TECHNICAL MANUAL

PowerPlex® 18D System for Use on the Applied Biosystems® Genetic Analyzers

Instructions for Use of Products DC1802 and DC1808
PowerPlex® 18D System for Use on the Applied Biosystems® Genetic Analyzers

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1. Description

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

The PowerPlex® 18D System (a–f) is used for human identification applications including forensic analysis, relationship testing and research use. The system allows co-amplification and four-color fluorescent detection of eighteen loci (seventeen STR loci and Amelogenin), including D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, Amelogenin, vWA, D8S1179, TPOX, FGA, D19S433 and D2S1338. The internal lane standard is labeled with a fifth dye, WEN.

The PowerPlex® 18D System and all system components are manufactured in accordance with ISO 18385:2016. All necessary materials are provided to amplify STR regions of human genomic DNA, including a hot-start thermostable DNA polymerase, which is a component of the PowerPlex® D 5X Master Mix. This manual contains a protocol for use of the PowerPlex® 18D System with the GeneAmp® PCR System 9700 and Veriti® 96-Well Thermal Cycler in addition to protocols to separate amplified products and detect separated material on the ABI PRISM® 3100 and 3100-Avant Genetic Analyzers and Applied Biosystems® 3130, 3130xl, 3500 and 3500xL Genetic Analyzers (Figure 1). 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.

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

Amplification Setup

*Sections 4, 9.D and 9.E.*

Thermal Cycling

*Sections 4, 9.D and 9.E.* GeneAmp® PCR System 9700 or Veriti® 96-Well Thermal Cycler

Instrument Setup and Sample Preparation

*Section 5.*

Applied Biosystems® 3500 or ABI PRISM® 3100 or 3100-Avant
3500xL Genetic Analyzer Genetic Analyzer
*Section 5.A* *Section 5.B*

Applied Biosystems® 3130 or Applied Biosystems® 3130 or 3130xl Genetic Analyzer 3130xl Genetic Analyzer
*Section 5.B* *Section 5.B*

Data Analysis

*Section 6.* GeneMapper® ID-X Software GeneMapper® ID Software

Figure 1. An overview of the PowerPlex® 18D System protocol.

2. Product Components and Storage Conditions

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>SIZE</th>
<th>CAT.#</th>
</tr>
</thead>
<tbody>
<tr>
<td>PowerPlex® 18D System</td>
<td>200 reactions</td>
<td>DC1802</td>
</tr>
</tbody>
</table>

Not For Medical Diagnostic Use. This system contains sufficient reagents for 200 reactions of 25μl each.

Includes:

**Pre-amplification Components Box**
- 1ml PowerPlex® D 5X Master Mix
- 1ml PowerPlex® 18D 5X Primer Pair Mix
- 25μl 2800M Control DNA, 10ng/μl
- 5 × 1,250μl Water, Amplification Grade

**Post-amplification Components Box**
- 100μl PowerPlex® 18D Allelic Ladder Mix
- 200μl WEN Internal Lane Standard 500
2. Product Components and Storage Conditions (continued)

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>SIZE</th>
<th>CAT.#</th>
</tr>
</thead>
<tbody>
<tr>
<td>PowerPlex® 18D System</td>
<td>800 reactions</td>
<td>DC1808</td>
</tr>
</tbody>
</table>

Not For Medical Diagnostic Use. This system contains sufficient reagents for 800 reactions of 25µl each. Includes:

**Pre-amplification Components Box**
- 4 × 1ml PowerPlex® D 5X Master Mix
- 4 × 1ml PowerPlex® 18D 5X Primer Pair Mix
- 25µl 2800M Control DNA, 10ng/µl
- 10 × 1,250µl Water, Amplification Grade

**Post-amplification Components Box**
- 4 × 50µl PowerPlex® 18D Allelic Ladder Mix
- 4 × 200µl WEN Internal Lane Standard 500

The PowerPlex® 18D Allelic Ladder Mix is provided in a separate, sealed bag for shipping. This component should be moved to the post-amplification box after opening. The Water, Amplification Grade, is provided in a separate, sealed bag for shipping. This component should be moved to the pre-amplification box after opening.

**Storage Conditions:** Upon receipt, store all components except the 2800M Control DNA at –30°C to –10°C in a nonfrost-free freezer. Store the 2800M Control DNA at 2–10°C. Make sure that the 2800M Control DNA is stored at 2–10°C for at least 24 hours before use. After the first use, store the WEN Internal Lane Standard (WEN ILS) 500 at 2–10°C, protected from light; do not refreeze. The PowerPlex® 18D 5X Primer Pair Mix, PowerPlex® 18D 5X Allelic Ladder Mix and WEN ILS 500 are light-sensitive and must be stored in the dark. We strongly recommend that pre-amplification and post-amplification reagents be stored and used separately with different pipettes, tube racks, etc.

