile was searched in the State and National CODIS databases and no matches were detected as of this report date. This profile will remain in the databases and searched weekly. You will be notified in the event of a future investigative lead.
36. This unknown DNA profile was searched against the State CODIS database and no matches were detected as of this report date. A search of the National CODIS database is pending. The results from this national search will be provided in a separate report. Additionally, this profile will remain in the databases and searched weekly. You will be notified in the event of a future investigative lead.
37. This unknown, partial DNA profile was searched against the State CODIS database and no matches were detected as of this report date. Additionally, this profile will remain in the State database and searched weekly. This partial DNA profile is not eligible for addition to the National database. You will be notified in the event of a future investigative lead.
38. This unknown DNA profile was searched against the CODIS database and a match was detected. The information being provided is an investigative lead only. In order to confirm this match, a blood or buccal sample from (Name) (DOB) must be submitted for DNA testing.
39. This DNA profile is not eligible for entry into the CODIS database.
40. The DNA profile previously obtained from this exhibit has been removed from the CODIS database. This profile does not meet the current CODIS eligibility requirements.
41. The DNA profile previously obtained from this exhibit has been removed from the CODIS database after comparison to the elimination standard noted above.



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42. During a search of the CODIS databases, an association was made to Lab # (agency/case#/officer). For additional information contact the undersigned analyst.
43. During a search of the CODIS databases, an association was made to Lab #. The information being provided is an investigative lead only. In order to confirm this, a blood or buccal sample from (Name) (DOB) must be submitted for DNA testing.

Reporting for Swabs and Cuttings

44. The _____ was swabbed for testing. The remaining swab(s) will be returned with the evidence.
45. A cutting was made from _____ for testing. The remaining portion of the cutting will be returned with the evidence.
46. The _____ was swabbed. No further testing was performed at this time. The swab(s) will be returned with the evidence.
47. A cutting was made from _____. No further testing was performed at this time. The cutting will be returned with the evidence.

Reporting Statements for Y-STR

Note: A Y-STR profile may be reported as a partial profile if one or more loci have no alleles detected at or above the analytical threshold.

1. For profiles generated with Yfiler® Plus:

DNA testing was performed using the polymerase chain reaction (PCR) technique at the following Y-STR loci: DYS576, DYS389I, DYS635, DYS389II, DYS627, DYS460, DYS458, DYS19, YGATAH4, DYS448, DYS391, DYS456, DYS390, DYS438, DYS392, DYS518, DYS570, DYS437, DYS385, DYS449, DYS393, DYS439, DYS481, DYF387S1, and DYS533.



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Note: Y-STR profiles with at least one locus having no alleles detected at or above the analytical threshold may be designated as a partial profile. Y-STR profiles with less than eight loci available for a database search may be reported as a limited profile.

2. Single Source Profile (Inclusion)

The Y-STR profile obtained matches exhibit # _____ (Name). Therefore, exhibit # _____ (Name) cannot be excluded as the source of this male DNA profile and, barring a mutation, neither can any of his paternal male relatives.

The partial Y-STR profile obtained is consistent with exhibit # _____ (Name). Therefore, exhibit # _____ (Name) cannot be excluded as the source of this male DNA profile and, barring a mutation, neither can any of his paternal male relatives.

3. Single Source Profile (Exclusion)

The Y-STR profile obtained is from an unknown male; therefore, exhibit # _____ (Name) is excluded as the source of this male DNA profile.

4. Mixture Profile (Exclusion)

The Y-STR profile obtained is consistent with a mixture of at least _____ males. Exhibit # _____ (Name) is excluded as a contributor to this mixture profile.

5. Mixture Profile (Inconclusive)

- The Y-STR profile obtained is consistent with a mixture of at least _____ males. Due to the number of contributors present in the profile, no conclusions were made regarding the inclusion of exhibit # _____ (Name).
- The Y-STR profile obtained is consistent with a mixture of at least _____ males. Due to the limited profile obtained and the unknown number of potential contributors, this profile is deemed to be inconclusive.

6. Single Source (Inconclusive)



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Due to the limited profile obtained, interpretation of the profile was deemed to be inconclusive.

