PowerPlex® ESI 17 Fast System for Use on the Applied Biosystems® Genetic Analyzers

Instructions for Use of Products
DC1720 and DC1721

PowerPlex® ESI 17 Fast System for Use on the Applied Biosystems® Genetic Analyzers

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: genetic@promega.com

1. Description

STR (short tandem repeat) loci consist of short, repetitive sequence elements 3–7 base pairs in length (1–4). These repeats are well distributed throughout the human genome and are a rich source of highly polymorphic markers, which may be detected using the polymerase chain reaction (5–9). Alleles of STR loci are differentiated by the number of copies of the repeat sequence contained within the amplified region and are distinguished from one another using fluorescence detection following electrophoretic separation.

The PowerPlex® ESI 17 Fast System[a,b] is used for human identification applications including forensic analysis, relationship testing and research use. This system allows co-amplification and four-color fluorescent detection of seventeen loci (sixteen STR loci and Amelogenin), including D22S1045, D2S1338, D19S433, D3S1358, Amelogenin, D2S441, D10S1248, D1S1656, D18S51, D16S539, D12S391, D21S11, vWA, TH01, SE33, FGA and D8S1179.

The PowerPlex® ESI 17 Fast System is designed with six of the original seven European Standard Set (ESS) loci (D3S1358, D18S51, TH01, vWA, D8S1179 and the more common FGA alleles) along with D16S539 and D19S433 as smaller amplicons (<250bp), while the loci recommended by the European Network of Forensic Science Institutes (ENFSI) and European DNA Profiling Group (EDNAP) (D1S1656, D2S441, D10S1248, D12S391 and D22S1045) are present as larger amplicons. A complementary system, the PowerPlex® ESX 17 Fast System, amplifies the same seventeen loci present in the PowerPlex® ESI 17 Fast System but with the new ENFSI/EDNAP loci designed as mini-STRs (<125bp; D2S441, D10S1248 and D22S1045) or midi-STRs (125–185bp; D1S1656 and D12S391). Therefore, these two STR systems can be used to complement each other when analyzing degraded or challenging samples to maximize recovery of allelic information from as many loci as possible and allow confirmation of results obtained with the other system.

The PowerPlex® ESI 17 Fast System and all system components are manufactured in accordance with ISO 18385:2016. All necessary materials are provided to amplify STR regions of human genomic DNA, including a hot-start thermostable DNA polymerase, which is a component of the PowerPlex® ESI/ESX Fast 5X Master Mix.

This manual contains a protocol for use of the PowerPlex® ESI 17 Fast System with the GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler and ProFlex® PCR System in addition to protocols to separate amplified products and detect separated material on the Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers. Protocols to operate the fluorescence-detection instruments should be obtained from the instrument manufacturer. Amplification and detection instrumentation may vary. You may need to optimize protocols including amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. In-house validation should be performed.

Information about other Promega fluorescent STR systems is available upon request from Promega or online at: **www.promega.com**

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
PowerPlex® ESI 17 Fast System	**100 reactions**	**DC1721**

Not For Medical Diagnostic Use. This system contains sufficient reagents for 100 reactions of 25µl each. Includes:

Pre-amplification Components Box
- 500µl   PowerPlex® ESI/ESX Fast 5X Master Mix
- 250µl   PowerPlex® ESI 17 Fast 10X Primer Pair Mix
- 25µl   2800M Control DNA, 10ng/µl
- 5 × 1,250µl   Water, Amplification Grade

Post-amplification Components Box
- 50µl   PowerPlex® ESI 17 Fast Allelic Ladder Mix
- 200µl   WEN Internal Lane Standard 500 ESS

PRODUCT	SIZE	CAT.#
PowerPlex® ESI 17 Fast System	**400 reactions**	**DC1720**

Not For Medical Diagnostic Use. This system contains sufficient reagents for 400 reactions of 25µl each. Includes:

Pre-amplification Components Box
- 4 × 500µl   PowerPlex® ESI/ESX Fast 5X Master Mix
- 4 × 250µl   PowerPlex® ESI 17 Fast 10X Primer Pair Mix
- 25µl   2800M Control DNA, 10ng/µl
- 10 × 1,250µl   Water, Amplification Grade

Post-amplification Components Box
- 4 × 50µl   PowerPlex® ESI 17 Fast Allelic Ladder Mix
- 2 × 200µl   WEN Internal Lane Standard 500 ESS

(!) The PowerPlex® ESI 17 Fast Allelic Ladder Mix is provided in a separate, sealed bag for shipping. This component should be moved to the post-amplification box after opening. The Water, Amplification Grade, is provided in a separate, sealed bag for shipping. This component should be moved to the pre-amplification box after opening.

Storage Conditions: Upon receipt, store all components at −30°C to −10°C in a nonfrost-free freezer. Make sure that the 2800M Control DNA is stored at +2°C to +10°C for **at least 24 hours** before use; do not refreeze. After the first use, store the WEN Internal Lane Standard (WEN ILS) 500 ESS at +2°C to +10°C, protected from light; do not refreeze. The PowerPlex® ESI 17 Fast 10X Primer Pair Mix, PowerPlex® ESI 17 Fast Allelic Ladder Mix and WEN ILS 500 ESS are light-sensitive and must be stored in the dark. We strongly recommend that pre-amplification and post-amplification reagents be stored and used separately with different pipettes, tube racks, etc.

Optional: The PowerPlex® ESI 17 Fast System components may be stored for up to 1 year at +2°C to +10°C without loss of activity.

Available Separately

PRODUCT	SIZE	CAT.#
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
5X AmpSolution™ Reagent	500µl	DM1231

The PunchSolution™ Kit is required to process nonlytic storage card punches prior to direct amplification. The SwabSolution™ Kit is required to process swabs prior to direct amplification. The 5X AmpSolution™ Reagent is required for direct amplification of DNA from storage card punches and swab extracts. Both the PunchSolution™ Kit and SwabSolution™ Kit contain the 5X AmpSolution™ Reagent.

The proper panels, bins and stutter text files and size standard .xml files for use with GeneMapper® *ID* and *ID*-X software can be downloaded at: **www.promega.com/resources/software-firmware/str-analysis/genemapper-id-software-panels-and-bin-sets/**

Matrix standards are required for initial setup of the color separation matrix. Matrix standards are provided separately and are available for the Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers (PowerPlex® 5C Matrix Standard, Cat.# DG4850).

3.   Before You Begin

3.A. Precautions

The application of PCR-based typing for forensic or paternity casework requires validation studies and quality-control measures that are not contained in this manual (10,11). Guidelines for the validation process are published in the *Internal Validation Guide of Autosomal STR Systems for Forensic Laboratories* (12).

The quality of purified DNA or direct-amplification samples, small changes in buffers, ionic strength, primer concentrations, reaction volume, choice of thermal cycler and thermal cycling conditions can affect PCR success. We suggest strict adherence to recommended procedures for amplification and fluorescence detection. Additional research and validation are required if any modifications to the recommended protocols are made.

PCR-based STR analysis is subject to contamination by very small amounts of human DNA. Extreme care should be taken to avoid cross-contamination when preparing template DNA, handling primer pairs, assembling amplification reactions and analyzing amplification products. Reagents and materials used prior to amplification (Master Mix, Primer Pair Mix, 2800M Control DNA and Water, Amplification Grade) are provided in a separate box and should be stored separately from those used following amplification (Allelic Ladder Mix and Internal Lane Standard). Always include a negative control reaction (i.e., no template) to detect reagent contamination. We highly recommend the use of gloves and aerosol-resistant pipette tips.

3.A. Precautions (continued)

Some reagents used in the analysis of STR products are potentially hazardous and should be handled accordingly. Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label, and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

3.B. Spectral Calibration

Proper spectral calibration is critical to evaluate multicolor systems with the Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers. A matrix must be generated for each individual instrument.

The PowerPlex® 5C Matrix Standard (Cat.# DG4850) is required for spectral calibration on the Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers.

For protocols and additional information about spectral calibration, see the *PowerPlex® 5C Matrix Standard Technical Manual* #TMD049. This manual is available online at: **www.promega.com/protocols/**

4. Protocols for DNA Amplification Using the PowerPlex® ESI 17 Fast System

The PowerPlex® ESI 17 Fast System was developed for amplification of extracted DNA and direct-amplification samples. Slight protocol variations are recommended for optimal performance with each template source. Protocols for amplification in a 25μl reaction volume using extracted DNA (Section 4.A), lytic and nonlytic storage card punches (Section 4.B) and swabs (Section 4.C) are included in the following amplification sections. Protocols for amplification in a 12.5μl reaction volume using lytic and nonlytic storage card punches and swabs are included in Sections 9.C and 9.D, respectively.

The PowerPlex® ESI 17 Fast System is compatible with the GeneAmp® PCR System 9700 thermal cycler with a gold-plated silver or silver sample block, the Veriti® 96-Well Thermal Cycler and the ProFlex® PCR System.

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is **not** recommended with the PowerPlex® ESI 17 Fast System.

The use of gloves and aerosol-resistant pipette tips is highly recommended to prevent cross-contamination. Keep all pre-amplification and post-amplification reagents in separate rooms. Prepare amplification reactions in a room dedicated for reaction setup. Use equipment and supplies dedicated for amplification setup.

Meticulous care must be taken to ensure successful amplification. A guide to amplification troubleshooting is provided in Section 7.

4.A. Amplification of Extracted DNA in a 25µl Reaction Volume

The PowerPlex® ESI 17 Fast System is optimized and balanced for 0.5ng of DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different. Testing at Promega shows that 30 cycles works well for 0.5ng of purified DNA templates. Developmental validation of the system showed routine generation of full profiles using 30 cycles of amplification with lower amounts of DNA template down to 62.5pg. Partial profiles were typically observed for DNA template amounts of 32pg and below (13). In-house optimization and validation should be performed to establish the performance of the system in your laboratory (12).

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block, Veriti® 96-Well Thermal Cycler or ProFlex® PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plates or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips

Amplification Setup

1. Thaw the PowerPlex® ESI/ESX Fast 5X Master Mix, PowerPlex® ESI 17 Fast 10X Primer Pair Mix and Water, Amplification Grade, completely.

   Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 10X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.

3. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.

4.A. Amplification of Extracted DNA in a 25µl Reaction Volume

4. Add the final volume of each reagent listed in Table 1 to a clean tube.

Table 1. PCR Amplification Mix for Amplification of Extracted DNA.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25.0µl	×		=	
PowerPlex® ESI/ESX Fast 5X Master Mix	5.0µl	×		=	
PowerPlex® ESI 17 Fast 10X Primer Pair Mix	2.5µl	×		=	
template DNA (0.5ng)[2,3,4]	up to 17.5µl				
total reaction volume	25.0µl				

[1]Add Water, Amplification Grade, to the tube first, and then add PowerPlex® ESI/ESX Fast 5X Master Mix and PowerPlex® ESI 17 Fast 10X Primer Pair Mix. The template DNA will be added at Step 6.

[2]Store DNA templates in TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE^{-4} buffer with 20µg/ml glycogen. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the volume of DNA added should not exceed 20% of the final reaction volume. PCR amplification efficiency and quality can be greatly altered by changes in pH (due to added Tris-HCl), available magnesium concentration (due to chelation by EDTA) or other PCR inhibitors, which may be present at low concentrations depending on the source of the template DNA and the extraction procedure used.

[3]Apparent DNA concentrations can differ, depending on the DNA quantification method used (14). We strongly recommend that you perform experiments to determine the optimal DNA amount based on your DNA quantification method and internal validation.

[4]The PowerPlex® ESI 17 Fast System is optimized and balanced for 0.5ng of DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different.

5. Vortex the PCR amplification mix for 5–10 seconds, and then pipet PCR amplification mix into each reaction well.

Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

Note: Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add DNA as soon as possible to each well and follow immediately by thermal cycling.

6. Add template DNA for each sample to the respective well containing PCR amplification mix.

Note: The PowerPlex® ESI 17 Fast System is optimized and balanced for 0.5ng of DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 0.5ng in the desired template volume. Add 0.5ng of diluted DNA to a reaction well containing PCR amplification mix.

8. For the negative amplification control, pipet Water, Amplification Grade, or TE^{-4} buffer instead of template DNA into a reaction well containing PCR amplification mix.

9. Seal or cap the plate, or close the tubes.
   Optional: Briefly centrifuge the plate to bring contents to the bottom of the wells and remove any air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You may need to optimize protocols including the amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 30 cycles work well for 0.5ng of purified DNA templates. In-house validation should be performed.

1. Place the reaction plate or tubes in the thermal cycler.

2. Select and run the recommended protocol below.

   Notes:

   a. When using the ProFlex®PCR System, use the 9700 Simulation Mode as the ramp speed.

   b. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.

   c. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol
96°C for 1 minute, then:
96°C for 5 seconds 60°C for 35 seconds 72°C for 5 seconds for 30 cycles, then:
60°C for 2 minutes
4°C soak

3. After completion of the thermal cycling protocol, proceed to fragment analysis or store amplified samples at −20°C protected from light.
   Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

4.B. Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block, Veriti® 96-Well Thermal Cycler or ProFlex® PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- PunchSolution™ Kit (Cat.# DC9271) for nonlytic storage card punches
- 5X AmpSolution™ Reagent (Cat.# DM1231, also supplied with the PunchSolution™ Kit)
- 1.2mm Harris Micro-Punch or equivalent manual punch and cutting mat or automated punch system

This section contains a protocol for direct amplification of DNA from storage card punches in a 25µl reaction volume using the PowerPlex® ESI 17 Fast System and GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler or ProFlex® PCR System. A protocol for direct amplification of DNA from storage card punches in a 12.5µl reaction volume is provided in Section 9.C.

When using the protocol detailed below, add the number of 1.2mm storage card punches indicated below to each 25µl amplification reaction.

Note: You will need to optimize and validate the number of storage card punches per reaction in your laboratory. See the PCR Optimization recommendations at the end of this section.

Lytic storage card sample types include:

- Buccal cells collected on FTA® cards with Whatman EasiCollect™ devices (one or two punches per 25µl amplification reaction)
- Buccal cells collected with swabs transferred to FTA® or Indicating FTA® cards (one or two punches per 25µl amplification reaction)
- Liquid blood (from collection or storage Vacutainer® tubes or finger sticks) spotted onto FTA® cards (one punch per 25µl amplification reaction)

Nonlytic storage card sample types include:

- Buccal samples on Bode Buccal DNA Collector™ devices (one punch per 25µl amplification reaction)
- Blood and buccal samples on nonlytic storage cards (e.g., S&S 903; one punch per 25µl amplification reaction)

Pretreat nonlytic sample types with the PunchSolution™ Kit (Cat.# DC9271) to lyse samples before adding the PCR amplification mix. For more information, see the *PunchSolution™ Kit Technical Manual* #TMD038. Failure to pretreat these samples may result in incomplete profiles.

Use a manual punch tool with a 1.2mm tip to manually create sample disks from a storage card. Place tip near the center of the sample spot, and with a twisting or pressing action, cut a 1.2mm sample disk. Use the plunger to eject the disk into the appropriate well of a reaction plate.

Automated punchers also can be used to create sample disks. Refer to the user's guide for your instrument for assistance with generating 1.2mm disks, technical advice and troubleshooting information.

Note: Static may be problematic when adding a punch to a well. For lytic storage card punches, adding PCR amplification mix to the well before adding the punch may help alleviate static problems. For nonlytic storage card punches, adding PunchSolution™ Reagent to the well before adding the punch during pretreatment may help alleviate static problems.

Amplification Setup

1. Thaw the PowerPlex® ESI/ESX Fast 5X Master Mix, PowerPlex® ESI 17 Fast 10X Primer Pair Mix and Water, Amplification Grade, completely.

    Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 10X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Vortex the 5X AmpSolution™ Reagent for 10–15 seconds.

    Note: The 5X AmpSolution™ Reagent should be thawed completely, mixed by vortexing and stored at +2°C to +10°C. The reagent may be turbid after thawing or storage at +2°C to +10°C. If this occurs, warm the buffer briefly at 37°C, and then vortex until clear. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Storing reagents in the refrigerator door can compromise stability.

3. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.

4. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.

4.B. Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume (continued)

5. Add the final volume of each reagent listed in Table 2 to a clean tube.

Table 2. PCR Amplification Mix for Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	12.5µl	×		=	
PowerPlex® ESI/ESX Fast 5X Master Mix	5.0µl	×		=	
PowerPlex® ESI 17 Fast 10X Primer Pair Mix	2.5µl	×		=	
5X AmpSolution™ Reagent	5.0µl	×		=	
total reaction volume	25.0µl				

[1]Add Water, Amplification Grade, to the tube first, and then add PowerPlex® ESI/ESX Fast 5X Master Mix, PowerPlex® ESI 17 Fast 10X Primer Pair Mix and 5X AmpSolution™ Reagent. For lytic storage card punches, the template DNA will be added at Step 7.

6. Vortex the PCR amplification mix for 5–10 seconds, and then pipet 25µl of PCR amplification mix into each reaction well.

⊘ Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

Note: Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the punches as soon as possible to each well and follow immediately by thermal cycling.

7. For lytic storage cards, add one or two 1.2mm punches from a card containing a buccal sample or one 1.2mm punch from a card containing whole blood to the appropriate wells of the reaction plate. For nonlytic storage card punches, add the PCR amplification mix to the PunchSolution™ Reagent-treated punches.

Note: It also is acceptable to add the lytic storage card punch first, and then add the PCR amplification mix.

8. For the positive amplification control, vortex the tube of 2800M Control DNA then add 1µl (10ng) to a reaction well containing 25µl of PCR amplification mix.

Notes:

   a. Do not include blank storage card punches in the positive control reactions.

   b. Optimization of the amount of 2800M Control DNA may be required, depending on thermal cycling conditions and laboratory preferences.

9. Reserve a well containing PCR amplification mix as a negative amplification control.

   Note: An additional negative control with a blank punch may be performed to detect contamination from the storage card or punch device.

10. Seal or cap the plate, or close the tubes. Briefly centrifuge the plate to bring storage card punches to the bottom of the wells and remove any air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including the number of storage card punches, cycle number, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 26 cycles works well for a variety of storage card sample types. Cycle number will need to be optimized in each laboratory for each sample type.

1. Place the reaction plate or tubes in the thermal cycler.

2. Select and run the recommended protocol below.

   Notes:

   a. When using the ProFlex® PCR System, use the 9700 Simulation Mode as the ramp speed.

   b. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.

   c. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol
96°C for 1 minute, then:
96°C for 5 seconds 60°C for 35 seconds 72°C for 5 seconds for 26 cycles, then:
60°C for 2 minutes
4°C soak

3. After completion of the thermal cycling protocol, proceed to fragment analysis or store amplified samples at −20°C protected from light.

   Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

PCR Optimization

Cycle number should be optimized based on the results of an initial experiment to determine the sensitivity with your collection method, sample types, number of punches and instrumentation.

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.

2. Depending on your preferred protocol, place one or two 1.2mm storage card punches containing a buccal sample or one 1.2mm storage card punch containing whole blood into each well of a reaction plate. Be sure to pretreat nonlytic storage card samples with the PunchSolution™ Kit (Cat.# DC9271).

3. Prepare three identical reaction plates with punches from the same samples.

4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (25, 26 and 27 cycles).

5. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type and number of storage card punches.