**Optional:** The PowerPlex® 18D System components may be stored for up to 1 year at 2–10°C without loss of activity.

**Available Separately**

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>SIZE</th>
<th>CAT.#</th>
</tr>
</thead>
<tbody>
<tr>
<td>PunchSolution™ Kit</td>
<td>100 preps</td>
<td>DC9271</td>
</tr>
<tr>
<td>SwabSolution™ Kit</td>
<td>100 preps</td>
<td>DC8271</td>
</tr>
<tr>
<td>5X AmpSolution™ Reagent</td>
<td>100 preps</td>
<td>DM1231</td>
</tr>
</tbody>
</table>

The PunchSolution™ Kit is required to process nonFTA punches prior to direct amplification. The SwabSolution™ Kit is required to process swabs prior to direct amplification. The 5X AmpSolution™ Reagent is required for direct amplification of DNA from storage card punches in a 12.5µl reaction volume. Both the PunchSolution™ Kit and SwabSolution™ Kit contain the 5X AmpSolution™ Reagent.
The proper panels, bins and stutter text files for use with GeneMapper® ID and ID-X software are available for download at: 
www.promega.com/resources/software-firmware/genemapper-id-software-panels-and-bin-sets/

Matrix standards are required for initial setup of the color separation matrix. The PowerPlex® 5C Matrix Standard is provided separately and is compatible with the ABI PRISM® 3100 and 3100-Avant Genetic Analyzers and Applied Biosystems® 3130, 3130xl, 3500 and 3500xL Genetic Analyzers (PowerPlex® 5C Matrix Standard, Cat.# DG4850).

3. Before You Begin

3.A. Precautions

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

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 (PowerPlex® D 5X Master Mix, PowerPlex® 18D 5X Primer Pair Mix, 2800M Control DNA and Water, Amplification Grade) are provided in a separate box and should be stored separately from those used following amplification (PowerPlex® 18D Allelic Ladder Mix and WEN Internal Lane Standard 500). Always include a negative control reaction to detect reagent contamination. We highly recommend the use of gloves and aerosol-resistant pipette tips.

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

3.B. Spectral Calibration

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

The PowerPlex® 5C Matrix Standard (Cat.# DG4850) is required for spectral calibration on the ABI PRISM® 3100 and 3100-Avant Genetic Analyzers and Applied Biosystems® 3130, 3130xl, 3500 and 3500xL Genetic Analyzers. The PowerPlex® 5C Matrix Standards, 310 (Cat. # DG5640), cannot be used to generate a matrix on these instruments.

For protocols and additional information about spectral calibration, see the PowerPlex® 5C Matrix Standard Technical Manual #TMD049. This manual is available online at: www.promega.com/protocols/
4. Protocols for Direct Amplification Using the PowerPlex® 18D System

The PowerPlex® 18D System was developed for amplification of direct-amplification samples. Slight protocol variations are recommended for optimal performance with each template source. Protocols for amplification in a 25µl reaction volume using FTA® and nonFTA storage card punches (Section 4.A) and swabs (Section 4.B) are included in the following amplification sections. A protocol for amplification in a 25µl reaction volume using extracted DNA is included in Section 9.C. Protocols for amplification in a 12.5µl reaction volume using FTA® and nonFTA storage card punches and swabs are included in Sections 9.D and 9.E, respectively.

The PowerPlex® 18D System is compatible with the GeneAmp® PCR System 9700 thermal cycler with a silver or gold-plated silver sample block and the Veriti® 96-Well Thermal Cycler.

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is NOT recommended with the PowerPlex® 18D System.

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

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

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

Materials to Be Supplied by the User

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

This section contains a protocol for direct amplification of DNA from storage card punches in a 25µl reaction volume using the PowerPlex® 18D System and GeneAmp® PCR System 9700 or Veriti® 96-Well Thermal Cycler. A protocol for direct amplification of DNA from storage card punches in a 12.5µl reaction volume is provided in Section 9.D.

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

Note: You will need to optimize and validate the number of storage card punches per reaction in your laboratory. See the PCR Optimization recommendations at the end of this section.
**FTA®-based sample types include:**

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

**NonFTA sample types include:**

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

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

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

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

**Note:** Static may be problematic when adding a punch to a well. For FTA® card punches, adding PCR amplification mix to the well before adding the punch may help alleviate static problems.

