PowerPlex® 16 System

Instructions for Use of Products
DC6530 and DC6531

PowerPlex® 16 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 (PCR; 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 System[a] 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 System provides all materials necessary to amplify STR regions of human genomic DNA except for AmpliTaq Gold® DNA polymerase. This manual contains protocols for use of the PowerPlex® 16 System with the GeneAmp® PCR System 9700 and ProFlex® PCR System in addition to protocols to separate amplified products and detect separated material on the Applied Biosystems® 3130, 3130*xl,* 3500 and 3500xL Genetic Analyzers. Protocols to operate the fluorescence-detection instruments should be obtained from the instrument manufacturer. Amplification and detection instrumentation may vary. You may need to optimize protocols including amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. In-house validation should be performed. Protocols to operate the fluorescence-detection instruments should be obtained from the instrument manufacturer.

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

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
PowerPlex® 16 System	**100 reactions**	**DC6531**

Not For Medical Diagnostic Use. Cat.# DC6531 contains sufficient reagents for 100 reactions of 25µl each. Includes:

Pre-amplification Components Box
*   1 × 300µl   Gold ST★R 10X Buffer
*   1 × 250µl   PowerPlex® 16 10X Primer Pair Mix
*   25µl   2800M Control DNA, 10ng/µl

Post-amplification Components Box
*   1 × 50µl   PowerPlex® 16 Allelic Ladder Mix
*   1× 150µl   Internal Lane Standard (ILS) 600

PRODUCT	SIZE	CAT.#
PowerPlex® 16 System	**400 reactions**	**DC6530**

Not For Medical Diagnostic Use. Cat.# DC6530 contains sufficient reagents for 400 reactions of 25µl each. Includes:

Pre-amplification Components Box
*   4 × 300µl   Gold ST★R 10X Buffer
*   4 × 250µl   PowerPlex® 16 10X Primer Pair Mix
*   25µl   2800M Control DNA, 10ng/µl

Post-amplification Components Box
*   4 × 50µl   PowerPlex® 16 Allelic Ladder Mix
*   4 × 150µl   Internal Lane Standard (ILS) 600

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

Storage Conditions: 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°C to +10°C. Make sure that the 2800M Control DNA is stored at +2°C to +10°C for at least 24 hours before use. The PowerPlex® 16 10X Primer Pair Mix, PowerPlex® 16 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.

The proper panels and bins text files for use with GeneMapper® *ID* and *ID*-X software are available for download at: **www.promega.com/resources/software-firmware/str-analysis/genemapper-id-software-panels-and-bin-sets/**

Matrix standards are required for initial setup of the color separation matrix. The matrix standards are sold separately and are available for the Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers (PowerPlex® 4C Matrix Standard; Cat. #DG4800). See Section 9.E 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 Guide of Autosomal STR Systems for Forensic Laboratories* (12).

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

PCR-based STR analysis is subject to contamination by very small amounts of human DNA. Extreme care should be taken to avoid cross-contamination when preparing template DNA, handling primer pairs, assembling amplification reactions and analyzing amplification products. Reagents and materials used prior to amplification (10X Buffer, 10X Primer Pair Mix and 2800M Control DNA) are provided in a separate box and should be stored separately from those used following amplification (Allelic Ladder Mix and Internal Lane Standard). Always include a negative control reaction (i.e., no template) to detect reagent contamination. We highly recommend the use of gloves and aerosol-resistant pipette tips.

3.B. Matrix Standardization or Spectral Calibration

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

The PowerPlex® 4C Matrix Standard (Cat.# DG4800) is required for spectral calibration on the Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers.

For protocols and additional information about matrix generation and spectral calibration, see the *PowerPlex® 4C Matrix Standard, Technical Manual* #TMD048. This manual is available online at:
www.promega.com/resources/protocols/

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

The PowerPlex® 16 System is optimized for the GeneAmp® PCR System 9700 thermal cycler. An amplification protocol for the ProFlex® PCR System 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.

4.A. Amplification of Extracted DNA

Materials to Be Supplied by the User
- GeneAmp® PCR System 9700 or ProFlex® PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- AmpliTaq Gold® DNA polymerase (Applied Biosystems)

The PowerPlex® 16 System is optimized for 0.5–1ng 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 system showed routine generation of full profiles using 10/22 cycles of amplification with lower amounts of DNA template down to 125pg (13). Partial profiles were typically observed for DNA template of 62pg and below. 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 system in your laboratory.

Amplification Setup
1. Thaw the Gold ST★R 10X Buffer and PowerPlex® 16 10X Primer Pair Mix completely.
   Notes:
   a. 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 after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.
   b. A precipitate may form in the Gold ST★R 10X Buffer. If this occurs, warm the solution briefly at 37°C, then vortex until the precipitate is in solution.
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 consumes 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 new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new, 0.2ml reaction tubes required, and label them appropriately.

4.A. Amplification of Extracted DNA (continued)

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

   Table 1. PCR Amplification Mix for Amplifying Extracted DNA.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
nuclease-free water	to a final volume of 25.0µl	×		=	
Gold ST★R 10X Buffer	2.5µl	×		=	
PowerPlex® 16 10X Primer Pair Mix	2.5µl	×		=	
AmpliTaq Gold® DNA polymerase[2]	0.8µl (4U)	×		=	
template DNA (0.5–1ng)[3,4,5]	up to 19.2µl				
total reaction volume	**25.0µl**				

   [1]Add nuclease-free water to the tube first, then add Gold ST★R 10X Buffer, PowerPlex® 16 10X Primer Pair Mix and AmpliTaq Gold® DNA polymerase. The template DNA will be added at Step 6.

   [2]Assumes the AmpliTaq Gold® DNA polymerase is at 5U/µl. If the enzyme concentration is different, the volume of enzyme must be adjusted accordingly.

   [3]Store DNA templates in TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE^{-4} buffer with 20µg/ml glycogen. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the volume of DNA added should not exceed 20% of the final reaction volume. 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.

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

   [5]The PowerPlex® 16 System is optimized and balanced for 0.5–1.0ng of DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different.

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

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

6. Add template DNA (0.5–1ng) for each sample to the respective well or tube 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 diluted DNA to a reaction well or tube containing PCR amplification mix.

8. For the negative amplification control, pipet nuclease-free water or TE⁻⁴ buffer instead of template DNA into a reaction well containing PCR amplification mix.

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

Thermal Cycling

This section contains protocols for use of the PowerPlex® 16 System with the GeneAmp® PCR system 9700 and ProFlex® PCR System thermal cyclers. For information about other thermal cyclers, contact Promega Technical Services at: **genetic@promega.com**

Amplification and detection instrumentation may vary. You may need to optimize protocols including the amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 10/22 cycles work well for 0.5–1ng of purified DNA templates. For higher template amounts 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 the plate or reaction tubes in the thermal cycler.

2. Select and run the recommended protocol. The preferred protocols for use with the GeneAmp® PCR System 9700 and ProFlex® PCR System thermal cyclers are provided below.
   Notes:
   a. When using the ProFlex® PCR System, the ramp rates indicated in the cycling program must be set. The remaining steps must be set to 1.5°C/second.
   b. When using the GeneAmp® PCR System 9700, 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** to adjust the ramp rates at each hold temperature.

      The ramp speed is set after the thermal cycling run is started. The 'Select Method Options' screen appears. Select **9600** for the ramp speed, and enter the reaction volume.

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

4.A. Amplification of Extracted DNA (continued)

Protocol for the GeneAmp® PCR System 9700 Thermal Cycler	Protocol for the ProFlex® PCR System
95°C for 11 minutes, then:	95°C for 11 minutes, then:
96°C for 1 minute, then:	96°C for 1 minute, 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 1.5°C/second to 94°C for 30 seconds ramp 1.2°C/second to 60°C for 30 seconds ramp 0.4°C/second 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 22 cycles, then:	ramp 1.5°C/second to 90°C for 30 seconds ramp 1.2°C/second to 60°C for 30 seconds ramp 0.4°C/second 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

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

Materials to Be Supplied by the User
- GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- AmpliTaq Gold® DNA polymerase (Applied Biosystems)
- Nuclease-Free Water (Cat.# P1193)
- PunchSolution™ Kit (Cat.# DC9271)
- 5X AmpSolution™ Reagent (Cat.# DM1231), also supplied with the PunchSolution™ Kit
- 1.2mm Harris Micro-Punch or equivalent manual punch and cutting mat

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

When using the protocol detailed below, add one 1.2mm storage card punch to each 25µl amplification reaction.

Nonlytic storage card sample types include:
- Buccal samples on Bode Buccal DNA Collector™ devices
- Buccal samples on nonlytic storage card punches (e.g., S&S 903)

We do not recommend amplification of DNA from blood samples on nonlytic storage cards using the PowerPlex® 16 System.

Pretreat nonlytic sample types with the PunchSolution™ Kit (Cat.# DC9271) to lyse nonlytic samples before adding the 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. Adding PunchSolution™ Reagent to the well before adding the punch during pretreatment may help alleviate static problems.

Amplification Setup

1. Thaw the Gold ST★R 10X Buffer and PowerPlex® 16 10X Primer Pair Mix completely.
   Notes:
   a. 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 after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.
   b. A precipitate may form in the Gold ST★R 10X Buffer. If this occurs, warm the solution briefly at 37°C, then vortex until the precipitate is in solution.

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

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

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

Table 2. PCR Amplification Mix for Directly Amplifying DNA from Nonlytic Storage Card Punches.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
nuclease-free water	14.2µl	×		=	
Gold ST★R 10X Buffer	2.5µl	×		=	
PowerPlex® 16 10X Primer Pair Mix	2.5µl	×		=	
AmpliTaq Gold® DNA polymerase[2]	0.8µl (4U)	×		=	
5X AmpSolution™ Reagent	5.0µl	×		=	
total reaction volume	**25.0µl**				

[1]Add nuclease-free water to the tube first, then add Gold ST★R 10X Buffer, PowerPlex® 16 10X Primer Pair Mix, AmpliTaq Gold® DNA polymerase and 5X AmpSolution™ Reagent. The template DNA will be added at Step 6.

[2]Assumes the AmpliTaq Gold® DNA polymerase is at 5U/µl. If the enzyme concentration is different, the volume of enzyme must be adjusted accordingly.

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

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

6. Pipet 25µl of PCR amplification mix into each reaction well with one pretreated 1.2mm punch from a nonlytic storage card containing a buccal sample. Pipet 25µl of PCR amplification mix into each reaction well for the control reactions.