4.C. Direct Amplification of DNA from Swabs in a 25µl Reaction Volume

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block, Veriti® 96-Well Thermal Cycler or ProFlex® PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- SwabSolution™ Kit (Cat.# DC8271)

This section contains a protocol for amplifying DNA from swab extracts in a 25µl reaction volume using the PowerPlex® ESI 17 Fast System and GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler or ProFlex® PCR System. A protocol for direct amplification of DNA from swab extracts in a 12.5µl reaction volume is provided in Section 9.D.

Pretreat OmniSwab™ (QIAGEN) or cotton swabs using the SwabSolution™ Kit (Cat.# DC8271) as described in the *SwabSolution™ Kit Technical Manual* #TMD037 to generate a swab extract.

Amplification Setup

1. Thaw the PowerPlex® ESI/ESX Fast 5X Master Mix, PowerPlex® ESI 17 Fast 10X Primer Pair Mix and Water, Amplification Grade, completely.

   Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 10X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Vortex the 5X AmpSolution™ Reagent for 10–15 seconds.

    Note: The 5X AmpSolution™ Reagent should be thawed completely, mixed by vortexing and stored at +2°C to +10°C. The reagent may be turbid after thawing or storage at +2°C to +10°C. If this occurs, warm the buffer briefly at 37°C, and then vortex until clear. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Storing reagents in the refrigerator door can compromise stability.

3. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.

4. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.

5. Add the final volume of each reagent listed in Table 3 to a clean tube.

    Table 3. PCR Amplification Mix for Direct Amplification of DNA from Swabs in a 25µl Reaction Volume.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	10.5µl	×		=	
PowerPlex® ESI/ESX Fast 5X Master Mix	5.0µl	×		=	
PowerPlex® ESI 17 Fast 10X Primer Pair Mix	2.5µl	×		=	
5X AmpSolution™ Reagent	5.0µl	×		=	
swab extract	2.0µl				
total reaction volume	25.0µl				

    [1]Add Water, Amplification Grade, to the tube first, and then add PowerPlex® ESI/ESX Fast 5X Master Mix, PowerPlex® ESI 17 Fast 10X Primer Pair Mix and 5X AmpSolution™ Reagent. The swab extract will be added at Step 7.

6. Vortex the PCR amplification mix for 5–10 seconds, and then pipet 23µl of PCR amplification mix into each reaction well.

    ⓘ Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

    Note: Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the swab extract as soon as possible to each well and follow immediately by thermal cycling.

7. Pipet 2µl of swab extract for each sample into the appropriate well of the reaction plate.

8. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 5ng/µl. Add 2µl (10ng) of the diluted 2800M Control DNA to a reaction well containing 23µl of PCR amplification mix.

    Note: Optimization of the amount of 2800M Control DNA may be required depending on thermal cycling conditions and laboratory preferences.

4.C. Direct Amplification of DNA from Swabs in a 25µl Reaction Volume (continued)

9. For the negative amplification control, pipet 2µl of Water, Amplification Grade, or TE^{-4} buffer instead of swab extract into a reaction well containing PCR amplification mix.

   Note: Additional negative controls can be included. Assemble a reaction containing the swab extract prepared from a blank swab, or assemble a reaction where the SwabSolution™ Reagent is processed as a blank without a swab.

10. Seal or cap the plate, or close the tubes.

    Optional: Briefly centrifuge the plate to bring contents to the bottom of the wells and remove any air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including the amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 26 cycles works well for a variety of swab sample types. Cycle number will need to be optimized in each laboratory for each sample type.

1. Place the reaction plate or tubes in the thermal cycler.

2. Select and run the recommended protocol below.

   Notes:

   a. When using the ProFlex® PCR System, use the 9700 Simulation Mode as the ramp speed.

   b. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.

   c. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol
96°C for 1 minute, then:
96°C for 5 seconds 60°C for 35 seconds 72°C for 5 seconds for 26 cycles, then:
60°C for 2 minutes
4°C soak

3. After completion of the thermal cycling protocol, proceed to fragment analysis or store amplified samples at −20°C protected from light.

   Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

PCR Optimization

Cycle number should be optimized based on the results of an initial experiment to determine the sensitivity with your collection method, sample types and instrumentation.

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.

2. Prepare three identical reaction plates with aliquots of the same swab extracts.

3. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (25, 26 and 27 cycles).

    Note: This recommendation is for 2µl of swab extract. If a different volume of swab extract is used, additional cycle number testing may be required.

4. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type.

5.   Instrument Setup and Sample Preparation

5.A.   Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer

Materials to Be Supplied by the User
*   95°C dry heating block, water bath or thermal cycler
*   crushed ice, ice-water bath or freezer plate block
*   centrifuge compatible with 96-well plates
*   aerosol-resistant pipette tips
*   3500/3500xL capillary array, 36cm
*   96-well retainer & base set (standard) (Applied Biosystems Cat.# 4410228)
*   POP-4® polymer for the Applied Biosystems® 3500 or 3500xL Genetic Analyzer
*   anode buffer container
*   cathode buffer container
*   MicroAmp® optical 96-well plate (or equivalent) and septa (Applied Biosystems)
*   Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)

(!) The quality of formamide is critical. Use only the recommended formamide. Freeze formamide in aliquots at −20°C. Multiple freeze-thaw cycles or long-term storage at 4°C may cause breakdown of formamide. Poor-quality formamide may contain ions that compete with DNA during injection, which results in lower peak heights and reduced sensitivity. A longer injection time may not increase the signal.

(!) Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label, and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

Sample Preparation

1. Prepare a loading cocktail by combining and mixing internal lane standard (ILS) and formamide as follows:

    $[(0.5\mu l\ ILS) \times (\#\ samples)] + [(9.5\mu l\ formamide) \times (\#\ samples)]$

    Note: The volume of internal lane standard used in the loading cocktail can be adjusted to change the intensity of the size standard peaks based on laboratory preferences.

2. Vortex for 10–15 seconds to mix.

3. Pipet 10µl of formamide/internal lane standard mix into each well.

4. Add 1µl of amplified sample (or 1µl of Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

    Notes:

    a. Instrument detection limits vary; therefore, injection time, injection voltage or the amount of product mixed with loading cocktail may need to be increased or decreased. To modify the injection time or injection voltage in the run module, select **Instrument Protocol** from the Library menu in the data collection software. If peak heights are higher than desired, use less DNA template in the amplification reactions or reduce the number of cycles in the amplification program to achieve the desired signal intensity.

    b. Use a volume of allelic ladder that results in peak heights that are all consistently above the peak amplitude threshold determined as part of your internal validation.

5. Centrifuge plate briefly to remove air bubbles from the wells.

6. Denature samples at 95°C for 3 minutes, and then immediately chill on crushed ice, a freezer plate block or in an ice-water bath for 3 minutes. Denature samples just prior to loading the instrument.

Instrument Preparation

Refer to the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide* for the instrument maintenance schedule and instructions to install the capillary array, buffers and polymer pouch and perform a spatial calibration. Samples may be analyzed as described in the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide*.

1. Open the 3500 Data Collection Software. The Dashboard screen will launch (Figure 1). To ensure you are viewing the most up-to-date information, press the **Refresh** button. Ensure that the Consumables Information and Maintenance Notifications are acceptable.

    Set the oven temperature to 60°C, and then select **Start Pre-heat**. When the Oven Temperature and Detection Cell Temperature turn green, you can proceed with the first injection.

Figure 1. The Dashboard.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

2. Prior to the first analysis using the PowerPlex® ESI 17 Fast System, you must create an Instrument Protocol, Size Standard, QC Protocol, Assay, File Name Convention and Results Group.

   2.a. To create a new Instrument Protocol, navigate to the Library, select **Instrument Protocol**, and then select **Create**. Alternatively, a previously created instrument protocol may be used.

   Figure 2 shows the settings used at Promega for the Applied Biosystems® 3500xL Genetic Analyzer for the application type, dye set, capillary length, polymer, run module and appropriate protocol information. The only setting that was changed from the default settings is dye set.

   When creating a new Instrument Protocol, be sure to select the same dye set that was used to perform the Promega 5-dye spectral calibration.

   Run time and other instrument settings should be optimized and validated in your laboratory.

   When optimizing injection conditions in your laboratory, you may choose to create specific Instrument Protocols for each condition tested. If a single Instrument Protocol is used, follow the instructions in the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide* to edit a library entry.

   Assign a descriptive protocol name.

   Note: For more detailed information refer to the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide.*
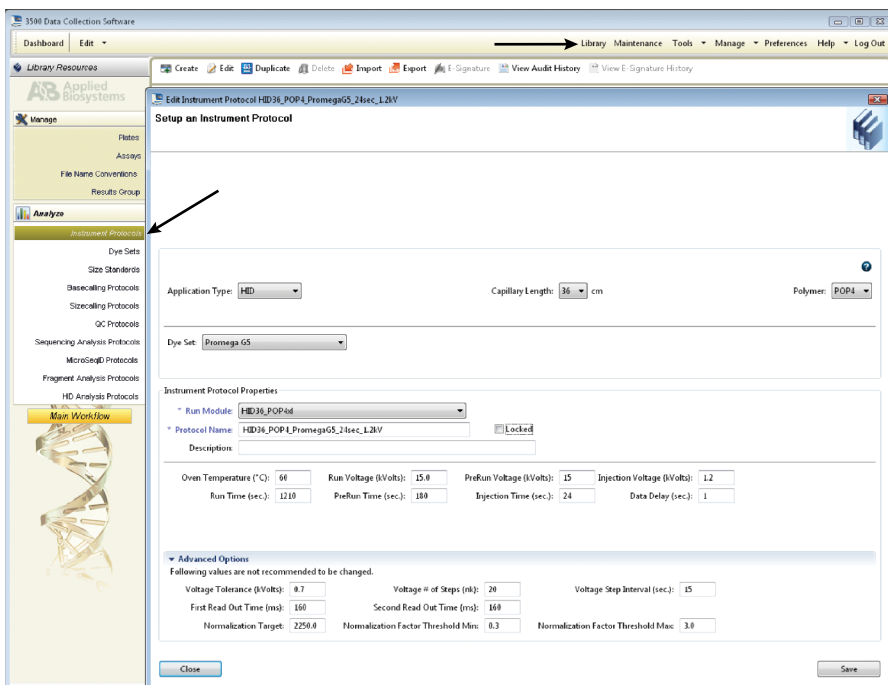
Figure 2. The Create New Instrument Protocol window.

The recommended settings are:

Application Type	HID
Capillary Length	36cm
Polymer	POP-4®
Dye Set	Promega G5
Run Module	HID36_POP4(xl)
Injection Time[1]	15 seconds for the Applied Biosystems® 3500 Genetic Analyzer 24 seconds for the Applied Biosystems® 3500xL Genetic Analyzer
Injection Voltage	1.2kV
Run Voltage	15kV
Run Time	1,210 seconds

[1]Injection time may be modified to increase or decrease peak heights.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

2.b. To create a new Size Standard for the QC protocol, navigate to the Library. Select **Size Standards**, and then select **Create**. Alternatively, a previously created Size Standard may be used.

Assign the Size Standard the name "ILS500" or another appropriate name. Choose **Orange** as the Dye Color. The fragments in the size standard are 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases. See Figure 3.
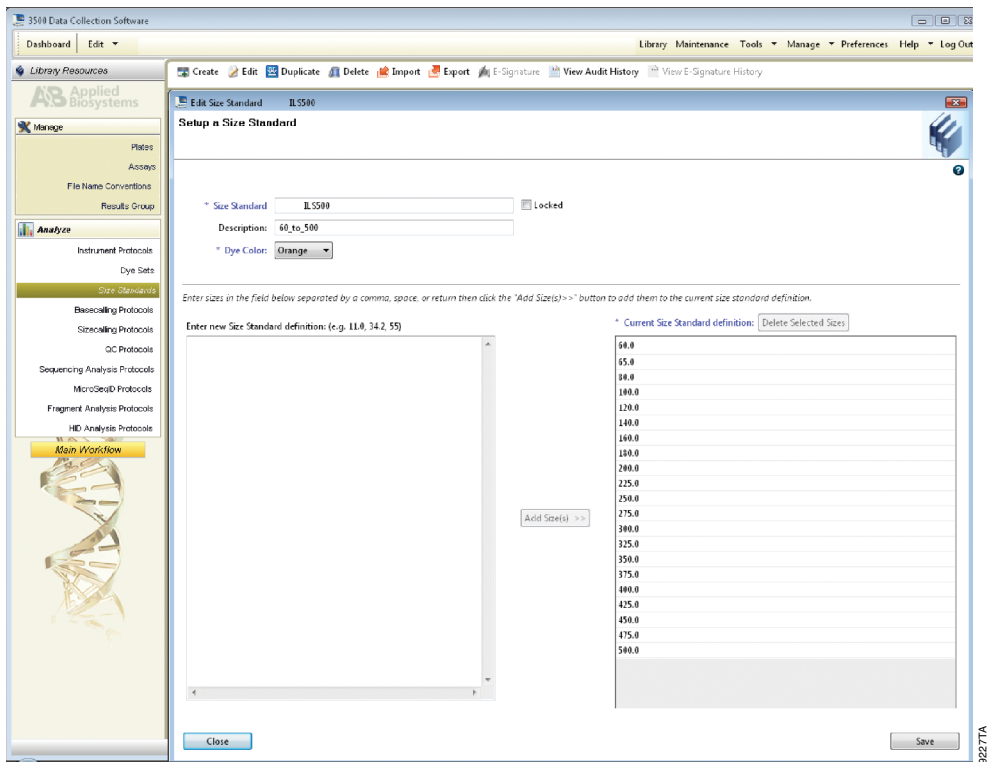
Figure 3. The Create New Size Standard window.

2.c. To create a new QC Protocol, navigate to the Library. Select **QC Protocols**, and then select **Create**. Alternatively, a previously created QC Protocol may be used.

Assign a descriptive protocol name. Select the size standard created in Step 2.b. The settings for the QC protocol should be based on the internally validated conditions for the PowerPlex® ESI 17 Fast System on the Applied Biosystems® 3500 or 3500xL Genetic Analyzer. Figure 4 shows one option for these settings.

Figure 4. The 'Create New QC Protocol' window.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

2.d. To create a new Assay, navigate to the Library. Select **Assays**, and then select **Create**. Alternatively, a previously created Assay may be used.

In the 'Create New Assay' window (Figure 5), select the application type **HID**. Choose the Instrument Protocol created in Step 2.a and the QC Protocol created in Step 2.c. Assign a descriptive assay name. An Assay is required for all named samples on a plate.

Note: If autoanalysis of a sample is desired, refer to the instrument user's manual for instructions.
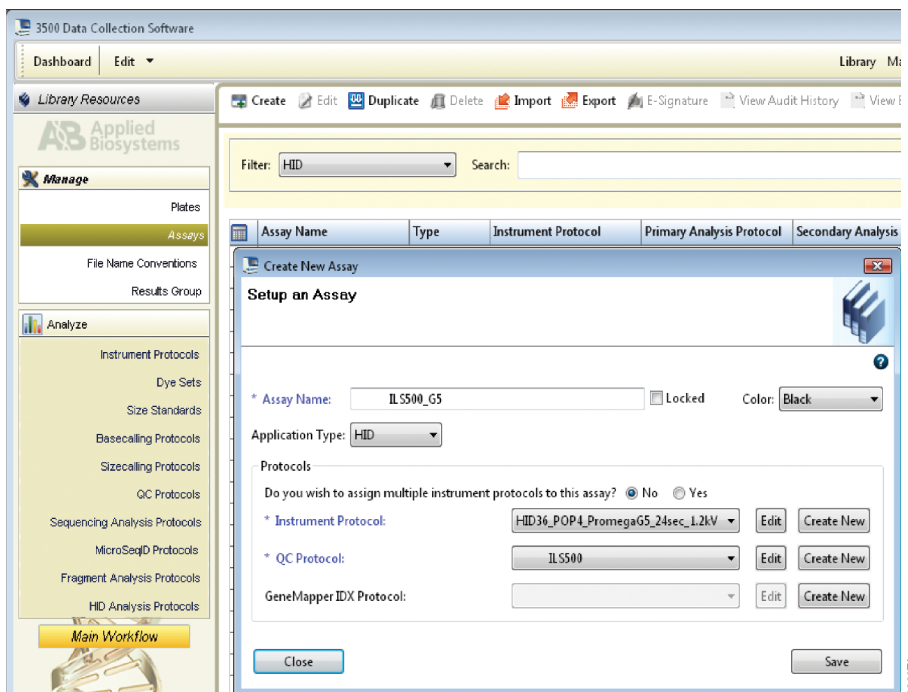
Figure 5. The 'Create New Assay' window.

2.e. To create a new File Name Convention (Figure 6), navigate to the Library. Select **File Name Conventions**, and then select **Create**. Alternatively, a previously created File Name Convention can be used.