**Amplification Setup**

1. Thaw the PowerPlex® D 5X Master Mix, PowerPlex® 18D 5X Primer Pair Mix and Amplification-Grade Water completely.
   **Note:** Centrifuge tubes briefly to bring contents to the bottom, then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

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

3. Use a clean plate for reaction assembly, and label appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.
4.A. Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume (continued)

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

Table 1. PCR Amplification Mix for Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume.

<table>
<thead>
<tr>
<th>PCR Amplification Mix Component¹</th>
<th>Volume Per Reaction</th>
<th>× Number of Reactions</th>
<th>= Final Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, Amplification Grade</td>
<td>15µl</td>
<td></td>
<td>25µl</td>
</tr>
<tr>
<td>PowerPlex® D 5X Master Mix</td>
<td>5.0µl</td>
<td></td>
<td>25µl</td>
</tr>
<tr>
<td>PowerPlex® 18D 5X Primer Pair Mix</td>
<td>5.0µl</td>
<td></td>
<td>25µl</td>
</tr>
<tr>
<td><strong>total reaction volume</strong></td>
<td></td>
<td></td>
<td><strong>25µl</strong></td>
</tr>
</tbody>
</table>

¹Add Water, Amplification Grade, to the tube first, then add PowerPlex® D 5X Master Mix and PowerPlex® 18D 5X Primer Pair Mix. For FTA® card punches, the template DNA will be added at Step 6.

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

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

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

6. For FTA® storage cards, add one or two 1.2mm punches from a card containing a buccal sample or one 1.2mm punch from a card containing whole blood to the appropriate wells of the reaction plate.

**Notes:**

1. It also is acceptable to add the FTA® card punch first, then add the PCR amplification mix.
2. For nonFTA card punches, add the PCR amplification mix to the plate containing the PunchSolution™ Reagent-treated punches.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, then dilute an aliquot to 5ng/µl. Pipet 1µl of diluted DNA into a reaction well containing 25µl of PCR amplification mix.

**Notes:**

1. Do not include blank storage card punches in the positive control reactions.
2. Optimization of the amount of 2800M Control DNA may be required, based on cycling conditions and laboratory preferences.

8. For the negative amplification control, pipet Amplification-Grade 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. Briefly centrifuge the plate to bring storage card punches to the bottom of the wells and remove any air bubbles.
Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including cycle number (26–29 cycles) and injection conditions for your laboratory instrument. Testing at Promega shows that 27 cycles works well for a variety of sample types. Buccal samples may require more amplification cycles than blood samples. Cycle number will need to be optimized in each laboratory for each sample type that is amplified.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol.

Notes:

1. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
2. When using the GeneAmp® PCR System 9700, the program must be run with 9600 as the ramp speed. 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.

<table>
<thead>
<tr>
<th>Thermal Cycling Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>96°C for 2 minutes, then:</td>
</tr>
<tr>
<td>94°C for 10 seconds</td>
</tr>
<tr>
<td>60°C for 1 minute</td>
</tr>
<tr>
<td>for 27 cycles, then:</td>
</tr>
<tr>
<td>60°C for 20 minutes, then:</td>
</tr>
<tr>
<td>4°C soak</td>
</tr>
</tbody>
</table>

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

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

PCR Optimization

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

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

2. Depending on your preferred protocol, place one or two 1.2mm storage card punches containing a buccal sample or one 1.2mm punch of a storage card containing whole blood in each well of a reaction plate.

3. Prepare four identical reaction plates with punches from the same samples.
4.A. Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume (continued)

4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (26–29 cycles).

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

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

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block or Veriti® 96-Well 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
- SwabSolution™ Kit (Cat.# DC8271)

This section contains a protocol for direct amplification of DNA from swabs in a 25µl reaction volume using the PowerPlex® 18D System and GeneAmp® PCR System 9700 or Veriti® 96-Well Thermal Cycler. A protocol for direct amplification of DNA from swabs in a 12.5µl reaction volume is provided in Section 9.E.

Pretreat cotton swabs or OmniSwabs™ (GE Healthcare) with the SwabSolution™ Kit (Cat.# DC8271) as described in the SwabSolution™ Kit Technical Manual TMD037 to generate a swab extract. Be sure to include a blank swab as a negative control when processing samples.