7. Mixture Profile (Major Contributor)

The Y-STR profile obtained is consistent with a mixture of at least _____ males. The major contributor profile matches exhibit # _____ (Name). Therefore, exhibit # _____ (Name) cannot be excluded as the source of this male DNA profile and, barring a mutation, neither can any of his paternal male relatives. Due to the limited minor contributor profile obtained, interpretation of the minor contributor profile is deemed to be inconclusive.

8. Mixture Profile (Deduced Profile)

The Y-STR profile is consistent with a mixture of _____ males. Assuming exhibit # _____ (Name) is a contributor to the mixture profile, the deduced male profile matches exhibit # _____. Therefore, exhibit # _____ (Name) cannot be excluded as the source of this male DNA profile and, barring a mutation, neither can any of his paternal male relatives.

9. Statistics

(Yfiler® loci only)

This Y-STR profile has been observed (X) times in _____ profiles in the National United States Database contained within the Y-STR Haplotype Reference Database (YHRD) and is expected to occur in approximately 1 in _____ African-American males, 1 in _____ Asian males, 1 in _____ Caucasian males, 1 in _____ Hispanic males, and 1 in _____ Native American males. Statistics were calculated for the following loci using Release: _____ of the YHRD: DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385, DYS393, DYS391, DYS439, DYS635, DYS392, YGATAH4, DYS437, DYS438, and DYS448.

(Yfiler® Plus loci)

This Y-STR profile has been observed (X) times in _____ profiles in the National United States Database contained within the Y-STR Haplotype Reference Database (YHRD) and is expected to occur in approximately 1 in _____ African-American males, 1 in _____ Asian males, 1 in _____ Caucasian males, 1 in _____ Hispanic males, and 1 in _____ Native American males.



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Statistics were calculated for the following loci using Release: _____ of the YHRD: DYS576, DYS389I, DYS635, DYS389II, DYS627, DYS460, DYS458, DYS19, YGATAH4, DYS448, DYS391, DYS456, DYS390, DYS438, DYS392, DYS518, DYS570, DYS437, DYS385, DYS449, DYS393, DYS439, DYS481, DYF387S1, and DYS533.

10. Request for Standards

Additional Y-STR DNA comparisons may be made upon submission of an appropriate male DNA standard(s).

11. Insufficient DNA for both GlobalFiler® and Yfiler® Plus

- This exhibit may be more suitable for Y-STR testing, therefore, no autosomal STR testing was performed. Y-STR testing may be performed upon receipt of a subject standard. Contact the undersigned analyst for additional information.

12. Inconclusive Minor Male Profile with GlobalFiler® Testing

- Y-STR testing may be performed upon request from the District Attorney General. Contact the undersigned analyst for additional information.
- Y-STR testing may be performed upon receipt of a subject standard and a request from the District Attorney General. Contact the undersigned analyst for additional information.

13. Penetration cases negative for screening and/or no probative GlobalFiler® result

- This case may be suitable for Y-STR testing. Y-STR testing may be performed upon receipt of a subject standard and a request from the District Attorney General. Contact the undersigned analyst for additional information.



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APPENDIX B

Critical Reagents

These reagents must be quality control checked prior to use in casework.

EZ1 DNA Investigator Kit:

Reagent Cartridges
Buffer G2 Lysis buffer (may be purchased separately from Qiagen)
Proteinase K (may be purchased separately from Qiagen)
Carrier RNA
Buffer MTL (purchased separately from Qiagen)
1.0M DTT (differential extraction procedure only)

Quantifiler® Trio DNA Quantification Kit:

Quantifiler® THP PCR Reaction Mix
Quantifiler® Trio Primer Mix
Quantifiler® THP DNA Standard
Quantifiler® THP Dilution Buffer

GlobalFiler® Amplification Kit:

GlobalFiler® Master Mix
GlobalFiler® Primer Set
DNA Control 007
GlobalFiler® Allelic Ladder

Yfiler® Plus Amplification Kit:

Yfiler® Plus Master Mix
Yfiler® Plus Primer Set
DNA Control 007
Yfiler® Plus Allelic Ladder

Extraction and Amplification Buffers and Reagents:

Sperm Extraction Buffer
Non-sperm Extraction Buffer
Sperm Wash Buffer
Stain Extraction Buffer
TE Buffer
PCI
0.39 M DTT
Proteinase K



Appendix C

Spatial Calibration: AB 3500 Genetic Analyzer

Introduction:

The 3500 Series Data Collection Software uses images collected during the spatial calibration to establish a relationship between the signal emitted by each capillary and the position where that signal falls on and is detected by the CCD camera.