Select the File Name Attributes according to laboratory practices, and save with a descriptive name.
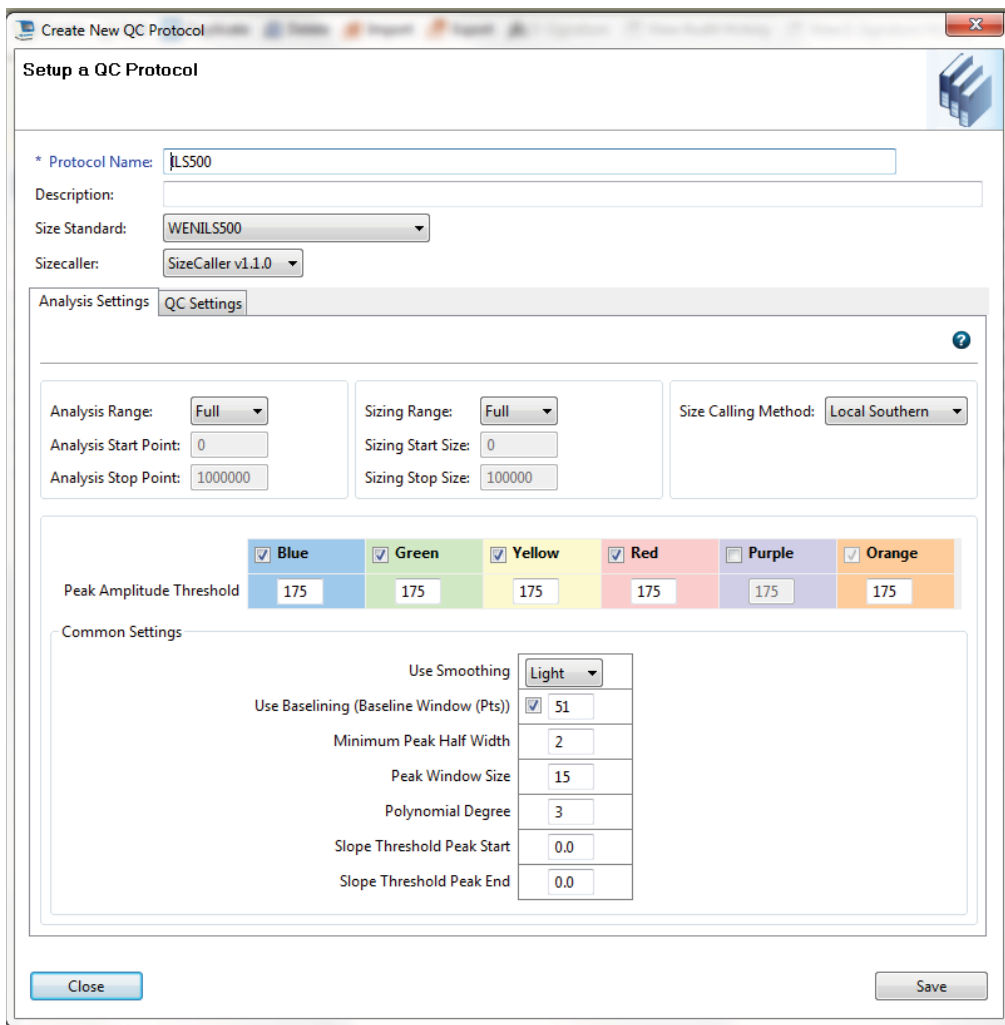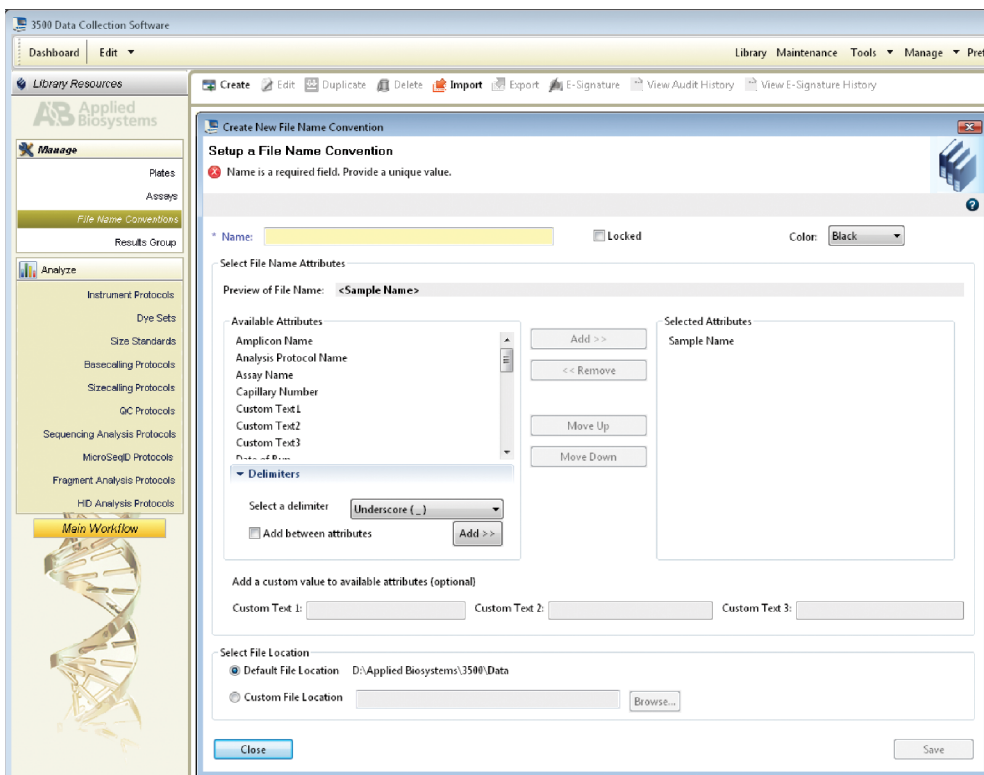

Figure 6. The 'Create New File Name Convention' window.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

2.f. To create a new Results Group (Figure 7), navigate to the Library. Select **Results Group**, and then select **Create**. Alternatively, a previously created Results Group can be used.

Select the Results Group Attributes according to laboratory practices. Save with a descriptive name.
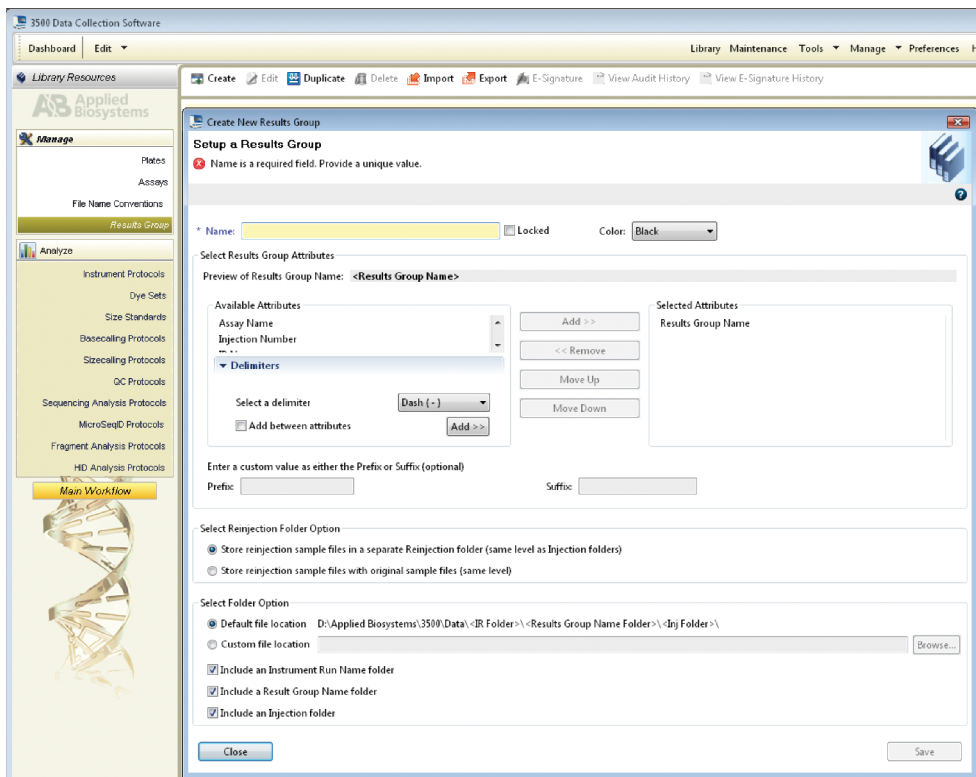
Figure 7. The 'Create New Results Group' window.

3. To create a New Plate, navigate to the Library, and from the Manage menu, select **Plates**, and then **Create**.

4. Assign a descriptive plate name. Select the plate type **HID** from the drop-down menu (Figure 8).
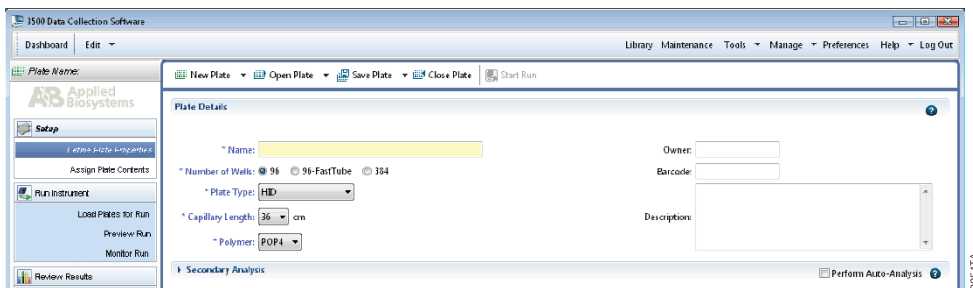
Figure 8. Defining plate properties.

5. Select **Assign Plate Contents** (Figure 9).
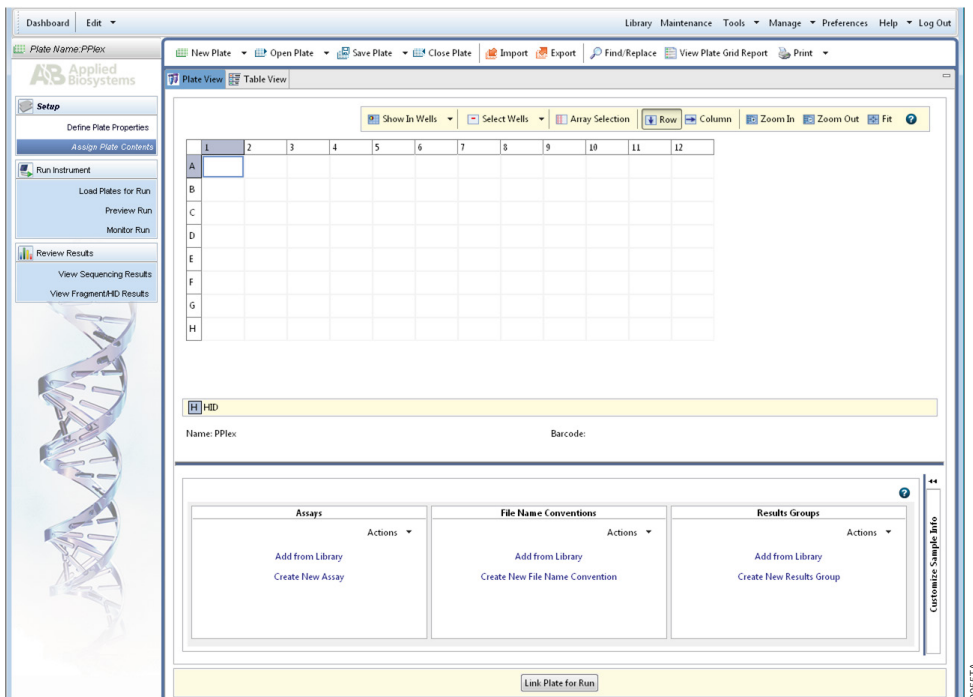
Figure 9. Assigning plate contents.

5.A.   Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

6.   Assign sample names to wells.

7.   In the lower left portion of the screen, under Assays, use the 'Add from Library' option to select the Assay created in Step 2.d or one previously created. Choose the **Add to Plate** button, and close the window.

8.   Under File Name Conventions, use the 'Add from Library' option to select the File Name Convention created in Step 2.e or one previously created. Choose the **Add to Plate** button, and close the window.

9.   Under Results Groups, use the 'Add from Library' option to select the Results Group created in Step 2.f or one previously created. Choose the **Add to Plate** button, and close the window.

10.   Highlight the sample wells, and then select the boxes in the Assays, File Name Conventions and Results Groups.

11.   Select **Link Plate for Run**.

12.   The 'Load Plate' window will appear. Select **Yes**.

13.   In the 'Run Information' window (Figure 10), assign a Run Name. Select **Start Run** (not shown).
      Note: Each injection will take approximately 40 minutes.

Figure 10. Assigning a run name.

5.B. Detection of Amplified Fragments Using the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with Data Collection Software, Version 3.0 or 4.0

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice, ice-water bath or freezer plate block
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- plate retainer and base set (standard)
- 3130 capillary array, 36cm
- POP-4® polymer for the Applied Biosystems® 3130/3130*xl* Genetic Analyzer
- 10X genetic analyzer buffer with EDTA
- MicroAmp® optical 96-well plate (or equivalent) and septa (Applied Biosystems)
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)

(!) The quality of formamide is critical. Use only the recommended formamide. Freeze formamide in aliquots at −20°C. Multiple freeze-thaw cycles or long-term storage at 4°C may cause breakdown of formamide. Poor-quality formamide may contain ions that compete with DNA during injection, which results in lower peak heights and reduced sensitivity. A longer injection time may not increase the signal.

(!) Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label, and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

Sample Preparation

1. Prepare a loading cocktail by combining and mixing internal lane standard (ILS) and formamide as follows:

   [(0.5µl ILS) × (# samples)] + [(9.5µl formamide) × (# samples)]

   Note: The volume of internal lane standard used in the loading cocktail can be adjusted to change the intensity of the size standard peaks based on laboratory preferences.

2. Vortex for 10–15 seconds to mix.

3. Pipet 10µl of formamide/internal lane standard mix into each well.
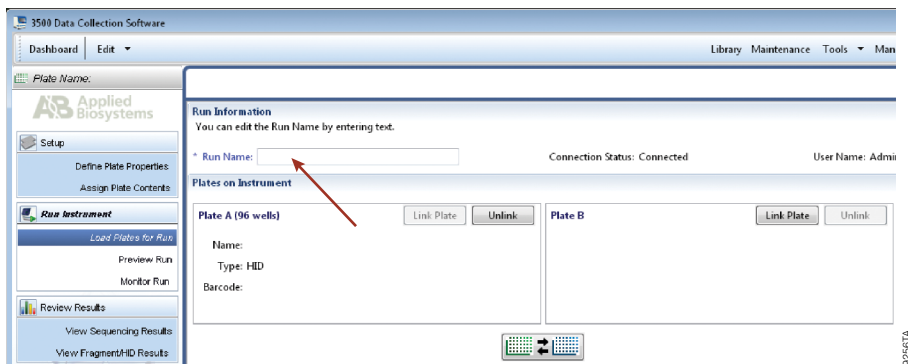
5.B. Detection of Amplified Fragments Using the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with Data Collection Software, Version 3.0 or 4.0 (continued)

4. Add 1µl of amplified sample (or 1µl of Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

    Notes:

    a. Instrument detection limits vary; therefore, injection time, injection voltage or the amount of product mixed with loading cocktail may need to be adjusted. Use the Module Manager in the data collection software to modify the injection time or voltage in the run module (see Instrument Preparation below).

    b. Use a volume of allelic ladder that results in peak heights that are all consistently above the peak amplitude threshold determined as part of your internal validation.

5. Centrifuge plate briefly to remove air bubbles from the wells.

6. Denature samples at 95°C for 3 minutes, and then immediately chill on crushed ice, a freezer plate block or in an ice-water bath for 3 minutes. Denature samples just prior to loading the instrument.

Instrument Preparation

Refer to the instrument user's manual for instructions on cleaning, installing the capillary array, performing a spatial calibration and adding polymer.

Analyze samples as described in the user's manual for the the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with the following exceptions.

1. In the Module Manager, select **New**. Select **Regular** in the 'Type' drop-down list, and select **HIDFragmentAnalysis36_POP4** in the 'Template' drop-down list. Confirm that the injection time is 5 seconds, the injection voltage is 3kV and the run time is 1,500 seconds. Give a descriptive name to your run module, and select **OK**.

    Note: Instrument sensitivities can vary. The injection time and voltage may be adjusted in the Module Manager. A suggested range for the injection time is 3–22 seconds and for the injection voltage is 1–3kV.

2. In the Protocol Manager, select **New**. Type a name for your protocol. Select **Regular** in the 'Type' drop-down list, and select the run module you created in the previous step in the 'Run Module' drop-down list. Lastly, select **G5** in the dye-set drop-down list. Select **OK**.

3. In the Plate Manager, create a new plate record as described in the instrument user's manual. In the dialog box that appears, select **GeneMapper—Generic** in the 'Application' drop-down list, and select the appropriate plate type (96-well). Add entries in the owner and operator fields, and select **OK**.

    Note: If autoanalysis of sample data is desired, refer to the instrument user's manual for instructions.

4. In the GeneMapper plate record, enter sample names in the appropriate cells. Scroll to the right. In the 'Results Group 1' column, select the desired results group. In the 'Instrument Protocol 1' column, select the protocol you created in Step 2. Be sure this information is present for each row that contains a sample name. Select **OK**.

   Note: To create a new results group, select **New** in the drop-down menu in the 'Results Group' column. Select the 'General' tab, and enter a name. Select the 'Analysis' tab, and select **GeneMapper—Generic** in the 'Analysis' type drop-down list.

5. Place samples in the instrument, and close the instrument doors.

6. In the spectral viewer, select **dye set G5**, and confirm that the active dye set is the file generated for the PowerPlex® 5-dye chemistry.

   ⊘ It is critical to select the correct G5 spectral for the PowerPlex® 5-dye chemistry.

   If the PowerPlex® 5-dye chemistry is not the active dye set, locate the PowerPlex® 5-dye spectral in the List of Calibrations for Dye Set G5, and select **Set**.

7. In the run scheduler, locate the plate record that you just created in Steps 3 and 4, and click once on the name to highlight it.

8. Once the plate record is highlighted, click the plate graphic that corresponds to the plate on the autosampler that contains your amplified samples.

9. When the plate record is linked to the plate, the plate graphic will change from yellow to green, and the green **Run Instrument** arrow becomes enabled.

10. Select the green **Run Instrument** arrow on the toolbar to start the sample run.

11. Monitor electrophoresis by observing the run, view, array or capillaries viewer window in the data collection software. Each injection will take approximately 40 minutes.

6. Data Analysis

The instructions in this section are for use with GeneMapper® *ID*-X software or GeneMapper® *ID* software, version 3.2. Due to potential differences between software versions, some of the instructions may not apply to all software versions.

6.A. Importing PowerPlex® ESI Fast Panels, Bins and Stutter Text Files into GeneMapper® *ID*-X Software

To facilitate analysis of data generated with the PowerPlex® ESI 17 Fast System, we have created panels, bins and stutter text files to allow automatic assignment of genotypes using GeneMapper® *ID*-X software. We recommend that users receive training from Applied Biosystems on the GeneMapper® *ID*-X software to familiarize themselves with proper operation of the software.

Getting Started

1. To obtain the proper panels, bins and stutter text files for the PowerPlex® ESI 17 Fast System go to: **www.promega.com/resources/software-firmware/str-analysis/genemapper-id-software-panels-and-bin-sets/**

2. Select the PowerPlex® System that you are using, and select **GeneMapper ID-X**. Enter your contact information, and select **Submit**.

3. Save the PowerPlex_ESI_Fast_Panels_IDX_vX.x.txt, PowerPlex_ESI_Fast_Bins_IDX_vX.x.txt and PowerPlex_ESI_Fast_Stutter_IDX_vX.x.txt files, where "X.x" refers to the most recent version of the panels, bins and stutter text files, to a known location on your computer.

4. Save the WEN_ILS_500_IDX.xml file to a known location on your computer.

Importing Panels, Bins and Stutter Text Files

1. Open the GeneMapper® *ID*-X software.

2. Select **Tools**, and then **Panel Manager**.

3. Highlight the **Panel Manager** icon in the upper left navigation pane.

4. Select **File**, and then **Import Panels**.

5. Navigate to the panels text file downloaded in the Getting Started section above. Select the file, and then **Import**.

6. Select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.

7. In the navigation pane, highlight the PowerPlex ESI Fast panels folder that you just imported in Step 5.

8. Select **File**, and then **Import Bin Set**.

9. Navigate to the bins text file downloaded in the Getting Started section above. Select the file, and then **Import**.

10. In the navigation pane, highlight the PowerPlex ESI Fast panels folder that you just imported in Step 5.

11. Select **File**, and then **Import Marker Stutter**. A warning box will appear asking if you want to overwrite current values. Select **Yes**.

12. Navigate to the stutter text file downloaded in the Getting Started section above. Select the file, and then **Import**.

13. At the bottom of the 'Panel Manager' window, select **OK**. This will save the panels, bins and stutter text files, and then close the window.

6.B. Importing the WEN ILS 500 ESS Size Standard into GeneMapper® *ID*-X Software

There are two options when creating a size standard. Use this protocol or the alternative protocol in Section 6.C.

The WEN_ILS_500_IDX.xml file can be used for the WEN ILS 500 ESS size standard supplied with the PowerPlex® ESI 17 Fast System.