Amplification Setup

1. Thaw the PowerPlex® D 5X Master Mix, PowerPlex® 18D 5X Primer Pair Mix and Amplification-Grade Water.
   
   **Note:** Centrifuge tubes briefly to bring contents to the bottom, then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

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

3. Use a clean plate for reaction assembly, and label appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.
4. Add the final volume of each reagent listed in Table 2 to a clean tube.

**Table 2. PCR Amplification Mix for Direct Amplification of DNA From Swabs in a 25µl Reaction Volume.**

<table>
<thead>
<tr>
<th>PCR Amplification Mix Component</th>
<th>Volume Per Reaction</th>
<th>×</th>
<th>Number of Reactions</th>
<th>=</th>
<th>Final Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, Amplification Grade</td>
<td>13µl ×</td>
<td>=</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PowerPlex® D 5X Master Mix</td>
<td>5.0µl ×</td>
<td>=</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PowerPlex® 18D 5X Primer Pair Mix</td>
<td>5.0µl ×</td>
<td>=</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>swab extract</td>
<td>2.0µl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>total reaction volume</strong></td>
<td><strong>25µl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Add Water, Amplification Grade, to the tube first, then add PowerPlex® D 5X Master Mix and PowerPlex® 18D 5X Primer Pair Mix. The swab extract will be added at Step 6.

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

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

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

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

7. For the positive amplification control, vortex the tube of 2800M Control DNA, then dilute an aliquot to 2.5ng/µl, and add 2µl to a reaction well containing 23µl of PCR amplification mix.

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

8. For the negative amplification control, pipet 2µl of Amplification-Grade Water 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™ or PunchSolution™ Reagent is processed as a blank without a swab.

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

**Thermal Cycling**

Amplification and detection instrumentation may vary. You will need to optimize protocols including cycle number (26–28 cycles) and injection conditions (or loading volume) for each laboratory instrument. Testing at Promega shows that 27 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.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol.

**Notes:**
1. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
2. When using the GeneAmp® PCR System 9700, the program must be run with 9600 as the ramp speed. 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.

<table>
<thead>
<tr>
<th>Thermal Cycling Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>96°C for 2 minutes, then:</td>
</tr>
<tr>
<td>94°C for 10 seconds</td>
</tr>
<tr>
<td>60°C for 1 minute</td>
</tr>
<tr>
<td>for 27 cycles, then:</td>
</tr>
<tr>
<td>60°C for 20 minutes</td>
</tr>
<tr>
<td>4°C soak</td>
</tr>
</tbody>
</table>

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

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

**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 (26, 27 and 28 cycles).

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

5. Instrument Setup and Sample Preparation

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

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice or ice-water bath
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- 3500/3500xL capillary array, 36cm
- plate 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
- 96-well plate and septa (e.g., Plate, Barcoded, Semi-Skirted, 96-Well [Cat.# V7845] and Septa Mat, 96-Well [Cat. # CE2696] or MicroAmp® optical 96-well plate [or equivalent] and septa [Applied Biosystems])
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)

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

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

Sample Preparation

1. Prepare a loading cocktail by combining and mixing WEN Internal Lane Standard 500 and formamide as follows:

   \[(0.5\mu l \text{ WEN ILS 500}) \times (\# \text{ samples}) + [(9.5\mu l \text{ formamide}) \times (\# \text{ samples})]\]

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

2. Vortex for 10–15 seconds to mix.

3. Pipet 10μl of formamide/internal lane standard mix into each well.
5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

4. Add 1μl of amplified sample (or 1μl of PowerPlex® 18D Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

   **Note:** Instrument detection limits vary; therefore, injection time 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, reduce the number of cycles in the amplification program to achieve the desired signal intensity.

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

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

**Instrument Preparation**

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

1. Open the 3500 Data Collection Software. The Dashboard screen will launch (Figure 2). Press the Refresh button. Ensure that the Consumables Information and Maintenance Notifications are acceptable.

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

![Figure 2. The Dashboard.](image-url)
5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)


2.a. 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 3 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.

![Figure 3. The Create New Instrument Protocol window.](image_url)
5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer
(continued)

The recommended settings are:

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

*Injection time may be modified to increase or decrease peak heights.*

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

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

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

Assign a descriptive protocol name.

**Note:** For more detailed information refer to the *Applied Biosystems® 3500/3500xL Genetic Analyzers User Guide.*
2.b. 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 “ILS500” or another appropriate name. Choose “Orange” as the Dye Color. The fragments in the size standard are 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases. See Figure 4.

Figure 4. The Create New Size Standard window.
5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

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

Assign a descriptive protocol name. Select the size standard created in Step 2.b. The settings for the QC protocol should be based on the internally validated conditions for the PowerPlex® 18D System on the Applied Biosystems® 3500 or 3500xL Genetic Analyzer. Figure 5 shows one option for these settings.