When to perform a spatial calibration

- After removing or replacing the capillary array
- After opening the detector door or moving the detection cell
- After moving the instrument

1. Access the Spatial Calibration screen by selecting **Maintenance**, then selecting **Spatial Calibration** in the navigation pane.
2. Select No Fill, or select Fill to fill the array with polymer before starting the calibration.

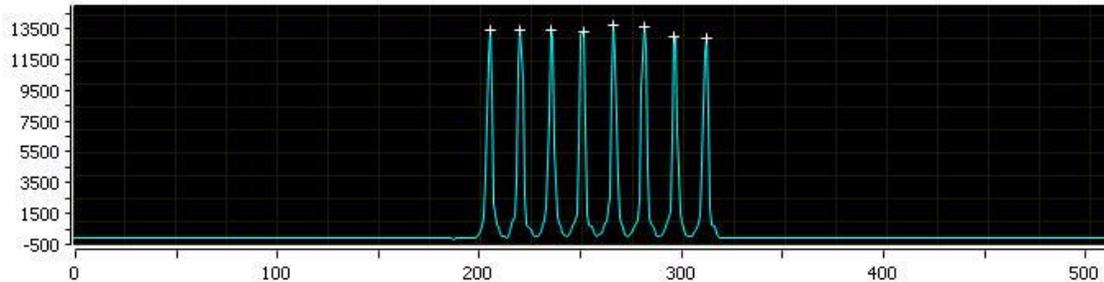
Select Perform QC Checks to check each capillary against the following specified ranges for spacing and intensity:

- Average peak height for 8-cap: 6400 RFU
 - Individual peak height: 1000 RFU
 - Uniformity (SD/average peak height): 0.2
 - Max. capillary spacing minus the Min. capillary spacing: 2 pixels
3. Click **Start Calibration**. The display will update as the run progresses. After completion of the run, evaluate the spatial calibration profile to ensure that you see:
 - One sharp peak for each capillary. Small shoulders are acceptable.
 - One marker (+) at the apex of every peak. No off-apex markers.
 - An even peak profile (all peaks about the same height).

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A spatial QC Check error will be displayed if:

- The average peak height or individual peak height is below threshold.
- Uniformity or capillary spacing exceeds the threshold.

The spacing between capillaries should be between 15 and 16.

Capillary	Position (pixels)	Spacing	Intensity
1	205	15	9014
2	220	16	9561
3	236	15	9253
4	251	15	9567
5	266	16	9883
6	282	15	9084
7	297	15	9700
8	312	0	8919

4. After completion of the run, evaluate the spatial calibration profile to ensure that you see:

- One sharp peak for each capillary. Small shoulders are acceptable.
- One marker (+) at the apex of every peak. No off-apex markers.
- An even peak profile (all peaks about the same height).

5. If the results meet the criteria above, click **Accept Results**.

Important: Do not close the software before clicking **Accept Results**. Spatial calibration results are not saved until you click Accept Results.

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6. If the results do not meet the criteria above, click **Reject Results** and rerun the spatial or consult the *Applied Biosystems 3500 Series User Guide* Rev. May 2012 page 268.

7. The spatial report may be saved by following these steps:
 - Click **View Spatial Calibration Report**.
 - Click **Print**.
 - In the Printer dialog box, select **CutePDF Writer** as the printer.
 - Specify a name and location for the report.

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Appendix D

AB 3500 Spectral Calibration Procedure Using Dye Set J6 (GlobalFiler® or Yfiler® Plus)

Introduction:

The DS-36 Matrix Standard (Dye Set J6) is used to perform the spectral calibration required to analyze 6-FAM™, VIC®, NED™, SID™, TAZ™, and LIZ® dye-labeled DNA fragments on the 3500 genetic analyzer. The Data Collection Software for these instruments uses the multicomponent matrix to automatically analyze the 6 different colored fluorescent dye-labeled samples in a single capillary. The DS-36 Matrix Standard (Dye Set J6) contains 6 specific sizes of a unique fluorescent dye label.

