TECHNICAL MANUAL

PowerPlex® 16 HS System

Instructions for Use of Products
DC2100 and DC2101

Revised 5/16
TMD022

PowerPlex® 16 HS System

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® 16 HS System[a–c] is used for human identification applications including forensic analysis, relationship testing and research use. The system allows co-amplification and three-color detection of sixteen loci (fifteen STR loci and Amelogenin), including Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Amelogenin, Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818. One primer for each of the Penta E, D18S51, D21S11, TH01 and D3S1358 loci is labeled with fluorescein (FL); one primer for each of the FGA, TPOX, D8S1179, vWA and Amelogenin loci is labeled with carboxy-tetramethylrhodamine (TMR); and one primer for each of the Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818 loci is labeled with 6-carboxy-4´,5´-dichloro-2´,7´-dimethoxy-fluorescein (JOE). All sixteen loci are amplified simultaneously in a single tube and analyzed in a single injection.

The PowerPlex® 16 Monoplex System, Penta E (Fluorescein) (Cat.# DC6591), and PowerPlex® 16 Monoplex System, Penta D (JOE) (Cat.# DC6651), are available to amplify the Penta E and Penta D loci, respectively. These monoplex systems allow amplification of a single locus to confirm results obtained with the PowerPlex® 16 System or PowerPlex® 16 HS System.

The PowerPlex® 16 HS System is compatible with the ABI PRISM® 310, 3100 and 3100-*Avant* Genetic Analyzers and Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers. The protocols presented in this manual were tested at Promega. Amplification and detection instrumentation may vary. You may need to optimize protocols including cycle number and injection conditions (or loading volume) for each laboratory instrument. In-house validation should be performed.

The PowerPlex® 16 HS System provides all materials necessary to amplify STR regions of human genomic DNA, including hot-start *Taq* DNA polymerase, which is a component of the PowerPlex® HS 5X Master Mix. This manual contains protocols for use of the PowerPlex® 16 HS System with GeneAmp® PCR system 9600 and 9700 thermal cyclers in addition to protocols to separate amplified products and detect separated material (Figure 1). Protocols to operate the fluorescence-detection instruments should be obtained from the instrument manufacturer.

Information on other Promega fluorescent STR systems, including the PowerPlex® 16 Monoplex Systems, is available upon request from Promega or online at: **www.promega.com**

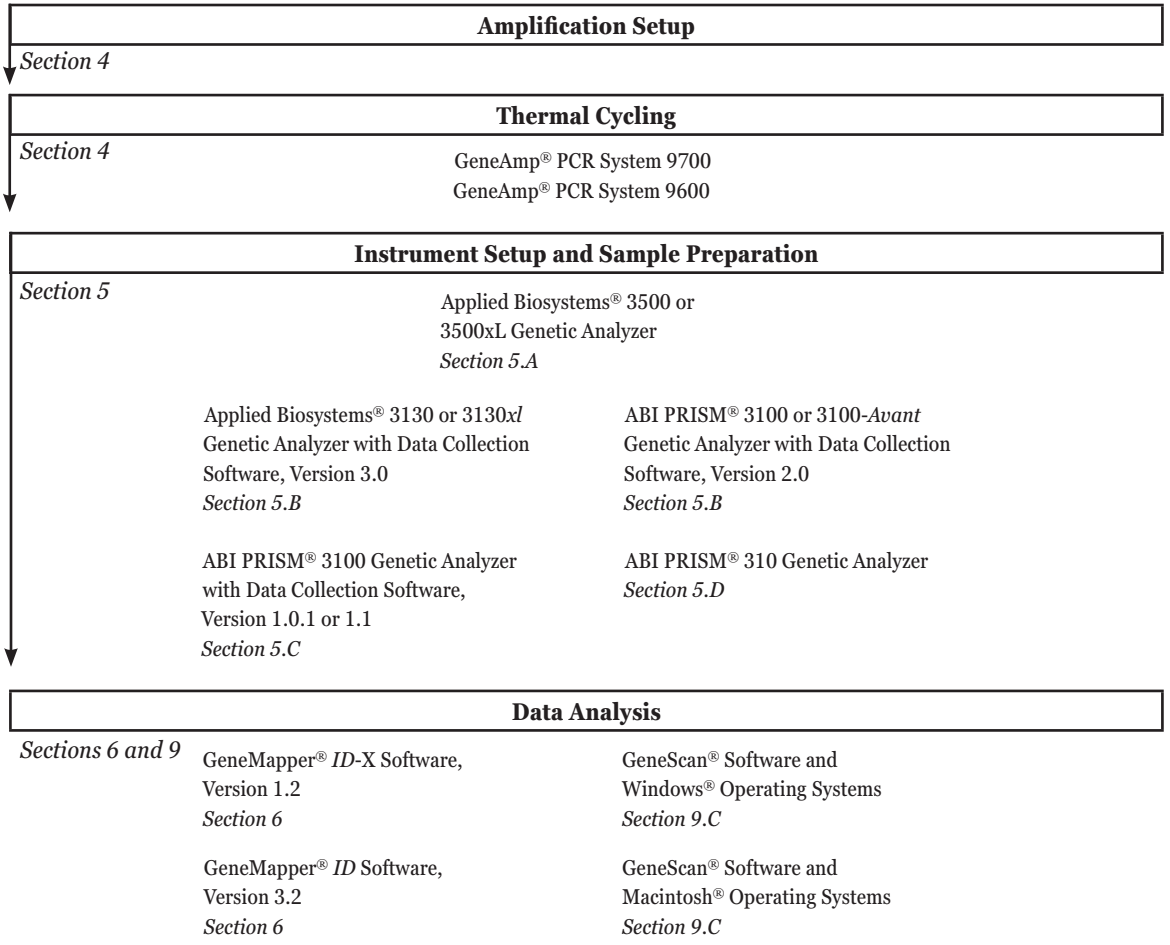

Figure 1. An overview of the PowerPlex® 16 HS System protocol.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
PowerPlex® 16 HS System	**100 reactions**	**DC2101**

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

Pre-amplification Components Box (Blue Label)

1 × 500µl	PowerPlex® HS 5X Master Mix
1 × 250µl	PowerPlex® 16 HS 10X Primer Pair Mix
25µl	2800M Control DNA, 10ng/µl
5 × 1,250µl	Water, Amplification Grade

Post-amplification Components Box (Yellow Label)

- 1 × 50µl PowerPlex® 16 HS Allelic Ladder Mix
- 1× 150µl Internal Lane Standard (ILS) 600

PRODUCT	SIZE	CAT.#
PowerPlex® 16 HS System	**400 reactions**	**DC2100**

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

Pre-amplification Components Box (Blue Label)

- 4 × 500µl PowerPlex® HS 5X Master Mix
- 4 × 250µl PowerPlex® 16 HS 10X Primer Pair Mix
- 25µl 2800M Control DNA, 10ng/µl
- 10 × 1,250µl Water, Amplification Grade

Post-amplification Components Box (Yellow Label)

- 4 × 50µl PowerPlex® 16 HS Allelic Ladder Mix
- 4 × 150µl Internal Lane Standard (ILS) 600

The PowerPlex® 16 HS Allelic Ladder Mix is provided in a separate, sealed bag for shipping. This component should be moved to the post-amplification box after opening.

PowerPlex® HS 5X Master Mix and PowerPlex® 16 HS 10X Primer Pair Mix are manufactured as a matched set for optimal performance. Do not combine components from kits with different lot numbers (printed on the boxes and Certificates of Analysis). If lots are mixed, locus-to-locus imbalance and variation in signal intensity may occur.

Storage Conditions: Upon receipt, store all components except the 2800M Control DNA at –30°C to –10°C in a nonfrost-free freezer. Store the 2800M Control DNA at 2–10°C. Make sure that the 2800M Control DNA is stored at 2–10°C for at least 24 hours before use. The PowerPlex® 16 HS 10X Primer Pair Mix, PowerPlex® 16 HS Allelic Ladder Mix and Internal Lane Standard 600 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. For daily use, the PowerPlex® 16 HS 10X Primer Pair Mix and PowerPlex® HS 5X Master Mix can be stored at 2–10°C for up to 1 week without loss of activity. The Water, Amplification Grade, can be stored at 2–10°C long-term.

Available Separately

PRODUCT	SIZE	CAT.#
PowerTyper™ Macros (Release 2.0)	**1 CD-ROM**	**DG3470**

Not For Medical Diagnostic Use.

The PowerTyper™ Macros (Release 2.0), for use with Genotyper® software, are available from Promega. This CD-ROM contains the file "PowerTyper™ 16 Macro (Release 2.0)" for use with the PowerPlex® 16 HS System. The macros also can be downloaded at: **www.promega.com/resources/software-firmware/powertyper-macros//**

The proper panels and bins text files for use with GeneMapper® *ID* and *ID*-X software can be obtained from the Promega web site at: **www.promega.com/resources/software-firmware/genemapper-id-software-panels-and-bin-sets/**

Matrix standards are required for initial setup of the color separation matrix. The matrix standards are sold separately and are available for the ABI PRISM® 310 Genetic Analyzer (PowerPlex® Matrix Standards, 310; Cat.# DG4640) and the ABI PRISM® 3100 and 3100-*Avant* Genetic Analyzers and Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers (PowerPlex® 4C Matrix Standard; Cat.# DG4800). See Section 9.G for ordering information.

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 of STR Systems Reference Manual* (12).

The quality of purified DNA, small changes in buffers, ionic strength, primer concentrations, 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 are made to the recommended protocols.

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 (PowerPlex® HS 5X Master Mix, PowerPlex® 16 HS 10X 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 (PowerPlex® 16 HS Allelic Ladder Mix and Internal Lane Standard 600). 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.

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.

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3.B. Matrix Standardization or Spectral Calibration

Proper spectral calibration is critical to evaluate multicolor systems with the ABI PRISM® 310, 3100 and 3100-*Avant* Genetic Analyzers and Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers. A matrix must be generated for each individual instrument.

The PowerPlex® Matrix Standards, 310 (Cat.# DG4640), is required for matrix standardization for the ABI PRISM® 310 Genetic Analyzer. The PowerPlex® 4C Matrix Standard (Cat.# DG4800) cannot be used to generate a matrix on the ABI PRISM® 310 Genetic Analyzer.

The PowerPlex® 4C Matrix Standard is required for spectral calibration on the ABI PRISM® 3100 and 3100-*Avant* Genetic Analyzers and Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers. The PowerPlex® Matrix Standards, 310, cannot be used to generate a matrix on these instruments.

For protocols and additional information on matrix standardization, see the *PowerPlex® Matrix Standards, 310, Technical Bulletin* #TBD021. For protocols and additional information on spectral calibration, see the *PowerPlex® 4C Matrix Standard Technical Bulletin* #TMD048. These manuals are available online at:
www.promega.com/resources/protocols/

4. Protocols for DNA Amplification Using the PowerPlex® 16 HS System

The PowerPlex® 16 HS System is optimized for the GeneAmp® PCR System 9700 thermal cycler. An amplification protocol for the GeneAmp® PCR Systems 9600 thermal cycler also is provided for extracted DNA.

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.A.

4.A. Amplification of Extracted DNA

Materials to Be Supplied by the User

- GeneAmp® PCR System 9600 or 9700 thermal cycler (Applied Biosystems)
- microcentrifuge
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips

The PowerPlex® 16 HS System is optimized for 0.5ng of purified DNA template in a 25µl reaction volume using 10/22 cycles of amplification. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different. Developmental validation of the kit showed routine generation of full profiles using 10/22 cycles of amplification with lower amounts of DNA template down to 62.5pg. Partial profiles were typically observed for DNA template amounts of 31.25pg (13). When the DNA template exceeds 1ng, preferential amplification of smaller loci can occur; reduce the amount of template DNA or the number of cycles to correct this. In-house optimization and validation should be performed to establish the performance of the kit in your laboratory (12).

Amplification Setup

1. Thaw the PowerPlex® HS 5X Master Mix and PowerPlex® 16 HS 10X Primer Pair Mix completely.

   Note: Centrifuge tubes briefly to bring contents to the bottom, 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.

   PowerPlex® HS 5X Master Mix and PowerPlex® 16 HS 10X Primer Pair Mix are manufactured as a matched set for optimal performance. Do not combine components from kits with different lot numbers (printed on the boxes and Certificates of Analysis). If lots are mixed, locus-to-locus imbalance and variation in signal intensity may occur.

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 MicroAmp® plate for reaction assembly, and label appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.

4.A. Amplification of Extracted DNA (continued)

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

   Note: In tests performed at Promega, we have found that reactions can remain at room temperature for up to 8 hours after reaction assembly and prior to thermal cycling with no adverse effect on amplification results.

Table 1. PCR Amplification Mix for the PowerPlex® 16 HS System.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25.0µl	×		=	
PowerPlex® HS 5X Master Mix	5.0µl	×		=	
PowerPlex® 16 HS 10X Primer Pair Mix	2.5µl	×		=	
template DNA (0.5–1ng)[2,3]	up to 17.5µl				
total reaction volume	**25µl**				

[1]Add Water, Amplification Grade, to the tube first, then add PowerPlex® HS 5X Master Mix and PowerPlex® 16 HS 10X Primer Pair Mix. The template DNA will be added at Step 6.

[2]Store DNA templates in nuclease-free water or TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA). 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. Amplification efficiency and quality can be altered greatly by changes in pH (due to added Tris-HCl), available magnesium concentration (due to chelation by EDTA) or 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). The amount of DNA template recommended here is based on DNA concentrations determined by measuring absorbance at 260nm. We strongly recommend that you perform experiments to determine the optimal DNA amount based on your DNA quantification method.

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

Failure to vortex the PCR amplification mix sufficiently can result in poor amplification, peak height imbalance and extra peaks.

6. Add the template DNA (0.5ng) for each sample to the respective well containing PCR amplification mix.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, then dilute an aliquot to 0.5ng in the desired template DNA volume. Add 0.5ng of the 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 the plate. **Optional:** Briefly centrifuge the plate to bring contents to the bottom and remove any air bubbles.

Amplification of >1.0ng of DNA template may result in an imbalance in peak heights from locus to locus. The smaller loci show greater amplification yield than the larger loci. Reducing the number of cycles in the amplification program by 2 to 4 cycles (i.e., 10/20 or 10/18 cycling) can improve locus-to-locus balance.

Thermal Cycling

This section contains protocols for use of the PowerPlex® 16 HS System with the GeneAmp® PCR system 9600 and 9700 thermal cyclers. For information on other thermal cyclers, contact Promega Technical Services by e-mail: **genetic@promega.com**

Amplification and detection instrumentation may vary. You may need to optimize protocols including cycle number and injection conditions (or loading volume) for each laboratory instrument. Testing at Promega shows that 10/22 cycles work well for 0.5ng of purified DNA templates. For higher amounts of input DNA (i.e., FTA® paper) or to decrease sensitivity, fewer cycles, such as 10/16, 10/18 or 10/20, should be evaluated. In-house validation should be performed.

1. Place reaction tubes or MicroAmp® plate in the thermal cycler.
2. Select and run the recommended protocol. The preferred protocols for use with the GeneAmp® PCR System 9600 and 9700 thermal cyclers are provided below.
3. After completion of the thermal cycling protocol, proceed to fragment analysis or store amplified samples at –20°C in a light-protected box.

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

Protocol for the GeneAmp® PCR System 9600 Thermal Cycler	Protocol for the GeneAmp® PCR System 9700 Thermal Cycler[1]
96°C for 2 minutes, then:	96°C for 2 minutes, then:
94°C for 30 seconds ramp 68 seconds to 60°C (hold for 30 seconds) ramp 50 seconds to 70°C (hold for 45 seconds) for 10 cycles, then:	ramp 100% to 94°C for 30 seconds ramp 29% to 60°C for 30 seconds ramp 23% to 70°C for 45 seconds for 10 cycles, then:
90°C for 30 seconds ramp 60 seconds to 60°C (hold for 30 seconds) ramp 50 seconds to 70°C (hold for 45 seconds) for 22 cycles, then:	ramp 100% to 90°C for 30 seconds ramp 29% to 60°C for 30 seconds ramp 23% to 70°C for 45 seconds for 22 cycles, then:
60°C for 30 minutes	60°C for 30 minutes
4°C soak	4°C soak

[1]When using the GeneAmp® PCR System 9700 thermal cycler, the ramp rates indicated in the cycling program must be set, and the program must be run in 9600 ramp mode.

The ramp rates are set in the Ramp Rate Modification screen. While viewing the cycling program, navigate to the Ramp Rate Modification screen by selecting “More”, then “Modify”. On the Ramp Rate Modification screen the default rates for each step are 100%. The rate under each hold step is the rate at which the temperature will change to that hold temperature. Figure 2 shows the appropriate ramp rates for the GeneAmp® PCR System 9700 thermal cycler.

The ramp mode is set after “start” has been selected for the thermal cycling run. A Select Method Options screen appears. Select 9600 ramp mode, and enter the reaction volume

4.A. Amplification of Extracted DNA (continued)

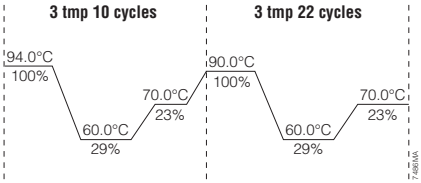

Figure 2. The ramp rates for the GeneAmp® PCR System 9700 thermal cycler.

4.B. Direct Amplification of DNA from Storage Card Punches

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems)
- microcentrifuge
- MicroAmp® optical 96-well reaction plate (Applied Biosystems)
- aerosol-resistant pipette tips
- 5X AmpSolution™ Reagent (Cat.# DM1231, also supplied with the PunchSolution™ Kit)
- PunchSolution™ Kit (Cat.# DC9271) for nonFTA punches
- 1.2mm Harris Micro-Punch or equivalent manual punch and cutting mat

This section contains a protocol for direct amplification of DNA from storage card punches using the PowerPlex® 16 HS System and GeneAmp® PCR System 9700 thermal cycler.

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.

FTA®-based sample types include:

- Buccal cells collected on FTA® cards with Whatman EasiCollect™ or Fitzco Sampact™ devices (one or two punch per 25µl amplification reaction)
- Buccal cells collected with swabs transferred to FTA® or Indicating FTA® cards (one or two punch 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)

NonFTA sample types include: (one punch per 25µl amplification reaction)

- Buccal samples on Bode Buccal DNA Collector™ devices
- Blood and buccal samples on nonFTA card punches (e.g., S&S 903)
  Pretreat these sample types with the PunchSolution™ Reagent (Cat.# DC9271) to lyse nonFTA 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 FTA® card punches, adding a PCR amplification mix to the well before adding the punch may help alleviate static problems.