![Figure 5. The Create New QC Protocol window.](image-url)
2.d. 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 6), select the instrument protocol created in Step 2.a and the QC protocol created in Step 2.c. Assign a descriptive assay name. Select the application type “HID”. An Assay is required for all named samples on a plate.

Figure 6. The Create New Assay window.
5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

2.e. To create a new File Name Convention (Figure 7), 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 7. The Create New File Name Convention window.
2.f. To create a new Results Group (Figure 8), 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.

Figure 8. The Create New Results Group window.
5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer (continued)

3. To create a New Plate (Figure 9), navigate to the Library and select “Plates” from the Manage menu, then “Create”.

![Figure 9. Defining plate properties.](image)

4. Assign a descriptive plate name. Select the plate type “HID” from the drop-down menu (Figure 9).

5. Select “Assign Plate Contents” (Figure 10).

![Figure 10. Assigning plate contents.](image)
6. Assign sample names to wells.

7. In the lower left portion of the screen, under “Assays”, use the Add from Library option to select the Assay created in Step 2.d or one previously created. Click on the Add to Plate button, and close the window. **Note:** Edit the Instrument Protocol using the instructions in Step 5.A, Step 2. Otherwise, changes to the Instrument Protocol, such as lengthening the run time, made in the Assay window may not be incorporated into the instrument run.

8. Under “File Name Convention”, use the Add from Library option to select the File Name Convention created in Step 2.e or one previously created. Click on the Add to Plate button, and close the window.

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

10. Highlight the sample wells, then select the boxes in the Assays, File Name Conventions and Results Groups that pertain to those samples.

11. Select “Link Plate for Run”.

12. The Load Plate window will appear. Select “Yes”.

13. In the Run Information window (Figure 11), assign a Run Name. Select “Start Run” (not shown).

---

**Figure 11. Assigning a run name.**
5.B Detection of Amplified Fragments Using the ABI PRISM® 3100 or 3100-Avant Genetic Analyzer with Data Collection Software, Version 2.0, or the Applied Biosystems® 3130 or 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 or ice-water bath
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- 3100 or 3130 capillary array, 36cm
- performance optimized polymer 4 (POP-4®) for the 3100 or 3130
- 10X genetic analyzer buffer with EDTA
- 96-well plate and septa (e.g., Plate, Barcoded, Semi-Skirted, 96-Well [Cat.# V7845] and Septa Mat, 96-Well [Cat.# CE2696] or MicroAmp® optical 96-well plate [or equivalent] and septa [Applied Biosystems])
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)

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

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

Sample Preparation

1. Prepare a loading cocktail by combining and mixing WEN Internal Lane Standard 500 and formamide as follows:
   
   
   \[(0.5\mu l \text{ WEN ILS 500}) \times (# \text{ samples})\] + \[(9.5\mu l \text{ formamide}) \times (# \text{ samples})\]

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

2. Vortex for 10–15 seconds to mix.

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

4. Add 1μl of amplified sample (or 1μl of PowerPlex® 18D 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 (see Instrument Preparation below).

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

6. Denature samples at 95°C for 3 minutes, then immediately chill on crushed ice or in an ice-water bath for 3 minutes. Denature samples just prior to loading the instrument.
**Instrument Preparation**

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

Analyze samples as described in the user's manual for the ABI PRISM® 3100 or 3100-Avant Genetic Analyzer with Data Collection Software, Version 2.0, and the Applied Biosystems® 3130 or 3130xl Genetic Analyzer with Data Collection Software, Version 3.0 or 4.0, with the following exceptions.

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

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

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

   The recommended settings are:

<table>
<thead>
<tr>
<th>Template</th>
<th>HIDFragmentAnalysis36_POP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Time¹</td>
<td>5 seconds</td>
</tr>
<tr>
<td>Injection Voltage¹</td>
<td>3kV</td>
</tr>
<tr>
<td>Run Time</td>
<td>1,500 seconds</td>
</tr>
<tr>
<td>Dye Set²</td>
<td>G5</td>
</tr>
</tbody>
</table>

   ¹Instrument sensitivities and migration times 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.

   ²The Dye Set setting is changed in the Protocol Manager.