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

    Notes:

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

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

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 or cap the plate, or close the tubes, and briefly centrifuge to bring the storage card punch 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 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 cycle number, injection conditions and loading volume for each laboratory instrument. Testing at Promega shows that 27 cycles works well for a variety of nonlytic sample types. Cycle number will need to be optimized in each laboratory for each sample type that is amplified.

1. Place the plate or tubes 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.

    Note: When using the GeneAmp® PCR System 9700, 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** to adjust the ramp rates at each hold temperature.

    The ramp speed is set after the thermal cycling run is started. The 'Select Method Options' screen appears. Select **9600** for the ramp speed, and enter the reaction volume.

    Thermal Cycling Protocol

    95°C for 11 minutes, then:

    96°C for 1 minute, 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

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

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

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

PCR Optimization

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

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

2. Place one 1.2mm nonlytic storage card punch in each well of a reaction plate. Be sure to pretreat nonlytic storage card samples with the PunchSolution™ Kit (Cat.# DC9271).

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

4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number.

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

4.C. Direct Amplification of DNA from Swabs

Materials to Be Supplied by the User
- GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate (Applied Biosystems)
- aerosol-resistant pipette tips
- AmpliTaq Gold® DNA polymerase (Applied Biosystems)
- Nuclease-Free Water (Cat.# P1193)
- SwabSolution™ Kit (Cat.# DC8271)

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

Pretreat OmniSwab™ (QIAGEN) or cotton 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 Gold ST★R 10X Buffer and PowerPlex® 16 10X Primer Pair Mix completely.

   Notes:

   a. 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 after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

   b. A precipitate may form in the Gold ST★R 10X Buffer. If this occurs, warm the solution briefly at 37°C, then vortex until the precipitate is in solution.

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

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

Table 3. PCR Amplification Mix for Directly Amplifying DNA from Swabs.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
nuclease-free water	12.2µl	×		=	
Gold ST★R 10X Buffer	2.5µl	×		=	
PowerPlex® 16 10X Primer Pair Mix	2.5µl	×		=	
AmpliTaq Gold® DNA polymerase[2]	0.8µl (4U)	×		=	
5X AmpSolution™ Reagent	5.0µl	×		=	
swab extract	2.0µl				
total reaction volume	**25.0µl**				

[1]Add nuclease-free water to the tube first, then add Gold ST★R 10X Buffer, PowerPlex® 16 10X Primer Pair Mix, AmpliTaq Gold® DNA polymerase and 5X AmpSolution™ Reagent. The swab extract will be added at Step 6.

[2]Assumes the AmpliTaq Gold® DNA polymerase is at 5U/µl. If the enzyme concentration is different, the volume of enzyme must be adjusted accordingly.

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 2µl 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 to 2.5ng/µl. Add 2µl (5ng) 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 2µl of Water, Amplification Grade, or TE^{-4} buffer instead of swab extract into a reaction well containing PCR amplification mix.

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

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

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

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including the amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 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 reaction plate or tubes 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.

   Note: When using the GeneAmp® PCR System 9700, 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** to adjust the ramp rates at each hold temperature.

   The ramp speed is set after the thermal cycling run is started. The 'Select Method Options' screen appears. Select **9600** for the ramp speed, and enter the reaction volume.

   Thermal Cycling Protocol

   95°C for 11 minutes, then:

   96°C for 1 minute, 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

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

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

PCR Optimization

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

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

2. 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, ice-water bath or freezer plate block
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- 3500/3500xL capillary array, 36cm
- 96-well retainer & base set (standard) (Applied Biosystems Cat.# 4410228)
- POP-4® polymer for the Applied Biosystems® 3500 or 3500xL Genetic Analyzer
- anode buffer container
- cathode buffer container
- MicroAmp® optical 96-well plate and septa, or equivalent (Applied Biosystems)
- 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 (ILS) and formamide as follows:

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

2. Vortex for 10–15 seconds to mix.

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

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

    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, an ice-water bath or freezer plate block for 3 minutes. Denature samples just prior to loading the instrument.

Instrument Preparation

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

1. Open the 3500 Data Collection Software. The Dashboard screen will launch (Figure 1). Ensure that the Consumables Information and Maintenance Notifications are acceptable.

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

Figure 1. The Dashboard.

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

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

   When creating a new Instrument Protocol, be sure to select the same dye set that was used to perform the Promega 4-dye spectral calibration.

   ⚠ 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*.
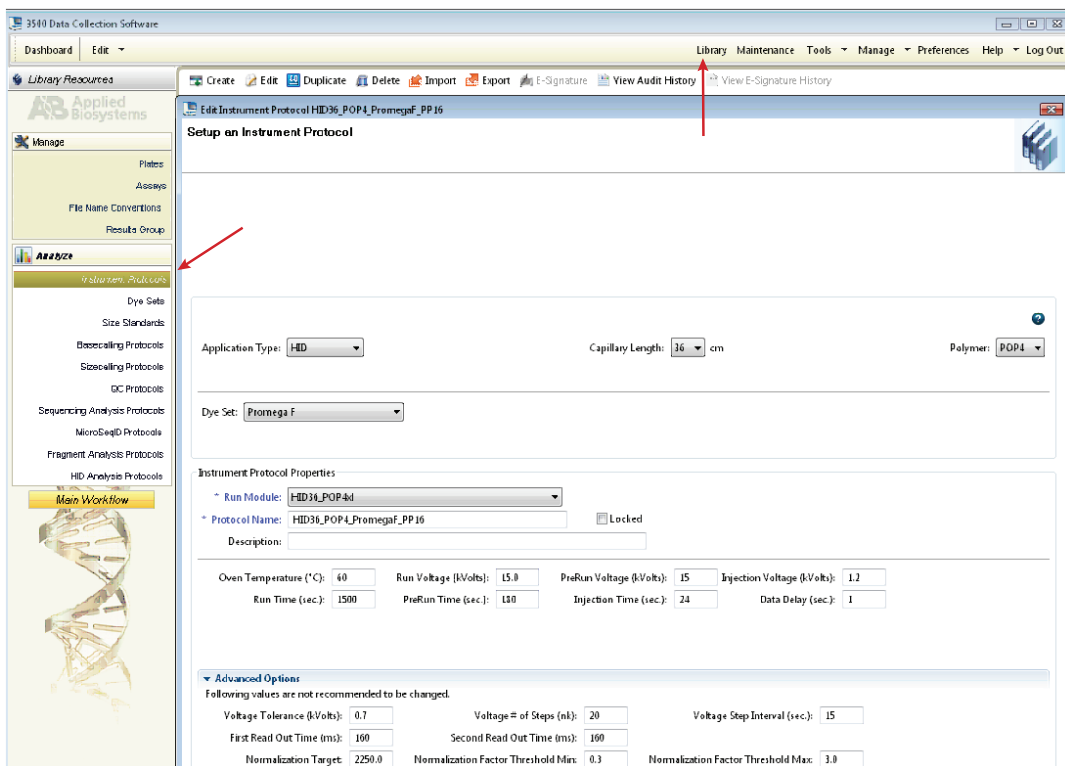

Figure 2. The Create New Instrument Protocol window.

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 3.

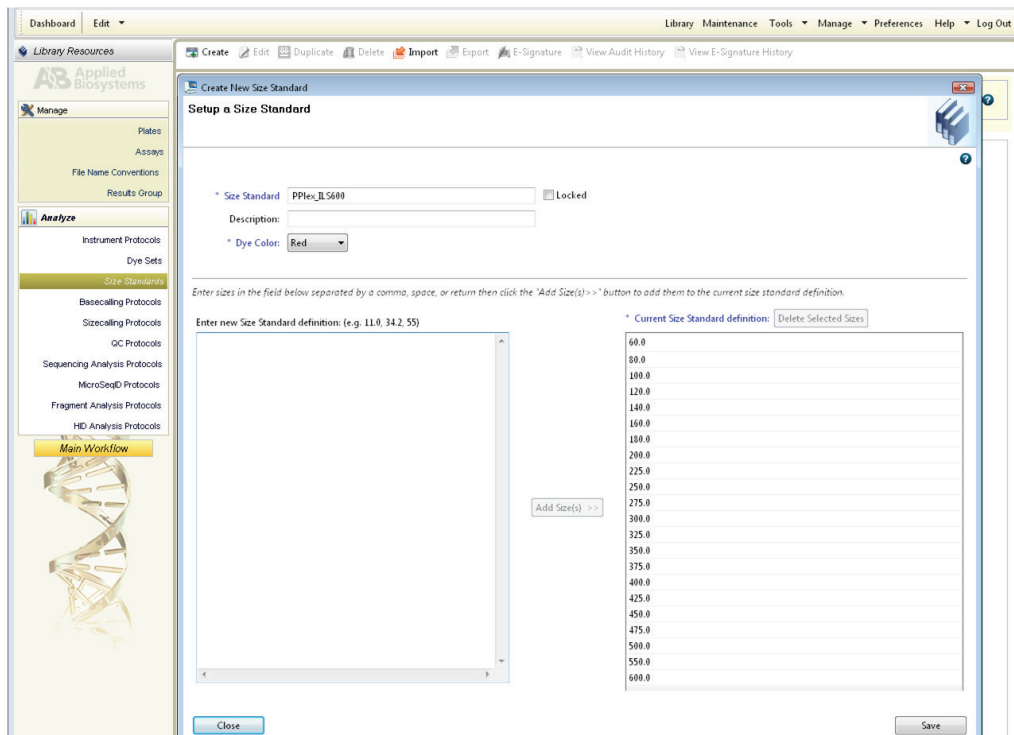**Note:** Definition and detection of the 600bp fragment is optional.

Figure 3. The 'Create New Size Standard' window.