When to perform a spectral calibration:

- Change the capillary array
- Have a service engineer perform an optical service procedure, such as re-aligning or replacing the laser or CCD camera or mirrors on the instrument
- See a decrease in spectral separation (pull-up/pull-down in peaks) in the raw or analyzed data

Sample Preparation:

1. Thoroughly mix the contents of the tube and spin briefly in a microcentrifuge.
2. Prepare the DS-36 matrix standard (Dye Set J6) by combining 6 µL of standard with 294 µL of Hi-Di Formamide in a 1.5 mL microcentrifuge tube. The amounts may be adjusted if necessary e.g. 5 µL of standard to 295 µL of Hi-Di Formamide.
3. Dispense 10 µL of the Matrix Standard / Hi-Di Formamide mixture into wells A1 thru H1 on a 96-well plate.
4. Centrifuge the plate to ensure that the samples are at the bottom of the wells. Heat the mixture at 95° C for 5 minutes to denature the DNA fragments and then chill in a -20° C freezer.

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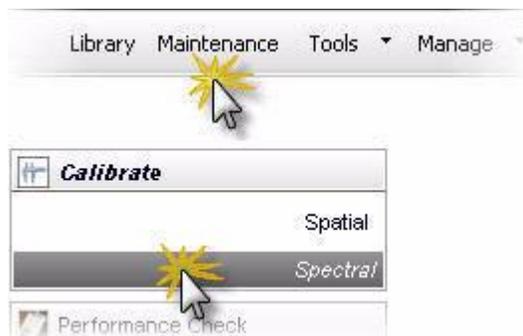
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NOTE: If the signal height of any one of the matrix standard fragments is saturated on the above instruments, prepare a new matrix standard mix with half the volume of matrix required in step 2. Rerun the spectral calibration.

Starting a Spectral Run on the 3500:

1. Access the Spectral Calibration screen:

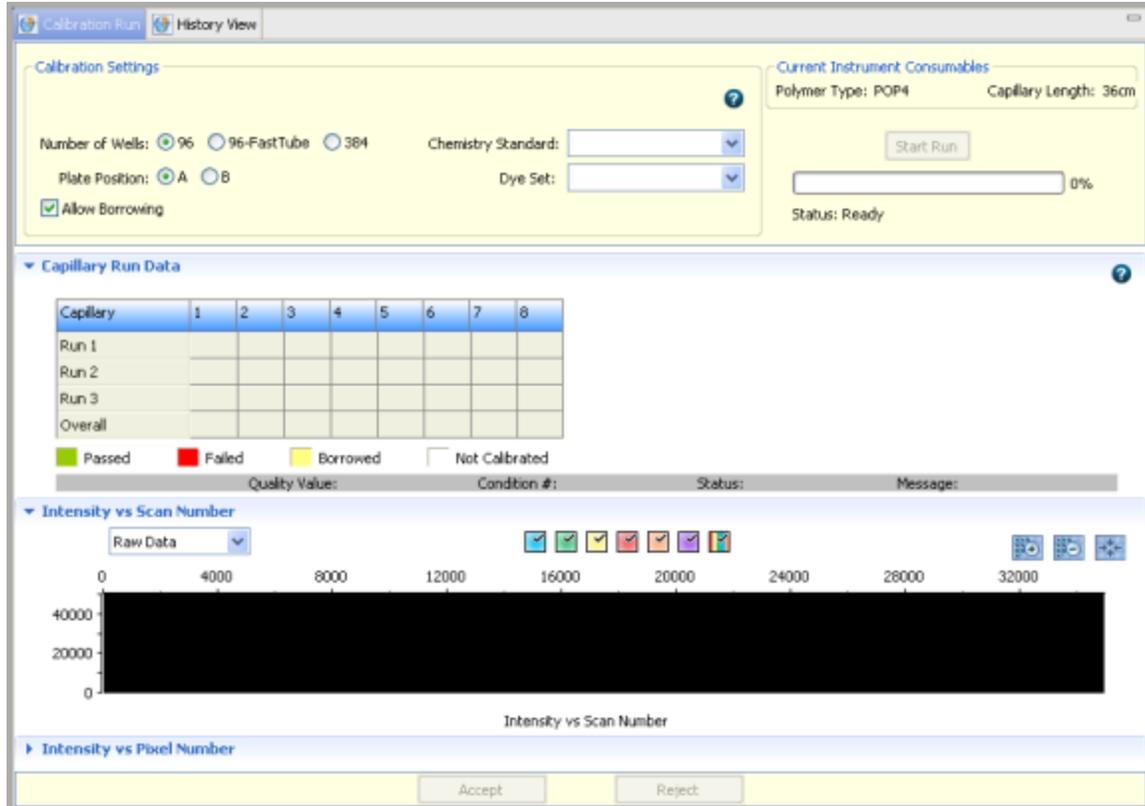
Select “**Maintenance**”, and then select “**Spectral Calibration**” in the Main Workflow.