Amplification Setup

1. Thaw the PowerPlex® HS 5X Master Mix and PowerPlex® 16 HS 10X Primer Pair Mix completely.

   Note: Centrifuge tubes briefly to bring contents to the bottom, 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 MicroAmp® plate for reaction assembly, and label appropriately.

4. Add the final volume of each reagent listed in Table 2 to a sterile tube.

Table 2. PCR Amplification Mix for Direct Amplification of DNA from Storage Card Punches.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	12.5µl	×		=	
PowerPlex® HS 5X Master Mix	5.0µl	×		=	
PowerPlex® 16 HS 10X Primer Pair Mix	2.5µl	×		=	
5X AmpSolution™ Reagent	5.0µl	×		=	
total reaction volume	**25µl**				

[1]Add Water, Amplification Grade, to the tube first, then add PowerPlex® HS 5X Master Mix, PowerPlex® 16 HS 10X Primer Pair Mix and 5X AmpSolution™ Reagent. For FTA® card punches, the template DNA will be added at Step 6.

5. Vortex the PCR amplification mix for 5–10 seconds, 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.

4.B. Direct Amplification of DNA from Storage Card Punches (continued)

6. For FTA® 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 nonFTA card punches, add the PCR amplification mix to the pretreated punches.

   Note: It also is acceptable to add the FTA® card punch first, then add the PCR amplification mix.

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

   Notes:

   1. Do not include blank storage card punches in the positive control reactions.
   2. Optimization of the amount of 2800M Control DNA may be required based on thermal cycling conditions and laboratory preferences. Typically, 10ng of 2800M Control DNA is sufficient to provide a robust profile using the cycle numbers recommended here. A one-cycle reduction in cycle number will require a twofold increase in mass of DNA template to generate similar signal intensity. Similarly, a one-cycle increase in cycle number will require a twofold reduction in the amount of 2800M Control DNA to avoid signal saturation.

8. 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.

9. Seal the plate, and briefly centrifuge the plate to bring storage card punches to the bottom of the wells and remove any air bubbles.

   Note: Place the amplification plate in the thermal cycler, and start the thermal cycling program as soon as the PowerPlex® System PCR amplification mix is added to all wells. Prolonged storage of assembled reactions prior to cycling may result in poor performance (i.e., lower peak heights for large amplicons).

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 27 cycles works well for a variety of sample types. Buccal samples may require more amplification cycles than blood samples. Cycle number will need to be optimized in each laboratory for each sample type that is amplified.

1. Place the MicroAmp® plate in the thermal cycler.

2. Select and run the recommended protocol. The preferred protocol for use with the GeneAmp® PCR System 9700 thermal cycler is provided below.

Thermal Cycling Protocol[1]
96°C for 2 minutes, then:
ramp 100% to 94°C for 30 seconds ramp 29% to 60°C for 30 seconds ramp 23% to 70°C for 45 seconds for 10 cycles, then:
ramp 100% to 90°C for 30 seconds ramp 29% to 60°C for 30 seconds ramp 23% to 70°C for 45 seconds for 17 cycles, then:
60°C for 30 minutes
4°C soak

[1]When using the GeneAmp® PCR System 9700 thermal cycler, the ramp rates indicated in the cycling program must be set, and the program must be run in 9600 ramp mode.

The ramp rates are set in the Ramp Rate Modification screen. While viewing the cycling program, navigate to the Ramp Rate Modification screen by selecting "More", then "Modify". On the Ramp Rate Modification screen the default rates for each step are 100%. The rate under each hold step is the rate at which the temperature will change to that hold temperature. Figure 2 shows the appropriate ramp rates for the GeneAmp® PCR System 9700 thermal cycler.

The ramp mode is set after "start" is selected for the thermal cycling run. A Select Method Options screen appears. Select 9600 ramp mode, and enter the reaction volume.

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

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

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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 punch of a storage card containing whole blood in each well of a reaction plate.
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.
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

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems)
- microcentrifuge
- MicroAmp® optical 96-well reaction plate (Applied Biosystems)
- aerosol-resistant pipette tips
- SwabSolution™ Kit (Cat.# DC8271)

This section contains a protocol for amplifying swab extracts using the PowerPlex® 16 HS System and GeneAmp® PCR System 9700 thermal cycler.

Pretreat cotton or OmniSwabs™ (GE Healthcare) swabs with 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® HS 5X Master Mix and PowerPlex® 16 HS 10X Primer Pair Mix completely.

   Note: Centrifuge tubes briefly to bring contents to the bottom, 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 MicroAmp® plate for reaction assembly, and label appropriately.

4. Add the final volume of each reagent listed in Table 3 to a sterile tube.

Table 3. PCR Amplification Mix for Direct Amplification of DNA From Swabs.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	10.5µl	×		=	
PowerPlex® HS 5X Master Mix	5.0µl	×		=	
PowerPlex® 16 HS 10X Primer Pair Mix	2.5µl	×		=	
5X AmpSolution™ Reagent	5.0µl	×		=	
swab extract	2.0µl				
total reaction volume	**25µl**				

[1]Add Water, Amplification Grade, to the tube first, then add PowerPlex® HS 5X Master Mix and PowerPlex® 16 HS 10X Primer Pair Mix. The swab extract will be added at Step 6.

5. Vortex the PCR amplification mix for 5–10 seconds, 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.

6. Pipet 2.0µl of swab extract for each sample into the appropriate well of the reaction plate.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, then dilute an aliquot to 2.5ng/µl, and add 2µl 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.

8. For the negative amplification control, pipet 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.

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

4.C. Direct Amplification of DNA from Swabs (continued)

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 28 cycles works well for a variety of sample types. Cycle number will need to be optimized in each laboratory for each sample type that is amplified (see below).

1. Place the MicroAmp® plate in the thermal cycler.
2. Select and run the recommended protocol. The preferred protocol for use with the GeneAmp® PCR System 9700 thermal cycler is provided below.

Thermal Cycling Protocol[1]
96°C for 2 minutes, then:
ramp 100% to 94°C for 30 seconds ramp 29% to 60°C for 30 seconds ramp 23% to 70°C for 45 seconds for 10 cycles, then:
ramp 100% to 90°C for 30 seconds ramp 29% to 60°C for 30 seconds ramp 23% to 70°C for 45 seconds for 18 cycles, then:
60°C for 30 minutes
4°C soak

[1]When using the GeneAmp® PCR System 9700 thermal cycler, the ramp rates indicated in the cycling program must be set, and the program must be run in 9600 ramp mode.

The ramp rates are set in the Ramp Rate Modification screen. While viewing the cycling program, navigate to the Ramp Rate Modification screen by selecting "More", then "Modify". On the Ramp Rate Modification screen the default rates for each step are 100%. The rate under each hold step is the rate at which the temperature will change to that hold temperature. Figure 2 shows the appropriate ramp rates for the GeneAmp® PCR System 9700 thermal cycler.

The ramp mode is set after "start" has been selected for the thermal cycling run. A Select Method Options screen appears. Select 9600 ramp mode, and enter the reaction volume.

3. After completion of the thermal cycling protocol, proceed to fragment analysis store amplified samples at –20°C in a light-protected box.

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

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 (27, 28 and 29 cycles).

   Note: This recommendation is for 2µl of swab extract. 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 or ice-water bath
- 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 and septa, or equivalent
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)

The quality of formamide is critical. Use Hi-Di™ 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

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

Sample Preparation

1. Prepare a loading cocktail by combining and mixing Internal Lane Standard 600 and Hi-Di™ formamide as follows:

   [(0.5µl ILS 600) × (# samples)] + [(9.5µl Hi-Di™ formamide) × (# samples)]

   Note: The volume of internal lane standard used in the loading cocktail can be increased or decreased to adjust the intensity of the size standard peaks. If peak heights are too low, we recommend altering the formamide/internal lane standard mix to contain 1.0µl of ILS 600 and 9.0µl of Hi-Di™ formamide. If peak heights are too high, we recommend altering the loading cocktail to contain 0.25µl of ILS 600 and 9.75µl of formamide.

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 PowerPlex® 16 HS Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

   Note: 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 by 2–4 cycles to achieve the desired signal intensity.

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

6. Denature samples at 95°C for 3 minutes, then immediately chill on crushed ice 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 3). Ensure that the Consumables Information and Maintenance Notifications are acceptable.

   Set the oven temperature to 60°C, then select "Start Pre-Heat" at least 30 minutes prior to the first injection to preheat the oven.

Figure 3. The Dashboard.

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

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

   Figure 4 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 settings that were changed from the default settings are dye set and run time.

   When creating an Instrument Protocol, be sure to select the same dye set that was used to perform the Promega 4-dye spectral calibration. We recommend using a run time of 1,500 seconds and the default injection conditions.

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

   Assign a descriptive protocol name.

   Note: For more detailed information refer to the *Applied Biosystems® 3500/3500xL Genetic Analyzers User Guide*.

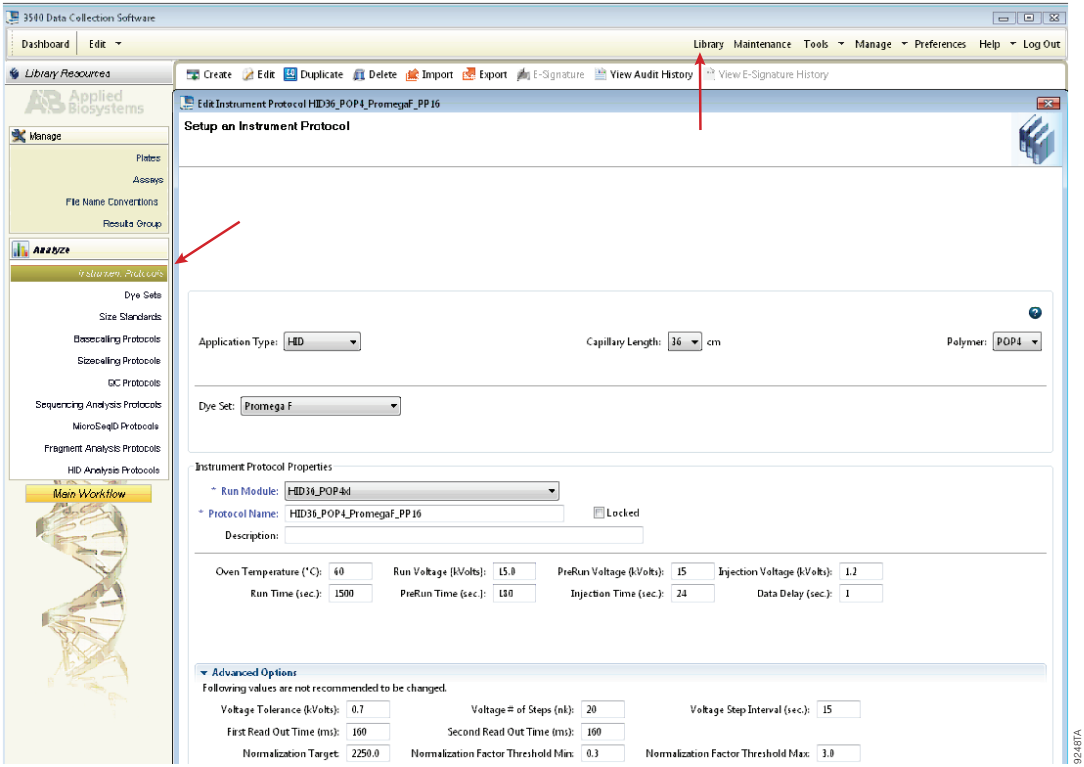

Figure 4. The Create New Instrument Protocol window.

3. To create a new Size Standard for the QC protocol, navigate to the Library. Select “Size Standards”, then select “Create”. Alternatively, a previously created Size Standard may be used.

   Assign the size standard the name “PPLX_ILS600” or another appropriate name. Choose “Red” as the Dye Color. The fragments in the size standard are 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550 and 600 bases. See Figure 5.

   Note: Definition and detection of the 600bp fragment is optional.

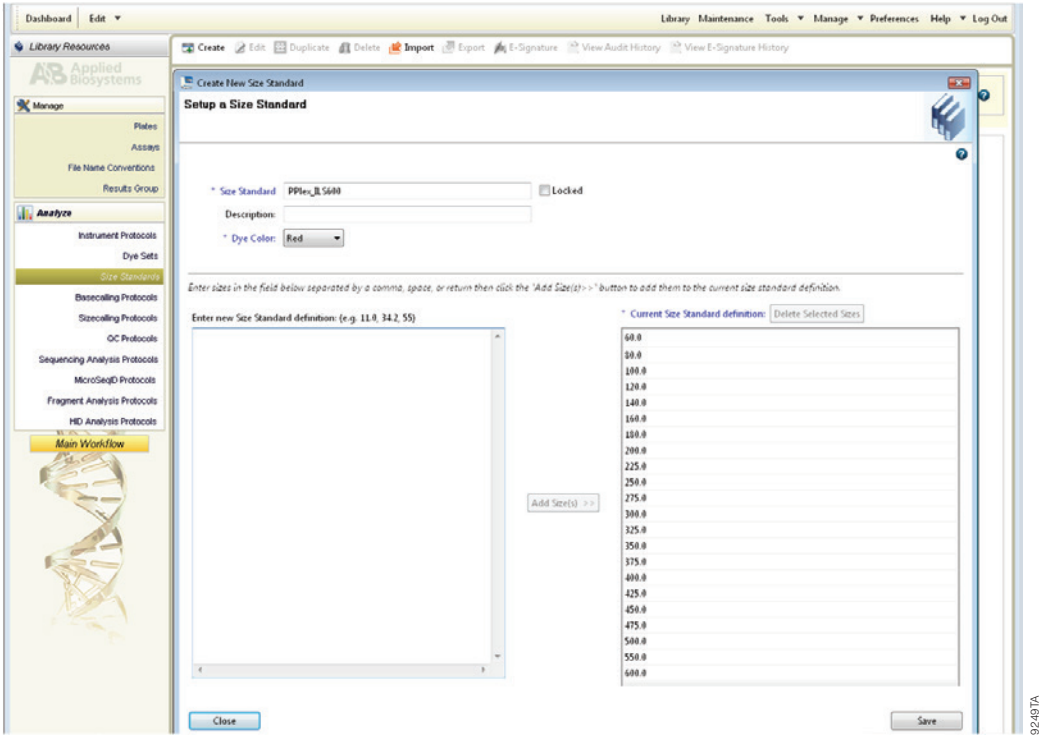

Figure 5. The Create New Size Standard window.

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

4. To create a new QC Protocol, navigate to the Library. Select "QC Protocols", then select "Create". A previously created QC Protocol may be used.

   Assign a descriptive protocol name. Select the size standard created in Step 3. The settings for the QC protocol should be based on the internally validated conditions for the PowerPlex® 16 HS System on the Applied Biosystems® 3500 or 3500xL Genetic Analyzer. Figure 6 shows one option for these settings.

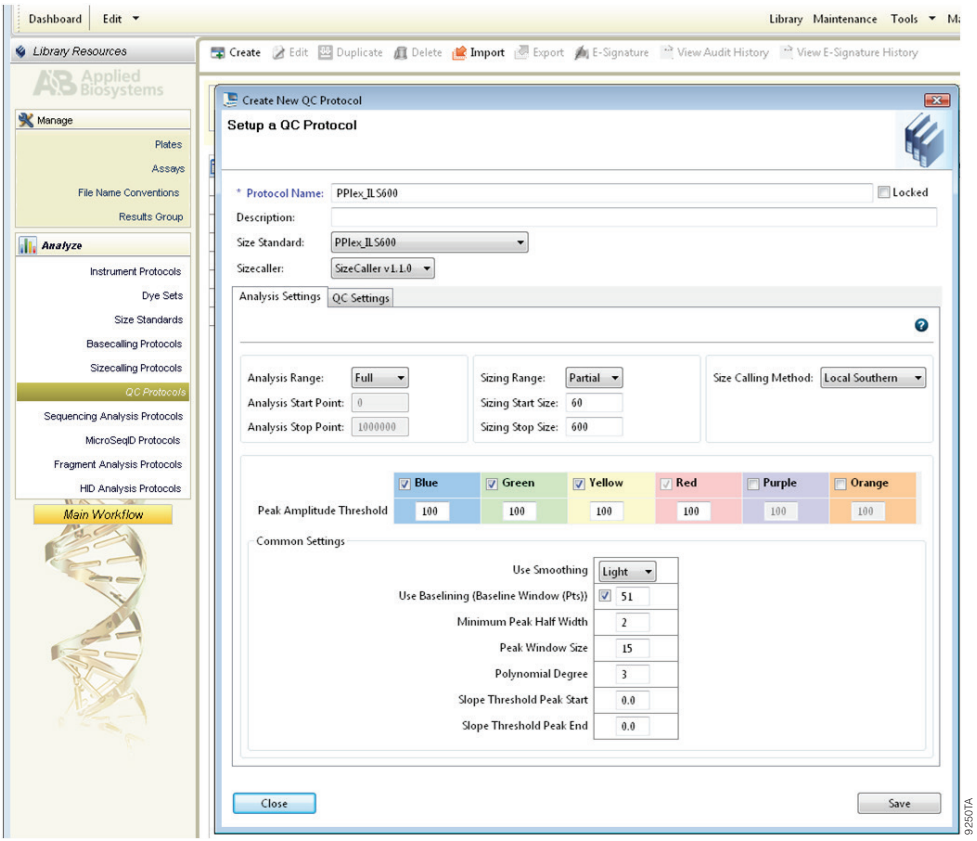

Figure 6. The Create New QC Protocol window.

5. To create a new Assay, navigate to the Library. Select “Assays”, then select “Create”. Alternatively, a previously created Assay may be used.

   In the Create New Assay window (Figure 7), select the instrument protocol created in Step 2 and the QC protocol created in Step 4. Assign a descriptive assay name. Select the application type “HID”. An Assay is required for all named samples on a plate.