1. Select **Tools**, and then **GeneMapper ID-X Manager**.

2. Select the 'Size Standard' tab.

3. Select **Import**.

4. Navigate to the location of the WEN_ILS_500_IDX.xml file downloaded in Section 6.A, Getting Started.

5. Highlight the file, and then select **Import**.

6. Select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.

7. Select **Done** to save changes and close the GeneMapper® *ID*-X Manager.

6.C. Creating a Size Standard with GeneMapper® *ID*-X Software

1. Select **Tools**, and then **GeneMapper ID-X Manager**.

2. Select the 'Size Standard' tab.

3. Select **New**.

4. In the 'Size Standard Editor' window (Figure 11), select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.

6.C. **Creating a Size Standard with GeneMapper® *ID*-X Software (continued)**
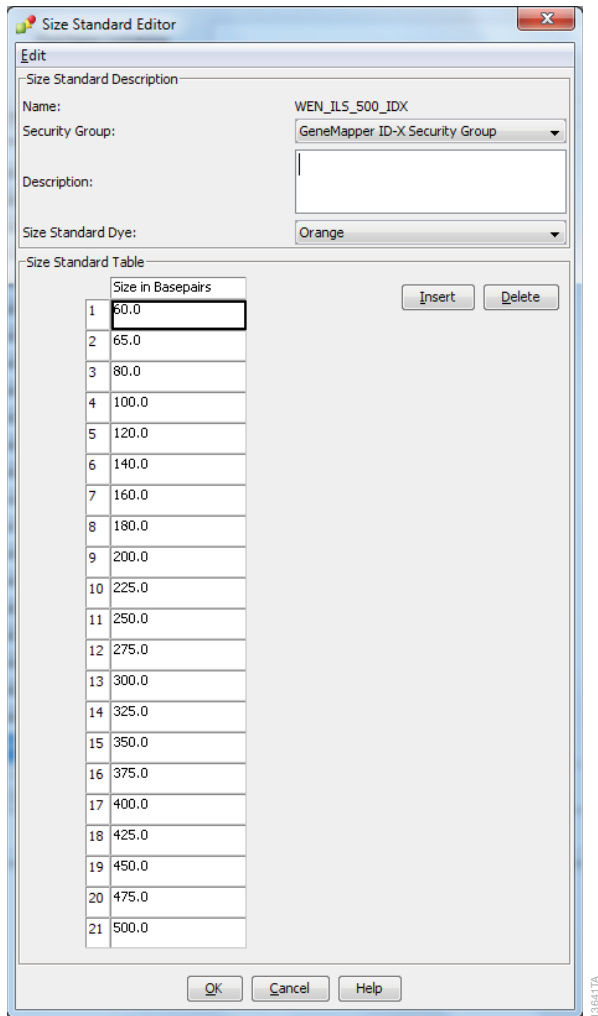
Figure 11. The GeneMapper® *ID*-X Size Standard Editor.

5.   Enter a detailed name, such as "WEN_ILS_500_IDX".

6.   Choose **Orange** for the Size Standard Dye.

7.   Enter the sizes of the internal lane standard fragments (60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases). See Section 9.B, Figure 23.

8.   Select **OK**.

6.D. Creating a Casework Analysis Method with GeneMapper® *ID*-X Software

These instructions are intended as a guide to start analyzing data in GeneMapper® *ID*-X Software. They are not intended as comprehensive instructions for using GeneMapper® *ID*-X software. We recommend that users contact Applied Biosystems for training on the software.

1. Select **Tools**, and then **GeneMapper ID-X Manager**.

2. Select the 'Analysis Methods' tab.

3. Select **New**, and a new analysis method dialog box will open.

4. In the 'Analysis Method Editor' window, select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.

5. Enter a descriptive name for the analysis method, such as "PowerPlex ESI 17 Fast".

6. Select the 'Allele' tab (Figure 12).

Figure 12. The GeneMapper® *ID*-X 'Allele' tab.

7.   Select the bins text file that was imported in Section 6.A.

8.   Ensure that the 'Use marker-specific stutter ratio and distance if available' box is checked. We recommend the values shown in Figure 15 for proper filtering of stutter peaks when using the PowerPlex® ESI 17 Fast System. You may need to optimize these settings. In-house validation should be performed.

9. Select the 'Peak Detector' tab (Figure 13). You will need to optimize these settings. In-house validation should be performed.

   Notes:

   a. Select full range or partial range for the analysis range. When using a partial range, choose an appropriate analysis range based on your data. Choose a start point after the primer peak and just before the first defined internal lane standard peak to help ensure proper sizing of the internal lane standard.

   b. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Individual laboratories should determine their peak amplitude thresholds from internal validation studies.

   c. The normalization box can be checked or unchecked.

   d. The peak window size shown in Figure 13 is not the default setting. It has been changed to 11 from the default value of 15. This ensures that the software can resolve alleles in the ladder that are 1 base apart for the larger loci such as D1S1656, D12S391 and D2S441.
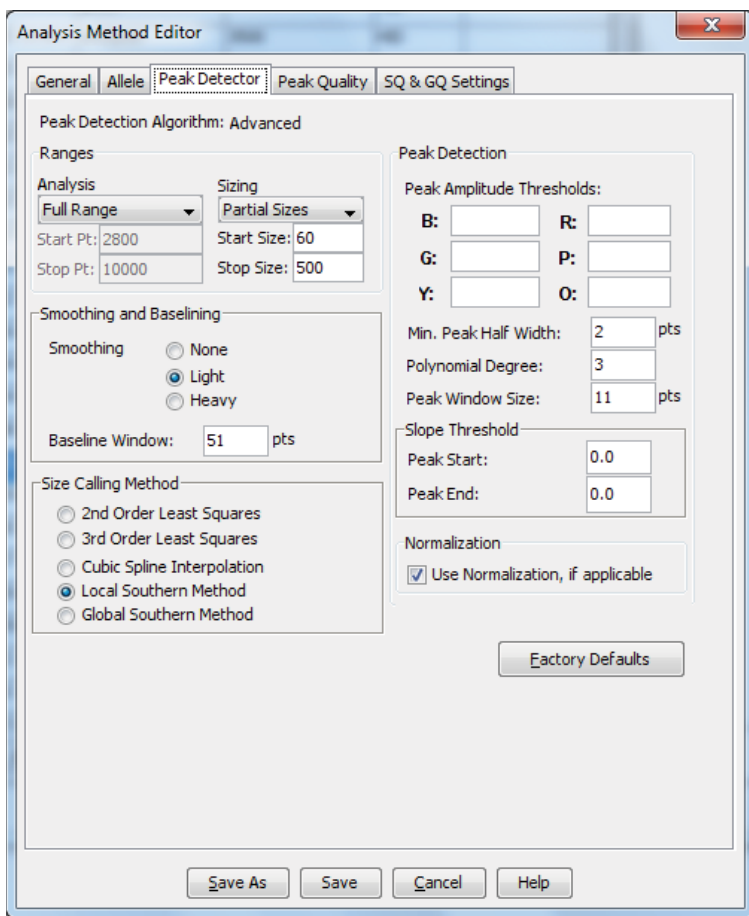
Figure 13. The GeneMapper® *ID*-X 'Peak Detector' tab.

10. Select the 'Peak Quality' tab. You may change the settings for peak quality.

11. Select the 'SQ & GQ Settings' tab. You may change these settings.

    Note: For Steps 10 and 11, see the GeneMapper® *ID*-X user's manual for more information. The settings in Steps 10 and 11 should be based on the results of your internal validation.

12. Select **Save** to save the new analysis method.

13. Select **Done** to exit the GeneMapper® *ID*-X Manager.

Processing Data for Casework Samples

1. Select **File**, and then **New Project**.

2. Select **Edit**, and then **Add Samples to Project**.

3. Browse to the location of the run files. Highlight desired files, and then select **Add to list** followed by **Add**.

4. In the 'Sample Type' column, use the drop-down menu to select **Allelic Ladder**, **Sample**, **Positive Control** or **Negative Control** as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as **Allelic Ladder** in the 'Sample Type' column for proper genotyping.

5. In the 'Analysis Method' column, select the analysis method created previously in this section.

6. In the 'Panel' column, select the panels text file that was imported in Section 6.A.

7. In the 'Size Standard' column, select the size standard that was imported in Section 6.B or created in Section 6.C.

8. Select **Analyze** (green arrow button) to start data analysis.

    Note: By default, the software is set to display the 'Analysis Requirements Summary' and 'Allelic Ladder Analysis Summary' windows if an analysis requirement is not met. After analysis is complete, the default setting is to show the 'Analysis Summary' tab. If the default settings are changed, manual troubleshooting may be necessary.

9. If all analysis requirements are met, the 'Save Project' window will open (Figure 14).
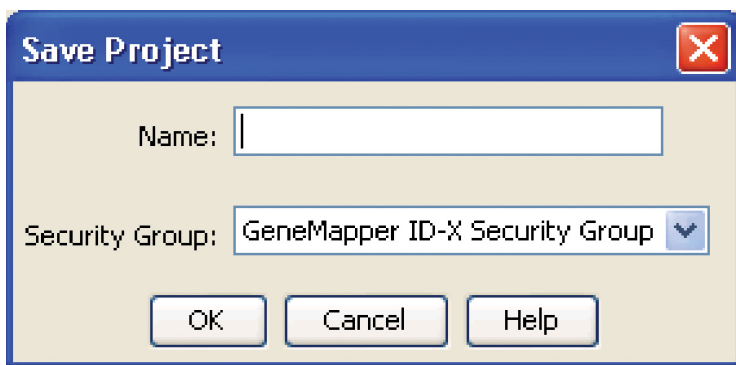
Figure 14. The 'Save Project' window.

10. Enter the project name.

11. Choose the applicable security group from the drop-down menu, and then select **OK**.

When the analysis is finished, the 'Analysis Summary' screen will appear. We recommend that you review any yellow or red marker header bars in the plots view and handle them according to laboratory standard operating procedures.

The values displayed in the 'Analysis Method Peak Quality' and 'SQ & GQ Settings' tabs are defaults and will affect the quality values displayed in the plot settings. We recommend that you modify the values in these tabs to fit your laboratory's data analysis protocols.

6.E. Creating a Databasing or Paternity Analysis Method with GeneMapper® *ID*-X Software

These instructions are intended as a guide to start analyzing data in GeneMapper® *ID*-X software. They are not intended as comprehensive instructions for using the GeneMapper® *ID*-X software. We recommend that users contact Applied Biosystems for training on the software.

1. Select **Tools**, and then **GeneMapper ID-X Manager**.

2. Select the 'Analysis Methods' tab.

3. Select **New**, and a new analysis method dialog box will open.

4. In the 'Analysis Method Editor' window, select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.

5. Enter a descriptive name for the analysis method, such as "PowerPlex ESI 17 Fast 20% Filter".

6. Select the 'Allele' tab (Figure 15).

7. Select the bins text file that was imported in Section 6.A.

8. We recommend the values shown in Figure 18 for proper filtering of stutter peaks when using the PowerPlex® ESI 17 Fast System. Ensure that the 'Use marker-specific stutter ratio and distance if available' box is checked. Doing this will assign locus-specific stutter filters and distances from the imported stutter file. Ensure that the appropriate global filter is applied to the analysis method. For example, for a 20% filter enter "0.20" for the Amelogenin Cutoff and Global Cutoff Value for Tri and Tetra repeats (Figure 15). In-house validation should be performed.

Analysis Method Editor

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

Bin Set: PowerPlex_ESI_Fast_Bins_IDX_v2.0

☑ Use marker-specific stutter ratio and distance if available

Marker Repeat Type:		Tri	Tetra	Penta	Hexa
Global Cut-off Value		0.2	0.2	0.0	0.0
MinusA Ratio		0.0	0.0	0.0	0.0
MinusA Distance	From	0.0	0.0	0.0	0.0
	To	0.0	0.0	0.0	0.0
Global Minus Stutter Ratio		0.0	0.0	0.0	0.0
Global Minus Stutter Distance	From	0.0	0.0	0.0	0.0
	To	0.0	0.0	0.0	0.0
Global Plus Stutter Ratio		0.0	0.0	0.0	0.0
Global Plus Stutter Distance	From	0.0	0.0	0.0	0.0
	To	0.0	0.0	0.0	0.0

Amelogenin Cutoff: 0.2

Range Filter...   Factory Defaults

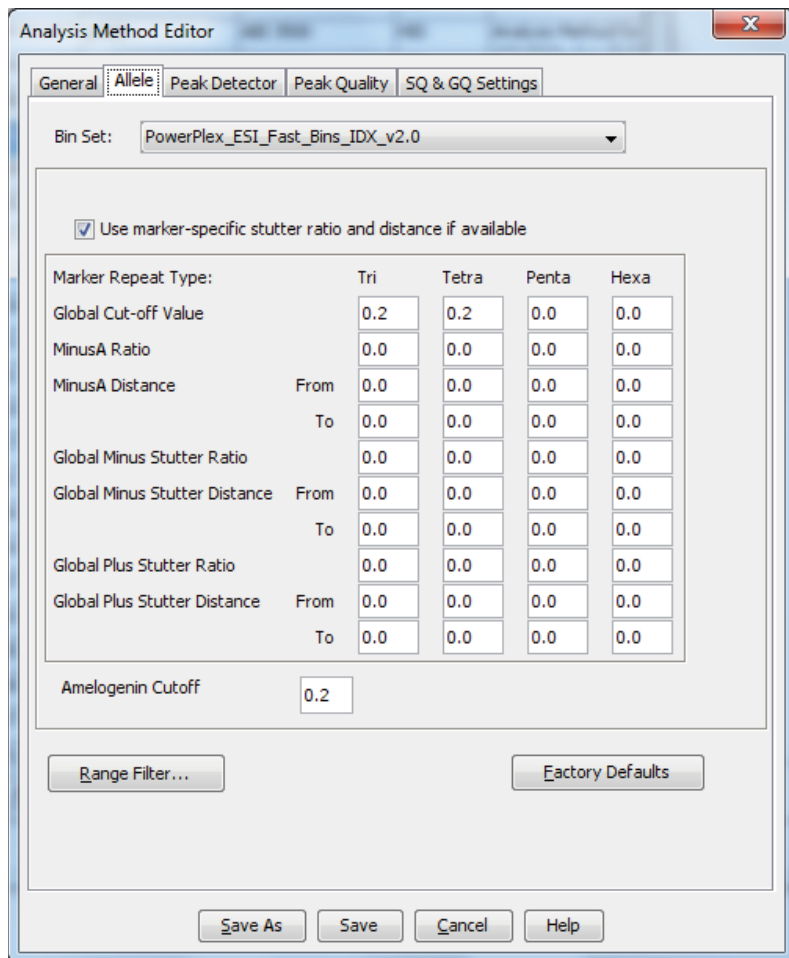Save As   Save   Cancel   Help

13707TA

Figure 15. The GeneMapper® ID-X 'Allele' tab with settings for using a 20% peak filter.

6.E. Creating a Databasing or Paternity Analysis Method with GeneMapper® *ID*-X Software (continued)

9. Select the 'Peak Detector' tab (Figure 13). You will need to optimize these settings. In-house validation should be performed.

    Notes:

    a. Select full range or partial range for the analysis range. When using a partial range, choose an appropriate analysis range based on your data. Choose a start point after the primer peak and just before the first defined internal lane standard peak to help ensure proper sizing of the internal lane standard.

    b. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Individual laboratories should determine their peak amplitude thresholds from internal validation studies.

    c. The normalization box can be checked or unchecked.

    d. The peak window size shown in Figure 15 is not the default setting. It has been changed to 11 from the default value of 15. This ensures that the software can resolve alleles in the ladder that are 1 base apart for the larger loci such as D1S1656, D12S391 and D2S441.

10. Select the 'Peak Quality' tab. You may change the settings for peak quality.

11. Select the 'SQ & GQ Settings' tab. You may change these settings.

    Note: For Steps 10 and 11, see the GeneMapper® *ID*-X user's manual for more information. The setttings in Steps 10 and 11 should be based on the results of your internal validation.

12. Select **Save** to save the new analysis method.

13. Select **Done** to exit the GeneMapper® *ID*-X Manager.

Processing Data for Databasing or Paternity Samples

1. Select **File**, and then **New Project**.

2. Select **Edit**, and then **Add Samples to Project**.

3. Browse to the location of run files. Highlight desired files, and then select **Add to list** followed by **Add**.

4. In the 'Sample Type' column, use the drop-down menu to select **Allelic Ladder**, **Sample**, **Positive Control** or **Negative Control** as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as **Allelic Ladder** in the 'Sample Type' column for proper genotyping.

5. In the 'Analysis Method' column, select the analysis method created previously in this section.

6. In the 'Panel' column, select the panels text file that was imported in Section 6.A.

7. In the 'Size Standard' column, select the size standard that was imported in Section 6.B or created in Section 6.C.

8. Select **Analyze** (green arrow button) to start data analysis.

   Note: By default, the software is set to display the 'Analysis Requirements Summary' and 'Allelic Ladder Analysis Summary' windows if an analysis requirement is not met. After analysis is complete, the default setting is to show the 'Analysis Summary' tab. If the default settings have been changed, manual troubleshooting may be necessary.

9. If all analysis requirements are met, the 'Save Project' window will open (Figure 14).

10. Enter the project name.

11. Choose the applicable security group from the drop-down menu, and then select **OK**.

When the analysis is finished, the 'Analysis Summary' screen will appear. We recommend that you review any yellow or red marker header bars in the plots view and handle them according to laboratory standard operating procedures.

The values displayed in the 'Analysis Method Peak Quality' and 'SQ & GQ Settings' tabs are defaults and will affect the quality values displayed in the plot settings. We recommend that you modify the values in these tabs to fit your laboratory's data analysis protocols.

6.F. Importing PowerPlex® ESI Fast Panels and Bins Text Files into GeneMapper® *ID* Software, Version 3.2

To facilitate analysis of data generated with the PowerPlex® ESI 17 Fast System, we have created panels and bins text files to allow automatic assignment of genotypes using GeneMapper® *ID* software, version 3.2. We recommend that users of GeneMapper® *ID* software, version 3.2, complete the *Applied Biosystems GeneMapper®* ID *Software Human Identification Analysis Tutorial* to familiarize themselves with proper operation of the software. For GeneMapper® *ID* software, version 3.1, users we recommend upgrading to version 3.2.

Note: Run files generated using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer cannot be analyzed using GeneMapper® *ID* software. You must analyze these files with GeneMapper® *ID*-X software.

Getting Started

1. To obtain the panels and bins text files for the PowerPlex® ESI 17 Fast System go to:
   www.promega.com/resources/software-firmware/str-analysis/genemapper-id-software-panels-and-bin-sets/

2. Select the PowerPlex® System that you are using, and select **GeneMapper ID**. Enter your contact information, and select **Submit**.

3. Save the PowerPlex_ESI_Fast_Panels_vX.x.txt and PowerPlex_ESI_Fast_Bins_vX.x.txt files, where "X.x" refers to the most recent version of the panels and bins text files, to a known location on your computer.

4. Save the WEN_ILS_500_IDX.xml file to a known location on your computer.

6.F. **Importing PowerPlex® ESI Fast Panels and Bins Text Files into GeneMapper®** *ID* **Software, Version 3.2 (continued)**

Importing Panels and Bins Text Files

These instructions loosely follow the Applied Biosystems GeneMapper® *ID* software tutorial, pages 1–4.