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2. Select the number of wells in the spectral calibration plate as “96” and specify the plate position on the instrument as “A” or “B”.

Note: You do not create a plate for the calibration. The software uses predetermined positions for the calibration. You cannot specify standard location on the plate.

3. Select “**Matrix Standard**” for the chemistry standard and “**J6**” for the dye set.
4. Select **Allow Borrowing**. Selecting this option instructs the software to automatically replace information from a failed capillary with information from an adjacent passing capillary with the highest Quality value. Only one adjacent capillary borrowing event is allowed.
5. Click **Start Run**.
The following occurs:
 - The system sets up three injections:

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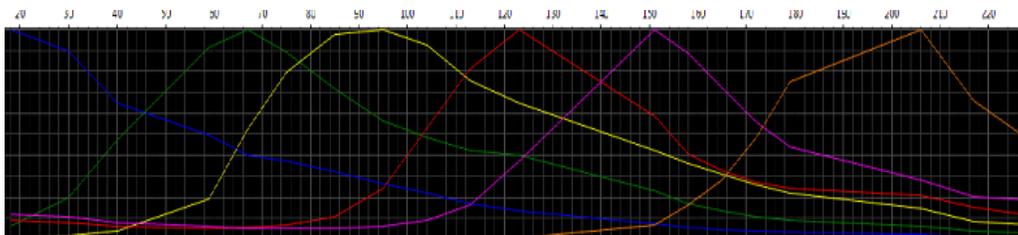
Injection 1: The software evaluates the Quality Value and Condition Number of all capillaries. If all capillaries pass, the calibration is complete, and injections 2 and 3 are not performed. If any capillaries fail in **Injection 1**, the software borrows from an adjacent capillary.

Injection 2: If, after borrowing, more than 1 capillary fails, **Injection 2** is performed. The software evaluates the quality values between adjacent capillaries in injection 2 and for each capillary across injections 1 and 2 and the information with the highest Quality Value for each capillary. If all capillaries pass, the calibration is complete and injection 3 is not performed.

Injection 3: If, after borrowing, more than 1 capillary from **Injection 1** or **2** do not pass, **Injection 3** is performed. The software evaluates the quality values between adjacent capillaries in **Injection 3** and for each capillary across **Injections 1, 2, and 3**, then the information with the highest Quality Value for each capillary. If all capillaries now pass, the calibration passes.

Note: If after borrowing, more than 1 capillary from **Injection 1, 2, or 3** do not pass, the calibration fails.

6. After completion of the injection(s), evaluate the spectral data. The minimum Quality Value for J6 is 0.95 and the Maximum Condition Number is 8.0.
7. Click each capillary to show the spectral and raw data. The order of the peaks in the spectral profile from left to right is blue-green-yellow-red-purple-orange.

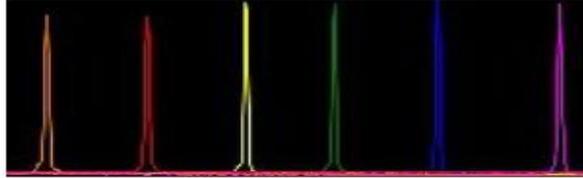


8. The order of the peaks in the raw data profile is orange-red-yellow-green-blue-purple.

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9. If the data for all capillaries meets the above criteria, click “**Accept Results**”.

10. If the spectral data does not meet the criteria above, click “**Reject Results**”. Refer to the *Applied Biosystems 3500 Series Users Guide* Revision date May 2012 pages 269-271.