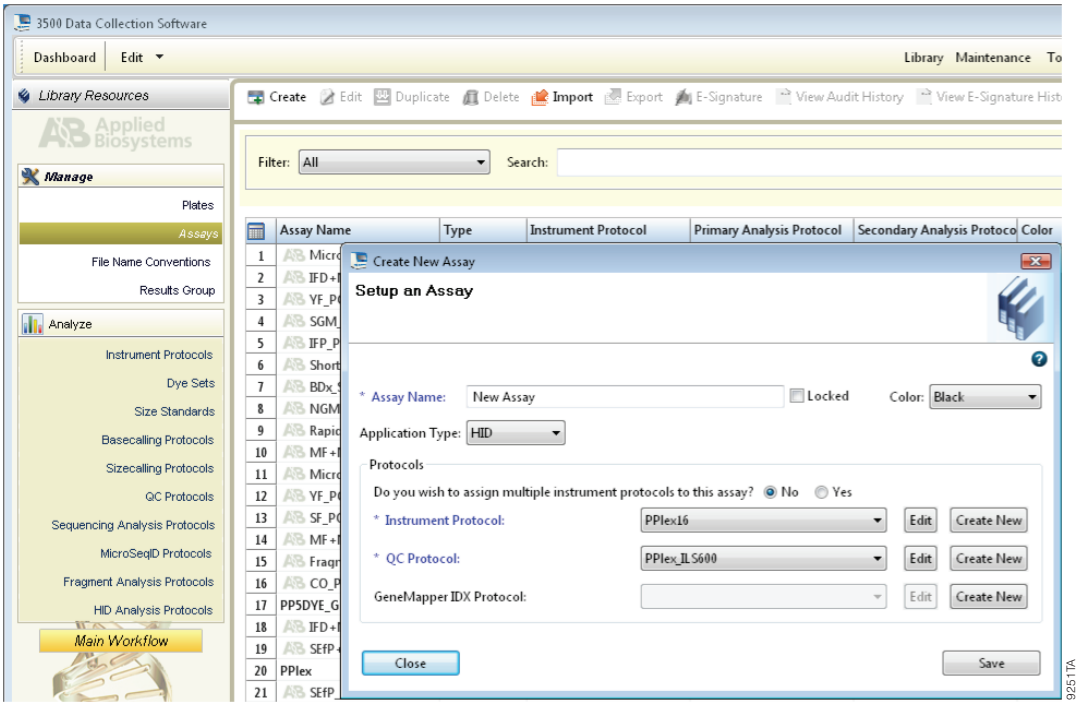

Figure 7. The Create New Assay window.

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

6. To create a new File Name Convention (Figure 8), navigate to the Library. Select "File Name Conventions", then select "Create". Alternatively, a previously created File Name Convention may be used.

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

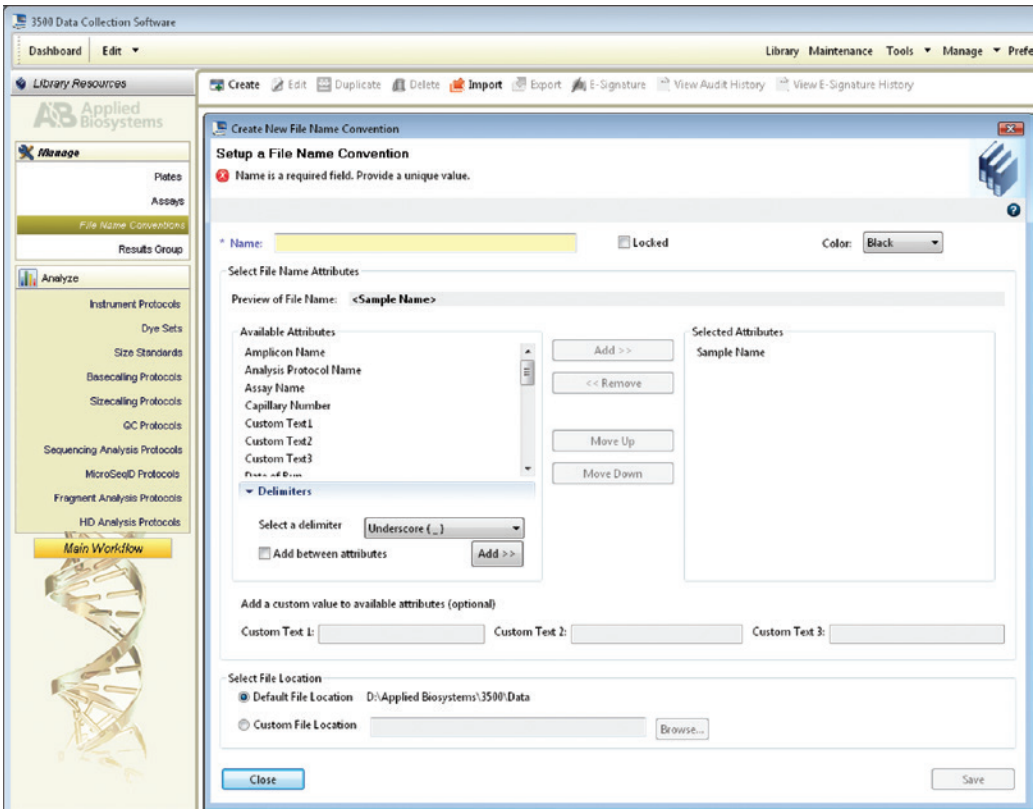

Figure 8. The Create New File Name Convention window.

7. To create a new Results Group (Figure 9), navigate to the Library. Select "Results Group", then select "Create". Alternatively, a previously created Results Groups may be used.

   Select the Results Group Attributes according to laboratory practices. Save with a descriptive name.

8. To create a New Plate, navigate to the Library, and from the Manage menu, select “Plates”, then “Create”.
9. Assign a descriptive plate name. Select the plate type “HID” from the drop-down menu (Figure 10).

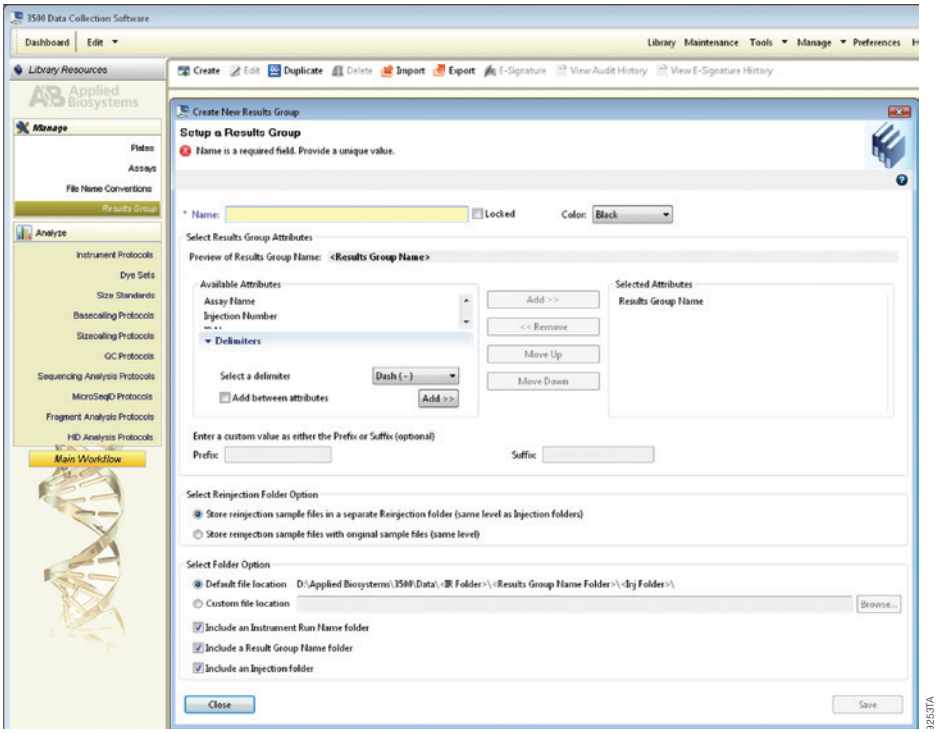

Figure 9. The Create New Results Group window.

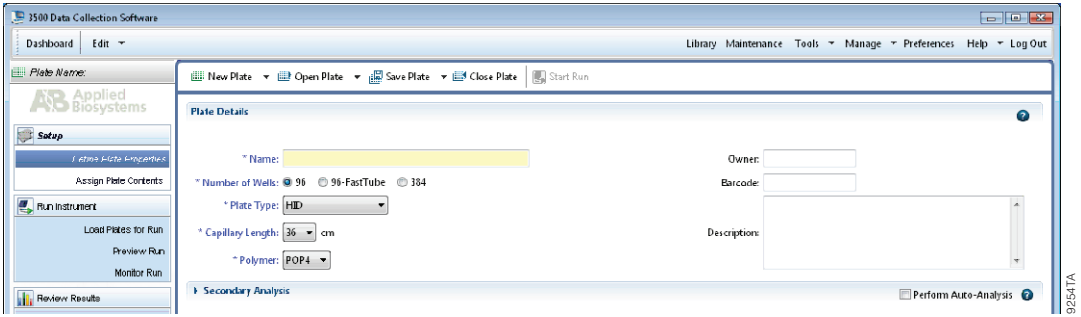

Figure 10. Defining plate properties.

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

10. Select "Assign Plate Contents" (Figure 11).
11. Assign sample names to wells.
12. In the lower left portion of the screen, under "Assays", use the Add from Library option to select the Assay created in Step 5 or one previously created. Click on the Add to Plate button, and close the window.
13. Under "File Name Conventions", use the Add from Library option to select the File Name Convention created in Step 6 or one previously created. Click on the Add to Plate button, and close the window.
14. Under "Results Groups", use the Add from Library option to select the Results Group created in Step 7 or one previously created. Click on the Add to Plate button, and close the window.
15. Highlight the sample wells, then select the boxes in the Assays, File Name Conventions and Results Groups that pertain to those samples.
16. Select "Link Plate for Run".
17. The Load Plate window will appear. Select "Yes".

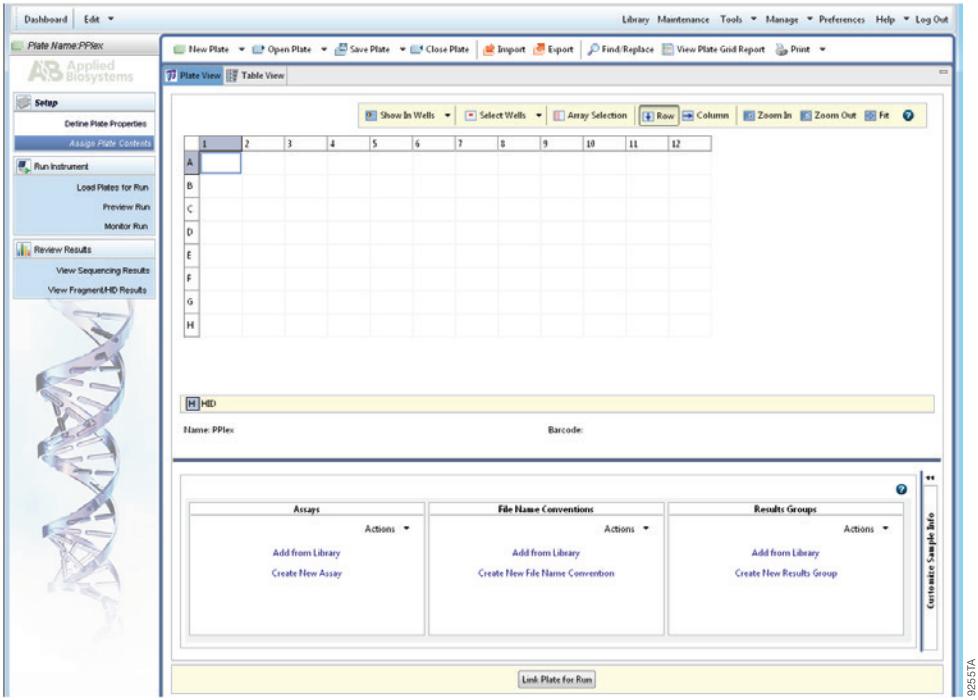

Figure 11. Assigning plate contents.

18. In the Run Information window (Figure 12), assign a Run Name. Select "Start Run" (not shown).

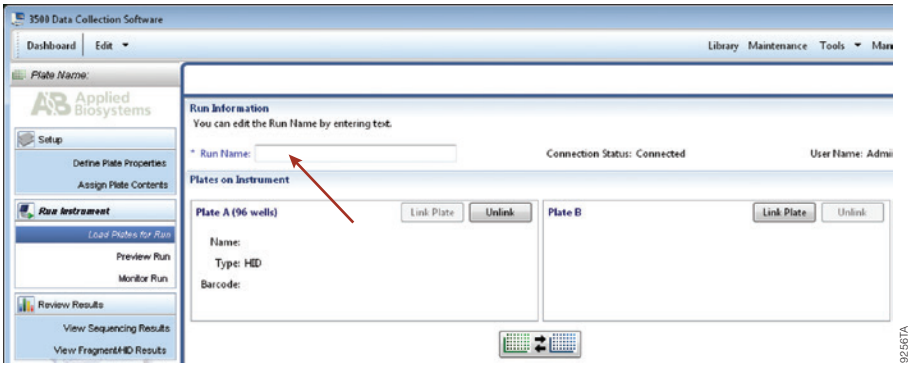

Figure 12. Assigning a run name.

5.B. Detection of Amplified Fragments Using the ABI PRISM® 3100 or 3100-*Avant* Genetic Analyzer with Data Collection Software, Version 2.0, or the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with Data Collection Software, Version 3.0

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice or ice-water bath
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- 3100 or 3130 capillary array, 36cm
- performance optimized polymer 4 (POP-4®) for the 3100 or 3130
- 10X genetic analyzer buffer with EDTA
- MicroAmp® optical 96-well plate and septa
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)

The quality of formamide is critical. Use Hi-Di™ 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.

5.B. Detection of Amplified Fragments Using the ABI PRISM® 3100 or 3100-Avant Genetic Analyzer with Data Collection Software, Version 2.0, or the Applied Biosystems® 3130 or 3130xl Genetic Analyzer with Data Collection Software, Version 3.0 (continued)

Sample Preparation

1. Thaw the Internal Lane Standard 600.

   Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex for 15 seconds before each use. Do not centrifuge after vortexing, as this may cause the size standard to be concentrated at the bottom of the tube.

2. Prepare a loading cocktail by combining and mixing Internal Lane Standard 600 and Hi-Di™ formamide as follows:

   [(0.5µl ILS 600) × (# samples)] + [(9.5µl Hi-Di™ formamide) × (# samples)]

   Note: The volume of internal lane standard used in the loading cocktail can be increased or decreased to adjust the intensity of the size standard peaks. If peak heights are too low, we recommend altering the formamide/internal lane standard mix to contain 1.0µl of ILS 600 and 9.0µl of Hi-Di™ formamide. If peak heights are too high, we recommend altering the loading cocktail to contain 0.25µl of ILS 600 and 9.75µl of formamide.

3. Vortex for 10–15 seconds to mix.

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

5. Add 1µl of amplified sample (or 1µl of PowerPlex® 16 HS Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

   Note: 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. 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 by 2–4 cycles to achieve the desired signal intensity.

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

7. Denature samples at 95°C for 3 minutes, then immediately chill on crushed ice 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 ABI PRISM® 3100 or 3100-*Avant* Genetic Analyzer with Data Collection Software, Version 2.0, and the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with Data Collection Software, Version 3.0, 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 and the injection voltage is 3kV. Lengthen the run time to 1,800 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 "F" 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 windows, 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, confirm that dye set F is active, and set the correct active spectral calibration for dye set F.

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. Click on 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 45 minutes.

5.C. Detection of Amplified Fragments Using the ABI PRISM® 3100 Genetic Analyzer with Data Collection Software, Version 1.0.1 or 1.1

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice or ice-water bath
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- 3100 capillary array, 36cm
- performance optimized polymer 4 (POP-4®) for the 3100
- 10X genetic analyzer buffer with EDTA
- MicroAmp® optical 96-well plate and septa for the 3100
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)

The quality of formamide is critical. Use Hi-Di™ 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 600 and Hi-Di™ formamide as follows:

   [(0.5µl ILS 600) × (# samples)] + [(9.5µl Hi-Di™ formamide) × (# samples)]

   Note: The volume of internal lane standard used in the loading cocktail can be increased or decreased to adjust the intensity of the size standard peaks. If peak heights are too low, we recommend altering the formamide/internal lane standard mix to contain 1.0µl of ILS 600 and 9.0µl of Hi-Di™ formamide. If peak heights are too high, we recommend altering the loading cocktail to contain 0.25µl of ILS 600 and 9.75µl of formamide.

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 PowerPlex® 16 HS Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

   Note: 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. Use the Module Manager in the data collection software to modify the injection time or voltage in the run module. 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 by 2–4 cycles to achieve the desired signal intensity.

5. Centrifuge plate briefly to remove air bubbles from the wells.
6. Denature samples at 95°C for 3 minutes, then immediately chill on crushed ice or in an ice-water bath for 3 minutes. Denature samples just prior to loading the instrument.

Instrument Preparation

Refer to the *ABI PRISM® 3100 Genetic Analyzer User Guide* for instructions on cleaning the blocks, installing the capillary array, performing a spatial calibration and adding polymer to the reserve syringe.

1. Open the ABI PRISM® 3100 Data Collection Software.
2. Change the GeneScan36_POP4DefaultModule module run time to 1,800 seconds.
3. Change the injection voltage to 3kV.
4. Change the injection time to 11 seconds.

   Note: Instrument sensitivities can vary. 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.
5. Save the module with a new name (e.g., GeneScan36_POP4PowerPlex16_3kV_11secs_1800). Use this as the initial run module for all runs.
6. Open a new plate record. Name the plate, and select "GeneScan". Select the plate size (96-well). Select "Finish".
7. Complete the plate record spreadsheet for the wells you have loaded. Enter appropriate information into the Sample Name and Color Info columns. For allelic ladder samples, insert the word "ladder" into the Color Info column for the blue, yellow and green dye colors. This information must be entered to successfully analyze data with the PowerTyper™ 16 Macro (Release 2.0).
8. In the BioLIMS Project column, select "3100_Project1" from the drop-down menu.
9. In the Dye Set column, select "Z" from the drop-down menu.
10. When using the ABI PRISM® 3100 Data Collection Software, Version 1.0.1 or 1.1, select "GeneScan36_POP4PowerPlex16_3kV_11secs_1800" from the drop-down menu in the Run Module 1 column.
11. To collect data without autoanalyzing, select "No Selection" in the Analysis Module 1 column. Analysis parameters can be applied after data collection and during data analysis using the GeneScan® software.
12. Select "OK". This new plate record will appear in the pending plate records table on the plate setup page of the collection software.
13. Place samples in the instrument, and close the instrument doors.
14. Locate the pending plate record that you just created, and click once on the name.
15. Once the pending plate record is highlighted, click on the plate graphic that corresponds to the plate on the autosampler that contains your amplified samples to link the plate to the plate record.
16. When the plate record is linked to the plate, the plate graphic will change from yellow to green, the plate record moves from the pending plate records table to the linked plate records table, and the Run Instrument button becomes enabled.