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 System on the Applied Biosystems® 3500 or 3500xL Genetic Analyzer. Figure 4 shows one option for these settings.
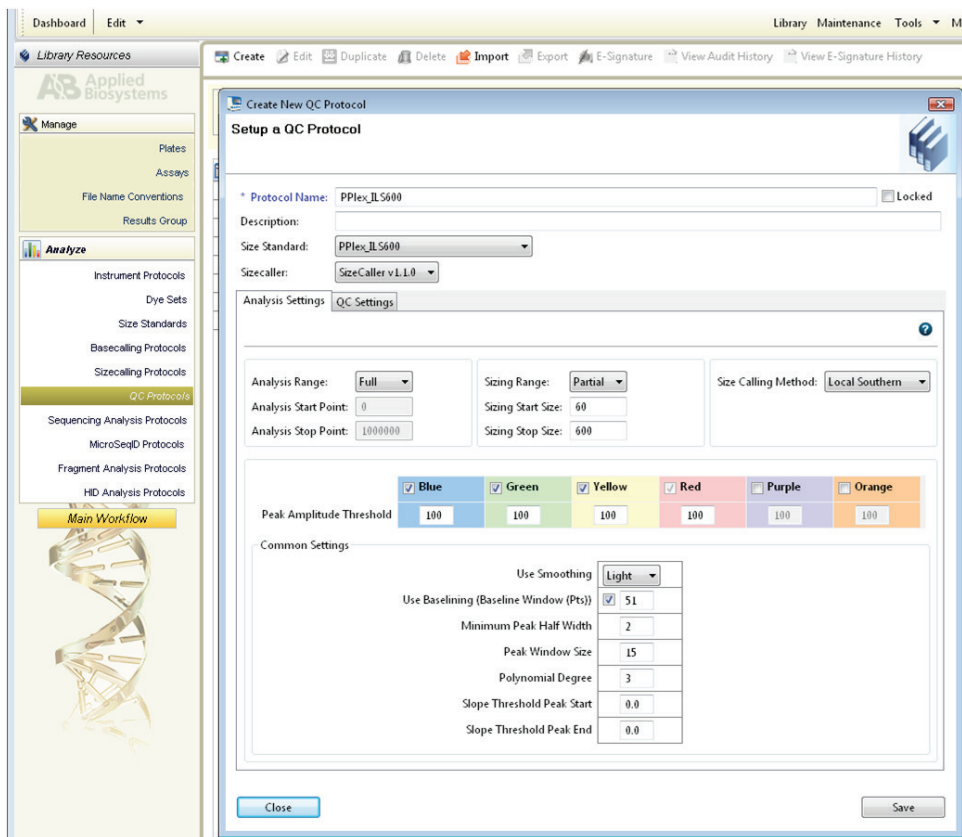

Figure 4. The 'Create New QC Protocol' window.

5. 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 5), select the application type **HID**. Choose the instrument protocol created in Step 2 and the QC protocol created in Step 4. Assign a descriptive assay name. An Assay is required for all named samples on a plate.
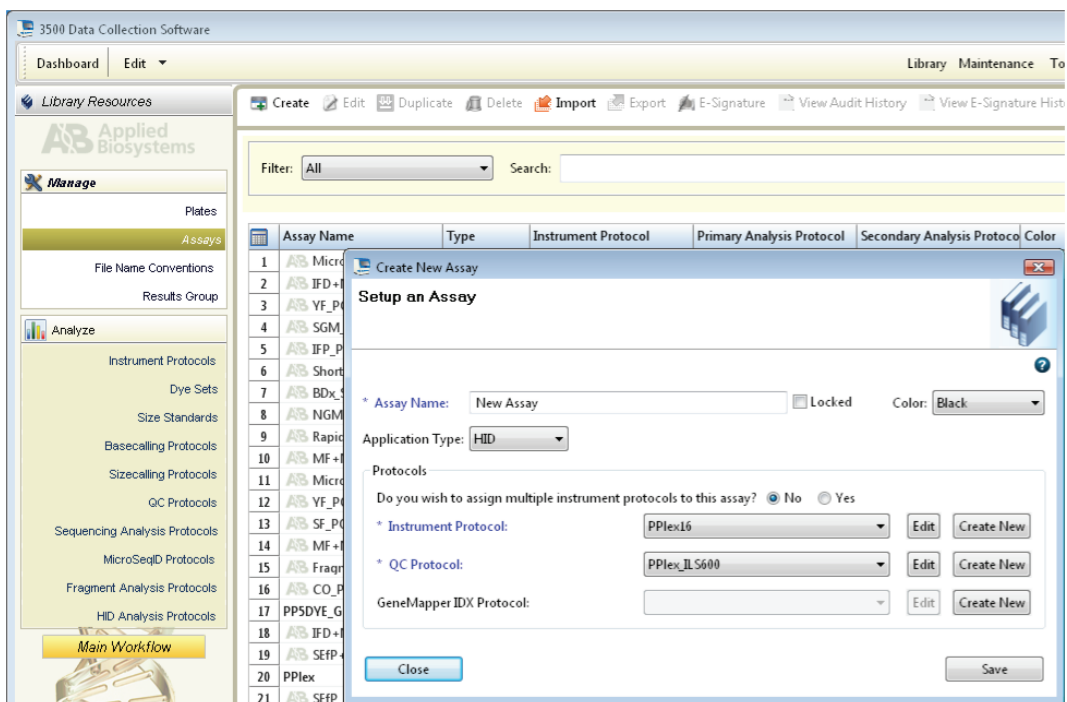

Figure 5. The 'Create New Assay' window.

6. To create a new File Name Convention (Figure 6), 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.
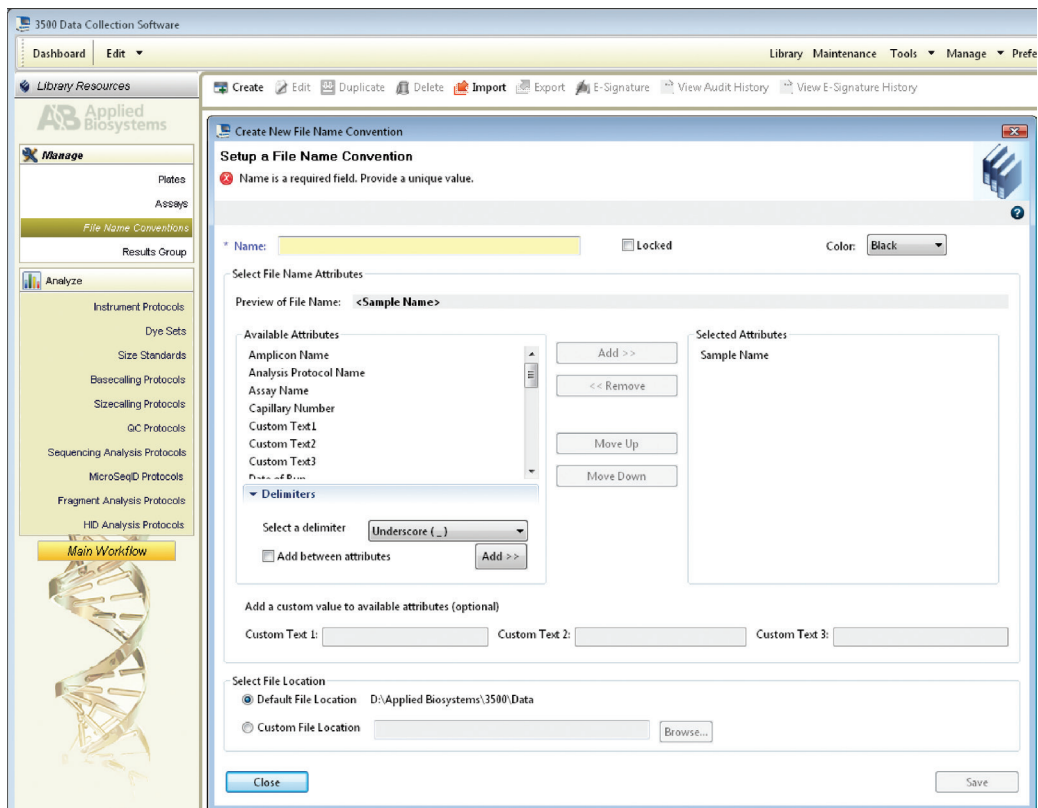

Figure 6. The 'Create New File Name Convention' window.

7. To create a new Results Group (Figure 7), 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**.

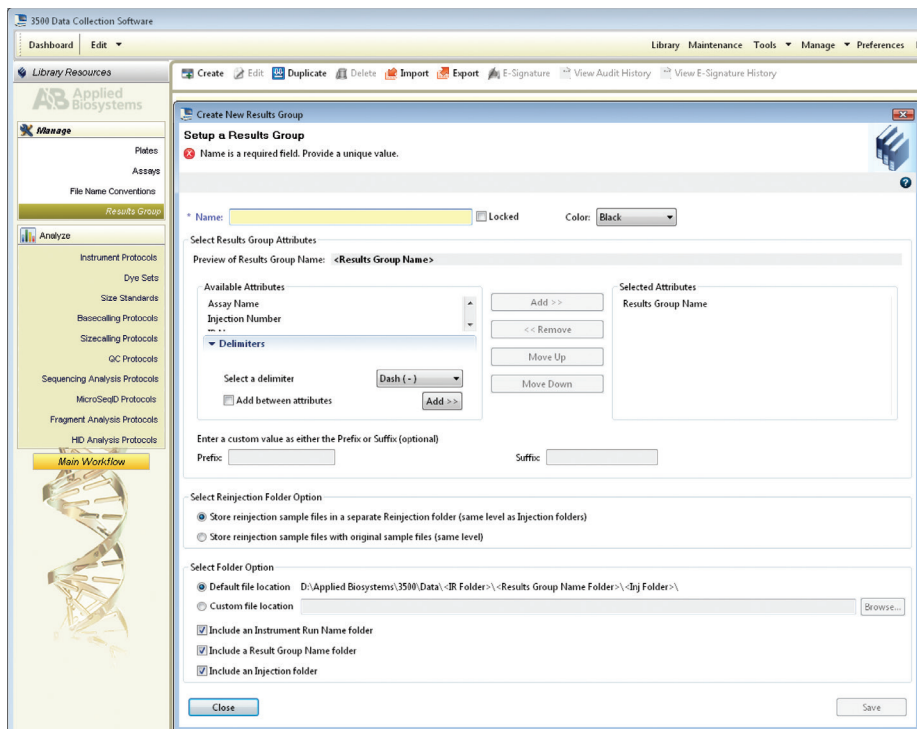9. Assign a descriptive plate name. Select the plate type "HID" from the drop-down menu (Figure 8).
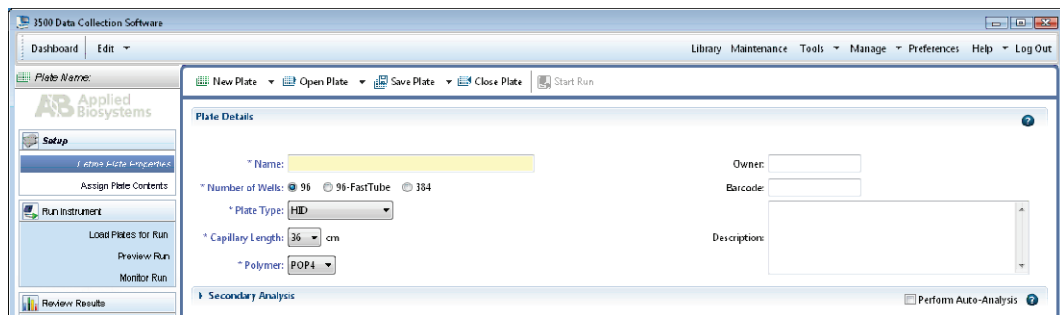
Figure 7. The 'Create New Results Group' window.