11. A copy of the Spectral Calibration Report may be saved for record keeping since only the most recent spectral calibration is maintained in the software:
 - Click **View Spectral Calibration Report**.
 - Click **Print**.
 - In the Printer dialog box, select **CutePDF Writer** as the printer.
 - Specify a name such as “Spectral Reports” and location such as
 - “Documents” for the report to be saved

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APPENDIX E

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STR ANALYSIS USING THE 3500 GENETIC ANALYZER

Applied Biosystems 3500 / 3500xL Genetic Analyzer User Guide Revision date
May 2012

Genemapper ID-X Software version 1.0 Getting Started Guide Copyright 2007

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APPENDIX F

QUANTIFILER® TRIO MASTER MIX

**Add 12 controls and an additional 4 samples to your calculations to account for master mix loss during transfer*

Samples	Primer Mix (µL)	Reaction Mix (µL)		Samples	Primer Mix (µL)	Reaction Mix (µL)		Samples	Primer Mix (µL)	Reaction Mix (µL)
1	8.0	10.0		33	264.0	330.0		65	520.0	650.0
2	16.0	20.0		34	272.0	340.0		66	528.0	660.0
3	24.0	30.0		35	280.0	350.0		67	536.0	670.0
4	32.0	40.0		36	288.0	360.0		68	544.0	680.0
5	40.0	50.0		37	296.0	370.0		69	552.0	690.0
6	48.0	60.0		38	304.0	380.0		70	560.0	700.0
7	56.0	70.0		39	312.0	390.0		71	568.0	710.0
8	64.0	80.0		40	320.0	400.0		72	576.0	720.0
9	72.0	90.0		41	328.0	410.0		73	584.0	730.0
10	80.0	100.0		42	336.0	420.0		74	592.0	740.0
11	88.0	110.0		43	344.0	430.0		75	600.0	750.0
12	96.0	120.0		44	352.0	440.0		76	608.0	760.0
13	104.0	130.0		45	360.0	450.0		77	616.0	770.0
14	112.0	140.0		46	368.0	460.0		78	624.0	780.0
15	120.0	150.0		47	376.0	470.0		79	632.0	790.0
16	128.0	160.0		48	384.0	480.0		80	640.0	800.0
17	136.0	170.0		49	392.0	490.0		81	648.0	810.0
18	144.0	180.0		50	400.0	500.0		82	656.0	820.0
19	152.0	190.0		51	408.0	510.0		83	664.0	830.0
20	160.0	200.0		52	416.0	520.0		84	672.0	840.0
21	168.0	210.0		53	424.0	530.0		85	680.0	850.0
22	176.0	220.0		54	432.0	540.0		86	688.0	860.0
23	184.0	230.0		55	440.0	550.0		87	696.0	870.0
24	192.0	240.0		56	448.0	560.0		88	704.0	880.0
25	200.0	250.0		57	456.0	570.0		89	712.0	890.0
26	208.0	260.0		58	464.0	580.0		90	720.0	900.0
27	216.0	270.0		59	472.0	590.0		91	728.0	910.0
28	224.0	280.0		60	480.0	600.0		92	736.0	920.0
29	232.0	290.0		61	488.0	610.0		93	744.0	930.0
30	240.0	300.0		62	496.0	620.0		94	752.0	940.0
31	248.0	310.0		63	504.0	630.0		95	760.0	950.0
32	256.0	320.0		64	512.0	640.0		96	768.0	960.0

QF Trio Primer Mix = 8.0 µL x Total # of samples **Reaction Mix = 10.0 µL x Total # of samples**
 QF Plate Set-up: 18 µL QF Master Mix + 2µL DNA
 (20 µL Total Reaction Volume)



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APPENDIX G

GLOBALFILER® AMPLIFICATION MASTER MIX

**Add negative/positive controls and an additional 3 samples to your calculations to account for master mix loss during transfer*