5.C. Detection of Amplified Fragments Using the ABI PRISM® 3100 Genetic Analyzer with Data Collection Software, Version 1.0.1 or 1.1 (continued)

17. Select "Run Instrument" on the toolbar to start the sample run.
18. Monitor electrophoresis by observing the run, status, array and capillary views windows in the collection software. Each injection will take approximately 45 minutes.

5.D. Detection of Amplified Fragments Using the ABI PRISM® 310 Genetic Analyzer

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice or ice-water bath
- 310 capillaries, 47cm × 50µm
- performance optimized polymer 4 (POP-4®)
- 10X genetic analyzer buffer with EDTA
- sample tubes and septa
- aerosol-resistant pipette tips
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)

The quality of formamide is critical. Use Hi-Di™ 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 Internal Lane Standard 600 (ILS 600) and Hi-Di™ formamide as follows:

   [(1.0µl ILS 600) × (# samples)] + [(24.0µl Hi-Di™ formamide) × (# samples)]

   Note: The volume of internal lane standard used in the loading cocktail can be increased or decreased to adjust the intensity of the size standard peaks. If peak heights are too high, we recommend altering the loading cocktail to contain 0.5µl of ILS 600 and 24.5µl of Hi-Di™ formamide.

2. Vortex for 10–15 seconds to mix.
3. Combine 25.0µl of prepared loading cocktail and 1.0µl of amplified sample (or 1.0µl of PowerPlex® 16 HS Allelic Ladder Mix).

   Note: 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. Modify the injection time or voltage in the injection list. If peak heights still are higher than desired, use less DNA template in the amplification reactions or reduce the number of cycles in the amplification program by 2–4 cycles to achieve the desired signal intensity.

4. Centrifuge tubes briefly to remove air bubbles from the wells.
5. Denature samples by heating at 95°C for 3 minutes, and immediately chill on crushed ice or in an ice-water bath for 3 minutes. Denature samples just prior to loading.
6. Place tubes in the appropriate autosampler tray.
7. Place the autosampler tray in the instrument, and close the instrument doors.

Instrument Preparation

Refer to the instrument user's manual for instructions on cleaning the pump block, installing the capillary, calibrating the autosampler and adding polymer to the syringe.

1. Open the ABI PRISM® 310 Data Collection Software.
2. Prepare a GeneScan® sample sheet as described in the *ABI PRISM® 310 Genetic Analyzer User Guide*. Enter the appropriate sample information in the Sample Info column.

   For rows containing PowerPlex® 16 HS Allelic Ladder Mix, insert the word "ladder" in the Sample Info column for the blue dye color, yellow dye color and green dye color. This information must be entered to successfully analyze your data using the PowerTyper™ 16 Macro (Release 2.0).
3. Create a new GeneScan® injection list. Select the appropriate sample sheet from the drop-down menu.
4. Select the GS STR POP4 (1ml) F Module using the drop-down menu. Change the injection time to 3 seconds and the run time to 30 minutes. Keep the settings for the remaining parameters as shown below:

Inj. Secs:	3
Inj. kV:	15.0
Run kV:	15.0
Run °C:	60
Run Time:	30

You may need to optimize the injection time for individual instruments. Injection times of 2–5 seconds are suggested for samples that contain 0.5–1ng of template DNA.

Note: Migration of fragments may vary slightly over the course of a long ABI PRISM® 310 Genetic Analyzer run. This may be due to changes in temperature or changes in the column. When analyzing many samples, injections of allelic ladder at different times throughout the run can aid in accurately genotyping samples.

5. Select the appropriate matrix file (see Section 3.B).
6. To analyze data automatically, select the auto analyze checkbox and the appropriate analysis parameters and size standard. Refer to the *ABI PRISM® 310 Genetic Analyzer User Guide* for specific information on these options.
7. Enter an operator name.
8. After loading the sample tray and closing the doors, select "Run" to start the capillary electrophoresis system.
9. Monitor electrophoresis by observing the raw data and status windows. Each sample will take approximately 40 minutes for syringe pumping, sample injection and sample electrophoresis.

6. Data Analysis

6.A. PowerPlex® 16 Panels, Bins and Stutter Text Files with GeneMapper® *ID*-X Software, Version 1.2

To facilitate analysis of data generated with the PowerPlex® 16 HS System, we have created panels and bins 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.

Note: The panels, bins and stutter text files mentioned here are compatible with earlier versions of the GeneMapper® *ID*-X software.

Getting Started

1. To obtain the proper panels, bins and stutter text files for the PowerPlex® 16 HS System, go to: **www.promega.com/resources/software-firmware/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_16_Panels_IDX_vX.x.txt, PowerPlex_16_Bins_IDX_vX.x.txt and PowerPlex_16_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.

Importing Panels, Bins and Stutter Text Files

1. Open the GeneMapper® *ID*-X software.
2. Select "Tools", then select "Panel Manager".
3. Highlight the Panel Manager icon in the upper left navigation pane.
4. Select "File", then "Import Panels".
5. Navigate to the panels text file imported in the Getting Started section. Select the file, then "Import".
6. In the navigation pane, highlight the PowerPlex 16 panels folder that you just imported in Step 5.
7. Select "File", then "Import Bin Set".
8. Navigate to the bins text file that was imported in the Getting Started section. Select the file, then "Import".
9. In the navigation pane, highlight the PowerPlex 16 panels folder that you just imported in Step 5.
10. Select "File", then "Import Marker Stutter" to import the marker-specific stutter ratio for the panels and bins text files. A warning box will appear asking if you want to overwrite current values. Select "Yes".
11. Navigate to the stutter file that was imported in the Getting Started section. Select the file, then "Import".
12. At the bottom of the Panel Manager window, select "OK". This will save the panels, bins and stutter text files and close the window.

6.B. Creating a Size Standard with GeneMapper® *ID*-X Software, Version 1.2

1. Select "Tools", then "GeneMapper ID-X Manager".
2. Select the Size Standard tab.
3. Select "New".
4. The Size Standard Editor box will open (Figure 13). Enter a detailed name, such as "ILS 600".
5. Choose a security group from the drop-down menu.
6. Choose "Red" for the Size Standard Dye.
7. Enter the sizes of the internal lane standard fragments (60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550 and 600 bases). See Section 9.D, Figure 28.

   Note: Definition and detection of the 600bp fragment is optional.
8. Select "OK".

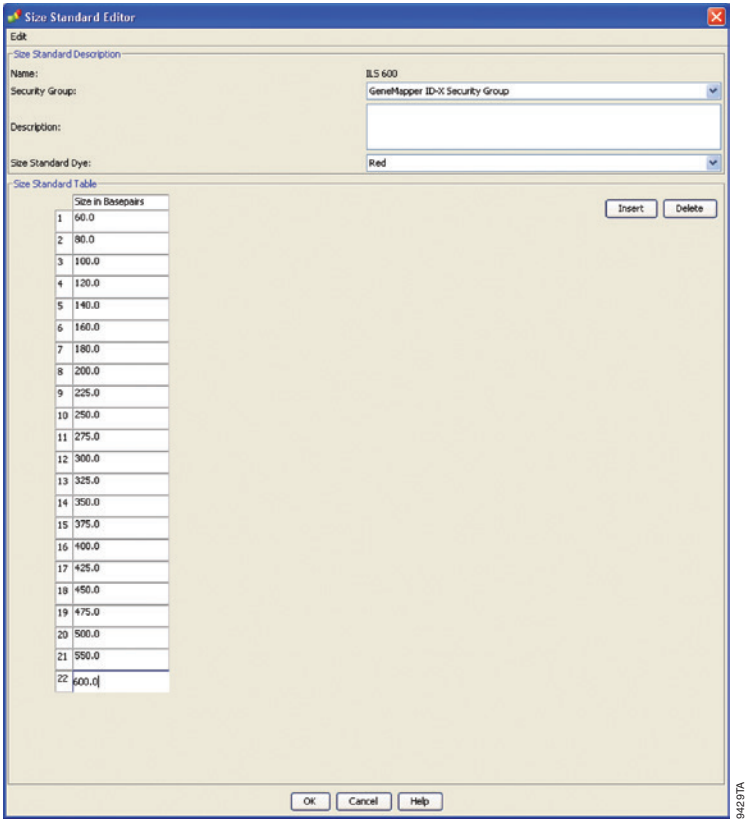

Figure 13. The GeneMapper® *ID*-X Size Standard Editor.

6.C. Creating a Casework Analysis Method with GeneMapper® *ID*-X Software, Version 1.2

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

1. Select "Tools", 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, enter a descriptive name for the analysis method, such as "PowerPlex 16 HS".
5. Choose a security group from the drop-down menu.
6. Select the Allele tab (Figure 14).

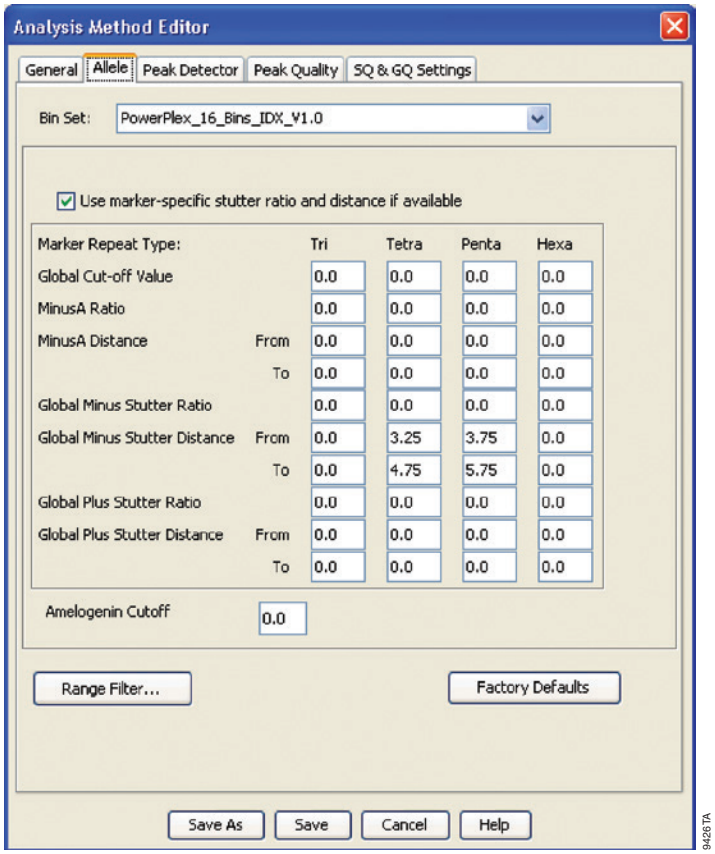

Figure 14. The GeneMapper® *ID*-X Allele tab.

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.
9. We recommend the values shown in Figure 14 for proper filtering of stutter peaks when using the PowerPlex® 16 HS System. You may need to optimize these settings. In-house validation should be performed.
10. Select the Peak Detector tab. Figure 15 shows an example of settings used at Promega. You may need to optimize these settings. In-house validation should be performed.

Notes:

1. In the Ranges section, select "Full Range" in the Analysis drop-down menu and "Partial Sizes" in the Sizing drop-down menu. If 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.

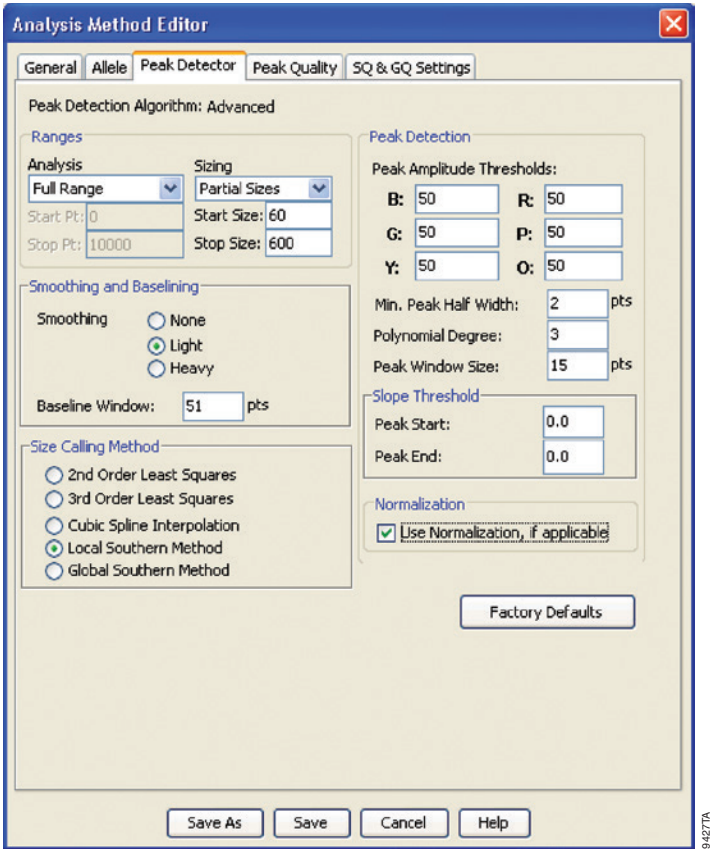

Figure 15. The GeneMapper® *ID*-X Peak Detector tab.

6.C. Creating a Casework Analysis Method with GeneMapper® *ID*-X Software, Version 1.2 (continued)

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

3. The normalization box can be checked regardless of whether normalization was or was not applied during data collection.

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

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

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

13. Select "Save" to save the new analysis method.

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

Processing Data for Casework Samples

1. Select "File", then "New Project".

2. Select "Edit", then "Add Samples to Project".

3. Browse to the location of the run files. Highlight desired files, 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 above.

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 created in Section 6.B.

8. If analyzing data from an ABI PRISM® 310 Genetic Analyzer, ensure that the appropriate matrix file is selected in the Matrix column.

9. Select "Analyze" (green arrow button) to start data analysis.

   Note: By default, the software displays the Analysis Requirement Summary, Allelic Ladder Analysis Summary and Analysis Summary windows after quality review by the software. Ensure that all requirements are met as each window appears. If you do not have the Analysis Requirement Summary window activated, you may need to do additional manual troubleshooting.

10. If all analysis requirements are met, the Save Project window will open (Figure 16).

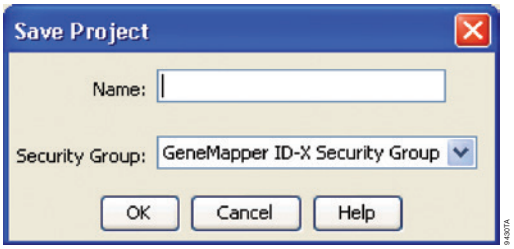

Figure 16. The Save Project window.

11. Enter the project name.
12. Choose the applicable security group from the drop-down menu, 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. Navigate to the Genotype tab or Samples tab. To assist the review of any low-quality samples, use the default Data Interpretation plot settings and review the contents in the Quality Value Details table.

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.D. Creating a Databasing or Paternity Analysis Method with GeneMapper® *ID*-X Software, Version 1.2

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

1. Select "Tools", then "GeneMapper ID-X Manager".
2. Select the Analysis Methods tab.
3. Select "New", and a new analysis method dialog box will open.

6.D. Creating a Databasing or Paternity Analysis Method with GeneMapper® *ID*-X Software, Version 1.2 (continued)

4. In the Analysis Method Editor, enter a descriptive name for the analysis method, such as "PowerPlex 16 HS_20% Filter".
5. Choose a security group from the drop-down menu.
6. Select the Allele tab (Figure 17).
7. Select the bins text file that was imported in Section 6.A.
8. We recommend the values shown in Figure 17 for proper filtering of stutter peaks when using the PowerPlex® 16 HS System. You may need to optimize these settings. In-house validation should be performed.

   Note: Ensure that the appropriate 20% filter is applied to this analysis method by entering "0.20" for the Global Cut-off Value for Tetra and Penta repeats.

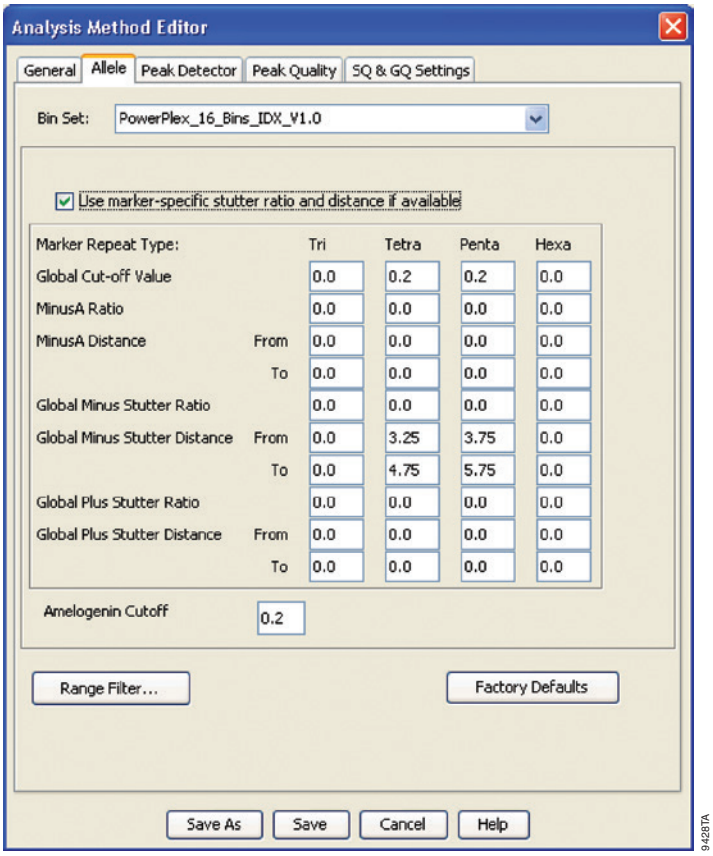

Figure 17. The Allele tab with settings for using a 20% peak filter.

9. Select the Peak Detector tab. Figure 15 shows an example of settings used at Promega. You may need to optimize these settings. In-house validation should be performed.

   Notes:

   1. In the Ranges section, select "Full Range" in the Analysis drop-down menu and "Partial Sizes" in the Sizing drop-down menu. 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.
   2. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Individual laboratories should determine peak amplitude thresholds from internal validation studies.
   3. The normalization box can be checked regardless of whether normalization was or was not applied during data collection.