Figure 8. 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 9).

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. Select 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. Select 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. Select 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**.

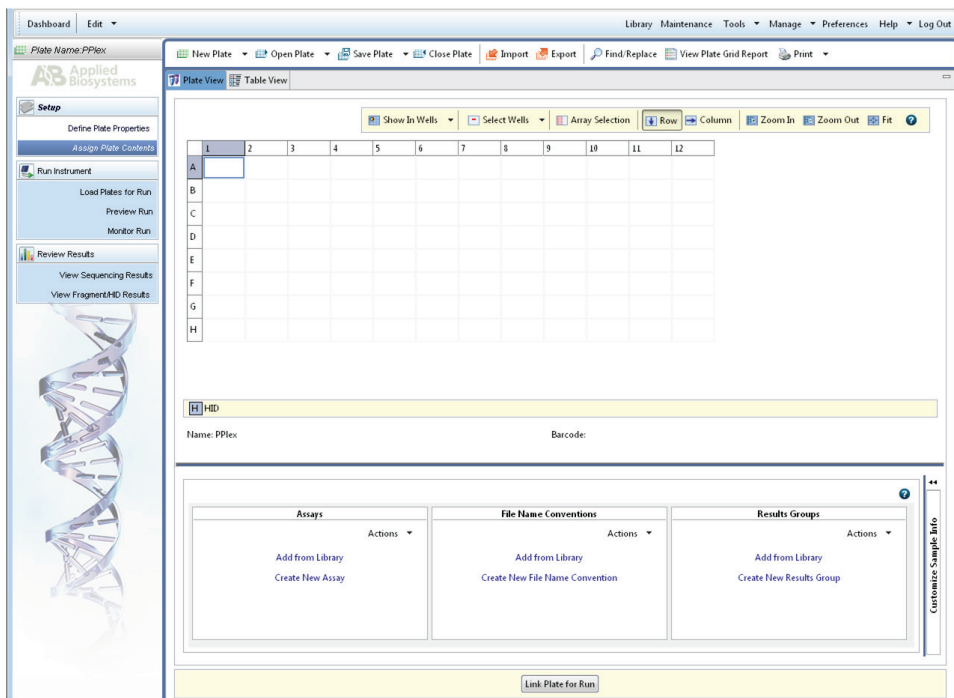17. The Load Plate window will appear. Select **Yes**.

Figure 9. Assigning plate contents.

18. In the Run Information window (Figure 10), assign a Run Name. Select **Start Run** (not shown).
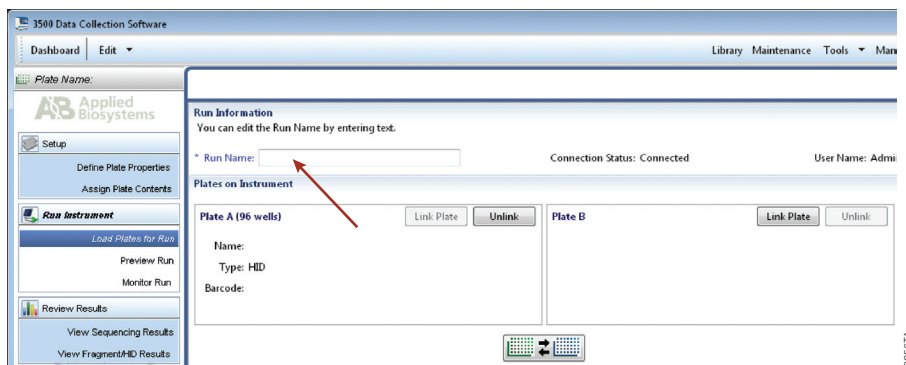

Figure 10. Assigning a run name.

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

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice, ice-water bath or freezer plate block
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- 3130 capillary array, 36cm
- POP-4® polymer for the Applied Biosystems® 3130/3130xl
- 10X genetic analyzer buffer with EDTA
- MicroAmp® optical 96-well plate (or equivalent) 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 Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with Data Collection Software, Version 3.0 or 4.0 (continued)

Sample Preparation

1. Thaw the Internal Lane Standard.

   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 (ILS) and formamide as follows:

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

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 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, in an ice-water bath or freezer plate block 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 Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with Data Collection Software 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 fields, and select **OK**.

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

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

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

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

6. In the spectral viewer, 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, select 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 changes 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.

6. Data Analysis

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

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.

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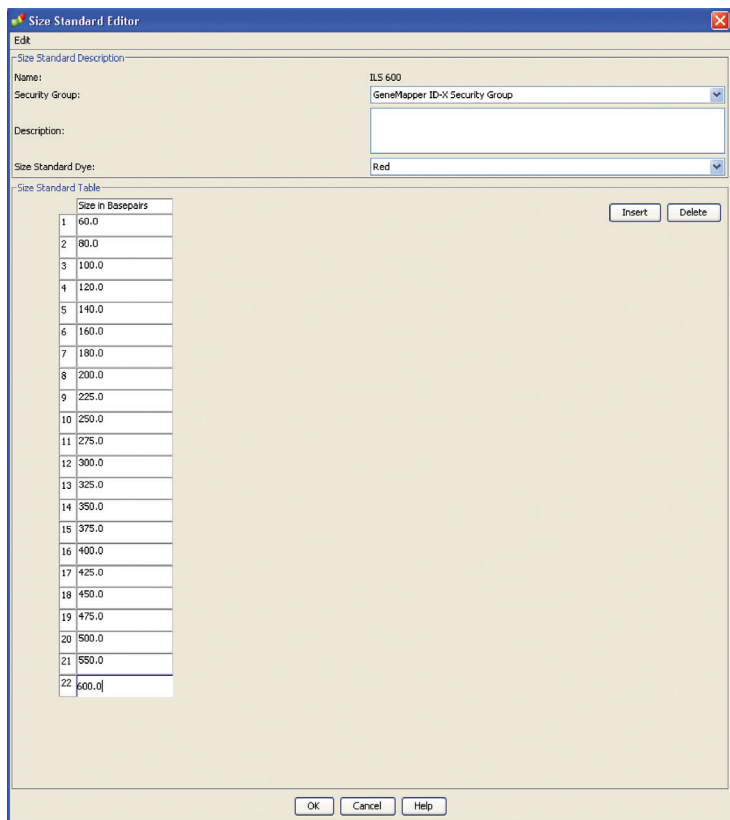
Getting Started

1. To obtain the proper panels, bins and stutter text files for the PowerPlex® 16 System, go to:
   www.promega.com/resources/software-firmware/str-analysis/genemapper-id-software-panels-and-bin-sets/

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

3. Save the PowerPlex_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**, and then **Panel Manager**.

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

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

5. Navigate to the panels text file downloaded in the Getting Started Section. 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**, and then **Import Bin Set**.

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

9. In the navigation pane, highlight the PowerPlex 16 panels folder that you just imported in Step 5.

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

11. Navigate to the stutter text file imported in the Getting Started section. Select the file, and 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

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

2. Select the 'Size Standard' tab.

3. Select **New**.

4. The 'Size Standard Editor' box will open (Figure 11). 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.C, Figure 23.

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

8. Select **OK**.

Figure 11. The GeneMapper® ID-X Size Standard Editor.

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

These instructions are intended as a guide to start analyzing data in GeneMapper® *ID*-X software. They are not intended as 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**, and then **GeneMapper ID-X Manager**.

2. Select the 'Analysis Methods' tab.

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

4. In the Analysis Method Editor, enter a descriptive name for the analysis method, such as "PowerPlex 16".

5. Choose a security group from the drop-down menu.

6. Select the 'Allele' tab (Figure 12).
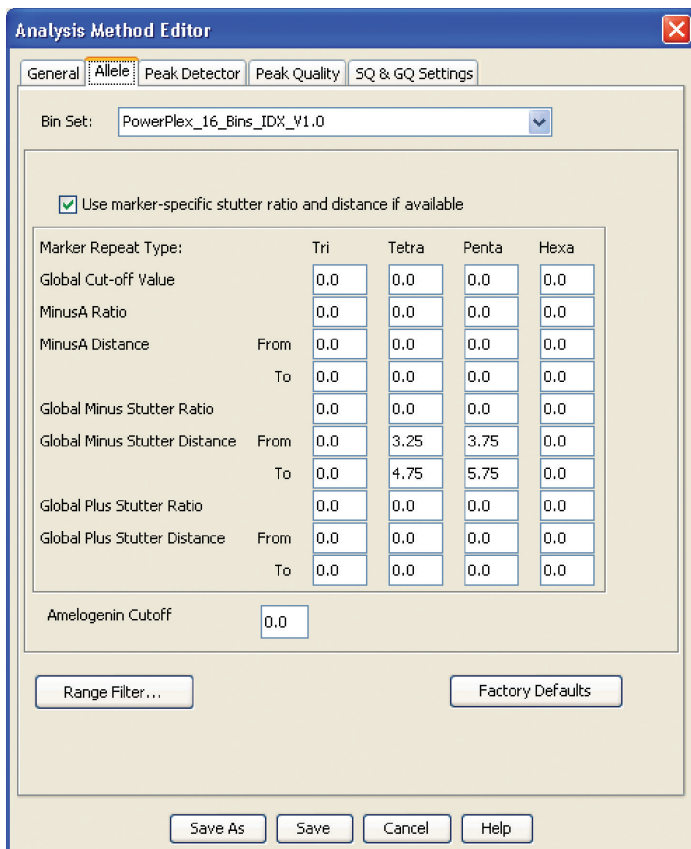
Figure 12. The GeneMapper® *ID*-X 'Allele' tab.

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

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

9. We recommend the values shown in Figure 12 for proper filtering of stutter peaks when using the PowerPlex® 16 System. You may need to optimize these settings. In-house validation should be performed.

10. Select the 'Peak Detector' tab. Figure 13 shows an example of settings used at Promega. You may need to optimize these settings. In-house validation should be performed.

   Notes:

   a. 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.

   b. 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.

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

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

Figure 13. The GeneMapper® *ID*-X 'Peak Detector' tab.