1. Open the GeneMapper® *ID* software, version 3.2.

2. Select **Tools**, and then **Panel Manager**.

3. Highlight the **Panel Manager** icon in the upper left navigation pane.

4. Select **File**, and then **Import Panels**.

5. Navigate to the panels text file downloaded in the Getting Started section above. Select the file, then **Import**.

6. In the navigation pane, highlight the PowerPlex ESI Fast panels folder that you just imported in Step 5.

7. Select **File**, and then **Import Bin Set**.

8. Navigate to the bins text file downloaded in the Getting Started section above. Select the file, and then **Import**.

9. At the bottom of the 'Panel Manager' window, select **OK**. This will save the panels and bins text files and close the window automatically.

6.G. **Importing the WEN ILS 500 ESS Size Standard into GeneMapper®** *ID* **Software, Version 3.2**

There are two options when creating a size standard. Use this protocol or the alternative protocol in Section 6.H.

The WEN_ILS_500.xml file can be used for the WEN ILS 500 ESS size standard supplied with the PowerPlex® ESI 17 Fast System.

1. Select **Tools**, and then **GeneMapper Manager**.

2. Select the 'Size Standard' tab.

3. Select **Import**.

4. Navigate to the WEN_ILS_500.xml file downloaded in Section 6.F.

5. Highlight the file, and then select **Import**.

6. Select **Done** to save changes and exit the GeneMapper® Manager.

6.H. Creating a Size Standard with GeneMapper® *ID* Software, Version 3.2

1. Select **Tools**, and then **GeneMapper Manager**.

2. Select the 'Size Standard' tab.

3. Select **New**.

4. Select **Basic or Advanced** (Figure 16). The type of analysis method selected must match the type of analysis method created earlier. Select **OK**.
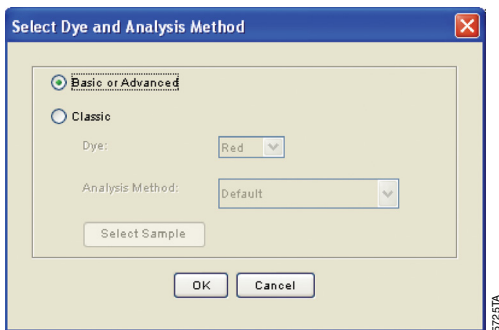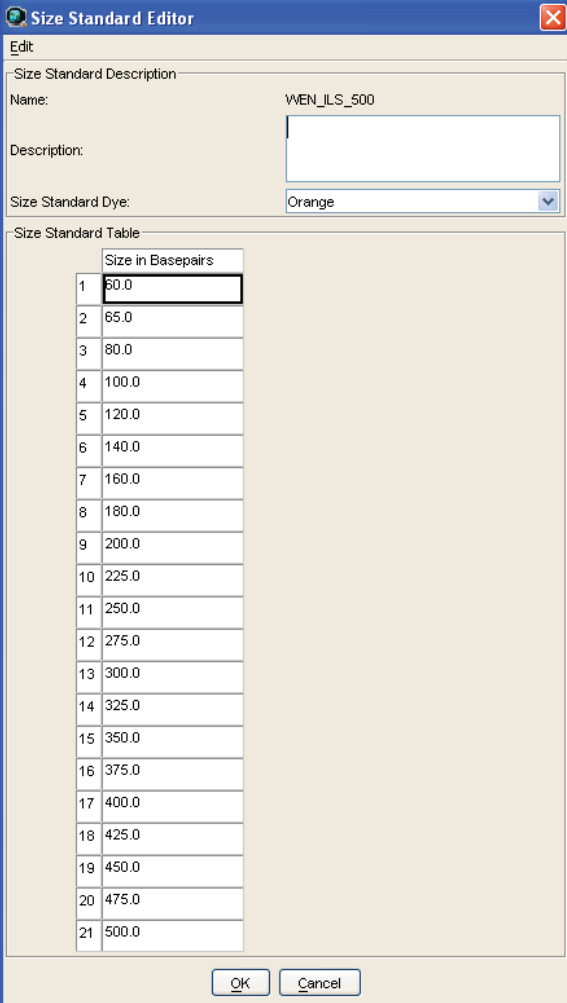
Figure 16. The 'Select Dye and Analysis Method' window.

6.H.   Creating a Size Standard with GeneMapper® *ID* Software, Version 3.2 (continued)

5.   Enter a detailed name, such as "WEN_ILS_500", in the Size Standard Editor (Figure 17).

Figure 17. The GeneMapper® *ID* Size Standard Editor.

6.   Choose **Orange** for the Size Standard Dye.

7.   Enter the sizes of the internal lane standard fragments (60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases). See Section 9.B, Figure 23.

8.   Select **OK**.

6.I. **Creating a Casework Analysis Method with GeneMapper® *ID* Software, Version 3.2**

These instructions are intended as a guide to start analyzing data in GeneMapper® *ID* Software. They are not intended as comprehensive instructions for using GeneMapper® *ID* Software. We recommend that users contact Applied Bisoystems for training on the software. These instructions loosely follow the Applied Biosystems GeneMapper® *ID* software tutorial, pages 5–11.

1.   Select **Tools**, and then **GeneMapper Manager**.

2.   Select the 'Analysis Methods' tab.

3.   Select **New**, and a new analysis method dialog box will open.

4.   Select **HID**, and select **OK**.

       Note: If you do not see the HID option, you do not have the GeneMapper® *ID* software. Contact Applied Biosystems.

5.   Enter a descriptive name for the analysis method, such as "PowerPlex ESI 17 Fast".

6.   Select the 'Allele' tab (Figure 18).
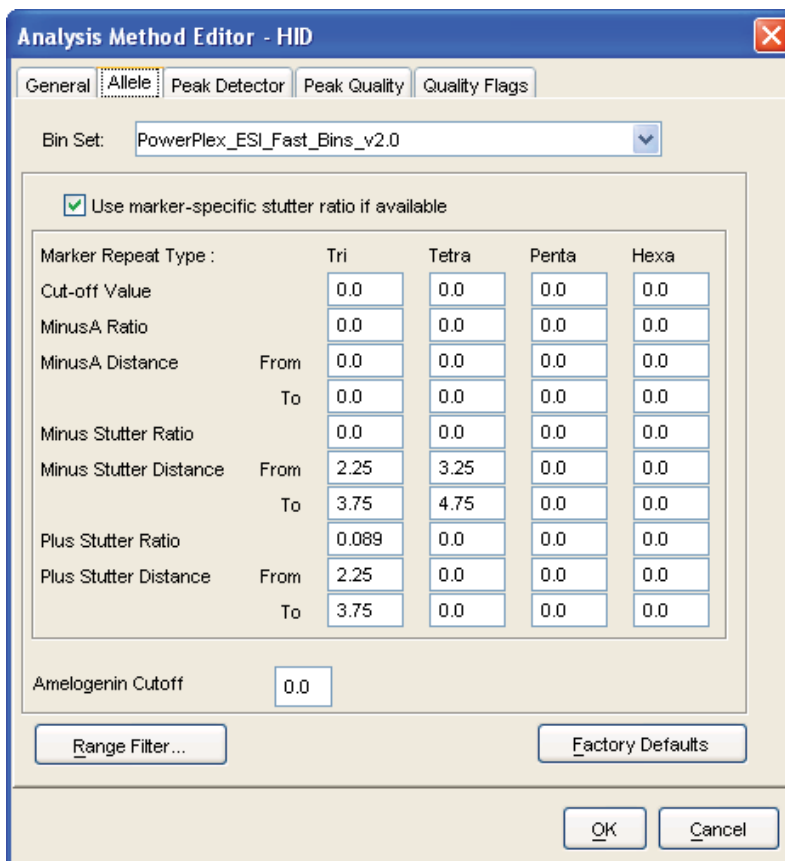
Figure 18. The GeneMapper® *ID* 'Allele' tab.

7.   Select the bins text file that was imported in Section 6.F.

8.   Ensure that the "Use marker-specific stutter ratio if available" box is checked.

9.   Enter the values shown in Figure 18 for proper filtering of stutter peaks when using the PowerPlex® ESI 17 Fast System. For an explanation of the proper usage and effects of these settings, refer to the Applied Biosystems user bulletin titled *"Installation Procedures and New Features for GeneMapper ID Software 3.2"*.

Note: Some of these settings have been optimized and are different from the recommended settings in the user bulletin. You may need to optimize these settings. In-house validation should be performed.

10. Select the 'Peak Detector' tab (Figure 19). You will need to optimize these settings. In-house validation should be performed.

    Notes:

    a. Select full range or partial range for the analysis range. When using a partial range, choose an appropriate analysis range based on your data. Choose a start point after the primer peak and just before the first defined internal lane standard peak to help ensure proper sizing of the internal lane standard.

    b. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Individual laboratories should determine their peak amplitude thresholds from internal validation studies.

    c. The peak window size shown in Figure 19 is not the default setting. It has been changed to 11 from the default value of 15. This ensures that the software can resolve alleles in the ladder that are 1 base apart for the larger loci, such as D1S1656, D12S391 and D2S441.

Figure 19. The GeneMapper® *ID* 'Peak Detector' tab.

11.   Select the 'Peak Quality' tab. You may change the settings for peak quality.

Note: For Steps 11 and 12, see the GeneMapper® *ID* user's manual for more information.

12.   Select the 'Quality Flags' tab. You may change these settings.

13.   Select **OK** to save your settings.

Processing Data for Casework Samples

1.   Select **File**, and then **New Project**.

2.   Select **Edit**, and then **Add Samples to Project**.

3.   Browse to the location of the run files. Highlight desired files, and then select **Add to list** followed by **Add**.

4. In the 'Sample Type' column, use the drop-down menu to select **Ladder**, **Sample**, **Positive Control** or **Negative Control** as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as **Ladder** in the 'Sample Type' column for proper genotyping.

5. In the 'Analysis Method' column, select the analysis method created previously in this section.

6. In the 'Panel' column, select the panels text file that was imported in Section 6.F.

7. In the 'Size Standard' column, select the size standard that was imported in Section 6.G or created in Section 6.H.

8. Select **Analyze** (green arrow button) to start data analysis.

6.J. Creating a Databasing or Paternity Analysis Method with GeneMapper® *ID* Software, Version 3.2

1. Select **Tools**, and then **GeneMapper Manager**.

2. Select the 'Analysis Methods' tab.

3. Select **New**, and a new analysis method dialog box will open.

4. Select **HID**, and select **OK**.

   Note: If you do not see the HID option, you do not have the GeneMapper® *ID* software. Contact Applied Biosystems.

5. Enter a descriptive name for the analysis method, such as "PowerPlex ESI 17 Fast 20%filter".

6. Select the 'Allele' tab (Figure 20).

7. Select the bins text file that was imported in Section 6.F.

8. Ensure that the 'Use marker-specific stutter ratio if available' box is checked. Doing this will assign locus-specific stutter filters and distances from the imported stutter file. Ensure that the appropriate global filter is applied to this analysis method. For example, for a 20% filter enter "0.20" for the Amelogenin Cutoff and the Global Cutoff Value for Tri and Tetra repeats (Figure 20).

6.J. Creating a Databasing or Paternity Analysis Method with GeneMapper® *ID* Software, Version 3.2 (continued)

9. Enter the values shown in Figure 20 for proper filtering of peaks when using the PowerPlex® ESI 17 Fast System. For an explanation of the proper usage and effect of these settings, refer to the Applied Biosystems user bulletin titled *"Installation Procedures and New Features for GeneMapper ID Software 3.2"*.

    Note: Some of these settings have been optimized and are different from the recommended settings in the user bulletin. You may need to optimize these settings. In-house validation should be performed.

Analysis Method Editor - HID

Tabs: General | **Allele** | Peak Detector | Peak Quality | Quality Flags

Bin Set: PowerPlex_ESI_Fast_Bins_v2.0

☑ Use marker-specific stutter ratio if available

Marker Repeat Type :		Tri	Tetra	Penta	Hexa
Cut-off Value		0.2	0.2	0.0	0.0
MinusA Ratio		0.0	0.0	0.0	0.0
MinusA Distance	From	0.0	0.0	0.0	0.0
	To	0.0	0.0	0.0	0.0
Minus Stutter Ratio		0.0	0.0	0.0	0.0
Minus Stutter Distance	From	2.25	3.25	0.0	0.0
	To	3.75	4.75	0.0	0.0
Plus Stutter Ratio		0.089	0.0	0.0	0.0
Plus Stutter Distance	From	2.25	0.0	0.0	0.0
	To	3.75	0.0	0.0	0.0

Amelogenin Cutoff   0.2

Range Filter...   Factory Defaults
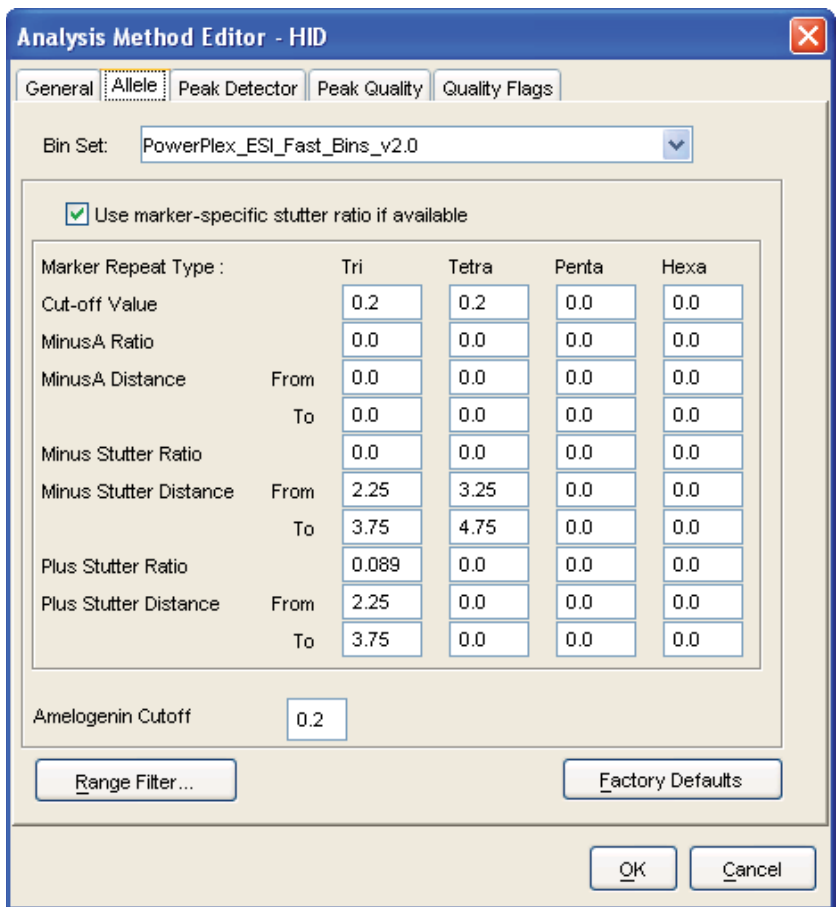
OK   Cancel

11403TA

Figure 20. The GeneMapper® *ID* 'Allele' tab with settings for using a 20% peak filter.

10. Select the 'Peak Detector' tab (Figure 19). You will need to optimize these settings. In-house validation should be performed.

    Notes:

    a. Select full range or partial range for the analysis range. When using a partial range, choose an appropriate analysis range based on your data. Choose a start point after the primer peak and just before the first defined internal lane standard peak to help ensure proper sizing of the internal lane standard.

    b. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Individual laboratories should determine their peak amplitude thresholds from internal validation studies.

    c. The peak window size shown in Figure 19 is not the default setting. It has been changed to 11 from the default value of 15. This ensures that the software can resolve alleles in the ladder that are 1 base apart for the larger loci such as D1S1656, D12S391 and D2S441.

11. Select the 'Peak Quality' tab. You may change the settings for peak quality.

    Note: For Steps 11 and 12, see the GeneMapper® *ID* user's manual for more information.

12. Select the 'Quality Flags' tab. You may change these settings.

13. Select **OK** to save your settings.

Processing Data for Databasing or Paternity Samples

1. Select **File**, and then **New Project**.

2. Select **Edit**, and then **Add Samples to Project**.

3. Browse to the location of the run files. Highlight desired files, and then select **Add to list** followed by **Add**.

4. In the 'Sample Type' column, use the drop-down menu to select **Ladder**, **Sample**, **Positive Control** or **Negative Control** as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as **Ladder** in the 'Sample Type' column for proper genotyping.

5. In the 'Analysis Method' column, select the analysis method created previously in this section.

6. In the 'Panel' column, select the panels text file that was imported in Section 6.F.

7. In the 'Size Standard' column, select the size standard that was imported in Section 6.G or created in Section 6.H.

8. Select **Analyze** (green arrow button) to start the data analysis.

6.K. Controls

1. Observe the results for the negative control. Using the protocols defined in this manual, the negative control should be devoid of amplification products.

2. Observe the results for the 2800M Control DNA. The expected 2800M DNA allele designations for each locus are listed in Table 8 (Section 9.A).

6.L. Results

Representative results of the PowerPlex® ESI 17 Fast System are shown in Figure 21. The PowerPlex® ESI 17 Fast Allelic Ladder Mix is shown in Figure 22.
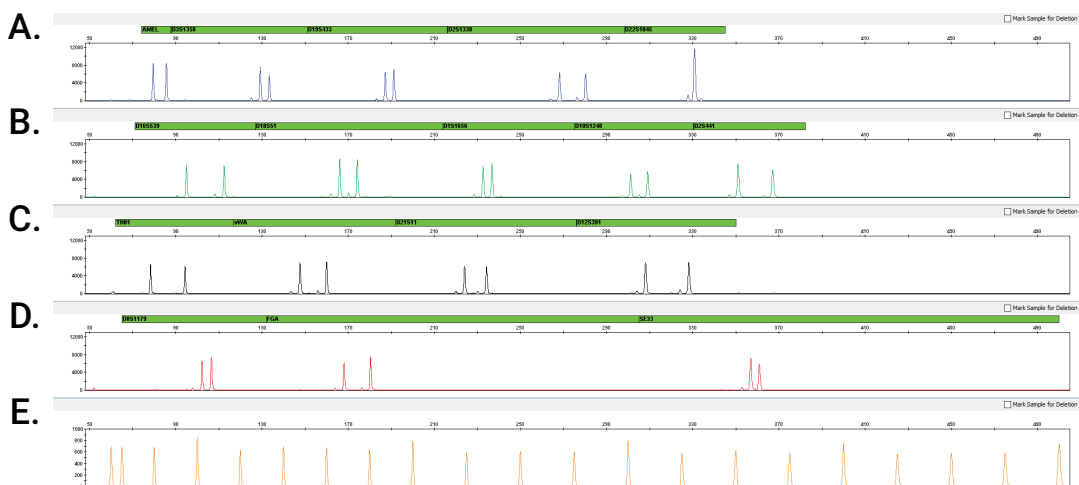
Figure 21. The PowerPlex® ESI 17 Fast System. The 2800M Control DNA (0.5ng) was amplified using the PowerPlex® ESI 17 Fast System. Amplification products were mixed with WEN Internal Lane Standard 500 ESS and analyzed with an Applied Biosystems® 3500 Genetic Analyzer using a 1.2kV, 12-second injection. Results were analyzed using GeneMapper® *ID*-X software, version 1.4. **Panel A.** An electropherogram showing the peaks of the fluorescein-labeled loci: Amelogenin, D3S1358, D19S433, D2S1338 and D22S1045. **Panel B.** An electropherogram showing the peaks of the JOE-labeled loci: D16S539, D18S51, D1S1656, D10S1248 and D2S441. **Panel C.** An electropherogram showing the peaks of the TMR-ET-labeled loci: TH01, vWA, D21S11 and D12S391. **Panel D.** An electropherogram showing the peaks of the CXR-ET-labeled loci: D8S1179, FGA and SE33. **Panel E.** An electropherogram showing the 60bp to 500bp fragments of the WEN Internal Lane Standard 500 ESS.