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

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

11. Select the SQ & GQ Settings tab. You may change these settings.
12. Select "Save" to save the new analysis method.
13. Select "Done" to exit the GeneMapper® *ID*-X Manager.

Processing Data for Databasing and Paternity Samples

1. Select "File", then "New Project".
2. Select "Edit", then "Add Samples to Project".
3. Browse to the location of the run files. Highlight desired files, 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 above.
6. In the Panel column, select the panels text file that was imported in Section 6.A.

6.D. Creating a Databasing or Paternity Analysis Method with GeneMapper® *ID*-X Software, Version 1.2 (continued)

7. In the Size Standard column, select the size standard that was created in Section 6.B.
8. If analyzing data from an ABI PRISM® 310 Genetic Analyzer, ensure that the appropriate matrix file is selected in the Matrix column.
9. Select "Analyze" (green arrow button) to start data analysis.

   Note: By default, the software displays the Analysis Requirement Summary, Allelic Ladder Analysis Summary and Analysis Summary windows after quality review by the software. Ensure that all requirements are met as each window appears. If you do not have the Analysis Requirement Summary window activated, you may need to do additional manual troubleshooting.
10. If all analysis requirements are met, the Save Project window will open (Figure 16).
11. Enter the project name.
12. Choose the applicable security group from the drop-down menu, 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. Navigate to the Genotype tab or Samples tab. To assist the review of any low-quality samples, use the default Data Interpretation plot settings and review the contents in the Quality Value Details table.

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. PowerPlex® Panels and Bins Text Files with GeneMapper® *ID* Software, Version 3.2

To facilitate analysis of data generated with the PowerPlex® 16 HS 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.

For analysis using GeneMapper® *ID* software, version 3.2, you will need the proper panels and bins text files: PowerPlex_16_Panels_vX.x.txt and PowerPlex_16_Bins_vX.x.txt files, where "X.x" refers to the most recent version of the panels and bins text files.

Getting Started

1. To obtain the panels and bins text files for the PowerPlex® 16 HS System go to: **www.promega.com/resources/software-firmware/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_16_Panels_vX.x.txt and PowerPlex_16_Bins_vX.x.txt files to a known location on your computer.

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", then "Panel Manager".
3. Highlight the Panel Manager icon in the upper left navigation pane.
4. Select "File", then "Import Panels".
5. Navigate to the panels text file that was imported in the Getting Started section above. Select the file, then "Import".
6. In the navigation pane, highlight the PowerPlex 16 panels folder that you just imported in Step 5.
7. Select "File", then "Import Bin Set".
8. Navigate to the bins text file that was imported in the Getting Started section above. Select the file, then "Import".
9. At the bottom of the Panel Manager window, select "OK". The Panel Manager window will close automatically.

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

1. Select "Tools", then "GeneMapper Manager".
2. Select the Size Standard tab.
3. Select "New".
4. Select "Basic or Advanced" (Figure 18). The type of analysis method selected must match the type of analysis method created earlier. Select "OK".

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

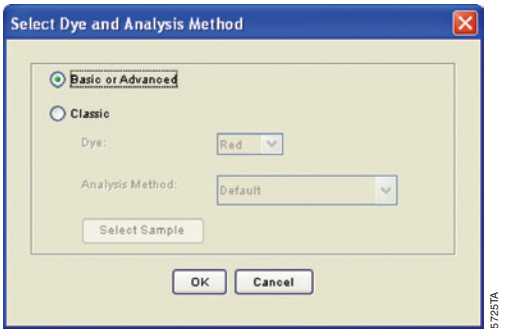

Figure 18. The Select Dye and Analysis Method window.

5. Enter a detailed name, such as "ILS 600 Advanced", in the Size Standard Editor (Figure 19).

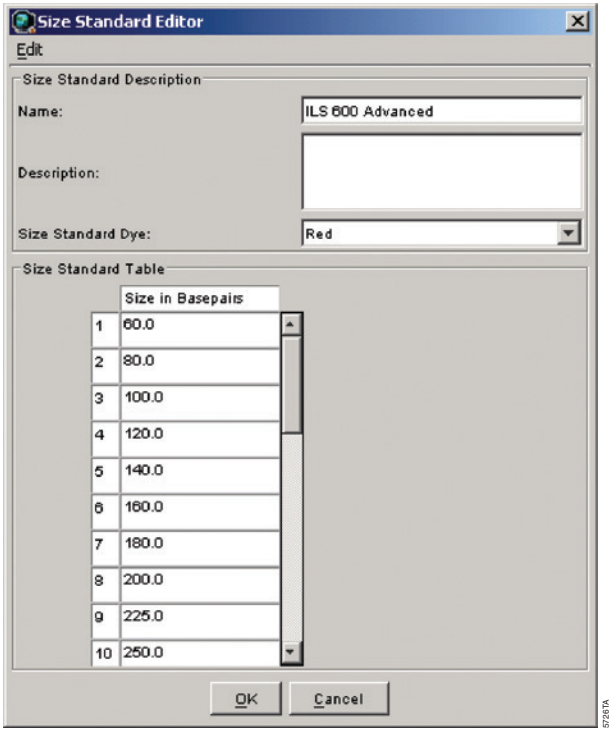

Figure 19. The Size Standard Editor

6. Choose “Red” for the Size Standard Dye.
7. Enter the sizes of the internal lane standard fragments (60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550 and 600 bases). See Section 9.D, Figure 28.

   Note: Definition and detection of the 600bp fragment is optional.
8. Select “OK”.

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

These instructions loosely follow the Applied Biosystems GeneMapper® *ID* software tutorial, pages 5–11.

1. Select “Tools”, 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. In the Analysis Method Editor, enter a descriptive name for the analysis method, such as “PowerPlex16HS advanced”.

6.G. Creating a Casework Analysis Method with GeneMapper® *ID* Software, Version 3.2 (continued)

6. Select the Allele tab (Figure 20).
7. Select the bins text file that was imported in Section 6.E.
8. Ensure that the "Use marker-specific stutter ratio if available" box is checked.

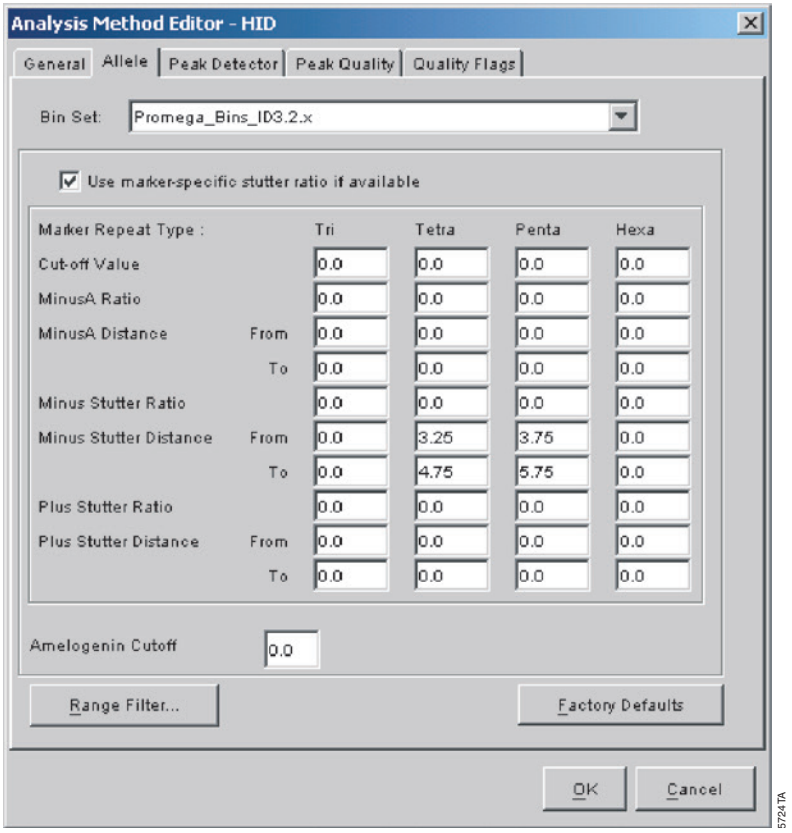

Figure 20. The GeneMapper® *ID* Allele tab.

9. Enter the values shown in Figure 20 for proper filtering of stutter peaks when using the PowerPlex® 16 HS 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.

10. Select the Peak Detector tab. We recommend the settings shown in Figure 21.

Notes:

1. 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.
2. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Values for peak amplitude thresholds should be determined by individual laboratories.

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.

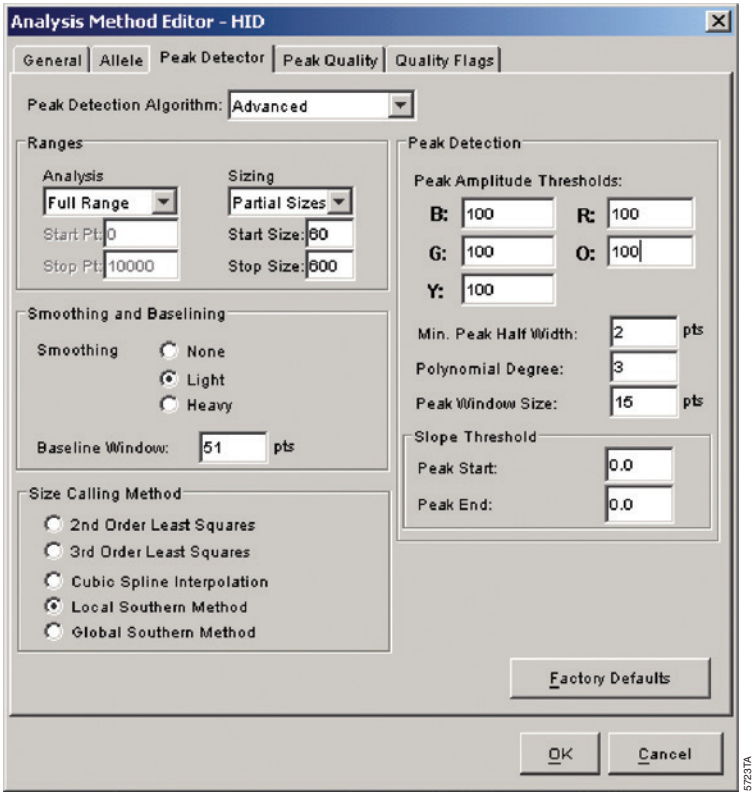

Figure 21. The GeneMapper® *ID* Peak Detector tab.

12. Select the Quality Flags tab. You may change these settings.
13. Select "OK" to save your settings.

6.G. Creating a Casework Analysis Method with GeneMapper® *ID* Software, Version 3.2 (continued)

Processing Data for Casework Samples

1. Select "File", then "New Project".
2. Select "Edit", then "Add Samples to Project".
3. Browse to the location of the run files. Highlight the desired files, 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.E.
7. In the Size Standard column, select the size standard that was created in Section 6.F.
8. If analyzing data from an ABI PRISM® 310 Genetic Analyzer, ensure that the appropriate matrix file is selected in the Matrix column.
9. Select "Analyze" (green arrow button) to start data analysis.

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

1. Select "Tools", 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. In the Analysis Method Editor, enter a descriptive name for the analysis method, such as "PowerPlex16_20%filter".
6. Select the Allele tab (Figure 22).
7. Select the bins text file that was imported in Section 6.E
8. Ensure that the "Use marker-specific stutter ratio if available" box is checked.

9. Enter the values shown in Figure 22 for proper filtering of peaks when using the PowerPlex® 16 HS 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*".

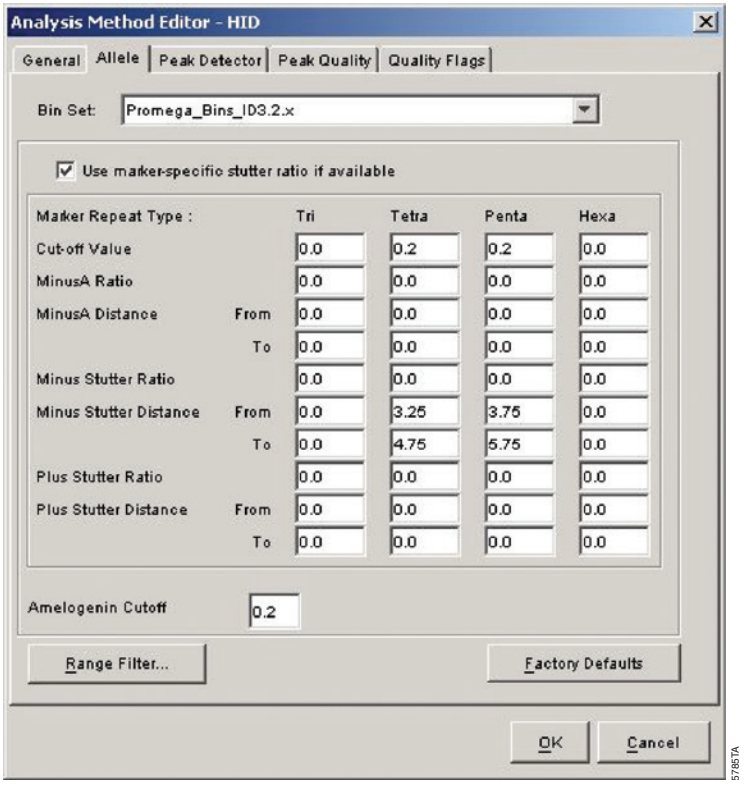

Figure 22. The GeneMapper® *ID* Allele tab with settings for using a 20% peak filter.

10. Select the Peak Detector tab. We recommend the settings shown in Figure 21.

    Notes:

    1. 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.
    2. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Values for peak amplitude thresholds should be determined by individual laboratories.

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.

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

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", then "New Project".
2. Select "Edit", then "Add Samples to Project".
3. Browse to the location of the run files. Highlight desired files, 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.E.
7. In the Size Standard column, select the size standard that was created in Section 6.F.
8. If analyzing data from an ABI PRISM® 310 Genetic Analyzer, ensure that the appropriate matrix file is selected in the Matrix column.
9. Select "Analyze" (green arrow button) to start the data analysis.

6.I. 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. Compare the 2800M Control DNA allelic repeat sizes with the locus-specific allelic ladder. The expected 2800M Control DNA allele designations for each locus are listed in Table 4 (Section 9.A).

6.J. Results

Representative results of the PowerPlex® 16 HS System are shown in Figure 23. The PowerPlex® 16 HS Allelic Ladder Mix is shown in Figure 24.

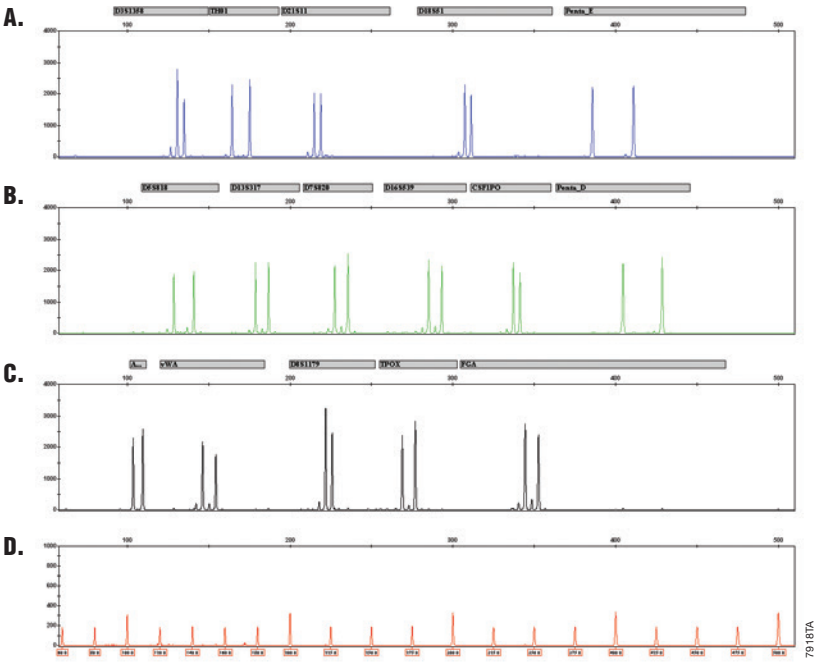

Figure 23. The PowerPlex® 16 HS System. A single-source template DNA (0.5ng) was amplified using the PowerPlex® 16 HS System. Amplification products were mixed with Internal Lane Standard 600 and analyzed with an Applied Biosystems® 3130 Genetic Analyzer using a 3kV, 5-second injection. Results were analyzed using GeneMapper® *ID* software, version 3.2. **Panel A**. An electropherogram showing the peaks of the fluorescein-labeled loci: D3S1358, TH01, D21S11, D18S51 and Penta E. **Panel B**. An electropherogram showing the peaks of the JOE-labeled loci: D5S818, D13S317, D7S820, D16S539, CSF1PO and Penta D. **Panel C**. An electropherogram showing the peaks of the TMR-labeled loci: Amelogenin, vWA, D8S1179, TPOX and FGA. **Panel D**. An electropherogram showing the 60bp to 500bp fragments of the Internal Lane Standard 600.

6.J. Results (continued)

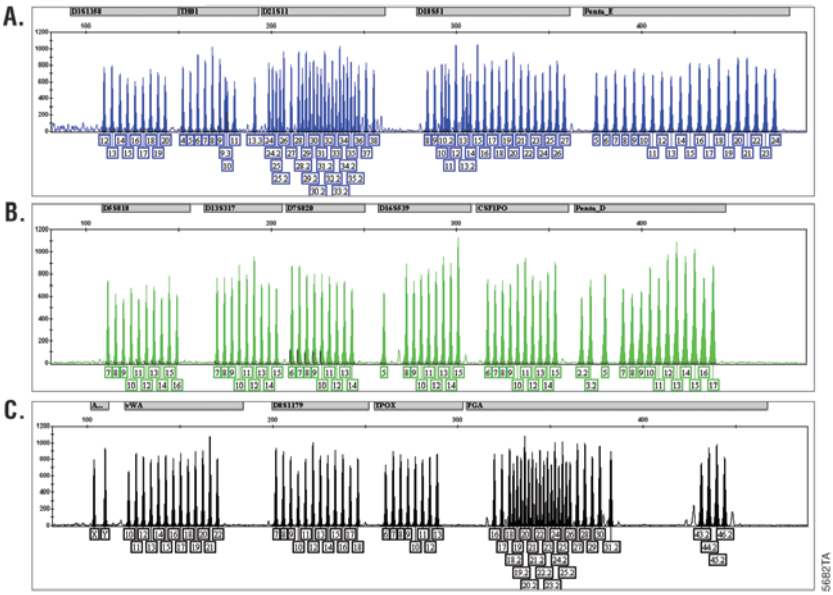

Figure 24. The PowerPlex® 16 HS Allelic Ladder Mix. The PowerPlex® 16 HS Allelic Ladder Mix was analyzed with an Applied Biosystems® 3130 Genetic Analyzer using a 3kV, 5-second injection. The sample file was analyzed with the GeneMapper® *ID* software, version 3.2, and PowerPlex® 16 HS panels and bins text files. **Panel A**. The fluorescein-labeled allelic ladder components and their allele designations. **Panel B**. The JOE-labeled allelic ladder components and their allele designations. **Panel C**. The TMR-labeled allelic ladder components and their allele designations.