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

12. Select the 'SQ & GQ Settings' tab. You may change these settings.
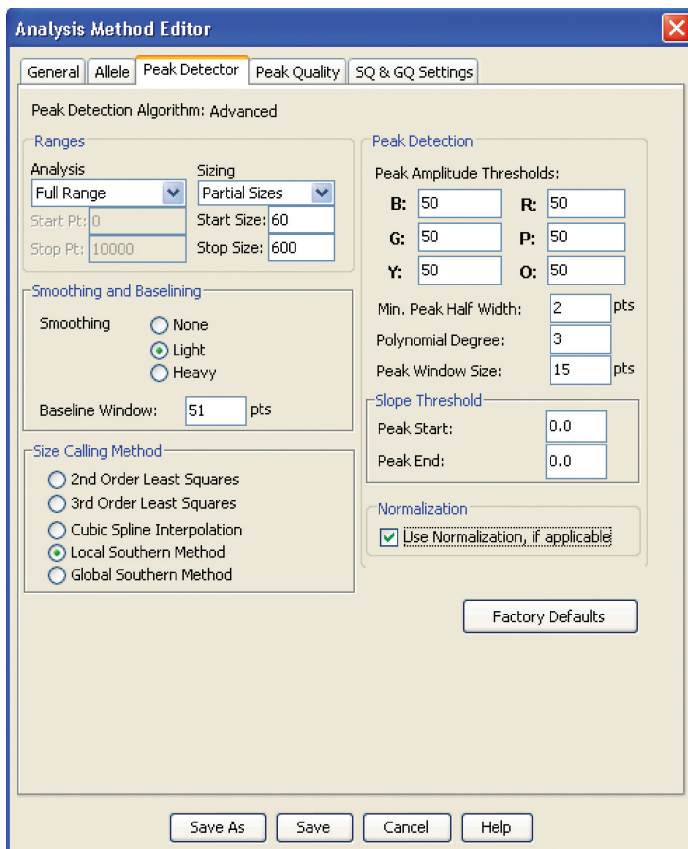
    Note: For Steps 11 and 12, see the GeneMapper® *ID*-X user's manual for more information.

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 previously in this section.

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

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

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

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

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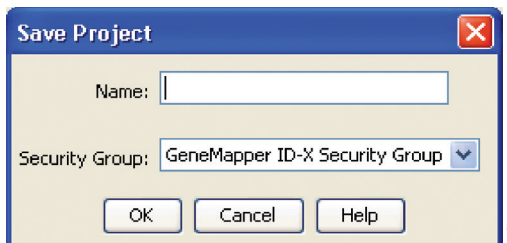
Figure 14. The 'Save Project' window.

10. Enter the project name.

11. Choose the applicable security group from the drop-down menu, 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' 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

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**, and then **GeneMapper ID-X Manager**.

2. Select the 'Analysis Methods' tab.

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

4. In the Analysis Method Editor, enter a descriptive name for the analysis method, such as "PowerPlex 16_20% Filter".

5. Choose a security group from the drop-down menu.

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

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. Doing this will assign locus-specific stutter filters and distances from the imported stutter file. Ensure that the appropriate global filter is applied to this analysis method. For example, for a 20% filter enter "0.20" for the Amelogenin Cutoff and Global Cutoff Value for Tri, Tetra and Penta repeats (Figure 15). You may need to optimize these settings. In-house validation should be performed.
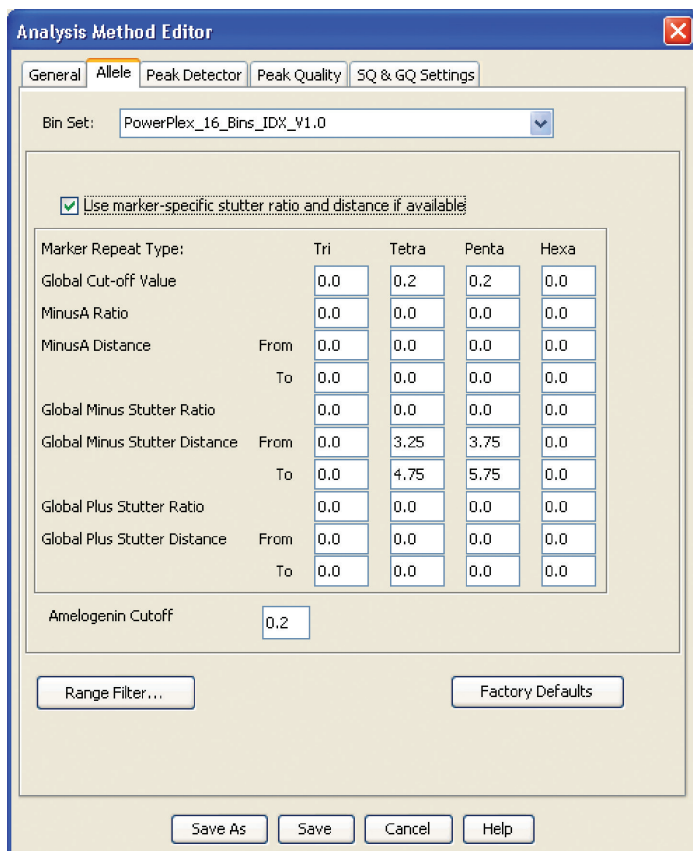
Figure 15. The 'Allele' tab with settings for using a 20% peak filter.

9. Select the 'Peak Detector' tab. Figure 13 shows an example of settings used at Promega. You may need to optimize these settings. In-house validation should be performed.

Notes:

a. 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.

b. 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.

c. The normalization box can be checked or unchecked.

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

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

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

   Note: For Steps 10 and 11, see the GeneMapper® *ID*-X user's manual for more information.

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 previously in this section.

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

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

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

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

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

10.   Enter the project name.

11.   Choose the applicable security group from the drop-down menu, 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' 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. Importing PowerPlex® Panels and Bins Text Files into GeneMapper® ID, Version 3.2

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

Getting Started

1. To obtain the panels and bins text files for the PowerPlex® 16 System go to:
    www.promega.com/resources/software-firmware/str-analysis/genemapper-id-software-panels-and-bin-sets/

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

3. Save the Promega_Panels_ID3.2.X.txt and Promega_Bins_ID3.2.X.txt files, where "X.x" refers to the most recent version of the panels and bins text files, to a known location on your computer.

Importing Panels and Bins Text Files

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

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

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

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

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

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

6. In the navigation pane, highlight the PowerPlex 16 panels folder that you just imported in Step 5.

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

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

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

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

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

2. Select the 'Size Standard' tab.

3. Select **New**.

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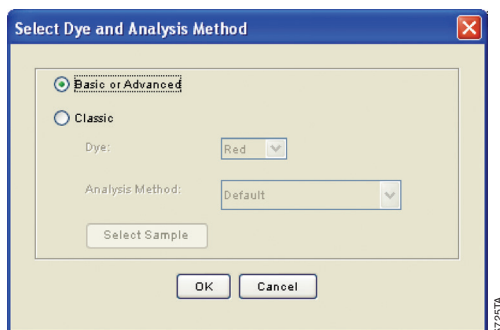
Figure 16. The 'Select Dye and Analysis Method' window.

5. Enter a detailed name, such as "ILS 600 Advanced", in the Size Standard Editor (Figure 17).
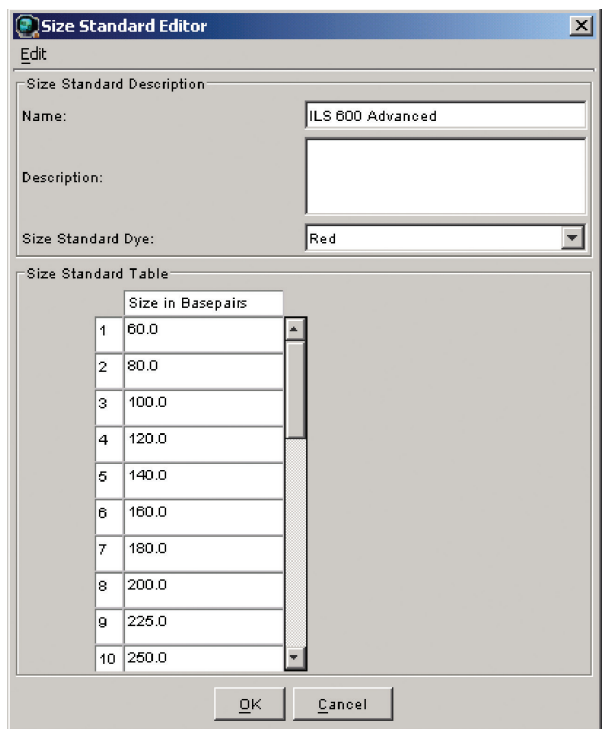
Figure 17. The Size Standard Editor

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.C, Figure 23.

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

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

2. Select the 'Analysis Methods' tab.

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

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

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

5. In the Analysis Method Editor, enter a descriptive name for the analysis method, such as "PowerPlex16 advanced".

6. Select the 'Allele' tab (Figure 18).

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 18 for proper filtering of stutter peaks when using the PowerPlex® 16 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.
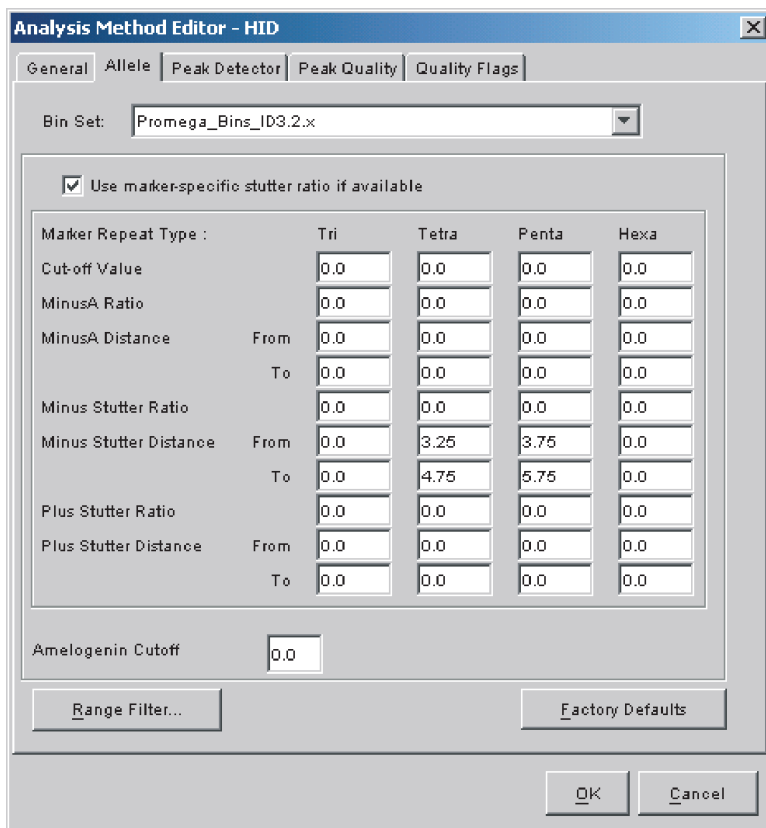
Figure 18. The GeneMapper® *ID* 'Allele' tab.