Figure 22. The PowerPlex® ESI 17 Fast Allelic Ladder Mix. The PowerPlex® ESI 17 Fast Allelic Ladder Mix was analyzed with an Applied Biosystems® 3500 Genetic Analyzer using a 1.2kV, 12-second injection. The sample file was analyzed with the GeneMapper® *ID*-X software, version 1.4, and PowerPlex® ESI Fast panels and bins text files. **Panel A.** The fluorescein-labeled allelic ladder components and their allele designations. **Pane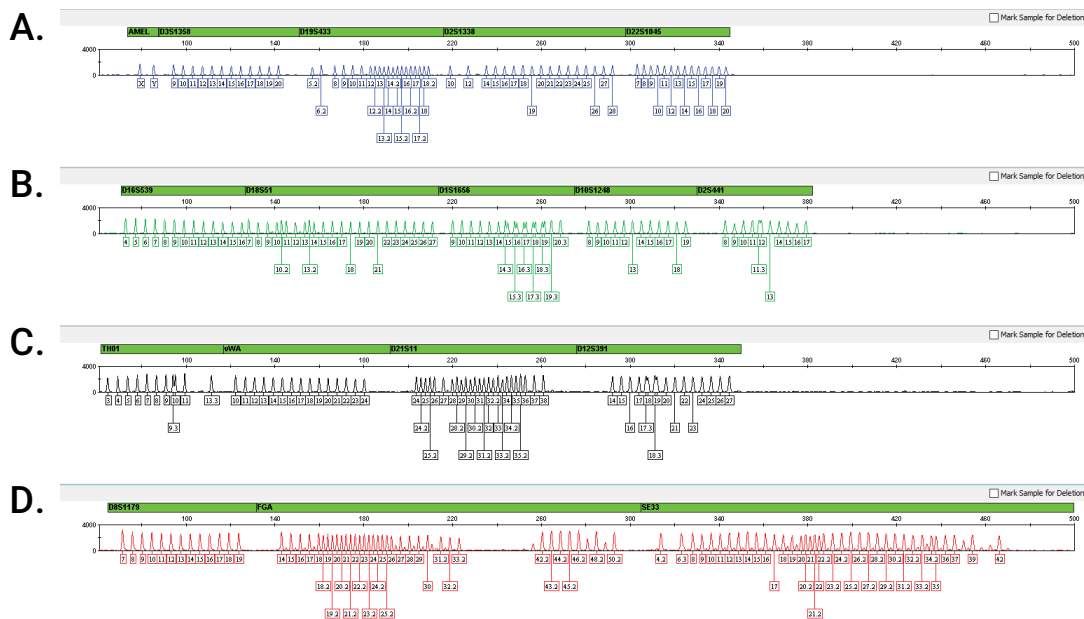l B.** The JOE-labeled allelic ladder components and their allele designations. **Panel C.** The TMR-ET-labeled allelic ladder components and their allele designations. **Panel D.** The CXR-ET-labeled allelic ladder components and their allele designations.

Artifacts and Stutter

Stutter products are a common amplification artifact associated with STR analysis (16,17). Stutter products are often observed one repeat unit below the true allele peak and, occasionally, two repeat units smaller or one repeat unit larger than the true allele peak. Frequently, alleles with a greater number of repeat units will exhibit a higher percent stutter. The pattern and intensity of stutter may differ slightly between primer sets for the same loci. Increased stutter often is associated with D22S1045, as it is a trinucleotide repeat marker.

The mean stutter plus three standard deviations observed at each locus is used in the PowerPlex® ESI Fast panels text file for locus-specific filtering in the GeneMapper® *ID* software, version 3.2, and in the stutter text file for locus-specific filtering in GeneMapper® *ID*-X software.

6.L. Results (continued)

In addition to stutter peaks, the following low-level artifact peaks may be observed with the PowerPlex® ESI 17 Fast System loci.

Table 4. DNA-Dependent Artifacts Observed in Amplification Reactions with Human Genomic DNA.

Dye Label or Locus	Artifact Size
Fluorescein	~65–68 bases and 69–72 bases[1,2] ~84–91 bases[1,3]
Amelogenin	n−1[4]
D19S433	n−10 to n−12[5]
D2S1338	n−16 to n−18[5]
D22S1045	n−10 to n−13[5]
D1S1656	n−2; n+2[6]
D2S441	n−2; n+2[6]
TH01	n−1[4]
D21S11	n−2; n+2[6]
FGA	n−2; n+2[6]
SE33	n−2; n+2[6]
SE33	n−13 to n−14 and n−17 to n−18[5]

[1]Artifact sizes may vary depending on CE instrumentation and environmental conditions in the laboratory.

[2]These variably sized peaks may represent double-stranded DNA derived from the Amelogenin amplicon (double-stranded DNA is known to migrate faster than single-stranded DNA on capillary electrophoresis instruments). This artifact is only seen with high peak heights for the X and Y alleles.

[3]Low-level, DNA-dependent artifact is noticeable with very high template amounts and allele peak heights. These peaks may be above or below analysis threshold, depending on sensitivity of your CE instrument.

[4]The n−1 artifact is more noticeable with high template amounts and allele peak heights.

[5]Low intensity peaks (50–200rfu) that migrate the indicated number of bases (n−) in front of the main allele may represent DNA secondary structure.

[6]Number of bases below (n−) or above (n+) the true allele peak, respectively.

Table 5. DNA-Independent Artifacts Observed in Amplification Reactions with and without Human Genomic DNA.

Dye Label or Locus	Artifact Size[1]
Fluorescein	~51–64 bases
JOE	~63–66 bases
TMR-ET	~59–60 bases ~60–62 bases[2]
D8S1179	~60–69 bases[3] ~69–71 bases ~78–82 bases ~94–96 bases

[1]Artifact sizes may vary depending on CE instrumentation and environmental conditions in the laboratory.

[2]Peak can vary in peak height.

[3]Artifact more likely to be seen if amplification reaction mix remains at ambient temperature for extended periods prior to thermal cycling. This artifact is not observed in no-amplification controls. Peak may be above or below analysis threshold depending on sensitivity of the capillary electrophoresis instrument.

7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com.** E-mail: **genetic@promega.com**

7.A. Amplification and Fragment Detection

This section provides information about general amplification and detection. For questions about amplification of extracted DNA, see Section 7.B. For questions about direct amplification, see Sections 7.C and 7.D.

Symptoms	Causes and Comments
Faint or absent allele peaks	The Master Mix was not vortexed well before use. Vortex the Master Mix for 15 seconds before dispensing into the PCR amplification mix.
	Primer concentration was too low. Use the recommended primer concentration. Vortex the Primer Pair Mix for 15 seconds before use.
	An air bubble formed at the bottom of the reaction tube. Use a pipette to remove the air bubble, or centrifuge the reactions briefly before thermal cycling.
	Thermal cycler, plate or tube problems. Review the thermal cycling protocols in Section 4 or 9. We have not tested reaction tubes, plates or thermal cyclers that are not listed. Calibrate the thermal cycler heating block if necessary.
	Repeat sample preparation using fresh formamide. Long-term storage of amplified sample in formamide can result in loss of signal.
	Poor capillary electrophoresis injection (ILS peaks also affected). Reinject the sample.
	Laser is starting to fail. Check laser power.
	Poor-quality formamide was used. Use only Hi-Di™ formamide when analyzing samples.
Faint or absent peaks for the positive control reaction	Improper storage of the 2800M Control DNA. Store the 2800M Control DNA at +2°C to +10°C. Make sure that the 2800M Control DNA is stored at +2°C to +10°C for at least 24 hours before use; do not refreeze. Vortex before use.

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels	Contamination with another template DNA or previously amplified DNA. Cross contamination can be a problem. Use aerosol-resistant pipette tips, and change gloves regularly.
	Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the DNA source as soon as possible to each well and follow immediately by thermal cycling.
	Samples were not denatured completely. Heat-denature samples for the recommended time, and then cool on crushed ice, a freezer plate block or in an ice-water bath immediately prior to capillary electrophoresis. Do not cool the samples in a thermal cycler set at 4°C, as this may lead to artifacts due to DNA re-annealing.
	Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis. Appearance of "shadow" peaks migrating in front of the main peaks (i.e., smaller in size), especially if the shadow peaks are separated by the same distance as the main peaks in a heterozygote, can indicate the presence of double-stranded DNA due to incomplete denaturation or post-injection re-annealing.
	CE-related artifacts ("spikes"). Minor voltage changes or urea crystals passing by the laser can cause "spikes" or unexpected peaks. Spikes sometimes appear in one color but often are easily identified by their presence in more than one color. Re-inject samples to confirm.
	CE-related artifacts (contaminants). Contaminants in the water used with the instrument or to dilute the 10X genetic analyzer buffer may generate peaks in the fluorescein and JOE channels. Use autoclaved, deionized water; change vials and wash buffer reservoir.
	Incorrect G5 spectral was active. Re-run samples, and confirm that the PowerPlex® G5 spectral calibration is set for G5. See instructions for instrument preparation in Section 5.

Symptoms	Causes and Comments
Extra peaks visible in one channels (continued)	Pull-up or bleedthrough. Pull-up can occur when peak heights are too high or if a poor or incorrect matrix is applied to the samples. • For the Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers, perform a new spectral calibration, and rerun the samples. • Confirm that the correct spectral was used for the sample run. • Instrument sensitivities can vary. Optimize the injection conditions. Refer to the instrument user manual. • Reboot the Applied Biosystems® 3500 or 3500xL Genetic Analyzer and the instrument's computer. Repeat the spectral calibration. Do not allow borrowing when running the spectral calibration on the Applied Biosystems® 3500 or 3500xL Genetic Analyzer.
	Dye blob artifacts. The signal strength of certain dye blob artifacts increases with storage of the amplification plate at 4°C, sometimes in as short a time period as overnight but more commonly when left at 4°C for a few days. We recommend storing amplification products at −20°C.
	An incorrect internal lane standard was used. Use the size standard provided in the kit.
	The CE polymer was beyond its expiration date, or polymer was stored at room temperature for more than one week.
	Maintain instrumentation on a daily or weekly basis as recommended by the manufacturer.
Allelic ladder not running the same as samples	Allelic ladder and primer pair mix were not compatible. Ensure that the allelic ladder is from the same kit as the primer pair mix.
	Be sure the allelic ladder and samples are from the same instrument run.
	Migration of samples changed slightly over the course of a CE run with many samples. This may be due to changes in temperature or the capillary over time. Use a different injection of allelic ladder to determine sizes.

Symptoms	Causes and Comments
Allelic ladder not running the same as samples (continued)	Poor injection of allelic ladder. Include more than one ladder per instrument run.
	Internal size standard was not assigned correctly. Evaluate the sizing labels on the ILS and correct if necessary.
Peak height imbalance	Miscellaneous balance problems. At the first use, thaw the Primer Pair Mix and Master Mix completely. Vortex the Primer Pair Mix and Master Mix for 15 seconds before use; do not centrifuge the Primer Pair Mix or Master Mix after mixing. Calibrate thermal cyclers and pipettes routinely.
	PCR amplification mix was not mixed well. Vortex the PCR amplification mix for 5–10 seconds before dispensing into the reaction tubes or plate.

7.B. Amplification of Extracted DNA

The following information is specific to amplification of purified DNA. For information about general amplification and detection, see Section 7.A.

Symptoms	Causes and Comments
Faint or absent allele peaks	Impure template DNA. Depending on the DNA purification procedure used and sample source, inhibitors might be present in the DNA sample. This may be more of an issue as DNA sample volume increases as a percentage of the total amplification reaction volume.
	Insufficient template. Use the recommended amount of template DNA if available. Quantify template DNA before use if possible.
	High salt concentration or altered pH. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the DNA volume should not exceed 20% of the total reaction volume. Carryover of K^+, Na^+, Mg^{2+} or EDTA from the DNA sample can negatively affect PCR. A change in pH also may affect PCR. Store DNA in TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE^{-4} buffer with 20µg/ml glycogen.
	The reaction volume was too low. This system is optimized for a final reaction volume of 25µl for extracted DNA. Decreasing the reaction volume may result in suboptimal performance.

7.B. Amplification of Extracted DNA (continued)

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels	Amplification of more than the recommended amount of purified DNA can result in a higher number of artifact peaks due to overamplification, resulting in saturating signal. Use the recommended amount of template DNA. See Section 6.L for additional information about stutter and artifacts. The amount of template will need to be optimized if you are using reduced reaction volumes.
	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´A residue. • Be sure to perform the recommended extension step at 60°C after thermal cycling. • Decrease the amount of template DNA. Using more than the recommended amount of template DNA can result in incomplete adenylation. • Decrease cycle number. • Increase the final extension time.
Peak height imbalance	Amplification of greater than the recommended amount of template DNA can result in an imbalance, with smaller loci showing more product than larger loci. Use less template or fewer cycles. The amount of template will need to be optimized if you are using reduced reaction volumes.
	Degraded DNA sample. DNA template was degraded, and larger loci showed diminished yield.
	Insufficient template DNA. Use the recommended amount of template DNA if available. Stochastic effects can occur when amplifying low amounts of template.
	Impure template DNA. Inhibitors that may be present in forensic samples can lead to allele dropout or imbalance.
	Imbalance may be seen more often when using the maximum template volume or a reduced amplification reaction volume.
	The reaction volume was too low. This system is optimized for a final reaction volume of 25µl. Decreasing the reaction volume can result in suboptimal performance.

7.C. Direct Amplification of DNA from Storage Card Punches

The following information is specific to direct amplification of DNA from storage card punches. For information about general amplification and detection, see Section 7.A.

Symptoms	Causes and Comments
Faint or absent allele peaks	For direct amplification reactions that require the addition of AmpSolution™ Reagent, its omission can result in inhibition of the amplification reaction. Review the amplification setup and confirm whether or not AmpSolution™ Reagent is required for your direct amplification reactions.
	DNA was not accessible on nonlytic material. Pretreat nonlytic materials with PunchSolution™ Reagent to ensure that DNA is released from cellular proteins.
	Poor sample deposition. Shedding and collection of donor cells was variable. Increase cycle number.
	Poor sample transfer to storage card or variable sampling from the storage card. Take punches from a different portion of the card. Increasing cycle number can increase low peak heights.
	Too much sample in the reaction can result in inhibition, decreasing intensity of peaks (especially larger amplicons). Be sure to use the recommended number of punches. Follow the manufacturer's recommendations when depositing sample onto the storage card.
	Amplification was inhibited when using more than one storage card punch with blood. Use only one 1.2mm storage card punch with blood.
	The reaction volume was too low. Decreasing the reaction volume from that recommended in the protocol may result in suboptimal performance. Use the recommended number of punches for the reaction volume used.
	Active PunchSolution™ Reagent carried over into the amplification reaction when using nonlytic storage card punches. Ensure that the heat block reached 70°C and samples were incubated for 30 minutes or until wells are dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. We have not tested longer incubation times.

Symptoms	Causes and Comments
Faint or absent allele peaks (continued)	Inactive PunchSolution™ Reagent was used to pretreat nonlytic storage card punches. Thaw the PunchSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
	Consider using PunchSolution™ Reagent with lytic storage card punches.
Faint or absent peaks for the positive control reaction	If the positive control reaction failed to amplify, check to make sure that the correct amount of 2800M Control DNA was added to the reaction. Due to the reduced cycle number it is necessary to increase the mass of 2800M Control DNA to obtain a profile. Use the recommended amount of 2800M Control DNA per amplification reaction. This mass of DNA should be reduced if the cycle number is increased and increased if the cycle number is decreased. Increase or decrease by twofold the mass of 2800M Control DNA for every one-cycle decrease or increase, respectively. We do not recommend including blank punches in the 2800M Control DNA reaction.
	Do not include a blank punch in the positive control reaction. Presence of a blank punch may inhibit amplification of 2800M Control DNA.
Extra peaks visible in one or or all color channels	Punch was contaminated with DNA from another sample. Perform punches on a blank storage card between samples to minimize potential for carryover between samples.
	Amplification of processed punches with high amounts of DNA can result in artifact peaks due to overamplification, resulting in saturating signal on the CE instrument. Be sure to use the recommended number of punches. Use of a larger punch size or a smaller reaction volume may result in overamplification and signal saturation. If the signal is saturated, repeat the amplification with a smaller punch, a larger reaction volume or reduced cycle number.

Symptoms	Causes and Comments
Extra peaks visible in one or or all color channels (continued)	Amplification of excess template for a given cycle number can result in overloading of the capillary upon electrokinetic injection. The presence of excess DNA in the capillary makes it difficult to maintain the DNA in a denatured single-stranded state. Some single-stranded DNA renatures and becomes double-stranded. Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis and appears as "shadow" peaks migrating in front of the main peaks (i.e., smaller in size). If this occurs at a heterozygous locus, it is sometimes possible to see two "shadow" peaks that differ in size from one another by approximately the same distance as the single-stranded alleles.
	Artifacts of STR amplification. Direct amplification of high amounts of template can result in a higher number of artifact peaks. Use the recommended punch size and number of punches. Optimize the cycle number. See Section 6.L for additional information on stutter and artifacts.
	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´A residue. • Be sure to perform the recommended extension step at 60°C after thermal cycling. • Decrease cycle number. • Increase the final extension time.
Peak height imbalance	Excessive amount of DNA. Amplification of high amounts of template can result in an imbalance, with smaller loci showing more product than larger loci. • Be sure to use the recommended number of punches. Follow the manufacturer's recommendation when depositing sample onto the card. • Decrease cycle number.
	The cycle number was too high. Decrease the cycle number by one cycle and repeat the amplification.
	For direct amplification reactions that require the addition of AmpSolution™ Reagent, its omission can result in inhibition of the amplification reaction. Review the amplification setup and confirm whether or not AmpSolution™ Reagent is required for your direct amplification reactions.

7.C. Direct Amplification of DNA from Storage Card Punches (continued)

Symptoms	Causes and Comments
Peak height imbalance (continued)	Amplification was inhibited when using more than one storage card punch with blood. Use only one 1.2mm storage card punch with blood.
	The reaction volume was too low. Decreasing the reaction volume can result in suboptimal performance due to inhibitors present in lytic cards and PunchSolution™ Reagent.
	DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat nonlytic materials with PunchSolution™ Reagent to ensure that DNA is released from cellular proteins.
	Active PunchSolution™ Reagent carried over into the amplification reaction. Larger loci are most susceptible to carryover and will drop out before the smaller loci. • Ensure that the heat block reached 70°C and samples were incubated for 30 minutes or until wells are dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. • We recommend treating one 1.2mm nonlytic storage card punch with 10µl of PunchSolution™ Reagent and using one punch per 25µl or 12.5µl amplification reaction. • Reducing the PunchSolution™ Reagent volume may improve results for reactions with reduced amplification volumes. Optimization and validation are required.
	Inactive PunchSolution™ Reagent was used to pretreat nonlytic storage card punches. Thaw the PunchSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
Extreme variability in sample-to-sample peak heights	There can be significant individual-to-individual variability in the number of cells on a storage card, resulting in peak height variability between samples. The PunchSolution™ Kit maximizes the recovery of amplifiable DNA from nonlytic storage card punches but does not normalize the amount of DNA present.