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

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

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

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

5. Place samples in the instrument, and close the instrument doors.
5.B Detection of Amplified Fragments Using the ABI PRISM® 3100 or 3100-Avant Genetic Analyzer with Data Collection Software, Version 2.0, or the Applied Biosystems® 3130 or 3130xl Genetic Analyzer with Data Collection Software Version 3.0 or 4.0 (continued)

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

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

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

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

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

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

10. 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 40 minutes.

6. Data Analysis

The instructions in this section were written using GeneMapper® ID-X software, version 1.2 or GeneMapper® ID software, version 3.2. Due to potential differences between individual software versions, some of the instructions may not apply to other software versions.

6.A. PowerPlex® 18D Panels, Bins and Stutter Text Files with GeneMapper® ID-X Software, Version 1.2

To facilitate analysis of data generated with the PowerPlex® 18D System, we have created panels, bins and stutter text files to allow automatic assignment of genotypes using GeneMapper® ID-X software. We recommend that users receive training from Applied Biosystems on the GeneMapper® ID-X software to familiarize themselves with proper operation of the software.

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

Getting Started

1. To obtain the proper panels, bins and stutter text files for the PowerPlex® 18D System go to: www.promega.com/resources/software-firmware/genemapper-id-software-panels-and-bin-sets/

2. Select the PowerPlex® System that you are using, and select “GeneMapper® ID-X”. Enter your contact information, and select “Submit”.
3. Save the PowerPlex_18D_Panels_IDX_vX.x.txt, PowerPlex_18D_Bins_IDX_vX.x.txt and PowerPlex_18D_Stutter_IDX_vX.x.txt files, where “X.x” refers to the most recent version of the panels, bins and stutter text files, to a known location on your computer.

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

**Importing Panels, Bins and Stutter Text Files**

1. Open the GeneMapper® ID-X software.
2. Select “Tools”, then “Panel Manager”.
3. Highlight the Panel Manager icon in the upper left navigation pane.
4. Select “File”, then “Import Panels”.
5. Navigate to the panels text file downloaded in the Getting Started Section. Select the file, then “Import”.
6. In the navigation pane, highlight the PowerPlex 18D panels folder that you just imported in Step 5.
7. Select “File”, then “Import Bin Set”.
8. Navigate to the bins text file downloaded in the Getting Started Section. Select the file, then “Import”.
9. In the navigation pane, highlight the PowerPlex 18D panels folder that you just imported in Step 5.
10. Select “File”, then “Import Marker Stutter”. A warning box will appear asking if you want to overwrite current values. Select “Yes”.
11. Navigate to the stutter file downloaded in the Getting Started Section. Select the file, then “Import”.
12. At the bottom of the Panel Manager window, select “OK”. This will save the panels, bins and stutter text files and close the window.

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

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

1. Select “Tools”, then “GeneMapper ID-X Manager”.
2. Select the Size Standard tab.
3. Select “New”.

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

4. In the Size Standard Editor window (Figure 12), select “GeneMapper ID-X Security Group” as the Security Group. This allows access for all users of the software. Other security groups may be used.

![Figure 12. The GeneMapper® ID-X Software Size Standard Editor.](image)

5. Enter a detailed name, such as “WEN_ILS_500_IDX”.


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

8. Select “OK”.

6.C. Importing the WEN ILS 500 IDX Size Standard into GeneMapper® ID-X Software, Version 1.2

The WEN_ILS_500_IDX.xml file is available for download at:
www.promega.com/resources/software-firmware/genemapper-id-software-panels-and-bin-sets/

1. Select “Tools”, then “GeneMapper ID-X Manager”.
2. Select the Size Standard tab.
3. Select “Import”.
4. Navigate to the location of the WEN_ILS_500_IDX.xml file on your computer.
5. Highlight the file, then select “Import”.
6. Select “Done” to save changes and close the GeneMapper® ID-X Manager.

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

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

1. Select “Tools”, then “GeneMapper ID-X Manager”.
2. Select the Analysis Methods tab.
4. In the Analysis Method Editor window, select “GeneMapper ID-X Security Group” as the Security Group. This allows access for all users of the software. Other security groups may be used.
5. Enter a descriptive name for the analysis method, such as “PowerPlex18D 20% Filter”.
6. Select the Allele tab (Figure 13).
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.
6.D. Creating a Databasing or Paternity Analysis Method with GeneMapper® ID-X Software, Version 1.2 (continued)

9. We recommend the values shown in Figure 13 for proper filtering of stutter peaks when using the PowerPlex® 18D System. You will need to optimize these settings. In-house validation should be performed.

![Figure 13. The GeneMapper® ID-X Software Allele tab.](image-url)
10. Select the Peak Detector tab. You will need to optimize these settings. In-house validation should be performed.