Artifacts and Stutter

Stutter products are a common amplification artifact associated with STR analysis. Stutter products often are 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.

In addition to stutter peaks, other artifact peaks can be observed at some of the PowerPlex® 16 HS System loci. Low-level products can be seen in the n−2 and n+2 positions (two bases below and above the true allele peak, respectively), with some loci such as D21S11. Samples may show low-level artifacts in the regions between D21S11 and D18S51, D7S820 and D16S539, and D8S1179 and TPOX. Occasionally, an off-ladder artifact can be seen in the 690–691bp position in the fluorescein dye channel. One or more extra peaks that are not directly related to amplification may be observed in the D3S1358, TH01, D21S11 and Penta E region of the fluorescein channel; D13S317 and D16S539 region of the JOE channel; and vWA region of the TMR channel. These extra peaks occur when the amplified peaks are particularly intense (high signal intensity or template amount); formamide, polymer or capillary was of poor quality; or denaturation was ineffective. See Section 7 for more information on how to minimize these artifacts.

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

Symptoms	Possible Causes and Comments
Faint or absent allele peaks	Impure template DNA. Because of the small amount of template used, this is rarely a problem. Depending on the DNA extraction procedure used and sample source, inhibitors might be present in the DNA sample.
	Insufficient template. Use the recommended amount of template DNA.
	Insufficient enzyme activity. Vortex the PowerPlex® HS 5X Master Mix before use, and use the recommended amount.
	Incorrect amplification program. Confirm the amplification program.
	The PowerPlex® HS 5X Master Mix was not vortexed well before use. Vortex the 5X Master Mix for 5–10 seconds before dispensing into reaction tubes or plates.
	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.
	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 nuclease-free water.
	Thermal cycler, plate or tube problems. Review the thermal cycling protocols in Section 4. We have not tested other reaction tubes, plates or thermal cyclers. Calibrate the thermal cycler heating block if necessary.
	Primer concentration was too low. Use the recommended primer concentration. Vortex the 10X PowerPlex® 16 HS Primer Pair for 15 seconds before use.
	Poor capillary electrophoresis injection (ILS 600 peaks also affected). Re-inject the sample. Check the syringe or pump system for leakage. Check the laser power.
	Samples were not denatured completely. Heat-denature samples for the recommended time, then cool on crushed ice or in an ice-water bath immediately prior to capillary electrophoresis. Do not cool samples in a thermal cycler set at 4°C, as this may lead to artifacts due to DNA re-annealing.
	Poor-quality formamide was used. Use only Hi-Di™ formamide when analyzing samples.

7.A. Amplification and Fragment Detection (continued)

Symptoms	Possible 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.
	Samples were not denatured completely. Heat denature samples for the recommended time, and cool on crushed ice or in an ice-water bath immediately prior to capillary electrophoresis. Do not cool samples in a thermal cycler set at 4°C, as this may lead to artifacts due to DNA re-annealing.
	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as faint peaks one repeat unit smaller than the allele. Stutter product peak heights can be high if samples are overloaded. See Section 6.J 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 30-minute extension step at 60°C after thermal cycling (Section 4).
	Excessive amount of DNA. Amplification of >1ng template can result in a higher number of stutter bands. Use less template DNA, or reduce the number of cycles in the amplification program by 2–4 cycles (10/20 or 10/18 cycling).
	High background. Load less amplification product, or decrease injection time. See Section 5.
	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 identified easily 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 blue and green dye colors. Use autoclaved deionized water; change vials and wash buffer reservoir.
	Pull-up or bleedthrough. Pull-up can occur when peak heights are too high or if a poor or incorrect matrix has been applied to the samples. • For the ABI PRISM® 310 Genetic Analyzer, generate a new matrix, and apply it to the samples. For the ABI PRISM® 3100 and 3100-*Avant* Genetic Analyzers and Applied Biosystems 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers, perform a new spectral calibration, and re-run the samples. • Instrument sensitivities can vary. Optimize the injection conditions. See Section 5.
	Long-term storage of amplified sample in formamide can result in degradation. Repeat sample preparation using fresh formamide.

Symptoms	Possible Causes and Comments
Extra peaks visible in one or all color channels (continued)	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.
	Poor-quality formamide. Use only Hi-Di™ formamide when analyzing samples.
	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 CE column over time. Use a different injection of allelic ladder to determine sizes.
	Poor injection of allelic ladder. Include more than one ladder per instrument run.
Peak height imbalance	Excessive amount of DNA. Amplification of >1ng of template can result in an imbalance, with smaller loci showing more product than larger loci. Use less template, or reduce the number of cycles in the amplification program by 2–4 cycles (10/20 or 10/18 cycling) to improve locus-to-locus balance. **Note:** Dilution of overamplified samples can result in dropout of larger loci.
	Degraded DNA sample. DNA template was degraded, and larger loci show diminished yield. Repurify template DNA.
	Insufficient template DNA. Use the recommended amount of template DNA. Stochastic effects can occur when amplifying low amounts of template.
	Miscellaneous balance problems. Thaw the 10X Primer Pair Mix and 5X Master Mix completely, and vortex for 15 seconds before use. Do not centrifuge the 10X Primer Pair Mix after mixing. Calibrate thermal cyclers and pipettes routinely. Using a 59°C annealing temperature instead of 60°C has been shown to improve balance in some instances.
	Impure DNA template. Inhibitors that may be present in forensic samples can lead to allele dropout or imbalance.
	Impure DNA template. Include a proteinase K digestion prior to DNA purification.
	PCR amplification mix prepared in Section 4 was not mixed well. Vortex the PCR amplification mix for 5–10 seconds before dispensing into the reaction tubes or plate.
	Tubes of 5X Master Mix and 10X Primer Pair Mix from different lots were used. The PowerPlex® HS 5X Master Mix and PowerPlex® 16 HS 10X Primer Pair Mix are manufactured as a matched set for optimal performance. If lots are mixed, locus-to-locus imbalance and variation in signal intensity may occur.

7.B. Direct Amplification 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	Possible Causes and Comments
Faint or absent allele peaks	The reaction volume was too low. This system is optimized for a final reaction volume of 25µl to overcome inhibitors present in storage cards. Decreasing the reaction volume may result in suboptimal performance, especially when amplifying DNA on storage card punches directly.
	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 also can improve low peak heights.
	Too much sample in the reaction. Use one or two 1.2mm storage card punches. Follow the manufacturer's recommendations when depositing sample onto the storage card. With storage cards, reducing the reaction volumes below 25µl may result in amplification failure.
	Amplification was inhibited when using more than one storage card punch with blood. Use only one 1.2mm storage card punch with blood.
	Make sure that the PCR amplification mix also contained AmpSolution™ Reagent. Omission of AmpSolution™ Reagent from amplification reactions will result in amplification failure.
	NonFTA card punches were not pretreated with PunchSolution™ Reagent.
	Active PunchSolution™ Reagent carried over into the amplification reaction when using nonFTA card punches. Ensure that the heat block reached 70°C and samples were incubated for 30 minutes. or until wells were dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. We have not tested longer incubation times.
	Inactive PunchSolution™ Reagent. Thaw the PunchSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze; avoid multiple freeze-thaw cycles, as this may reduce activity.
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. • Do not include a blank punch in the positive control reaction. Presence of a blank punch may inhibit amplification of 2800M Control DNA. • Optimize the amount of 2800M Control DNA for your thermal cycling conditions and laboratory preferences.

Symptoms	Possible Causes and Comments
Extra peaks visible in one or all color channels	Punch was contaminated. Take punches from blank paper samples 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. We recommend one or two 1.2mm punches per 25µl reaction. 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.
	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. 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 >20ng of template can result in a higher number of artifact peaks. Use the recommended punch size and number of punches. Optimize the cycle number. Do not reduce the reaction volume below 25µl. 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 a 30-minute extension step at 60°C after thermal cycling (Section 4).
Peak height imbalance	Excessive amount of DNA. Amplification of >20ng of template can result in an imbalance with smaller loci showing more product than larger loci. • Use one or two 1.2mm punches from a storage card containing a buccal sample or one 1.2mm punch from a storage card containing whole blood. Follow the manufacturer's recommendations when depositing sample onto the storage card. • Decrease number of cycles.
	The reaction volume was too low. This system is optimized for a final reaction volume of 25µl to overcome inhibitors present in storage cards and PunchSolution™ Reagent. Decreasing the reaction volume can result in suboptimal performance.
	Amplification was inhibited when using more than one storage card punch with blood. Use only one 1.2mm storage card punch with blood.

7.B. Direct Amplification from Storage Card Punches (continued)

Symptoms	Possible Causes and Comments
Peak height imbalance (continued)	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 were dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. • Using a smaller amplification reaction volume may compromise performance when using 10µl of PunchSolution™ Reagent. Reducing the PunchSolution™ Reagent volume may improve results for reactions with reduced amplification volumes. Optimization and validation are required.
	Inactive PunchSolution™ Reagent. Thaw the PunchSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze; avoid multiple freeze-thaw cycles, as this may reduce activity.
	Carryover of excess PunchSolution™ Reagent into amplification reaction. We recommend treating one 1.2mm nonFTA card punch with 10µl of PunchSolution™ Reagent, and using one punch per 25µl amplification reaction. Use of a smaller amplification reaction volume may compromise performance if using 10µl of PunchSolution™ Reagent. Reducing the PunchSolution™ Reagent volume may improve results when using a reduced amplification reaction volume. Laboratory optimization and validation are required.
Extreme variability in sample-to-sample peak heights	There can be significant individual-to-individual variability in the deposition of cells onto a punch, resulting in peak height variability between samples. The PunchSolution™ Kit increases the recovery of amplifiable DNA from samples but does not normalize the amount of DNA present.

7.C. Amplification of DNA from Swabs

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

Symptoms	Possible 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 the SwabSolution™ Reagent completely in a 37°C water bath, and mix by gentle inversion. Store the SwabSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze; avoid multiple freeze-thaw cycles, as this may reduce activity.

Symptoms	Possible Causes and Comments
Faint or absent allele peaks (continued)	Active SwabSolution™ Reagent carried over into the amplification reaction. Ensure that the heat block is heating to 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. Only use 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.
	Make sure that the PCR amplification mix also contained AmpSolution™ Reagent. Omission of AmpSolution™ Reagent from amplification reactions will result in amplification failure.
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 numbers used with swab extracts, it is necessary to increase the mass of 2800M Control DNA to obtain a profile. We recommend 5ng of 2800M Control DNA per 25µl amplification reaction. This mass of DNA should be reduced if the cycle number used is increased and decreased if the cycle number is increased. 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 as a blank without a swab.
	Artifacts of STR amplification. Amplification of swab extracts with high concentrations of DNA can result in artifact peaks due to overamplification, resulting in saturated signal on the CE instrument. We recommend 2µl of swab extract per 25µl reaction. Using more than 2µl in a 25µl reaction or using 2µl with a smaller reaction volume may result in overamplification and signal saturation. If signal is saturated, repeat the amplification with less swab extract or a reduced cycle number.
	Amplification of excess template for a given cycle number resulted in overloading of the capillary upon electrokinetic injection. In addition to signal saturation, 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. 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.
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.

7.C. Amplification of DNA from Swabs (continued)

Symptoms	Possible Causes and Comments
Peak height imbalance (continued)	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 is heating to 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 protease inactivation. Do not use an incubator set at 70°C to incubate tubes or plates. Heat transfer is inefficient and will result in poor performance. Only use 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 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not re-freeze; avoid multiple freeze-thaw cycles, as this may reduce activity.
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, quantitate the DNA using a fluorescence-based double-stranded DNA quantitation method or qPCR-based quantitation method. The quantitation values can be used to normalize input template amounts to minimize variation in signal intensity.

7.D. GeneMapper® *ID*-X Software

Symptoms	Possible Causes and Comments
Stutter peaks not filtered	Stutter file was not imported into the Panel Manager when the panels and bins text files were imported.
	Stutter distance was not defined in the Analysis Method Allele tab.
	Be sure that the "Use marker-specific stutter ratio and distance if available" box is checked.
Samples in the project not analyzed	The Analysis Requirement Summary window is not active, and there is an analysis requirement that has not been met. Turn on Analysis Requirement Summary in the Options menu, 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, then view the label edit table.
Marker header bars for some loci	When an edit is made to a locus, the quality flags and are gray 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.

Symptoms	Possible Causes and Comments
Alleles not called (continued)	An insufficient number of ILS 600 fragments were defined. Be sure to define at least two ILS 600 fragments smaller than the smallest sample peak or allelic ladder peak and at least two ILS 600 fragments larger than the largest sample peak or allelic ladder peak. In this instance, the allelic ladder would have failed the allelic ladder quality check.
	Run was too short, and larger peaks in ILS were not captured. Not all ILS 600 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.
	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.
	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.C or 6.D.
	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.
	An allelic ladder from a different run than the samples was used. Re-analyze samples with an allelic ladder from the same run.
Size standard not called correctly	Starting data point was incorrect for the partial range chosen in Section 6.D. Adjust the starting data point in the analysis method. Alternatively, 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”.
	Run was too short, and larger peaks in ILS were not captured. Not all ILS 600 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 are below threshold, decrease the peak amplitude threshold in the analysis method for the red channel to include peaks.
	If peaks are low-quality, redefine the size standard for the sample to skip these peaks.

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

Symptoms	Possible Causes and Comments
Significantly raised baseline	• Poor spectral calibration for the ABI PRISM® 3100 and 3100-*Avant* Genetic Analyzers and Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers. Perform a new spectral calibration, and re-run the samples. • Poor matrix for the ABI PRISM® 310 Genetic Analyzer. Re-run and optimize the matrix. Make sure that the matrix applied was generated on the same instrument.
	Incorrect spectral was active. Re-run samples, and confirm that the PowerPlex® 4-dye F spectral calibration is set for F. See instructions for instrument preparation in Section 5.

7.E. GeneMapper® *ID* Software

Symptoms	Possible Causes and Comments
Alleles not called	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 (Figure 25).
	To analyze samples with GeneMapper® *ID* software, at least one allelic ladder must be defined.
	An insufficient number of ILS 600 fragments was defined. Be sure to define at least two ILS 600 fragments smaller than the smallest sample peak or allelic ladder peak and at least two ILS 600 fragments larger than the largest sample peak or allelic ladder peak.
	Run was too short, and larger peaks in ILS were not captured. Not all ILS 600 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.

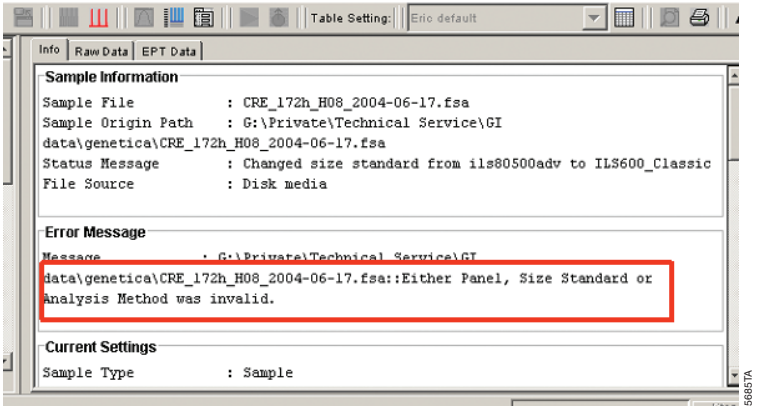

Figure 25. The error message that appears in the GeneMapper® *ID* software when the analysis parameters and size standard have different analysis types.

Symptoms	Possible Causes and Comments
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.
	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 re-analyze, as described in Section 6.G or 6.H.
	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.
Size standard not called correctly (Figure 26)	Starting data point was incorrect for the partial range chosen in Section 6.G. Adjust the starting data point in the analysis method. Alternatively, use a full range for the analysis.
	Extra peaks in advanced mode size standard. Open the Size Match Editor. Highlight the extra peak, select "Edit" and select "delete size label". Select "auto adjust sizes".
	Run was too short, and larger peaks in ILS were not captured. Not all ILS 600 peaks defined in the size standard were detected during the run. Definition and detection of the 600bp fragment is optional. • Create a new size standard using the internal lane standard fragments present in the sample. • Re-run samples using a longer run time.

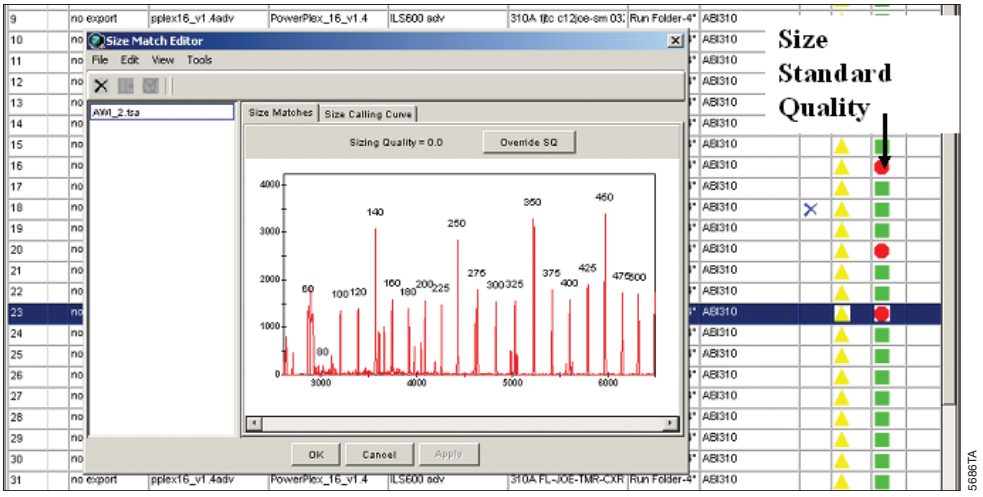

Figure 26. An example showing improper assignment of size standard fragments in the GeneMapper® *ID* Software.