7.D. Direct Amplification of DNA from Swabs

The following information is specific to amplification of DNA from swabs after pretreatment using the SwabSolution™ Kit. For information about general amplification and detection, see Section 7.A.

Symptoms	Causes and Comments
Faint or absent allele peaks	Poor sample deposition. Shedding and collection of donor cells was variable. Increase cycle number.
	Inactive SwabSolution™ Reagent. Thaw SwabSolution™ Reagent completely in a 37°C water bath, and mix by gentle inversion. Store the SwabSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
	Active SwabSolution™ Reagent carried over into the amplification reaction. Ensure that the heat block reached 70°C (90°C if using a 2.2ml, Square-Well Deep Well Plate) and samples were incubated for the full 30 minutes. Incubation for shorter time periods may result in incomplete reagent inactivation. Do not use an incubator to incubate tubes or plates; heat transfer is inefficient and will result in poor performance. Use only a heat block to maintain efficient heat transfer. We have tested 60-minute incubation times and observed no difference in performance compared to a 30-minute incubation.
	For direct amplification reactions that require the addition of AmpSolution™ Reagent, its omission can result in inhibition of the amplification reaction. Review the amplification setup and confirm whether or not AmpSolution™ Reagent is required for your direct amplification reactions.
	DNA was not accessible on nonlytic material. Pretreat swabs with SwabSolution™ Reagent to ensure that DNA is released from cellular proteins.

Symptoms	Causes and Comments
Faint or absent peaks for the positive control reaction	If the positive control reaction failed to amplify, check to make sure that the correct amount of 2800M Control DNA was added to the reaction. Due to the reduced cycle number used with swab extracts, it is necessary to increase the mass of 2800M Control DNA to obtain a profile. Use the recommended amount of 2800M Control DNA per amplification reaction. This mass of DNA should be reduced if the cycle number is increased and increased if the cycle number is decreased. Increase or decrease by twofold the mass of 2800M Control DNA for every one-cycle decrease or increase, respectively.
Extra peaks visible in one or all color channels	Swab extract was contaminated. Assemble a reaction containing the swab extract prepared from a blank swab, or assemble a reaction where the SwabSolution™ Reagent is processed and incubated as a blank without a swab.
	Artifacts of STR amplification. Amplification of swab extracts with high DNA concentrations can result in artifact peaks due to overamplification, resulting in saturated signal on the CE instrument.
	We recommend 2µl of swab extract per reaction. Using more than 2µl may result in overamplification and signal saturation. If signal is saturated, repeat amplification with less swab extract or reduced cycle number.
	Amplification of excess template for a given cycle number resulted in overloading of the capillary upon electrokinetic injection. Excess DNA in the capillary is difficult to maintain in a denatured single-stranded state. Some single-stranded DNA renatures and becomes double-stranded. Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis and appears as "shadow" peaks migrating in front of the main peaks (i.e., smaller in size). If this occurs at a heterozygous locus it is possible to observe the presence of two "shadow" peaks that differ in size by approximately the same distance as the single-stranded alleles.

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels (continued)	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´A residue.
	• Be sure to perform the recommended extension step at 60°C after thermal cycling.
	• Use 2µl of swab extract in an amplification reaction. A larger volume of swab extract may contain more than the recommended amount of DNA template, resulting in incomplete adenylation.
	• Decrease cycle number.
	• Increase the final extension time.
Peak height imbalance	Excess DNA in the amplification reaction can result in locus-to-locus imbalance within a dye channel such that the peak heights at the smaller loci are greater than those at the larger loci (ski-slope effect). Use less swab extract or reduce the cycle number.
	Active SwabSolution™ Reagent carried over from swab extracts into the amplification reaction. Larger loci are most susceptible to reagent carryover and will drop out before the smaller loci. Ensure that the heat block reached 70°C (90°C if using 2.2ml, Square-Well Deep Well Plates) and samples were incubated for the full 30 minutes. Incubation for shorter time periods may result in incomplete reagent inactivation. Do not use an incubator to incubate tubes or plates; heat transfer is inefficient and will result in poor performance. Use only a heat block to maintain efficient heat transfer.
	Inactive SwabSolution™ Reagent. Thaw the SwabSolution™ Reagent completely in a 37°C water bath and mix by gentle inversion. Store the SwabSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
	DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat swabs with SwabSolution™ Reagent to ensure that DNA is released from cellular proteins.

Symptoms	Causes and Comments
Extreme variability in sample-to-sample peak heights	There can be significant individual-to-individual variability in cell deposition onto buccal swabs. This will appear as variability in peak heights between swab extracts. The extraction process maximizes recovery of amplifiable DNA from buccal swabs but does not normalize the amount of DNA present. If variability is extreme, quantify the DNA using a fluorescence-based double-stranded DNA quantification method or qPCR-based quantification method. The quantification values can be used to normalize input template amounts to minimize variation in signal intensity.

7.E. GeneMapper® *ID*-X Software

Symptoms	Causes and Comments
Stutter peaks not filtered	Stutter text file was not imported into the Panel Manager when the panels and bins text files were imported.
	Be sure that the "Use marker-specific stutter ratio and distance if available" box is checked. If the 'Use marker-specific stutter ratio and distance if available' box is not checked, stutter distance must be defined in the Analysis Method 'Allele' tab.
Samples in the project not analyzed	The 'Analysis Requirements Summary' window was not active, and there was an analysis requirement that was not met. Turn on Analysis Requirements Summary in the 'Project Options' menu under File, and correct the necessary analysis requirements to continue analysis.
Edits in label edit viewer cannot be viewed	To view edits made to a project, the project first must be saved. Close the plot view window, return to the main GeneMapper® *ID*-X page and save the project. Display the plot window again, and then view the label edit table.
Marker header bar for some loci are gray	When an edit is made to a locus, the quality flags and marker header bar automatically change to gray. To change the GQ and marker header bar for a locus to green, override the GQ in the plot window.
Alleles not called	To analyze samples with GeneMapper® *ID*-X software, at least one allelic ladder must be defined per folder of sample files being analyzed in the project.

Symptoms	Causes and Comments
Alleles not called (continued)	Alleles were not resolved in D12S391 or D2S441. Change polymer and capillary array to resolve the 17.3 and 18 alleles and 18.3 and 19 alleles in the D12S391 allelic ladder and the 11.3 and 12 alleles in the D2S441 allelic ladder.
	Run was too short and larger peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Rerun samples using a longer run time.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.
Off-ladder alleles	An allelic ladder from a different run than the samples was used. Re-analyze samples with an allelic ladder from the same run.
	Alleles were not resolved in D12S391 or D2S441. Change polymer and capillary array to resolve the 17.3 and 18 alleles and 18.3 and 19 alleles in the D12S391 allelic ladder and the 11.3 and 12 alleles in the D2S441 allelic ladder.
	The GeneMapper® *ID*-X software requires that the allelic ladder be imported from the same folder as the sample. Be sure that the allelic ladder is in the same folder as the sample. Create a new project and re-analyze, as described in Section 6.D, Processing Data for Casework Samples or Section 6.E, Processing Data for Databasing or Paternity Samples.
	Panels text file selected for analysis was incorrect for the STR system used. Assign correct panels text file that corresponds to the STR system used for amplification.
	The allelic ladder was not identified as an allelic ladder in the 'Sample Type' column.
	The internal lane standard was not properly identified in the sample. Manually redefine the sizes of the size standard fragments in the sample.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.

7.E. GeneMapper® *ID*-X Software (continued)

Symptoms	Causes and Comments
Size standard not called correctly	Starting data point was incorrect for the partial range chosen for the analysis in the GeneMapper® *ID*-X Analysis Method Editor 'Peak Detector' tab as described in Section 6.D, Creating a Casework Analysis Method or Section 6.E, Creating a Databasing or Paternity Analysis Method. Adjust the starting data point in the analysis method or use a full range for the analysis.
	Extra peaks in size standard. Open the Size Match Editor. Highlight the extra peak, select **Edit** and select **Delete Size Label**. Select **Auto Adjust Sizes**.
	An incorrect internal lane standard was used.
	Run was too short, and larger peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Rerun samples using a longer run time.
Peaks in size standard missing	If peaks are low-quality, redefine the size standard for the sample to skip these peaks.
	An incorrect internal lane standard was used.
Significantly raised baseline	Poor spectral calibration for the Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers. Perform a new spectral calibration, and rerun the samples.
	Incorrect G5 spectral was active. Rerun samples, and confirm that the PowerPlex® G5 spectral calibration is set for G5. See instructions for instrument preparation in Section 5.

7.F. GeneMapper® *ID* Software

Symptoms	Causes and Comments
Alleles not called	Alleles were not resolved in D12S391 or D2S441. Change polymer and capillary array to resolve the 17.3 and 18 alleles and 18.3 and 19 alleles in the D12S391 allelic ladder and the 11.3 and 12 alleles in the D2S441 allelic ladder.
	To analyze samples with GeneMapper® *ID* software, the analysis parameters and size standard must both have 'Basic or Advanced' as the analysis type. If they are different, an error is obtained.

Symptoms	Causes and Comments
Alleles not called (continued)	To analyze samples with GeneMapper® *ID* software, at least one allelic ladder must be defined per folder of sample files being analyzed in the project.
	Run was too short and larger peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Rerun samples using a longer run time.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.
Off-ladder alleles	An allelic ladder from a different run than the samples was used. Reanalyze samples using an allelic ladder from the same run.
	Alleles were not resolved in D12S391 or D2S441. Change polymer and capillary array to resolve the 17.3 and 18 alleles and 18.3 and 19 alleles in the D12S391 allelic ladder and the 11.3 and 12 alleles in the D2S441 allelic ladder.
	The GeneMapper® *ID* software requires that the allelic ladder be imported from the same folder as the sample. Be sure that the allelic ladder is in the same folder as the sample. Create a new project and reanalyze as described in Section 6.I or 6.J.
	Panels text file selected for analysis was incorrect for the STR system used. Assign correct panels text file that corresponds to the STR system used for amplification.
	The allelic ladder was not identified as an allelic ladder in the 'Sample Type' column.
	The wrong analysis type was chosen for the analysis method. Be sure to use the HID analysis type.
	The internal lane standard was not properly identified in the sample. Manually redefine the sizes of the size standard fragments in the sample.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.
Size standard not called correctly	Starting data point was incorrect for the partial range chosen in Section 6.J or 6.I. Adjust the starting data point in the analysis method. Alternatively, use a full range for the analysis.

7.F. GeneMapper® *ID* Software (continued)

Symptoms	Causes and Comments
Size standard not called correctly	Extra peaks in size standard. Open the Size Match Editor. Highlight the extra peak, select **Edit** and select **Delete Size Label**. Select **Auto Adjust Sizes**.
	An incorrect size standard was used.
	Peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Re-run samples using a longer run time.
Peaks in size standard missing	If peaks were low-quality, redefine the size standard for the sample to skip these peaks.
	An incorrect size standard was used.
Error message: "Either panel, size standard, or analysis method is invalid"	The size standard and analysis method were not in the same mode ('Classic' vs. 'Basic or Advanced'). Be sure both files are set to the same mode, either 'Classic' or 'Basic or Advanced' mode.
No alleles called, but no error message appears	Panels text file was not selected for sample. In the 'Panel' column, select the appropriate panels text file for the STR system that was used.
	No size standard was selected. In the 'Size Standard' column, be sure to select the appropriate size standard.
	Size standard was not correctly defined, or size peaks were missing. Redefine size standard to include only peaks present in your sample. Terminating analysis early or using short run times will cause larger ladder peaks to be missing. This will cause your sizing quality to be flagged as "red", and no allele sizes will be called.
Error message: "Both the Bin Set used in the Analysis Method and the Panel must belong to the same Chemistry Kit"	The bins text file assigned to the analysis method was deleted. In the GeneMapper Manager, select the 'Analysis Methods' tab, and open the analysis method of interest. Select the 'Allele' tab, and select an appropriate bins text file.
	The wrong bins text file was chosen in the analysis method 'Allele' tab. Be sure to choose the appropriate bins text file, as shown in Figure 21.

Symptoms	Causes and Comments
Significantly raised baseline	Poor spectral calibration for the Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers. Perform a new spectral calibration, and rerun the samples.
	Use of 'Classic' mode analysis method. Use of 'Classic' mode analysis on samples can result in baselines with more noise than those analyzed using the 'Basic or Advanced' mode analysis method. 'Advanced' mode analysis methods and size standards are recommended.
	Incorrect G5 spectral was active. Rerun samples, and confirm that the PowerPlex® G5 spectral calibration is set for G5. See instructions on instrument preparation in Section 5.
Error message after attempting to import panels and bins text files: "Unable to save panel data: java.SQLEEException: ORA-00001: unique constraint (IFA.CKP_NNN) violated".	There was a conflict between different sets of panels and bins text files. Check to be sure that the bins are installed properly. If not, delete all panels and bins text files, and reimport files in a different order.
Allelic ladder peaks labeled off-ladder	GeneMapper® *ID* software was not used, or microsatellite analysis settings were used instead of HID analysis settings. GeneMapper® software does not use the same algorithms as GeneMapper® *ID* software and cannot correct for sizing differences using the allelic ladder. We recommend using GeneMapper® *ID* software to analyze PowerPlex® reactions. If using GeneMapper® *ID* software, version 3.2, be sure that the analysis method selected is an HID method. This can be verified by opening the analysis method using the GeneMapper Manager, and then selecting the 'General' tab. The analysis type cannot be changed. If the method is not HID, it should be deleted and a new analysis method created.

Symptoms	Causes and Comments
Allelic ladder peaks labeled off-ladder (continued)	Alleles were incorrectly labeled in allelic ladder at one or all of the following loci: D1S1656, D2S441 and D12S391. The D1S1656, D2S441 and D12S391 allelic ladders contain alleles that differ in size by one base. Because these loci are large in the PowerPlex® ESI 17 Fast System, these alleles are sensitive to poor resolution, unlike alleles separated by more than one base. If there is poor resolution of alleles separated by one base, the GeneMapper® *ID* software is not able to distinguish these alleles and cannot call alleles correctly. Poor resolution is usually due to an old capillary, old capillary array or use of expired POP-4® polymer. Install a new array or polymer on your CE instrument, and repeat the injection of those samples.

8.   References

1.   Edwards, A. *et al.* (1991) DNA typing with trimeric and tetrameric tandem repeats: Polymorphic loci, detection systems, and population genetics. In: *The Second International Symposium on Human Identification 1991*, Promega Corporation, 31–52.

2.   Edwards, A. *et al.* (1991) DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *Am. J. Hum. Genet.* **49**, 746–56.

3.   Edwards, A. *et al.* (1992) Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. *Genomics* **12**, 241–53.

4.   Warne, D. *et al.* (1991) Tetranucleotide repeat polymorphism at the human b-actin related pseudogene 2 (actbp2) detected using the polymerase chain reaction. *Nucleic Acids Res.* **19**, 6980.

5.   Ausubel, F.M. *et al.* (1996) Unit 15: The polymerase chain reaction. In: *Current Protocols in Molecular Biology*, Vol. 2, John Wiley and Sons, NY.

6.   Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Chapter 14: In vitro amplification of DNA by the polymerase chain reaction. In: *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

7.   PCR Technology: Principles and Applications for DNA Amplification (1989) Erlich, H.A., ed., Stockton Press, New York, NY.

8.   *PCR Protocols: A Guide to Methods and Applications* (1990) Innis, M.A. *et al.* eds., Academic Press, San Diego, CA.

9.   Butler, J.M. (2005) *Forensic DNA Typing*, 2nd ed. Elsevier Academic Press, London.

10. Presley, L.A. *et al.* (1992) The implementation of the polymerase chain reaction (PCR) HLA DQ alpha typing by the FBI laboratory. In: *The Third International Symposium on Human Identification 1992*, Promega Corporation, 245–69.

11. Hartmann, J.M. *et al.* (1991) Guidelines for a quality assurance program for DNA analysis. *Crime Laboratory Digest* **18**, 44–75.

12. *Internal Validation Guide of Autosomal STR Systems for Forensic Laboratories*. #GE053, Promega Corporation.

13. McLaren, R.S. *et al.* (2014) Developmental validation of the PowerPlex® ESI 16/17 Fast and PowerPlex® ESX 16/17 Fast Systems. *Forensic Sci. Int. Genet.* **13**, 195–205.

14. Kline, M.C. *et al.* (2005) Results from the NIST 2004 DNA quantitation study. *J. Forensic Sci.* **50**, 570–8.

15. McLaren, R.S. *et al.* (2008) Post-injection hybridization of complementary DNA strands on capillary electrophoresis platforms: A novel solution for dsDNA artifacts. *Forensic Sci. Int. Genet.* **2**, 257–73.

16. Levinson, G. and Gutman, G.A. (1987) Slipped-strand mispairing: A major mechanism for DNA sequence evolution. *Mol. Biol. Evol.* **4**, 203–21.

17. Schlotterer, C. and Tautz, D. (1992) Slippage synthesis of simple sequence DNA. *Nucleic Acids Res*. **20**, 211–5.

18. Smith, J.R. *et al*. (1995) Approach to genotyping errors caused by nontemplated nucleotide addition by *Taq* DNA polymerase. *Genome Res.* **5**, 312–7.

19. Magnuson, V.L. *et al*. (1996) Substrate nucleotide-determined non-templated addition of adenine by Taq DNA polymerase: Implications for PCR-based genotyping. *BioTechniques* **21**, 700–9.

20. Walsh, P.S., Fildes, N.J. and Reynolds, R. (1996) Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. *Nucleic Acids Res*. **24**, 2807–12.

21. Griffiths, R. *et al.* (1998) New reference allelic ladders to improve allelic designation in a multiplex STR system. *Int. J. Legal Med*. **111**, 267–72.

22. Butler, J.M. (2006) Genetics and genomics of core STR loci used in human identity testing. *J. Forensic Sci.* **51**, 253–65.

23. Hill, C.R. *et al.* (2008) Characterization of 26 miniSTR loci for improved analysis of degraded DNA samples. *J. Forensic Sci*. **53**, 73–80.

24. Bär, W. *et al.* (1997) DNA recommendations. Further report of the DNA Commission of the ISFH regarding the use of short tandem repeat systems. *Int. J. Legal Med*. **110**, 175–6.

25. Gill, P. *et al.* (1997) Considerations from the European DNA Profiling Group (EDNAP) concerning STR nomenclature. *Forensic Sci. Int.* **87**, 185–92.

9. Appendix

9.A. Advantages of Using the Loci in the PowerPlex® ESI 17 Fast System

The loci included in the PowerPlex® ESI 17 Fast System (Tables 6 and 7) were selected because they meet the recommendations of the European Network of Forensic Science Institutes (ENFSI). The PowerPlex® ESI 17 Fast System amplifies all ENFSI core loci plus SE33 in a single reaction. Table 8 lists the PowerPlex® ESI 17 Fast System alleles amplified from the 2800M Control DNA.

We have carefully selected primers to avoid or minimize artifacts, including those associated with DNA polymerases, such as repeat slippage and terminal nucleotide addition (16,17). Repeat slippage, sometimes called "n−4 peaks", "stutter" or "shadow bands", is due to the loss of a repeat unit during DNA amplification, somatic variation within the DNA or both. The amount of this artifact observed depends primarily on the locus and the DNA sequence being amplified.