Notes:

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

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

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

Figure 14. The GeneMapper® ID-X Software Peak Detector tab.
6.D. Creating a Databasing or Paternity Analysis Method with GeneMapper® ID-X Software, Version 1.2 (continued)

11. Select the Peak Quality tab. You may change the settings for peak quality.
    **Note:** For Steps 11 and 12, see the GeneMapper® ID-X user's manual for more information.

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

13. Select “Save” to save the new analysis method.

14. Select “Done” to exit the GeneMapper® ID-X Manager.

**Processing Data for Databasing or Paternity Samples**

1. Select “File”, then “New Project”.

2. Select “Edit”, then “Add Samples to Project”.

3. Browse to the location of 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.

   In the Analysis Method column, select the analysis method created above.

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

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

7. Select “Analyze” (green arrow button) to start data analysis.

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

6.E. Creating a No-Global-Filter Analysis Method with GeneMapper® ID-X Software, Version 1.2

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

1. Select “Tools”, then “GeneMapper ID-X Manager”.

2. Select the Analysis Methods tab.


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

5. Enter a descriptive name for the analysis method, such as “PowerPlex18D”.

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 if available” box is checked.

9. We recommend the values shown in Figure 15 for proper filtering of stutter peaks when using the PowerPlex® 18D System. You will need to optimize these settings. In-house validation should be performed.

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

**Notes:**

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

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

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

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

**Note:** For Steps 11 and 12, see the GeneMapper® ID-X user’s manual for more information.
6.E. Creating a No-Global-Filter Analysis Method with GeneMapper® ID-X Software, Version 1.2 (continued)

12. Select the Quality Flags tab. You may change these settings.
13. Select “OK” to save your settings.
14. Select “Done” to exit the GeneMapper® ID-X Manager.

Processing Data for Samples with No Global Filter

1. Select “File”, then “New Project”.
2. Select “Edit”, then “Add Samples to Project”.
3. Browse to the location of the run files. Highlight desired files, then select “Add to list” followed by “Add”.
4. In the Sample Type column, use the drop-down menu to select “Allelic Ladder”, “Sample”, “Positive Control” or “Negative Control” as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as “Allelic Ladder” in the Sample Type column for proper genotyping.
5. In the Analysis Method column, select the analysis method created above.
6. In the Panel column, select the panels text file that was imported in Section 6.A.
7. In the Size Standard column, select the size standard that was created in Section 6.B or imported in Section 6.C.
8. Select “Analyze” (green arrow button) to start data analysis.

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

6.F. Importing PowerPlex® 18D Panels and Bins Text Files into GeneMapper® ID Software, Version 3.2

To facilitate analysis of data generated with the PowerPlex® 18D System, we have created panels and bins text files to allow automatic assignment of genotypes using GeneMapper® ID software, version 3.2. We recommend that users of GeneMapper® ID software, version 3.2, complete the Applied Biosystems GeneMapper® ID Software Human Identification Analysis Tutorial to familiarize themselves with proper operation of the software. For GeneMapper® ID software, version 3.1, users we recommend upgrading to version 3.2.

For analysis using GeneMapper® ID software, version 3.2, you will need the proper panels and bins text files:
PowerPlex_18D_Panels_vX.x.txt and PowerPlex_18D_Bins_vX.x.txt files, where “X.x” refers to the most recent version of the panels and bins text files.

Getting Started

1. To obtain the panels and bins text files for the PowerPlex® 18D System go to:
   www.promega.com/resources/software-firmware/genemapper-id-software-panels-and-bin-sets/
2. Select the PowerPlex® System that you are using, and select “GeneMapper® ID-X”. Enter your contact information, and select “Submit”.

www.promega.com
3. Save the PowerPlex_18D_Panels_X.x.txt and PowerPlex_18D_Bins_X.x.txt files to a known location on your computer.

4. Save the WEN_ILS_500.xml file to a known location on your computer.

### Importing Panels and Bins Text Files

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

1. Open the GeneMapper® ID software, version 3.2.
2. Select “Tools”, then “Panel Manager”.
3. Highlight the Panel Manager icon in the upper left navigation pane.
4. Select “File”, then “Import Panels”.
5. Navigate to the panels text file downloaded in the Getting Started section. Select the file, then “Import”.
6. In the navigation pane, highlight the PowerPlex 18D panels folder that you just imported in Step 5.
7. Select “File”, then “Import Bin Set”.
8. Navigate to the bins text file downloaded in the Getting Started section above. Select the file, then “Import”.
9. At the bottom of the Panel Manager window, select “OK”. This will save the panels and bins text files and close the window.