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

Symptoms	Possible Causes and Comments
Peaks in size standard missing	If peaks are below threshold, decrease the peak amplitude threshold in the analysis method for the red channel to include peaks.
	If peaks are low-quality, redefine the size standard for the sample to skip these peaks.
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	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 20.
Significantly raised baseline	• Poor spectral calibration for the ABI PRISM® 3100 and 3100-*Avant* Genetic Analyzers and Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers. Perform a new spectral calibration, and re-run the samples. • Poor matrix for the ABI PRISM® 310 Genetic Analyzer. Re-run and optimize the matrix.
	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 spectral was active. Re-run samples, and confirm that the PowerPlex® 4-dye F spectral is set for F. See instructions for instrument preparation in Section 5.
Red bar appears during analysis of samples, and the following error message appears when data are displayed: "Some selected sample(s) do not contain analysis data. Those sample(s) will not be shown".	If none of the samples had matrices applied when run on the ABI PRISM® 310 Genetic Analyzer, no data will be displayed. Apply a matrix file during analysis in the GeneMapper® *ID* software and re-analyze.

Symptoms	Possible Causes and Comments
Error message after attempting to import panels and bins text files: "Unable to save panel data: java.SQLEException: 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 re-import 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. Promega recommends 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, 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.

7.F. PowerTyper™ 16 Macro

Symptoms	Possible Causes and Comments
File does not open on your computer	Genotyper® software was not installed. Be certain that the Genotyper® software, version 2.5 (Macintosh®) or version 3.6 or higher (Windows NT®), is installed.
	Incorrect version of Genotyper® software. The PowerTyper™ 16 Macro will not work with Genotyper® software versions prior to version 2.5.
Error message: "Could not complete the "Run Macro" command because no dye/lanes are selected"	Allelic ladder sample files were not identified. Be certain the Sample Info or Color Info column for each lane containing PowerPlex® 16 HS Allelic Ladder Mix contains the word "ladder". The macro uses the word "ladder" to identify sample files containing allelic ladder.
	All four dye colors were not imported. For Genotyper® software, versions 2.5 and 3.5 or higher, set preferences (in the Edit menu) to import the blue, green, yellow and red colors.
Error message: "Could not complete the "Run Macro" command because the labeled peak could not be found"	Peak heights for one or more alleles in the allelic ladder sample file were below 150RFU. The allelic ladder categories are defined as having a minimum peak height of 150RFU. If peak heights of ladder alleles are below 150RFU, the software will not be able to locate the allele peak. Re-run the allelic ladder using more sample or longer injection time to assure peak heights above 150RFU.
	CE spikes in the allelic ladder sample were identified as alleles by the macro. Use a different injection of allelic ladder.

7.F. PowerTyper™ 16 Macro (continued)

Symptoms	Possible Causes and Comments
Error message: "Could not complete the "Run Macro" command because the labeled peak could not be found" (continued)	TH01 9.3 and 10 alleles were not separated when using heavy smoothing in the GeneScan® analysis parameters. Use light smoothing in the GeneScan® analysis parameters.
	Allelic ladder data were not compatible with the PowerTyper™ file used. Confirm that the PowerTyper™ Macro file matches the allelic ladder being used.
	The base-pair size of alleles in the allelic ladder are outside of the defined category range. Be sure internal lane standard fragments are correctly sized. Redefine internal lane standard fragments, and re-analyze the sample using GeneScan® software. Compare the size of the smallest allele in the allelic ladder with the base-pair size and range listed in the categories for the same alleles. If necessary, increase the category start range (in the category window) to greater than ±6bp, and save the macro under a new name.
	Allelic ladder peaks were too high, causing stutter peaks to be called as allele peaks. Use a shorter injection time, decrease the amount of allelic ladder used or re-analyze the allelic ladder sample using increased peak amplitude thresholds in the GeneScan® analysis parameters.
	Allelic ladder data were not compatible with the PowerTyper™ Macro file used. Confirm that the PowerTyper™ Macro file matches the allelic ladder being used.
The plots window or allele table does not display all data	The macros were not run in the proper order. Use the POWER or POWER 20% Filter macro option.
	All four dye colors were not imported. For Genotyper® software, versions 2.5 and 3.5 or higher, set preferences (in the Edit menu) to import the blue, green, yellow and red colors.
The Check ILS macro displays an empty plot window	All four dye colors were not imported. For Genotyper® software, versions 2.5 and 3.5 or higher, set preferences (in the Edit menu) to import the blue, green, yellow and red colors.
Off-ladder peaks	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 CE column over time. Use a different injection of allelic ladder to determine sizes in the PowerTyper™ 16 Macro (Release 2.0). Do not use the first injection on a new column for the ladder sample.
	The base-pair size of alleles was incorrect because incorrect fragment sizes were assigned to the internal lane standard. Confirm that internal lane standard fragment sizes are assigned correctly. Re-analyze the sample using GeneScan® software, and redefine the internal lane standard fragments.

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 β-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. Ensenberger, M.G. *et al*. (2010) Developmental validation of the PowerPlex® 16 HS System: An improved 16-locus fluorescent STR multiplex. *Forensic Sci. Int. Genet*. **4**, 257–64.
14. Kline, M.C. *et al*. (2005) Results from the NIST 2004 DNA quantitation study. *J. Forensic Sci*. **50**, 570–8.
15. Levinson, G. and Gutman, G.A. (1987) Slipped-strand mispairing: A major mechanism for DNA sequence evolution. *Mol. Biol. Evol*. **4**, 203–21.
16. Schlotterer, C. and Tautz, D. (1992) Slippage synthesis of simple sequence DNA. *Nucleic Acids Res*. **20**, 211–5.
17. Smith, J.R. *et al*. (1995) Approach to genotyping errors caused by nontemplated nucleotide addition by *Taq* DNA polymerase. *Genome Res*. **5**, 312–7.
18. 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.
19. 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.

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8. References (continued)

20. Moller, A., Meyer, E. and Brinkmann, B. (1994) Different types of structural variation in STRs: HumFES/FPS, HumVWA and HumD21S11. *Int. J. Leg. Med.* **106**, 319–23.
21. Brinkmann, B., Moller, A. and Wiegand, P. (1995) Structure of new mutations in 2 STR systems. *Int. J. Leg. Med.* **107**, 201–3.
22. 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.
23. 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.
24. Gill, P. *et al.* (1997) Considerations from the European DNA Profiling Group (EDNAP) concerning STR nomenclature. *Forensic Sci. Int.* **87**, 185–92.
25. Levadokou, E.N. *et al.* (2001) Allele frequencies for fourteen STR loci of the PowerPlex® 1.1 and 2.1 multiplex systems and Penta D locus in Caucasians, African-Americans, Hispanics, and other populations of the United States of America and Brazil. *J. Forensic Sci.* **46**, 736–61.
26. Lins, A.M. *et al.* (1998) Development and population study of an eight-locus short tandem repeat (STR) multiplex system. *J. Forensic Sci.* **43**, 1168–80.
27. Puers, C. *et al.* (1993) Identification of repeat sequence heterogeneity at the polymorphic STR locus HUMTH01[AATG]n and reassignment of alleles in population analysis using a locus-specific allelic ladder. *Am. J. Hum. Genet.* **53**, 953–8.
28. Hammond, H. *et al.* (1994) Evaluation of 13 short tandem repeat loci for use in personal identification applications. *Am. J. Hum. Genet.* **55**, 175–89.
29. Bever, R.A. and Creacy, S. (1995) Validation and utilization of commercially available STR multiplexes for parentage analysis. In: *Proceedings from the Fifth International Symposium on Human Identification 1994*. Promega Corporation, 61–8.
30. Sprecher, C.J. *et al.* (1996) General approach to analysis of polymorphic short tandem repeat loci. *BioTechniques* **20**, 266–76.
31. Lins, A.M. *et al.* (1996) Multiplex sets for the amplification of polymorphic short tandem repeat loci—silver stain and fluorescent detection. *BioTechniques* **20**, 882–9.
32. Jones, D.A. (1972) Blood samples: Probability of discrimination. *J. Forensic Sci. Soc.* **12**, 355–9.
33. Brenner, C. and Morris, J.W. (1990) In: *Proceedings from the International Symposium on Human Identification 1989*, Promega Corporation, 21–53.
34. Mandrekar, P.V., Krenke, B.E. and Tereba, A. (2001) DNA IQ™: The intelligent way to purify DNA. *Profiles in DNA* **4**(3), 16.
35. Gill, P., Jeffreys, A.J. and Werrett, D.J. (1985) Forensic application of DNA 'fingerprints'. *Nature* **318**, 577–9.
36. Krenke, B.E. *et al.* (2005) Development of a novel, fluorescent, two-primer approach to quantitative PCR. *Profiles in DNA* **8**(1), 3–5.
37. Krenke, E. *et al.* (2008) Developmental validation of a real-time PCR assay for the simultaneous quantification of total human and male DNA. *Forensic Sci. Int. Genet.* **3**, 14–21.

9. Appendix

9.A. Advantages of Using the Loci in the PowerPlex® 16 HS System

The loci included in the PowerPlex® 16 HS System (Tables 2 and 3) were selected because they satisfy the needs of several major standardization bodies throughout the world. For example, the United States Federal Bureau of Investigation (FBI) has selected 13 STR core loci for typing prior to searching or including (submitting) samples in CODIS (Combined DNA Index System), the U.S. national database of convicted offender profiles. The PowerPlex® 16 HS System amplifies all CODIS core loci in a single reaction.

The PowerPlex® 16 HS System also contains two low-stutter, highly polymorphic pentanucleotide repeat loci, Penta E and Penta D. These additional loci add significantly to the discrimination power of the system, making the PowerPlex® 16 HS System a single-amplification system with a power of exclusion sufficient to resolve paternity disputes definitively. In addition, the extremely low level of stutter seen with Penta E and Penta D makes them ideal loci to evaluate DNA mixtures often encountered in forensic casework. Finally, the Amelogenin locus is included in the PowerPlex® 16 HS System to allow gender identification of each sample. Table 4 lists the PowerPlex® 16 HS System alleles revealed in Promega control DNA templates.

We have carefully selected STR loci and primers to avoid or minimize artifacts, including those associated with *Taq* DNA polymerase, such as repeat slippage and terminal nucleotide addition. Repeat slippage (15,16), sometimes called "n–4 bands", "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 (17,18) occurs when *Taq* 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 band 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 for 30 minutes (19) to the amplification protocols to provide conditions for essentially complete terminal nucleotide addition when recommended amounts of template DNA are used.

The presence of microvariant alleles (alleles differing from one another by lengths other than the repeat length) complicates interpretation and assignment of alleles. There appears to be a correlation between a high degree of polymorphism, a tendency for microvariants and increased mutation rate (20,21). Thus, FGA and D21S11 display numerous, relatively common microvariants. For reasons yet unknown, the highly polymorphic Penta E locus does not display frequent microvariants (Table 4).

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Table 2. The PowerPlex® 16 HS System Locus-Specific Information.

STR Locus	Label	Chromosomal Location	GenBank® Locus and Locus Definition	Repeat Sequence[1] 5´→ 3´
Penta E	FL	15q	NA	AAAGA
D18S51	FL	18q21.3	HUMUT574	AGAA (22)
D21S11	FL	21q11–21q21	HUMD21LOC	TCTA Complex (22)
TH01	FL	11p15.5	HUMTH01, human tyrosine hydroxylase gene	AATG (22)
D3S1358	FL	3p	NA	TCTA Complex
FGA	TMR-ET	4q28	HUMFIBRA, human fibrinogen alpha chain gene	TTTC Complex (22)
TPOX	TMR-ET	2p24–2pter	HUMTPOX, human thyroid peroxidase gene	AATG
D8S1179	TMR-ET	8q24.13	NA	TCTA Complex (22)
vWA	TMR-ET	12p13.31	HUMVWFA31, human von Willebrand factor gene	TCTA Complex (22)
Amelogenin[2]	TMR-ET	Xp22.1–22.3 and Y	HUMAMEL, human Y chromosomal gene for Amelogenin-like protein	NA
Penta D	JOE	21q	NA	AAAGA
CSF1PO	JOE	5q33.3–34	HUMCSF1PO, human c-fms proto-oncogene for CSF-1 receptor gene	AGAT
D16S539	JOE	16q24.1	NA	GATA
D7S820	JOE	7q11.21–22	NA	GATA
D13S317	JOE	13q22–q31	NA	TATC
D5S818	JOE	5q23.3–32	NA	AGAT

[1]The August 1997 report (23,24) 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".

[2]Amelogenin is not an STR.

TMR = carboxy-tetramethylrhodamine

FL = fluorescein

JOE = 6-carboxy-4´,5´-dichloro-2´,7´-dimethoxyfluorescein

NA = not applicable

Table 3. The PowerPlex® 16 HS System Allelic Ladder Information.

STR Locus	Label	Size Range of Allelic Ladder Components[1,2] (bases)	Repeat Numbers of Allelic Ladder Components[3]
Penta E	FL	379–474	5–24
D18S51	FL	290–366	8–10, 10.2, 11–13, 13.2, 14–27
D21S11	FL	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
TH01	FL	156–195	4–9, 9.3, 10–11, 13.3
D3S1358	FL	115–147	12–20
FGA	TMR	322–444	16–18, 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, 43.2, 44.2, 45.2, 46.2
TPOX	TMR	262–290	6–13
D8S1179	TMR	203–247	7–18
vWA	TMR	123–171	10–22
Amelogenin[4]	TMR	106, 112	X, Y
Penta D	JOE	376–449	2.2, 3.2, 7–17
CSF1PO	JOE	321–357	6–15
D16S539	JOE	264–304	5, 8–15
D7S820	JOE	215–247	6–14
D13S317	JOE	176–208	7–15
D5S818	JOE	119–155	7–16

[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 Internal Lane Standard 600, 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 also affects 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: **www.cstl.nist.gov/div831/strbase/**

[4]Amelogenin is not an STR.

Table 4. The PowerPlex® 16 HS System Allele Determinations in Commonly Available Standard DNA Templates.

	Standard DNA Templates[1]	
STR Locus	**K562[2]**	**2800M**
Penta E	5, 14	7, 14
D18S51	15, 16	16, 18
D21S11	29, 30, 31	29, 31.2
TH01	9.3, 9.3	6, 9.3
D3S1358	16, 16	17, 18
FGA	21, 24	20, 23
TPOX	8, 9,	11, 11
D8S1179	12, 12	14, 15
vWA	16, 16	16, 19
Amelogenin	X, X	X, Y
Penta D	9, 13	12, 13
CSF1PO	9, 10	12, 12
D16S539	11, 12	9, 13
D7S820	9, 11	8, 11
D13S317	8, 8	9, 11
D5S818	11, 12	12, 12

[1]Strain K562 is available from the American Type Culture Collection: **www.atcc.org** (Manassas, VA).

[2]Strain K562 displays three alleles at the D21S11 locus.

9.B. Power of Discrimination

The fifteen STR loci amplified with the PowerPlex® 16 HS System provide powerful discrimination. Population statistics for these loci are displayed in Table 5. These data were generated as part of a collaboration (25) with The Bode Technology Group (Springfield, VA), North Carolina Bureau of Investigation (Raleigh, NC), Palm Beach County Sheriff's Office (West Palm Beach, FL), Virginia Division of Forensic Science (Richmond, VA) and Charlotte/Mecklenburg Police Department Laboratory (NC). Data generation included analysis of over 200 individuals from African-American, Caucasian-American and Hispanic-American populations. Data for Asian-Americans include analysis of more than 150 individuals. For additional population data for STR loci, see references 26–31 and the Short Tandem Repeat DNA Internet DataBase at: **www.cstl.nist.gov/div831/strbase/**

Table 5 shows the matching probability (32) for the PowerPlex® 16 HS System in various populations. The matching probability of the PowerPlex® 16 HS System ranges from 1 in 1.83×10^{17} for Caucasian-Americans to 1 in 1.41×10^{18} for African-Americans.

A measure of discrimination often used in paternity analyses is the paternity index (PI), a means for presenting the genetic odds in favor of paternity given the genotypes for the mother, child and alleged father (33). The typical paternity indices for the PowerPlex® 16 HS System are shown in Table 5. The PowerPlex® 16 HS System provides typical paternity indices exceeding 500,000 in each population group. An alternative calculation used in paternity analyses is the power of exclusion (33). This value, calculated for the PowerPlex® 16 HS System, exceeds 0.999998 in all populations tested (Table 5).

Table 5. Matching Probabilities, Paternity Indices and Power of Exclusion of the PowerPlex® 16 HS System in Various Populations.

	African-American	Caucasian-American	Hispanic-American	Asian-American
Matching Probability	1 in 1.41×10^{18}	1 in 1.83×10^{17}	1 in 2.93×10^{17}	1 in 3.74×10^{17}
Paternity Index	2,510,000	1,520,000	522,000	4,110,000
Power of Exclusion	0.9999996	0.9999994	0.9999983	0.9999998

9.C. Sample Analysis Using the GeneScan® and Genotyper® Software

Sample Analysis Using the GeneScan® Software and Windows® Operating Systems

1. Analyze data using the GeneScan® software.
2. Review the raw data for one or more sample runs. Highlight the sample file name, then in the Sample menu, select "raw data". Move the cursor so that the crosshair is on the baseline to the right of the large primer peak (before the first internal lane standard peak [red]). Use the X-value number shown at the bottom left of the window for the start position in the analysis parameters.
3. The recommended analysis parameters are shown in Figure 27.