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

10. Select the 'Peak Detector' tab. We recommend the settings shown in Figure 19.

    Notes:

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

    b. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Values for peak amplitude thresholds should be determined by individual laboratories.
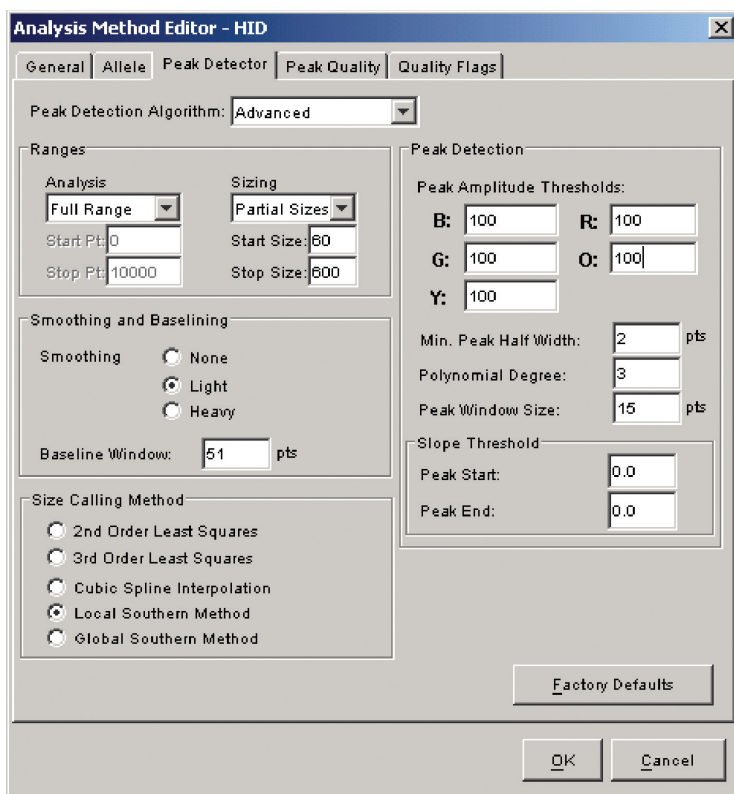
Figure 19. The GeneMapper® *ID* 'Peak Detector' tab.

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

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

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

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

Processing Data for Casework Samples

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

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

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

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

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

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

7. In the 'Size Standard' column, select the size standard that was created in Section 6.F.

8. 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

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

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

2. Select the 'Analysis Methods' tab.

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

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

5. In the Analysis Method Editor, enter a descriptive name for the analysis method, such as "PowerPlex16_20%filter".

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.

9. Enter the values shown in Figure 20 for proper filtering of peaks when using the PowerPlex® 16 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*".
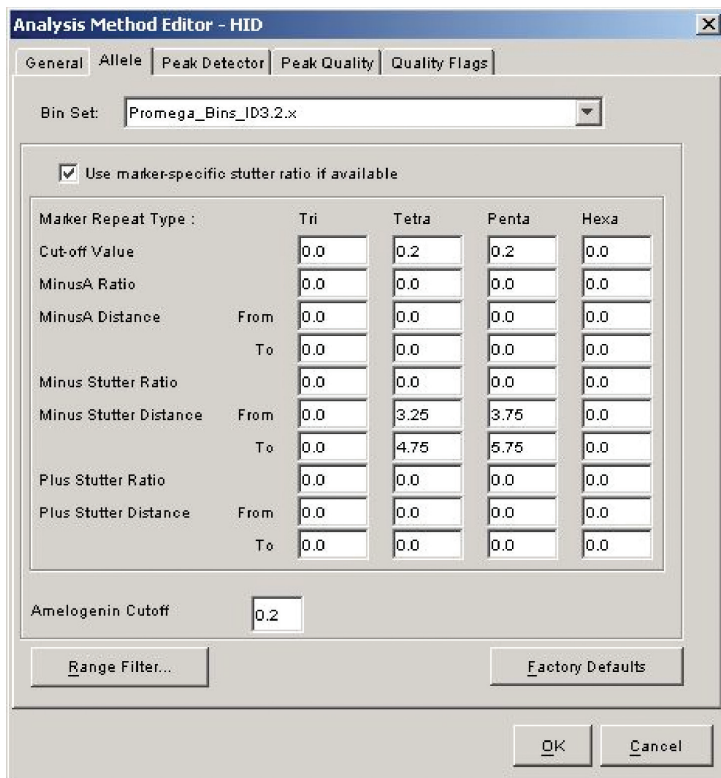
Figure 20. The GeneMapper® ID 'Allele' tab with settings for using a 20% peak filter.

10. Select the 'Peak Detector' tab. We recommend the settings shown in Figure 19.

    Notes:

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

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

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

12. Select the 'Quality Flags' tab to change these settings.

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

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

Processing Data for Databasing or Paternity Samples

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

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

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

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

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

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

7. In the 'Size Standard' column, select the size standard that was created in Section 6.F.

8. 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 System are shown in Figure 21. The PowerPlex® 16 Allelic Ladder Mix is shown in Figure 22.
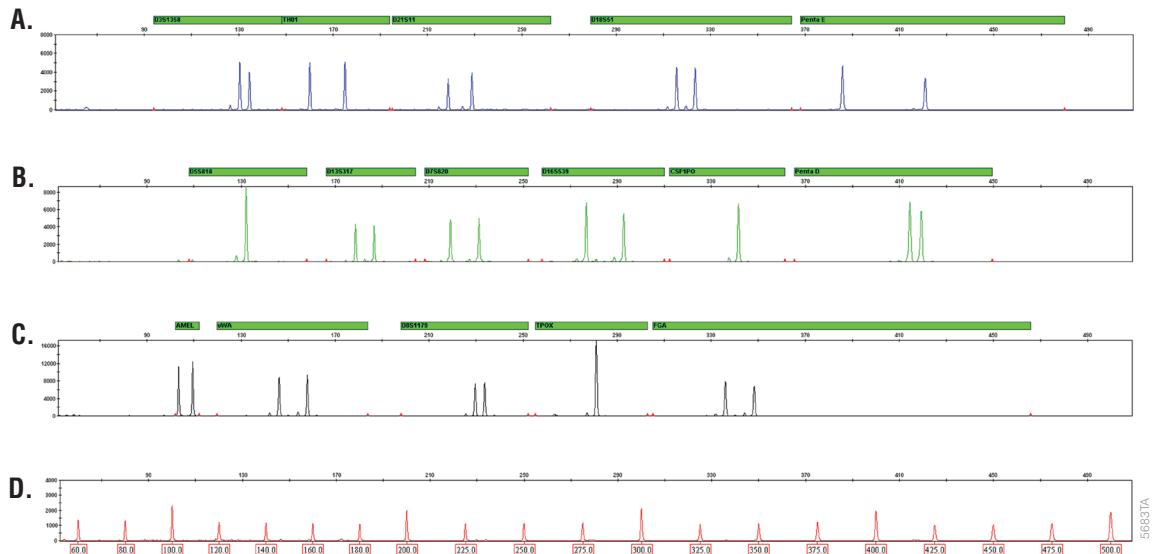
Figure 21. The PowerPlex® 16 System. A single-source template DNA (0.5ng) was amplified using the PowerPlex® 16 System. Amplification products were mixed with Internal Lane Standard 600 and analyzed with an Applied Biosystems 3500 Genetic Analyzer using a 1.2kV, 12-second injection. Results were analyzed using GeneMapper® *ID*-X software, version 1.4. **Panel A.** An electropherogram showing the peaks of the fluorescein-labeled loci: 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.
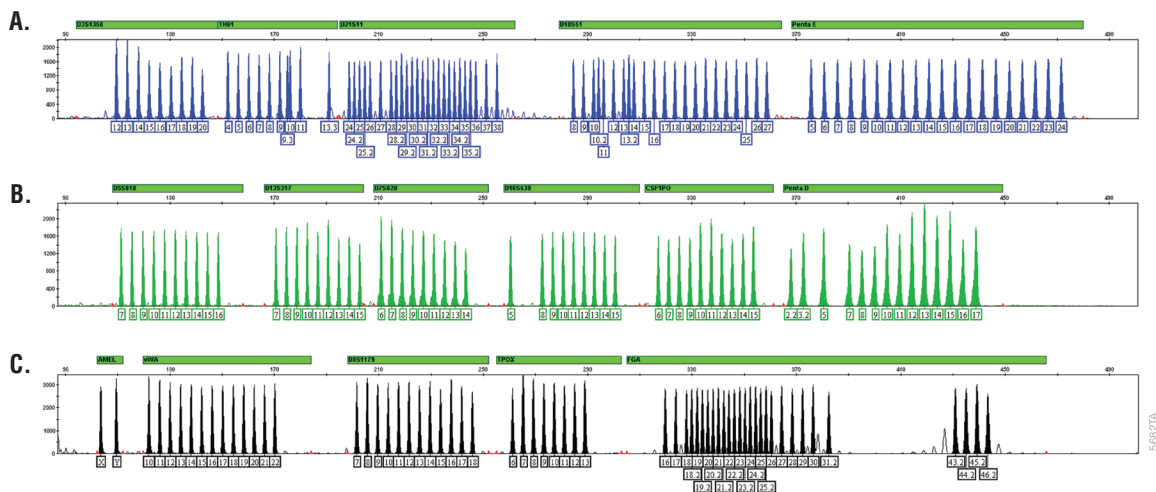
Figure 22. The PowerPlex® 16 Allelic Ladder Mix. The PowerPlex® 16 Allelic Ladder Mix was analyzed with an Applied Biosystems 3500 Genetic Analyzer using a 1.2kV, 12-second injection. Results were analyzed using GeneMapper® *ID*-X software, version 1.4, and PowerPlex® 16 panels and bins text files. **Panel 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 (15,16). 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. The level of stutter was determined and published as part of the PowerPlex® 16 System validation (13).

In addition to stutter peaks, other artifact peaks can be observed at some of the PowerPlex® 16 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.

In addition to these peaks, you may observe the following DNA-dependent (Table 2) and DNA-independent (with or without human genomic DNA) artifact peaks (Table 3).

6.J. Results (continued)

Table 2. DNA-Dependent Artifacts Observed in Amplification Reactions with Human Genomic DNA.