Samples	GF Master Mix (µL)	GF Primer Set (µL)	Samples	GF Master Mix (µL)	GF Primer Set (µL)	Samples	GF Master Mix (µL)	GF Primer Set (µL)
1	7.5	2.5	33	247.5	82.5	65	487.5	162.5
2	15.0	5.0	34	255.0	85.0	66	495.0	165.0
3	22.5	7.5	35	262.5	87.5	67	502.5	167.5
4	30.0	10.0	36	270.0	90.0	68	510.0	170.0
5	37.5	12.5	37	277.5	92.5	69	517.5	172.5
6	45.0	15.0	38	285.0	95.0	70	525.0	175.0
7	52.5	17.5	39	292.5	97.5	71	532.5	177.5
8	60.0	20.0	40	300.0	100.0	72	540.0	180.0
9	67.5	22.5	41	307.5	102.5	73	547.5	182.5
10	75.0	25.0	42	315.0	105.0	74	555.0	185.0
11	82.5	27.5	43	322.5	107.5	75	562.5	187.5
12	90.0	30.0	44	330.0	110.0	76	570.0	190.0
13	97.5	32.5	45	337.5	112.5	77	577.5	192.5
14	105.0	35.0	46	345.0	115.0	78	585.0	195.0
15	112.5	37.5	47	352.5	117.5	79	592.5	197.5
16	120.0	40.0	48	360.0	120.0	80	600.0	200.0
17	127.5	42.5	49	367.5	122.5	81	607.5	202.5
18	135.0	45.0	50	375.0	125.0	82	615.0	205.0
19	142.5	47.5	51	382.5	127.5	83	622.5	207.5
20	150.0	50.0	52	390.0	130.0	84	630.0	210.0
21	157.5	52.5	53	397.5	132.5	85	637.5	212.5
22	165.0	55.0	54	405.0	135.0	86	645.0	215.0
23	172.5	57.5	55	412.5	137.5	87	652.5	217.5
24	180.0	60.0	56	420.0	140.0	88	660.0	220.0
25	187.5	62.5	57	427.5	142.5	89	667.5	222.5
26	195.0	65.0	58	435.0	145.0	90	675.0	225.0
27	202.5	67.5	59	442.5	147.5	91	682.5	227.5
28	210.0	70.0	60	450.0	150.0	92	690.0	230.0
29	217.5	72.5	61	457.5	152.5	93	697.5	232.5
30	225.0	75.0	62	465.0	155.0	94	705.0	235.0
31	232.5	77.5	63	472.5	157.5	95	712.5	237.5
32	240.0	80.0	64	480.0	160.0	96	720.0	240.0

GF Master Mix = 7.5 µL x Total # of samples GF Primer Set = 2.5 µL x Total # of samples
GF Amplification Set-up: 10 µL (GF Master Mix & Primer Set) + 15 µL (TE Buffer & DNA)
(25 µL Total Reaction Volume)



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Appendix H

Yfiler® Plus AMPLIFICATION MASTER MIX

**Add negative/positive controls and an additional 3 samples to your calculations to account for master mix loss during transfer*

Samples	YFP Master Mix (µL)	YFP Primer Set (µL)		Samples	YFP Master Mix (µL)	YFP Primer Set (µL)		Samples	YFP Master Mix (µL)	YFP Primer Set (µL)
1	10.0	5.0		33	330.0	165.0		65	650.0	325.0
2	20.0	10.0		34	340.0	170.0		66	660.0	330.0
3	30.0	15.0		35	350.0	175.0		67	670.0	335.0
4	40.0	20.0		36	360.0	180.0		68	680.0	340.0
5	50.0	25.0		37	370.0	185.0		69	690.0	345.0
6	60.0	30.0		38	380.0	190.0		70	700.0	350.0
7	70.0	35.0		39	390.0	195.0		71	710.0	355.0
8	80.0	40.0		40	400.0	200.0		72	720.0	360.0
9	90.0	45.0		41	410.0	205.0		73	730.0	365.0
10	100.0	50.0		42	420.0	210.0		74	740.0	370.0
11	110.0	55.0		43	430.0	215.0		75	750.0	375.0
12	120.0	60.0		44	440.0	220.0		76	760.0	380.0
13	130.0	65.0		45	450.0	225.0		77	770.0	385.0
14	140.0	70.0		46	460.0	230.0		78	780.0	390.0
15	150.0	75.0		47	470.0	235.0		79	790.0	395.0
16	160.0	80.0		48	480.0	240.0		80	800.0	400.0
17	170.0	85.0		49	490.0	245.0		81	810.0	405.0
18	180.0	90.0		50	500.0	250.0		82	820.0	410.0
19	190.0	95.0		51	510.0	255.0		83	830.0	415.0
20	200.0	100.0		52	520.0	260.0		84	840.0	420.0
21	210.0	105.0		53	530.0	265.0		85	850.0	425.0
22	220.0	110.0		54	540.0	270.0		86	860.0	430.0
23	230.0	115.0		55	550.0	275.0		87	870.0	435.0
24	240.0	120.0		56	560.0	280.0		88	880.0	440.0
25	250.0	125.0		57	570.0	285.0		89	890.0	445.0
26	260.0	130.0		58	580.0	290.0		90	900.0	450.0
27	270.0	135.0		59	590.0	295.0		91	910.0	455.0
28	280.0	140.0		60	600.0	300.0		92	920.0	460.0
29	290.0	145.0		61	610.0	305.0		93	930.0	465.0
30	300.0	150.0		62	620.0	310.0		94	940.0	470.0
31	310.0	155.0		63	630.0	315.0		95	950.0	475.0
32	320.0	160.0		64	640.0	320.0		96	960.0	480.0