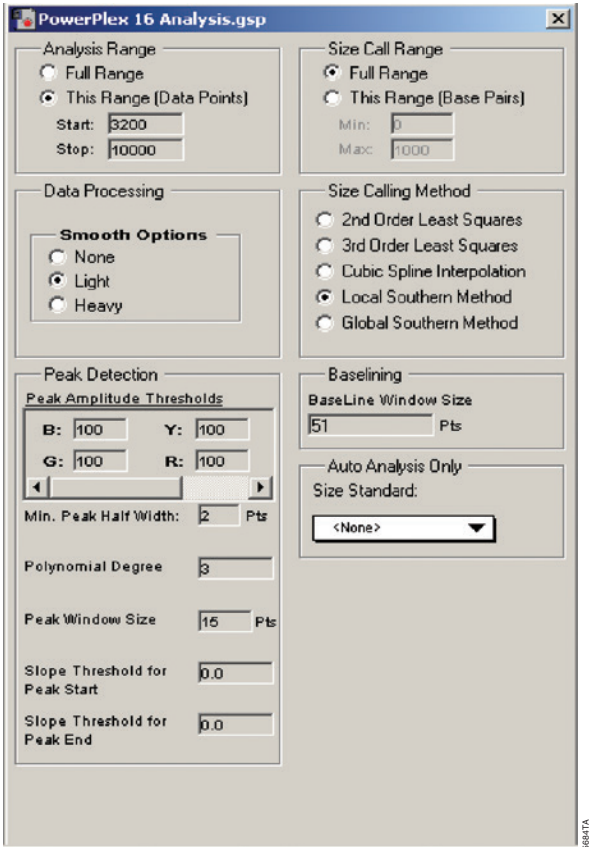

Figure 27. The Analysis Parameters window. The start point of the analysis range, which will vary, is defined in Step 2.

4. The analysis parameters can be saved in the Params folder; in most installations this is located at: C:\AppliedBio\Shared\Analysis\Sizecaller\Params
5. Apply the stored analysis parameters file to the samples.
6. Assign a new size standard. Select a sample file, and highlight the arrow next to size standard. Select "define new". Assign the size standard peaks as shown in Figure 28 in Section 9.D. Store the size standard in the Size Standards folder at: C:\AppliedBio\Shared\Analysis\Sizecaller\SizeStandards\
7. Apply the size standard file to the samples, then analyze the sample files. See below for additional information on the use of the PowerTyper™ 16 Macro (Release 2.0) and Genotyper® software.

Notes:

1. Peak heights outside the linear range of the instrument may generate artifact peaks due to instrument saturation (i.e., overloading the sample). Bleedthrough (pull-ups) from one color to another may be observed. Saturated signal also may appear as two peaks (split peak).
2. If peak heights are not within the linear range of detection of the instrument, the ratio of stutter peaks to real allele peaks increases, and allele designations become difficult to interpret. The balance of peak heights also may appear less uniform.
3. There can be variation between instruments regarding the relative fluorescence levels detected using the same sample. Furthermore, different instruments vary in the relative efficiency of color detection, affecting the dye color-to-dye color balance.

Sample Analysis Using the GeneScan® Software and Macintosh® Operating Systems

1. Analyze data using the GeneScan® software.
2. Review the raw data for one or more sample runs. Highlight the sample file name, then in the Sample menu, select "raw data". Move the cursor so that the crosshair is on the baseline to the right of the large primer peak (before the first internal lane standard peak [red]). Use the X-value number shown at the bottom left of the window for the start position in the analysis parameters.

Sample Analysis Using the GeneScan® Software and Macintosh® Operating Systems (continued)

3. The recommended analysis parameters are:

Analysis Range	Start: Defined in Step 2 Stop: 10,000
Data Processing	Baseline: Checked Multicomponent: Checked Smooth Options: Light[1]
Peak Detection	Peak Amplitude Thresholds[2]: B: Y: G: R: Min. Peak Half Width: 2pt
Size Call Range	Min: 60 Max: 600
Size Calling Method	Local Southern Method
Split Peak Correction	None

[1]Smooth options should be determined by individual laboratories. Occasionally the TH01 alleles 9.3 and 10 will not be distinguished using heavy smoothing.

[2]The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Values for peak amplitude thresholds should be determined by individual laboratories.

4. The analysis parameters can be saved in the Params folder.
5. Apply the stored analysis parameters file to the samples.
6. Assign a new size standard. Select a sample file, highlight the arrow next to size standard, then select "define new". Assign the size standard peaks as shown in Figure 28 in Section 9.D. Store the size standard in the Size Standards folder.
7. Apply the size standard file to the samples, then analyze the sample files. See below for additional information on the use of the PowerTyper™ 16 Macro (Release 2.0) and Genotyper® software.

   For additional information regarding the GeneScan® software, refer to the *GeneScan® Analysis Software User's Manual*.

Notes:

1. Peak heights outside the linear range of the instrument may generate artifact peaks due to instrument saturation (i.e., overloading the sample). Bleedthrough (pull-ups) from one color to another may be observed. Saturated signal also may appear as two peaks (split peak).
2. If peak heights are not within the linear range of detection of the instrument, the ratio of stutter peaks to real allele peaks increases, and allele designations become difficult to interpret. The balance of peak heights also may appear less uniform.

3. There can be variation between instruments regarding the relative fluorescence levels detected using the same sample. Furthermore, different instruments vary in the relative efficiency of color detection, affecting the dye color-to-dye color balance.

Sample Analysis Using the Genotyper® Software and PowerTyper™ 16 Macro

To facilitate analysis of data generated with the PowerPlex® 16 HS System, we have created a file to allow automatic assignment of genotypes using the Genotyper® software. After samples are amplified, detected using the ABI PRISM® 310 or 3100 Genetic Analyzer (using Data Collection Software, Version 1.0.1 or 1.1) and analyzed using the GeneScan® software, sample files can be imported into the Genotyper® program and analyzed using the PowerTyper™ 16 Macro (Release 2.0).

The PowerTyper™ 16 Macro (Release 2.0) is available for download from the Promega web site at: **www.promega.com/resources/software-firmware/powertyper-macros/**

The PowerTyper™ 16 Macro (Release 2.0) is used in conjunction with Macintosh® Genotyper® software, version 2.5, and Windows NT® Genotyper® software, version 3.6, or later. The Genotyper® software must be installed on your computer before the PowerTyper™ 16 Macro (Release 2.0) can be used.

Be certain the Sample Info (Macintosh® computers) or Color Info (Windows NT® operating systems) column for each lane containing allelic ladder mix contains the word "ladder". The macro uses the word "ladder" to identify the sample file(s) containing allelic ladder. Sample info can be added or modified after importing into the PowerTyper™ Macro. Highlight the sample, then select "show dye/lanes window" in the Views menu.

1. Transfer the PowerTyper™ 16 Macro (Release 2.0) to a designated location on your computer hard drive.
2. Open the Genotyper® software, then the PowerTyper™ 16 Macro (Release 2.0). For questions about the Genotyper® software, refer to the *Genotyper® Analysis Software User's Manual*.
3. In the File menu, select "Import", and import the GeneScan® project or sample files to be analyzed. Import the blue, yellow, green and red dye colors.

   Note: To select the dye colors to be imported, select "Set Preferences" in the Edit menu.
4. Double-click on the Check ILS macro. The macros are listed at the bottom left corner of the active window. A plots window will be displayed to show the internal lane standard (i.e., ILS 600) in the red dye color. Scroll down to view, and confirm that the internal lane standard fragment sizes are correct. If necessary, re-analyze samples using the GeneScan® software and redefine internal lane standard fragments.

   Note: The software uses one ladder sample to determine allele sizes. The macro uses the first ladder sample imported for allele designations.

Sample Analysis Using the Genotyper® Software and PowerTyper™ 16 Macro (continued)

5. For casework, double-click on the POWER macro. The POWER macro identifies alleles in the ladder sample and calculates offsets for all loci. This process may take several minutes. When completed, a plots window will open to display the allelic ladders (i.e., Penta E, D18S51, D21S11, TH01 and D3S1358).

   Alternatively, for databasing or paternity, double-click on the POWER 20% Filter macro. This macro has a higher level of filtering than the standard POWER macro to reduce the need for manual editing of peak labels. The POWER 20% Filter should not be used if mixtures may exist.

   In general, allelic ladders contain fragments of the same lengths as many known alleles for the locus. Allelic ladder sizes and repeat units are listed in Table 3 (Section 9.A). Analysis using GeneScan® and Genotyper® software allows allele determination by comparing amplified sample fragments with allelic ladders and internal lane standards. When using an internal lane standard, the calculated lengths of allelic ladder components might differ from those listed in the table. This is due to differences in migration resulting from sequence differences between the allelic ladder fragments and internal size standard and is not a matter of concern.

6. Double-click on the Allelic Ladders macro. A plots window will open to display the blue (fluorescein) dye allelic ladders (i.e., Penta E, D18S51, D21S11, TH01 and D3S1358), green (JOE) dye allelic ladders (i.e., Penta E, CSF1PO, D16S539, D7S820, D13S317 and D5S818) and yellow (TMR) dye allelic ladders (i.e., FGA, TPOX, D8S1179, vWA and Amelogenin). Confirm that the correct allele designations were assigned to the allelic ladders (Figure 24 in Section 6.J).

   Note: The software uses one ladder sample to determine allele sizes. The macro uses the first ladder sample imported for allele designations. If the POWER macro is run a second time, the software will use the second ladder; if the POWER macro is run a third time, the software will use the third ladder, etc., until all ladders in the project are used. If an allelic ladder fails to be analyzed or if many off-ladder alleles are found in the samples, samples should be re-analyzed using another ladder from the project.

7. Double-click on the Display Fluorescein Data macro to display the blue dye for all sample injections. Scroll down to observe and edit as needed.

8. Double-click on the Display TMR Data macro to display the yellow dye for all sample injections. Scroll down to observe and edit as needed.

9. Double-click on the Display JOE Data macro to display the green dye for all sample injections. Scroll down to observe and edit as needed.

10. Create the appropriate table by selecting the PowerTable, Make Allele Table or Make CODIS Table macro. The three available table formats are shown below. The PowerTable option allows up to four alleles per sample file. Additional information such as low peak signal or high peak signal also is included. The Allele Table and CODIS Table options include only two alleles per locus. If more than two alleles are present at a locus, the smallest alleles identified are included. The Allele Table format displays the categories (loci) in columns, while the CODIS table format displays the categories in rows. These tables can be customized to fit needs. To save data in tables, go to the Table drop-down menu, highlight "Export to File..." and save the file with the desired name and location. The saved file can be viewed and analyzed using Microsoft® Excel®.

11. Save the analyzed data. Go to the File menu, and select "Save as".

! The PowerTyper™ Macro is a Genotyper® file and can be overwritten if "Save" is used instead of "Save as".

PowerTable Format

Sample Info	Sample Comment	Category	Peak 1	Peak 2	Peak 3	Peak 4	Overflow	Low Signal	Saturation	Edited Label	Edited Row

Allele Table Format

Sample Info	Category Allele 1	Category Allele 2	Category Allele 1	Category Allele 2	Category Allele 1	Category Allele 2	Category Allele 1	Category Allele 2

CODIS Table Format

Sample Info	Category	Peak 1	Peak 2

9.D. The Internal Lane Standard 600

The Internal Lane Standard (ILS) 600 contains 22 DNA fragments of 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550 and 600 bases in length (Figure 28). Each fragment is labeled with carboxy-X-rhodamine (CXR) and can be detected separately (as a fourth color) in the presence of PowerPlex® 16 HS-amplified material. The ILS 600 is designed for use in each CE injection to increase precision in analyses when using the PowerPlex® 16 HS System. Protocols for preparation and use of this internal lane standard are provided in Section 5.

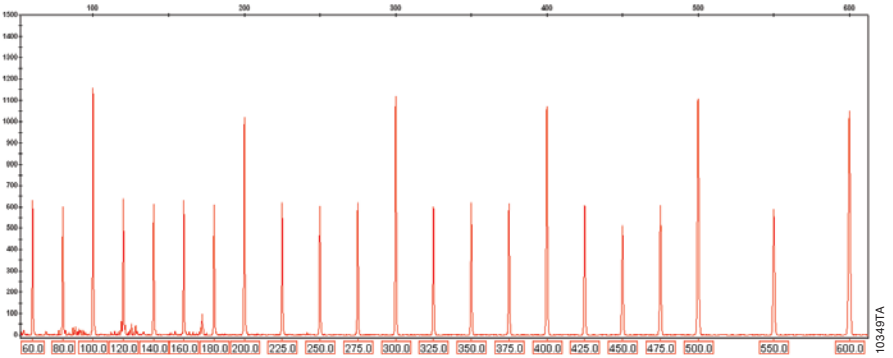

Figure 28. Internal Lane Standard 600. An electropherogram showing the Internal Lane Standard 600 fragments.

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 ($Na_2EDTA \cdot 2H_2O$)

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 ($Na_2EDTA \cdot 2H_2O$)
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. DNA Extraction and Quantitation Methods and Automation Support

Promega offers a wide variety of reagents and automated methods for sample preparation, DNA purification and DNA quantitation prior to STR amplification.

For analysis of database, reference and other single-source samples, we recommend direct amplification from FTA® punches or preprocessing of swabs and nonFTA punches with the SwabSolution™ Kit or PunchSolution™ Kit. The SwabSolution™ Kit (Cat.# DC8271) contains reagents for rapid DNA preparation from buccal swab samples prior to amplification. The procedure lyses cells contained on the swab head and releases into solution sufficient DNA for STR amplification. A small volume of the final swab extract is added to the PowerPlex® reaction. The PunchSolution™ Kit is used to process punches from nonFTA storage cards containing blood or buccal samples prior to direct amplification.

For casework or samples that require DNA purification, we recommend the DNA IQ™ System (Cat.# DC6700), which is a DNA isolation system designed specifically for forensic samples (34). This system uses paramagnetic particles to prepare clean samples for STR analysis easily and efficiently and can be used to extract DNA from stains or liquid samples, such as blood or solutions. The DNA IQ™ Resin eliminates PCR inhibitors and contaminants frequently encountered in casework samples. With larger samples, the DNA IQ™ System delivers a consistent amount of total DNA. The system has been used to isolate and quantify DNA from routine sample types including buccal swabs, stains on FTA® paper and liquid blood. Additionally, DNA has been isolated from casework samples such as tissue, differentially separated sexual assault samples and stains on support materials. The DNA IQ™ System has been tested with the PowerPlex® Systems to ensure a streamlined process. See Section 9.G for ordering information.

To process sexual assault samples, differential extraction can be used to enrich sperm cells in the presence of an excess of epithelial cells (35). Traditionally, these samples are processed by performing a controlled lysis of epithelial cells in the absence of a reducing agent and centrifuging samples to separate the pellet of intact sperm and cell debris from the buffer containing DNA from lysed epithelial cells. However, this method is time-consuming and labor-intensive, and several washings and centrifugations often are required to obtain sperm free of epithelial DNA. The Differex™ System simplifies differential extraction. This system uses a standard proteinase K digestion and a combination of phase separation and differential centrifugation to separate sperm and epithelial DNA quickly and easily. The Differex™ System offers similar recovery as the standard method commonly used for differential extraction. The Differex™ System, in combination with the DNA IQ™ System, can be automated to extract up to 48 differential extractions in less than 5 hours, including incubation time, and less than 1 hour of hands-on laboratory time.

9.F. DNA Extraction and Quantitation Methods and Automation Support (continued)

For applications requiring human-specific DNA quantification, the Plexor® HY System (Cat.# DC1000) was developed (36,37). See Section 9.G for ordering information.

For information about automation of Promega chemistries on automated workstations using Identity Automation™ solutions, contact your local Promega Branch Office or Distributor, contact information available at:
www.promega.com/support/worldwide-contacts/
e-mail: **genetic@promega.com** or visit: **www.promega.com/idautomation/**

9.G. Related Products

STR Systems

Product	Size	Cat.#
PowerPlex® 16 Monoplex System, Penta E (Fluorescein)	100 reactions	DC6591
PowerPlex® 16 Monoplex System, Penta D (JOE)	100 reactions	DC6651
PowerPlex® 18D System	200 reactions	DC1802
	800 reactions	DC1808
PowerPlex® 21 System	200 reactions	DC8902
PowerPlex® ESX 16 System	100 reactions	DC6711
	400 reactions	DC6710
PowerPlex® ESX 17 System	100 reactions	DC6721
	400 reactions	DC6720
PowerPlex® ESI 16 System	100 reactions	DC6771
	400 reactions	DC6770
PowerPlex® ESI 17 Pro System	100 reactions	DC7781
	400 reactions	DC7780
PowerPlex® CS7 System	100 reactions	DC6613
PowerPlex® Y23 System	50 reactions	DC2305
	200 reactions	DC2320

Not for Medical Diagnostic Use.

Accessory Components

Product	Size	Cat.#
PowerPlex® Matrix Standards, 310*	50µl (each dye)	DG4640
PowerPlex® 4C Matrix Standard*	5 preps	DG4800
Internal Lane Standard 600	150µl	DG1071
Water, Amplification Grade*	5 × 1,250µl	DW0991
2800M Control DNA (10ng/µl)*	25µl	DD7101
2800M Control DNA (0.25ng/µl)*	500µl	DD7251
PunchSolution™ Kit*	100 preps	DC9271
SwabSolution™ Kit*	100 preps	DC8271
5X AmpSolution™ Reagent*	500µl	DM1231

*Not for Medical Diagnostic Use.

Sample Preparation and Quantification Systems

Product	Size	Cat.#
PowerQuant™ System	200 reactions	PQ5002
	800 reactions	PQ5008
DNA IQ™ System	100 reactions	DC6701
	400 reactions	DC6700
Differex™ System*	50 samples	DC6801
	200 samples	DC6800
Tissue and Hair Extraction Kit (for use with DNA IQ™)	100 reactions	DC6740
Maxwell® 16 Forensic Instrument*	1 each	AS3060
DNA IQ™ Reference Sample Kit for Maxwell® 16	48 preps	AS1040
DNA IQ™ Casework Pro Kit for Maxwell® 16*	48 preps	AS1240
Plexor® HY System*	200 reactions	DC1001
	800 reactions	DC1000
Slicprep™ 96 Device	10 pack	V1391

*Not for Medical Diagnostic Use.

10. Summary of Changes

The following changes were made to the 5/16 revision of this document:

1. Description of quantification method for 2800M Control DNA was removed.
2. Other general updates were incorporated.

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[a]U.S. Pat. No. 6,238,863, Chinese Pat. No. ZL99802696.4, European Pat. No. 1058727, Japanese Pat. No. 4494630 and other patents pending.

[b]Australian Pat. No. 724531, Canadian Pat. No. 2,251,793, Korean Pat. No. 290332, Singapore Pat. No. 57050, Japanese Pat. Nos. 3602142 and 4034293, Chinese Pat. Nos. ZL99813729.4 and ZL97194967.0, European Pat. No. 0960207 and other patents pending.

[c]Allele sequences for one or more of the loci vWA, FGA, D8S1179, D21S11 and D18S51 in allelic ladder mixtures is licensed under U.S. Pat. Nos. 7,087,380 and 7,645,580, Australia Pat. No. 2003200444 and corresponding patent claims outside the US.

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