Terminal nucleotide addition (18,19) occurs when a thermostable nonproofreading DNA polymerase adds a nucleotide, generally adenine, to the 3´ ends of amplified DNA fragments in a template-independent manner. The efficiency with which this occurs varies with different primer sequences. Thus, an artifact peak one base shorter than expected (i.e., missing the terminal addition) is sometimes seen. We have modified primer sequences and added a final extension step of 60°C (20) to the amplification protocols to provide conditions for essentially complete terminal nucleotide addition when recommended amounts of template DNA are used.

Table 6. The PowerPlex® ESI 17 Fast System Locus-Specific Information.

STR Locus	Label	Chromosomal Location[1]	Repeat Sequence[2] 5´→3´
D22S1045	Fluorescein	22q12.3 (35.779Mb)	ATT
D2S1338	Fluorescein	2q35 (218.705Mb)	TGCC/TTCC
D19S433	Fluorescein	19q12 (35.109Mb)	AAGG Complex
D3S1358	Fluorescein	3p21.31 (45.557Mb)	TCTA Complex
Amelogenin[3]	Fluorescein	Xp22.1−22.3 and Y	NA
D2S441	JOE	2p14 (68.214Mb)	TCTA
D10S1248	JOE	10q26.3 (130.567Mb)	GGAA
D1S1656	JOE	1q42 (228.972Mb)	TAGA Complex
D18S51	JOE	18q21.33 (59.1Mb)	AGAA (21)
D16S539	JOE	16q24.1 (84.944Mb)	GATA
D12S391	TMR-ET	12p12 (12.341Mb)	AGAT/AGAC Complex
D21S11	TMR-ET	21q21.1(19.476Mb)	AAAGA
vWA	TMR-ET	12p13.31 (5.963Mb)	TCTA Complex (21)
TH01	TMR-ET	11p15.5 (2.149Mb)	AATG (21)
SE33	CXR-ET	6q14 (89.043Mb)	AAAG Complex
FGA	CXR-ET	4q28 (155.866Mb)	TTTC Complex (21)
D8S1179	CXR-ET	8q24.13 (125.976Mb)	TCTA Complex (21)

[1]Information about chromosomal location of these loci can be found in references 22 and 23 and at:
https://strbase.nist.gov//chrom.htm

[2]The August 1997 report (24,25) of the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) states, "1) for STR loci within coding genes, the coding strand shall be used and the repeat sequence motif defined using the first possible 5´ nucleotide of a repeat motif; and 2) for STR loci not associated with a coding gene, the first database entry or original literature description shall be used".

[3]Amelogenin is not an STR.

NA = Not applicable.

Table 7. The PowerPlex® ESI 17 Fast System Allelic Ladder Information.

STR Locus	Label	Size Range of Allelic Ladder Components[1,2] (bases)	Repeat Numbers of Allelic Ladder Components[3]
D22S1045	Fluorescein	306–345	7–20
D2S1338	Fluorescein	223–295	10, 12, 14–28
D19S433	Fluorescein	163–215	5.2, 6.2, 8–12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2, 18, 18.2
D3S1358	Fluorescein	103–147	9–20
Amelogenin[4]	Fluorescein	89, 95	X, Y
D2S441	JOE	347–383	8–11, 11.3, 12–17
D10S1248	JOE	286–330	8–19
D1S1656	JOE	226–273	9–14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18, 18.3, 19, 19.3, 20.3
D18S51	JOE	134–214	7–10, 10.2, 11–13, 13.2, 14–27
D16S539	JOE	84–132	4–16
D12S391	TMR-ET	291–343	14–17, 17.3, 18, 18.3, 19–27
D21S11	TMR-ET	203–259	24, 24.2, 25, 25.2, 26–28, 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
vWA	TMR-ET	124–180	10–24
TH01	TMR-ET	72–115	3–9, 9.3, 10–11, 13.3
SE33	CXR-ET	309–459	4.2, 6.3, 8–20, 20.2, 21, 21.2, 22, 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, 35–37, 39, 42
FGA	CXR-ET	143–289	14–18, 18.2, 19, 19.2, 20, 20.2, 21, 21.2, 22, 22.2, 23, 23.2, 24, 24.2, 25, 25.2, 26–30, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 48.2, 50.2
D8S1179	CXR-ET	76–124	7–19

[1]The length of each allele in the allelic ladder has been confirmed by sequence analyses.

[2]When using an internal lane standard, such as the WEN Internal Lane Standard 500 ESS, the calculated sizes of allelic ladder components may differ from those listed. This occurs because different sequences in allelic ladder and ILS components may cause differences in migration. The dye label and linker also affect migration of alleles.

[3]For a current list of microvariants, see the Variant Allele Report published at the U.S. National Institute of Standards and Technology (NIST) web site at: **https://strbase.nist.gov/var_tab.htm**

[4]Amelogenin is not an STR.

Table 8. The PowerPlex® ESI 17 Fast System Allele Determinations for the 2800M Control DNA.

STR Locus	2800M
D22S1045	16, 16
D2S1338	22, 25
D19S433	13, 14
D3S1358	17, 18
Amelogenin	X, Y
D2S441	10, 14
D10S1248	13, 15
D1S1656	12, 13
D18S51	16, 18
D16S539	9, 13
D12S391	18, 23
D21S11	29, 31.2
vWA	16, 19
TH01	6, 9.3
SE33	15, 16
FGA	20, 23
D8S1179	14, 15

9.B. The WEN Internal Lane Standard 500 ESS

The WEN Internal Lane Standard 500 ESS contains 21 DNA fragments of 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases in length (Figure 26). Each fragment is labeled with WEN dye and can be detected separately (as a fifth color) in the presence of PowerPlex® ESI 17 Fast-amplified material. The WEN ILS 500 ESS is designed for use in each CE injection to increase precision in analyses when using the PowerPlex® ESI 17 Fast System. Protocols to prepare and use this internal lane standard are provided in Section 5.
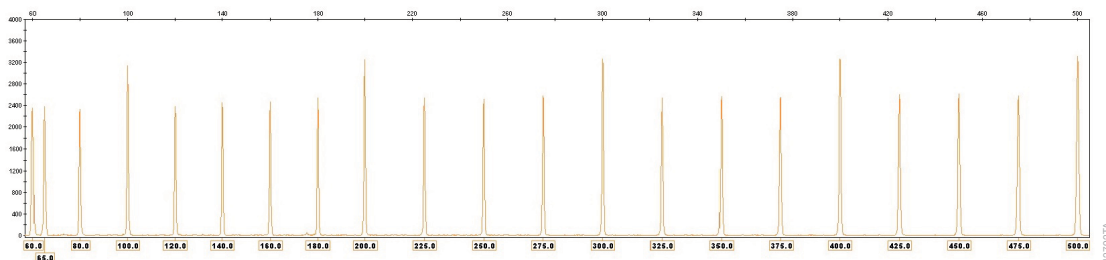

Figure 23. WEN Internal Lane Standard 500 ESS. An electropherogram showing the WEN Internal Lane Standard 500 ESS fragments.

9.C. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume

Testing at Promega has shown successful direct amplification of DNA from storage card punches in a 12.5µl reaction volume (13). This section contains a protocol for direct amplification of DNA from storage card punches in a 12.5µl reaction volume using the PowerPlex® ESI 17 Fast System and GeneAmp® PCR System 9700 or ProFlex® PCR System. When using the protocol detailed below, add only one 1.2mm storage card punch to each 12.5µl amplification reaction.

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block or ProFlex® PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- PunchSolution™ Kit (Cat.# DC9271) for nonlytic storage card punches
- 5X AmpSolution™ Reagent (Cat.# DM1231, also supplied with the PunchSolution™ Kit)
- 1.2mm Harris Micro-Punch or equivalent manual punch and cutting mat or automated punch system

Notes:

a. It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is **not** recommended with the PowerPlex® ESI 17 Fast System.

b. You will need to optimize and validate the number of storage card punches per reaction in your laboratory. See the PCR Optimization recommendations at the end of this section.

Lytic storage card sample types include:

- Buccal cells collected on FTA® cards with Whatman EasiCollect™ devices (one punch per 12.5µl amplification reaction)
- Buccal cells collected with swabs transferred to FTA® or Indicating FTA® cards (one punch per 12.5µl amplification reaction)
- Liquid blood (from collection or storage Vacutainer® tubes or finger sticks) spotted onto FTA® cards (one punch per 12.5µl amplification reaction)

Nonlytic storage card sample types include:

- Buccal samples on Bode Buccal DNA Collector™ devices (one punch per 12.5µl amplification reaction)
- Blood and buccal samples on nonlytic storage cards (e.g., S&S 903; one punch per 12.5µl amplification reaction)

Pretreat nonlytic sample types with the PunchSolution™ Kit (Cat.# DC9271) to lyse samples before adding the PCR amplification mix. For more information, see the *PunchSolution™ Kit Technical Manual* #TMD038. Failure to pretreat these samples may result in incomplete profiles.

Use a manual punch tool with a 1.2mm tip to manually create sample disks from a storage card. Place tip near the center of the sample spot, and with a twisting or pressing action, cut a 1.2mm sample disk. Use the plunger to eject the disk into the appropriate well of a reaction plate.

Automated punchers also can be used to create sample disks. Refer to the user's guide for your instrument for assistance with generating 1.2mm disks, technical advice and troubleshooting information.

Note: Static may be problematic when adding a punch to a well. For lytic storage card punches, adding PCR amplification mix to the well before adding the punch may help alleviate static problems. For nonlytic storage card punches, adding PunchSolution™ Reagent to the well before adding the punch during pretreatment may help alleviate static problems.

9.C. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume (continued)

Amplification Setup

1. Thaw the PowerPlex® ESI/ESX Fast 5X Master Mix, PowerPlex® ESI 17 Fast 10X Primer Pair Mix and Water, Amplification Grade, completely.

    Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 10X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Vortex the 5X AmpSolution™ Reagent for 10–15 seconds.

    Note: The 5X AmpSolution™ Reagent should be thawed completely, mixed by vortexing and stored at +2°C to +10°C. The reagent may be turbid after thawing or storage at +2°C to +10°C. If this occurs, warm the buffer briefly at 37°C, and then vortex until clear. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Storing reagents in the refrigerator door can compromise stability.

3. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.

4. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.

5. Add the final volume of each reagent listed in Table 9 to a clean tube.

Table 9. PCR Amplification Mix for Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume.

PCR Amplification Mix Component[1]	Volume Per Reaction	x	Number of Reactions	=	Final Volume
Water, Amplification Grade	6.25µl	×		=	
PowerPlex® ESI/ESX Fast 5X Master Mix	2.50µl	×		=	
PowerPlex® ESI 17 Fast 10X Primer Pair Mix	1.25µl	×		=	
5X AmpSolution™ Reagent	2.50µl	×		=	
total reaction volume	12.50µl				

[1]Add Water, Amplification Grade, to the tube first, and then add PowerPlex® ESI/ESX Fast 5X Master Mix, PowerPlex® ESI 17 Fast 10X Primer Pair Mix and 5X AmpSolution™ Reagent. For lytic storage card punches, the template DNA will be added at Step 7.

6. Vortex the PCR amplification mix for 5–10 seconds, and then pipet 12.5µl of PCR amplification mix into each reaction well.

(!) Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

Note: Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the punches as soon as possible to each well and follow immediately by thermal cycling.

7. For lytic storage cards, add one 1.2mm punch from a card containing buccal cells or one 1.2mm punch from a card containing whole blood to the appropriate wells of the reaction plate. For nonlytic storage card punches, add the PCR amplification mix to the PunchSolution™ Reagent-treated punches.

Note: It also is acceptable to add the lytic storage card punch first, and then add the PCR amplification mix.

8. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 5ng/µl. Add 1µl (5ng) of the diluted 2800M Control DNA to a reaction well containing 12.5µl of PCR amplification mix.

   Notes:
   a. Do not include blank storage card punches in the positive control reactions.
   b. Optimization of the amount of 2800M Control DNA may be required depending on thermal cycling conditions and laboratory preferences.

9. Reserve a well containing PCR amplification mix as a negative amplification control.

   Note: An additional negative control with a blank punch may be performed to detect contamination from the storage card or punch device.

10. Seal or cap the plate, or close the tubes. Briefly centrifuge the plate to bring storage card punches to the bottom of the wells and remove any air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols, including cycle number, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 25 cycles works well for a variety of storage card sample types. Cycle number will need to be optimized in each laboratory for each sample type.

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is **not** recommended with the PowerPlex® ESI 17 Fast System.

9.C. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume (continued)

1. Place the reaction plate or tubes in the thermal cycler.

2. Select and run the recommended protocol below.

    Notes:

    a. When using the ProFlex® PCR System, use the 9700 Simulation Mode as the ramp speed.

    b. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol
96°C for 1 minute, then:
96°C for 5 seconds 60°C for 35 seconds 72°C for 5 seconds for 25 cycles, then:
60°C for 2 minutes
4°C soak

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at −20°C protected from light.

    Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

PCR Optimization

Cycle number should be optimized based on the results of an initial experiment to determine the sensitivity with your collection method, sample types and instrumentation.

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.

2. Depending on your preferred protocol, place one 1.2mm storage card punch containing a buccal sample or one 1.2mm punch of a storage card containing whole blood into each well of a reaction plate. Be sure to pretreat nonlytic storage card samples with the PunchSolution™ Kit (Cat.# DC9271).

3. Prepare three identical reaction plates with punches from the same samples.

4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (24, 25 and 26 cycles).

5. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type.

9.D. Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume

Testing at Promega has shown successful direct amplification of DNA from swabs in a 12.5µl reaction volume (13). This section contains a protocol for amplifying DNA from swab extracts in a 12.5µl reaction volume using the PowerPlex® ESI 17 Fast System and GeneAmp® PCR System 9700 or ProFlex® PCR System.

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block or ProFlex® PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- SwabSolution™ Kit (Cat.# DC8271)

Pretreat OmniSwab™ (QIAGEN) or cotton swabs using the SwabSolution™ Kit (Cat.# DC8271) as described in the *SwabSolution™ Kit Technical Manual* #TMD037 to generate a swab extract.

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is **not** recommended with the PowerPlex® ESI 17 Fast System.

Amplification Setup

1. Thaw the PowerPlex® ESI/ESX Fast 5X Master Mix, PowerPlex® ESI 17 Fast 10X Primer Pair Mix and Water, Amplification Grade, completely.

   Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 10X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Vortex the 5X AmpSolution™ Reagent for 10−15 seconds.

   Note: The 5X AmpSolution™ Reagent should be thawed completely, mixed by vortexing and stored at +2°C to +10°C. The reagent may be turbid after thawing or storage at +2°C to +10°C. If this occurs, warm the buffer briefly at 37°C, and then vortex until clear. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Storing reagents in the refrigerator door can compromise stability.

3. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.

4. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.

9.D. Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume (continued)

5. Add the final volume of each reagent listed in Table 10 to a clean tube.

Table 10. PCR Amplification Mix for Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	4.25µl	×		=	
PowerPlex® ESI/ESX Fast 5X Master Mix	2.50µl	×		=	
PowerPlex® ESI 17 Fast 10X Primer Pair Mix	1.25µl	×		=	
5X AmpSolution™ Reagent	2.50µl	×		=	
swab extract	2.00µl				
total reaction volume	12.50µl				

[1]Add Water, Amplification Grade, to the tube first, and then add PowerPlex® ESI/ESX Fast 5X Master Mix, PowerPlex® ESI 17 Fast 10X Primer Pair Mix and 5X AmpSolution™ Reagent. The swab extract will be added at Step 7.

6. Vortex the PCR amplification mix for 5−10 seconds, and then pipet 10.5µl of PCR amplification mix into each reaction well.

    ⓘ Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

    Note: Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the swab extract as soon as possible to each well and follow immediately by thermal cycling.

7. Pipet 2µl of swab extract for each sample into the appropriate well of the reaction plate.

8. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 2.5ng/µl. Add 2µl (5ng) of the diluted 2800M Control DNA to a reaction well containing 10.5µl of PCR amplification mix.

    Note: Optimization of the amount of 2800M Control DNA may be required depending on thermal cycling conditions and laboratory preferences.

9. For the negative amplification control, pipet 2µl of Water, Amplification Grade, or TE[−4] buffer instead of swab extract into a reaction well containing PCR amplification mix.

    Note: Additional negative controls can be included. Assemble a reaction containing the swab extract prepared from a blank swab, or assemble a reaction where the SwabSolution™ Reagent is processed as a blank without a swab.

10. Seal or cap the plate, or close the tubes.

    Optional: Briefly centrifuge the plate to bring contents to the bottom of the wells and remove any air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including the amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 25 cycles works well for a variety of swab sample types. Cycle number will need to be optimized in each laboratory for each sample type.

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is **not** recommended with the PowerPlex® ESI 17 Fast System.

1. Place the reaction plate or tubes in the thermal cycler.

2. Select and run the recommended protocol below.

    Notes:

    a. When using the ProFlex® PCR System, use the 9700 Simulation Mode as the ramp speed.

    b. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol
96°C for 1 minute, then:
96°C for 5 seconds 60°C for 35 seconds 72°C for 5 seconds for 25 cycles, then:
60°C for 2 minutes
4°C soak

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at −20°C protected from light.

    Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

9.D. Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume (continued)

PCR Optimization

Cycle number should be optimized based on the results of an initial experiment to determine the sensitivity with your collection method, sample types and instrumentation.

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.

2. Prepare three identical reaction plates with aliquots of the same swab extracts.

3. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (24, 25 and 26 cycles).

    Note: This recommendation is for 2µl of swab extract. If a different volume of swab extract is used, additional cycle number testing may be required.

4. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type.

9.E. Composition of Buffers and Solutions

TE⁻⁴ buffer (10mM Tris-HCl, 0.1mM EDTA [pH 8.0])

  1.21g   Tris base
  0.037g   EDTA (Na$_2$EDTA • 2H$_2$O)

Dissolve Tris base and EDTA in 900ml of deionized water. Adjust to pH 8.0 with HCl. Bring the final volume to 1 liter with deionized water.

TE⁻⁴ buffer with 20µg/ml glycogen

  1.21g   Tris base
  0.037g   EDTA (Na$_2$EDTA • 2H$_2$O)
  20µg/ml   glycogen

Dissolve Tris base and EDTA in 900ml of deionized water. Adjust to pH 8.0 with HCl. Add glycogen. Bring the final volume to 1 liter with deionized water.

9.F.   Related Products

Product	Size	Cat.#
PowerPlex® 5C Matrix Standard	5 preps	DG4850
WEN Internal Lane Standard 500 ESS	200µl	DG5101
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
2800M Control DNA (10ng/µl)	25µl	DD7101
2800M Control DNA (0.25ng/µl)	500µl	DD7251
Water, Amplification Grade	6,250µl (5 × 1,250µl)	DW0991
5X AmpSolution™ Reagent	500µl	DM1231

Not for Medical Diagnostic Use.

9.G.   Summary of Changes

The following changes were made to the 10/23 revision of this document:

1.   Removed any mention of ABI PRISM® Genetic Analyzers.

2.   Updated Section 9.F.

3.   Replaced cover image and document font.

4.   Made minor text edits.

5.   Updated patent statements.