Dye Label or Locus	Artifact Size
Fluorescein	One or more peaks in D21S11[1] One or more peaks in Penta E[1] Low-level artifacts between D3S1358 and TH01[2]
JOE	~147–169 bases[2] ~192–213 bases[2] Low-level artifacts between D13S317 and D7S820[2] Off ladder at ~270–271 bases[2] One or more peaks in Penta D[1]
TMR	~96–98 bases[2] ~184–198 bases[2] ~252–255 bases[2]
TH01	n−3[3]
Penta E	n−3[3]
D13S317	n−3[3]
TPOX	n−8 to n−26[3]
vWA	n−6 to n−21[3]

[1]Peaks not directly related to amplification. 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.

[2]Low-level artifact has been observed in amplifications with large amounts of template DNA.

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

Table 3. DNA-Independent Artifacts Observed in Amplification Reactions with and without Human Genomic DNA.

Dye Label	Artifact Size[1]
Fluorescein	~64–69 bases
JOE	~114–120 bases
TMR	~81–83 bases ~142–144 bases ~400–405 bases

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

7. Troubleshooting

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

7.A. Amplification and Fragment Detection

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

Symptoms	Causes and Comments
Faint or absent allele peaks	The PCR amplification mix was not vortexed well before use. Vortex the PCR amplification mix for 5–10 seconds before dispensing into the reaction tubes or plate.
	Primer concentration was too low. Use the recommended primer concentration. Vortex the Primer Pair Mix for 15 seconds before use.
	An air bubble formed at the bottom of the reaction tube. Use a pipette to remove the air bubble or centrifuge the reactions briefly before thermal cycling.
	Thermal cycler, plate or tube problems. Review the thermal cycling protocols. We have not tested reaction tubes, plates or thermal cyclers that are not listed. Calibrate the thermal cycler heating block if necessary.
	Repeat sample preparation using fresh formamide. Long-term storage of amplified sample in formamide can result in loss of signal.
	Poor capillary electrophoresis injection (ILS peaks also affected). Re-inject the sample.
	Laser is starting to fail. Check laser power.
	Poor-quality formamide was used. Use only Hi-Di™ formamide when analyzing samples.
Faint or absent peaks for the positive control reaction	Improper storage of the 2800M Control DNA. Store the 2800M Control DNA at +2°C to +10°C. Make sure that the 2800M Control DNA is stored at +2°C to +10°C for at least 24 hours before use; do not refreeze. Vortex before use.

7.A. Amplification and Fragment Detection (continued)

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels	Contamination with another template DNA or previously amplified DNA. Cross-contamination can be a problem. Use aerosol-resistant pipette tips and change gloves regularly.
	Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the DNA as soon as possible to each well and follow immediately by thermal cycling.
	Samples were not denatured completely. Heat-denature samples for the recommended time, and cool on crushed ice, a freezer plate block or in an ice-water bath immediately prior to capillary electrophoresis. Do not cool the samples in a thermal cycler set at 4°C, as this may lead to artifacts due to DNA re-annealing.
	Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis. Appearance of "shadow" peaks migrating in front of the main peaks, especially if the shadow peaks are separated by the same distance as the main peaks in a heterozygote, can indicate the presence of double-stranded DNA due to incomplete denaturation or post-injection re-annealing.
	CE-related artifacts ("spikes"). Minor voltage changes or urea crystals passing by the laser can cause "spikes" or unexpected peaks. Spikes sometimes appear in one color but often are easily identified by their presence in more than one color. Re-inject samples to confirm.
	CE-related artifacts (contaminants). Contaminants in the water used with the instrument or to dilute the 10X genetic analyzer buffer may generate peaks in the fluorescein and JOE channels. Use autoclaved deionized water; change vials and wash buffer reservoir.
	Incorrect spectral was active when analyzing samples with the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer. Re-run samples, and confirm that the PowerPlex 4C spectral calibration is set for dye set F. See instructions for instrument preparation in Section 5.B.

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

7.A. Amplification and Fragment Detection (continued)

Symptoms	Causes and Comments
Peak height imbalance	Miscellaneous balance problems. At the first use, thaw the Primer Pair Mix and Buffer completely. Vortex the Primer Pair Mix and Buffer for 15 seconds before use; do not centrifuge the Primer Pair Mix or Buffer after mixing. Calibrate thermal cyclers and pipettes routinely.
	PCR amplification mix was not mixed well. Vortex the PCR amplification mix for 5–10 seconds before dispensing into the reaction tubes or plate.

7.B. Amplification of Extracted DNA

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

Symptoms	Causes and Comments
Faint or absent allele peaks	Impure template DNA. Depending on the DNA purification procedure used and sample source, inhibitors might be present in the DNA sample. As DNA sample volume increases relative to the total amplification reaction volume, this may be more likely.
	Insufficient template. Use the recommended amount of template DNA if available. Quantify template DNA before use if possible.
	High salt concentration or altered pH. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the DNA volume should not exceed 20% of the total reaction volume. Carryover of K^+, Na^+, Mg^{2+} or EDTA from the DNA sample can negatively affect PCR. A change in pH may also affect PCR. Store DNA in TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE^{-4} buffer with 20µg/ml glycogen.
	The reaction volume was too low. This system is optimized for a final reaction volume of 25µl for extracted DNA. Decreasing the reaction volume may result in suboptimal performance.
Extra peaks visible in one or all color channels	Amplification of more than the recommended amount of purified DNA can result in a higher number of artifact peaks due to overamplification, resulting in saturating signal. Use the recommended amount of template DNA. See Section 6.J, Results, for additional information about stutter and artifacts. The amount of template will need to be optimized if you are using reduced reaction volumes.

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

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

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

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

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

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels (continued)	Artifacts of STR amplification. Direct amplification of high amounts of template can result in a higher number of artifact peaks. Use the recommended punch size and number of punches. Optimize the cycle number. See Section 6.J, Results, for additional information on stutter and artifacts.
	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´A residue. • Be sure to perform the recommended extension step at 60°C after thermal cycling. • Decrease cycle number. • Increase the final extension time.
Peak height imbalance	Excessive amount of DNA. Amplification of high amounts of template can result in an imbalance, with smaller loci showing more product than larger loci. • Be sure to use the recommended number of punches. Follow the manufacturer's recommendation when depositing sample onto the card. • Decrease cycle number.
	The cycle number was too high. Decrease the cycle number by one cycle and repeat the amplification.
	For direct amplification reactions that require the addition of AmpSolution™ Reagent, its omission can result in inhibition of the amplification reaction. Review the amplification setup and confirm whether or not AmpSolution™ Reagent is required for your direct amplification reactions.
	The reaction volume was too low. Decreasing the reaction volume can result in suboptimal performance due to inhibitors present in lytic cards and PunchSolution™ Reagent.
	DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat nonlytic materials with PunchSolution™ Reagent to ensure that DNA is liberated from cellular proteins.

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

7.D. Direct Amplification of DNA from Swabs

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

Symptoms	Causes and Comments
Faint or absent allele peaks	Poor sample deposition. Shedding and collection of donor cells was variable. Increase cycle number.
	Inactive SwabSolution™ Reagent. Thaw the SwabSolution™ Reagent completely in a 37°C water bath and mix by gentle inversion. Store the SwabSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.

7.D. Direct Amplification of DNA from Swabs (continued)

Symptoms	Causes and Comments
Faint or absent allele peaks (continued)	Active SwabSolution™ Reagent carried over into the amplification reaction. Ensure that the heat block reached 70°C (90°C if using a 2.2ml, Square-Well Deep Well Plate) and samples were incubated for the full 30 minutes. Incubation for shorter time periods may result in incomplete reagent inactivation. Do not use an incubator to incubate tubes or plates; heat transfer is inefficient and will result in poor performance. Use only a heat block to maintain efficient heat transfer. We have tested 60-minute incubation times and observed no difference in performance compared to a 30-minute incubation.
	For direct amplification reactions that require the addition of AmpSolution™ Reagent, its omission can result in inhibition of the amplification reaction. Review the amplification setup and confirm whether or not AmpSolution™ Reagent is required for your direct amplification reactions.
	DNA was not accessible on nonlytic material. Pretreat swabs with SwabSolution™ Reagent to ensure that DNA is liberated from cellular proteins.
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. Use the recommended amount of 2800M Control DNA per amplification reaction. This mass of DNA should be reduced if the cycle number is increased and increased if the cycle number is decreased. Increase or decrease by twofold the mass of 2800M Control DNA for every one-cycle decrease or increase, respectively.
Extra peaks visible in one or all color channels	Swab extract was contaminated. Assemble a reaction containing the swab extract prepared from a blank swab, or assemble a reaction where the SwabSolution™ Reagent is processed and incubated as a blank without a swab.
	Artifacts of STR amplification. Amplification of swab extracts with high DNA concentrations can result in artifact peaks due to overamplification, resulting in saturated signal on the CE instrument. We recommend 2µl of swab extract per reaction. Using more than 2µl may result in overamplification and signal saturation. If signal is saturated, repeat amplification with less swab extract or reduced cycle number.

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels (continued)	Amplification of excess template for a given cycle number resulted in overloading of the capillary upon electrokinetic injection. Excess DNA in the capillary is difficult to maintain in a denatured single-stranded state. Some single-stranded DNA renatures and becomes double-stranded. Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis and appears as "shadow" peaks migrating in front of the main peaks. 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.
	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´A residue.
	• Be sure to perform the recommended extension step at 60°C after thermal cycling.
	• Use 2µl of swab extract in an amplification reaction. A larger volume of swab extract may contain more than the recommended amount of DNA template, resulting in incomplete adenylation.
	• Decrease cycle number.
	• Increase the final extension time.
Peak height imbalance	Excess DNA in the amplification reaction can result in locus-to-locus imbalance within a dye channel such that the peak heights at the smaller loci are greater than those at the larger loci (ski-slope effect). Use less swab extract or reduce the cycle number.
	Active SwabSolution™ Reagent carried over from swab extracts into the amplification reaction. Larger loci are most susceptible to reagent carryover and will drop out before the smaller loci. Ensure that the heat block reached 70°C (90°C if using 2.2ml, Square-Well Deep Well Plates) and samples were incubated for the full 30 minutes. Incubation for shorter time periods may result in incomplete reagent inactivation. Do not use an incubator to incubate tubes or plates; heat transfer is inefficient and will result in poor performance. Use only a heat block to maintain efficient heat transfer.