YFP Master Mix = 10.0 µL x Total # of samples **YFP Primer Set = 5.0 µL x Total # of samples**
YFP Amplification Set-up: 15 µL (YFP Master Mix & Primer Set) + 10 µL (TE Buffer & DNA)
(25 µL Total Reaction Volume)



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APPENDIX I

GlobalFiler® and Yfiler® Plus 3500 STR MASTER MIX

**Add 4 samples to your calculations to account for master mix loss during transfer*

Samples	HiDi Formamide (µL)	LIZ600 (µL)	Samples	HiDi Formamide (µL)	LIZ600 (µL)	Samples	HiDi Formamide (µL)	LIZ600 (µL)
1	9.6	0.4	33	316.8	13.2	65	624.0	26.0
2	19.2	0.8	34	326.4	13.6	66	633.6	26.4
3	28.8	1.2	35	336.0	14.0	67	643.2	26.8
4	38.4	1.6	36	345.6	14.4	68	652.8	27.2
5	48.0	2.0	37	355.2	14.8	69	662.4	27.6
6	57.6	2.4	38	364.8	15.2	70	672.0	28.0
7	67.2	2.8	39	374.4	15.6	71	681.6	28.4
8	76.8	3.2	40	384.0	16.0	72	691.2	28.8
9	86.4	3.6	41	393.6	16.4	73	700.8	29.2
10	96.0	4.0	42	403.2	16.8	74	710.4	29.6
11	105.6	4.4	43	412.8	17.2	75	720.0	30.0
12	115.2	4.8	44	422.4	17.6	76	729.6	30.4
13	124.8	5.2	45	432.0	18.0	77	739.2	30.8
14	134.4	5.6	46	441.6	18.4	78	748.8	31.2
15	144.0	6.0	47	451.2	18.8	79	758.4	31.6
16	153.6	6.4	48	460.8	19.2	80	768.0	32.0
17	163.2	6.8	49	470.4	19.6	81	777.6	32.4
18	172.8	7.2	50	480.0	20.0	82	787.2	32.8
19	182.4	7.6	51	489.6	20.4	83	796.8	33.2
20	192.0	8.0	52	499.2	20.8	84	806.4	33.6
21	201.6	8.4	53	508.8	21.2	85	816.0	34.0
22	211.2	8.8	54	518.4	21.6	86	825.6	34.4
23	220.8	9.2	55	528.0	22.0	87	835.2	34.8
24	230.4	9.6	56	537.6	22.4	88	844.8	35.2
25	240.0	10.0	57	547.2	22.8	89	854.4	35.6
26	249.6	10.4	58	556.8	23.2	90	864.0	36.0
27	259.2	10.8	59	566.4	23.6	91	873.6	36.4
28	268.8	11.2	60	576.0	24.0	92	883.2	36.8
29	278.4	11.6	61	585.6	24.4	93	892.8	37.2
30	288.0	12.0	62	595.2	24.8	94	902.4	37.6
31	297.6	12.4	63	604.8	25.2	95	912.0	38.0
32	307.2	12.8	64	614.4	25.6	96	921.6	38.4

HiDi Formamide = 9.6 µL x Total # of samples LIZ 600 v.2.0 = 0.4 µL x Total # of samples
GF and YFP 3500 STR Set-up: 10.0 µL (HiDi Formamide & LIZ600 v.2.0) to 1.0 µL (DNA or Allelic Ladder)
(11 µL Total Volume)