7.D. Direct Amplification of DNA from Swabs (continued)

Symptoms	Causes and Comments
Peak height imbalance (continued)	Inactive SwabSolution™ Reagent. Thaw the SwabSolution™ Reagent completely in a 37°C water bath and mix by gentle inversion. Store the SwabSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
	DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat swabs with SwabSolution™ Reagent to ensure that DNA is liberated from cellular proteins.
Extreme variability in sample-to-sample peak heights	There can be significant individual-to-individual variability in cell deposition onto buccal swabs. This will appear as variability in peak heights between swab extracts. The extraction process maximizes recovery of amplifiable DNA from buccal swabs but does not normalize the amount of DNA present. If variability is extreme, quantify the DNA using a fluorescence-based double-stranded DNA quantification method or qPCR-based quantification method. The quantification values can be used to normalize input template amounts to minimize variation in signal intensity.

7.E. GeneMapper® *ID*-X Software

Symptoms	Causes and Comments
Stutter peaks not filtered	Stutter text file was not imported into the Panel Manager when the panels and bins text files were imported.
	Be sure that the "Use marker-specific stutter ratio and distance if available" box is checked. If the "Use marker-specific stutter ratio and distance if available" box is not checked, stutter distance must be defined in the Analysis Method 'Allele' tab.
Samples in the project not analyzed	The 'Analysis Requirements Summary' window was not active, and there was an analysis requirement that was not met. Turn on Analysis Requirements Summary in the Project Options menu under File, and correct the necessary analysis requirements to continue analysis.
Edits in label edit viewer cannot be viewed	To view edits made to a project, the project first must be saved. Close the plot view window, return to the main GeneMapper® *ID*-X page and save the project. Display the plot window again, then view the label edit table.

Symptoms	Causes and Comments
Marker header bar for some loci are gray	When an edit is made to a locus, the quality flags and marker header bar automatically change to gray. To change the GQ and marker header bar for a locus to green, override the GQ in the plot window.
Alleles not called	To analyze samples with GeneMapper® *ID*-X software, at least one allelic ladder must be defined per folder of sample files being analyzed in the project.
	Run was too short and larger peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Rerun samples using a longer run time.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.
Off-ladder alleles	An allelic ladder from a different run than the samples was used. Re-analyze samples with an allelic ladder from the same run.
	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 reanalyze, as described in the "Processing Data for Casework Samples" section or "Processing Data for Databasing or Paternity Samples".
	Panels text file selected for analysis was incorrect for the STR system used. Assign correct panels text file that corresponds to the STR system used for amplification.
	The allelic ladder was not identified as an allelic ladder in the 'Sample Type' column.
	The internal lane standard was not properly identified in the sample. Manually redefine the sizes of the size standard fragments in the sample.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.
	Incorrect polymer was used. Use of a polymer other than POP-4® may change migration of the fragments. Alleles may migrate outside of the panel range established using POP-4®.

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

Symptoms	Causes and Comments
Size standard not called correctly	Starting data point was incorrect for the partial range chosen for the analysis in the GeneMapper® *ID*-X 'Peak Detector' tab as described in sections "Creating a Casework Analysis Method" or "Creating a Databasing or Paternity Analysis Method." Adjust the starting data point in the analysis method or use a full range for the analysis.
	Extra peaks in size standard. Open the Size Match Editor. Highlight the extra peak, select **Edit** and select **Delete Size Labe**l. Select **Auto Adjust Sizes**.
	An incorrect internal lane standard was used.
	Run was too short, and larger peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Re-run samples using a longer run time.
Peaks in size standard missing.	If peaks are low-quality, redefine the size standard for the sample to skip these peaks.
	An incorrect internal lane standard was used.
Significantly raised baseline	Poor spectral calibration. Perform a new spectral calibration and re-run the samples.
	Incorrect spectral calibration was active. Confirm that the correct spectral was used to run the samples.

7.F. GeneMapper® *ID* Software

Symptoms	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.
	To analyze samples with GeneMapper® *ID* software, at least one allelic ladder must be defined per folder of sample files being analyzed in the project.

Symptoms	Causes and Comments
Alleles not called (continued)	Run was too short and larger peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run.
	• Create a new size standard using the internal lane standard fragments present in the sample.
	• Rerun samples using a longer run time.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.
Off-ladder alleles	An allelic ladder from a different run than the samples was used. Re-analyze samples using 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.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis
Size standard not called correctly	Starting data point was incorrect for the partial range chosen in Section 6.G or 6.H. 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**.

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

Symptoms	Causes and Comments
Size standard not called correctly (continued)	Peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Rerun samples using a longer run time.
Peaks in size standard missing	If peaks are low-quality, redefine the size standard for the sample to skip these peaks.
	An incorrect size standard was used.
Error message: "Either panel, size standard, or analysis method is invalid"	The size standard and analysis method were not in the same mode ('Classic' vs. 'Basic or Advanced'). Be sure both files are set to the same mode, either 'Classic' or 'Basic or Advanced' mode.
No alleles called, but no error message appears	Panels text file was not selected for sample. In the Panel column, select the appropriate panels text file for the STR system that was used.
	No size standard was selected. In the Size Standard column, be sure to select the appropriate size standard.
	Size standard was not correctly defined, or size peaks were missing. Redefine size standard to include only peaks present in your sample. Terminating analysis early or using short run times will cause larger ladder peaks to be missing. This will cause your sizing quality to be flagged as red, and no allele sizes will be called.
Error message: "Both the Bin Set used in the Analysis Method and the Panel must belong to the same Chemistry Kit"	The bins text file assigned to the analysis method was deleted. In the GeneMapper Manager, select the 'Analysis Methods' tab, and open the analysis method of interest. Select the 'Allele' tab, and select an appropriate bins text file.
	The wrong bins text file was chosen in the analysis method 'Allele' tab. Be sure to choose the appropriate bins text file, as shown in Figure 20.
Significantly raised baseline	Poor spectral calibration. Perform a new spectral calibration and rerun the samples.

Symptoms	Causes and Comments
Significantly raised baseline (continued)	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 when analyzing samples with the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer. Rerun samples, and confirm that the Promega 4C spectral calibration is set for dye set F. See instructions for instrument preparation in Section 5.B.
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.

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9. Appendix

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

The loci included in the PowerPlex® 16 System (Tables 4 and 5) 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 System amplifies all of the original CODIS core loci in a single reaction.

The PowerPlex® 16 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 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 System to allow gender identification of each sample. Table 6 lists the PowerPlex® 16 System allele determinations for the 2800M Control DNA.

We have carefully selected STR loci and primers to avoid or minimize artifacts, including those associated with DNA polymerases, such as repeat slippage (15,16) and terminal nucleotide addition (17,18). Repeat slippage, sometimes called "n−4 peaks", "stutter" or "shadow bands", is due to the loss of a repeat unit during DNA amplification, somatic variation within the DNA, or both. The amount of this artifact observed depends primarily on the locus and the DNA sequence being amplified.

Terminal nucleotide addition (17,18) occurs when a thermostable nonproofreading DNA polymerase adds a nucleotide, generally adenine, to the 3′ ends of amplified DNA fragments in a template-independent manner. The efficiency with which this occurs varies with different primer sequences. Thus, an artifact 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 6).

Table 4. The PowerPlex® 16 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	4q28	HUMFIBRA, human fibrinogen alpha chain gene	TTTC Complex (22)
TPOX	TMR	2p24–2pter	HUMTPOX, human thyroid peroxidase gene	AATG
D8S1179	TMR	8q24.13	NA	TCTA Complex (22)
vWA	TMR	12p13.31	HUMVWFA31, human von Willebrand factor gene	TCTA Complex (22)
Amelogenin[2]	TMR	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 5. The PowerPlex® 16 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: **https://strbase.nist.gov/var_tab.htm**

[4]Amelogenin is not an STR.

Table 6. The PowerPlex® 16 System Allele Determinations for the 2800M Control DNA.

STR Locus	2800M
Penta E	7, 14
D18S51	16, 18
D21S11	29, 31.2
TH01	6, 9.3
D3S1358	17, 18
FGA	20, 23
TPOX	11, 11
D8S1179	14, 15
vWA	16, 19
Amelogenin	X, Y
Penta D	12, 13
CSF1PO	12, 12
D16S539	9, 13
D7S820	8, 11
D13S317	9, 11
D5S818	12, 12

9.B.   Power of Discrimination

The fifteen STR loci amplified with the PowerPlex® 16 System provide powerful discrimination. Population statistics for these loci and their various multiplex combinations are displayed in Table 7. 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: **https://strbase.nist.gov/var_tab.htm**

Table 5 shows the matching probability (32) for the PowerPlex® 16 System in various populations. The matching probability 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 System are shown in Table 7. The PowerPlex® 16 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 System, exceeds 0.999998 in all populations tested.

Table 7. 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. 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 23). Each fragment is labeled with carboxy-X-rhodamine (CXR) and can be detected separately (as a fourth color) in the presence of PowerPlex® 16-amplified material. The ILS 600 is designed for use in each CE injection to increase precision in analyses when using the Power-Plex® 16 System. The protocol to prepare and use this internal lane standard is provided in Section 5.
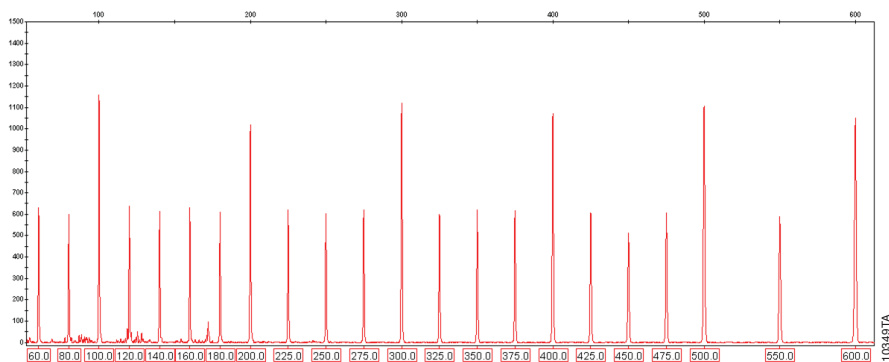
Figure 23. Internal Lane Standard 600. An electropherogram showing the Internal Lane Standard 600 fragments.

9.D. Composition of Buffers and Solutions

Gold ST★R 10X Buffer

500mM	KCl
100mM	Tris-HCl (pH 8.3 at 25°C)
15mM	$MgCl_2$
1%	Triton® X-100
2mM	each dNTP
1.6mg/ml	BSA

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.E.   Related Products

Product	Size	Cat.#
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
Gold ST★R 10X Buffer	1.2ml	DM2411

*Not for Medical Diagnostic Use.

10.   Summary of Changes

The following changes were made to the 1/23 revision of this document:

1.   Updated information in amplification table, Section 4.A.

2.   Changed listing of "Notes" from numerical to alphabetical.

3.   Updated font.