### 6.G. Creating a Size Standard with GeneMapper® ID Software, Version 3.2

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

1. Select “Tools”, 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.](image)

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


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

8. Select “OK”.

Figure 17. The GeneMapper® ID-X Software Size Standard Editor.
6.H. Importing the WEN ILS 500 Size Standard into GeneMapper® ID Software, Version 3.2

The WEN_ILS_500.xml file is available for download at: www.promega.com/resources/software-firmware/genemapper-id-software-panels-and-bin-sets/

Save the WEN_ILS_500.xml file to a known location on your computer.

1. Select “Tools”, then “GeneMapper Manager”.
2. Select the Size Standard tab.
3. Select “Import”.
4. Browse to the location of the WEN_ILS_500.xml file.
5. Highlight the file, then select “Import”.
6. Select “Done” to save changes and exit the GeneMapper Manager.

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

1. Select “Tools”, then “GeneMapper Manager”.
2. Select the Analysis Methods tab.
4. Select “HID”, and select “OK”.
   **Note:** If you do not see the HID option, you do not have GeneMapper® ID software. Contact Applied Biosystems.
5. Enter a descriptive name for the analysis method, such as “PowerPlex18D_20%filter”.


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

6. Select the Allele tab (Figure 18).

![GeneMapper® ID-X Software Allele tab](image)

Figure 18. The GeneMapper® ID-X Software Allele tab with settings for using a 20% peak filter.

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

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

9. Enter the values shown in Figure 18 for proper filtering of peaks when using the PowerPlex® 18D 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”.

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

**Notes:**

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

2. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Individual laboratories should determine their peak amplitude thresholds from internal validation studies.
11. Select the Peak Quality tab. You may change the settings for peak quality.
   
   **Note:** For Steps 11 and 12, see the GeneMapper® ID user’s manual for more information.

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

13. Select “OK” to save your settings.

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

**Processing Data for Databasing or Paternity Samples**

1. Select “File”, then “New Project”.

2. Select “Edit”, then “Add Samples to Project”.

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

4. In the Sample Type column, use the drop-down menu to select “Ladder”, “Sample”, “Positive Control” or “Negative Control” as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as “Ladder” in the Sample Type column for proper genotyping.
6.I. Creating a Databasing or Paternity Analysis Method with GeneMapper® ID Software, Version 3.2 (continued)

5. In the Analysis Method column, select the analysis method created previously in this section.
6. In the Panel column, select the panels text file that was imported in Section 6.F.
7. In the Size Standard column, select the size standard that was created in Section 6.G or imported in Section 6.H.
8. Select “Analyze” (green arrow button) to start the data analysis.


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

1. Select “Tools”, then “GeneMapper Manager”.
2. Select the Analysis Methods tab.
4. Select “HID”, and select “OK”.
   **Note:** If you do not see the HID option, you do not have GeneMapper® ID software. Contact Applied Biosystems.
5. Enter a descriptive name for the analysis method, such as “PowerPlex18D”.
6. Select the Allele tab (Figure 20).
7. Select the bins text file that was imported in Section 6.F.
8. Ensure that the “Use marker-specific stutter ratio if available” box is checked.
9. Enter the values shown in Figure 20 for proper filtering of stutter peaks when using the PowerPlex® 18D 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 20. The GeneMapper® ID Software Allele tab.

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

Notes:

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

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

11. Select the Peak Quality tab. You may change the settings for peak quality.
   **Note:** For Steps 11 and 12, see the GeneMapper® ID user's manual for more information.

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

13. Select “OK” to save your settings.

**Processing Data for Samples with No Global Filter**

1. Select “File”, then “New Project”.
2. Select “Edit”, then “Add Samples to Project”.
3. Browse to the location of the run files. Highlight desired files, then select “Add to list” followed by “Add”.
4. In the Sample Type column, use the drop-down menu to select “Ladder”, “Sample”, “Positive Control” or “Negative Control” as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as “Ladder” in the Sample Type column for proper genotyping.
5. In the Analysis Method column, select the analysis method created previously in this section.
6. In the Panel column, select the panels text file that was imported in Section 6.F.
7. In the Size Standard column, select the size standard that was created in Section 6.G or imported in Section 6.H.
8. Select “Analyze” (green arrow button) to start data analysis.

6.K. Controls

1. Observe the results for the negative control. Using the protocols defined in this manual, the negative control should be devoid of amplification products.
2. Observe the results for the 2800M Control DNA. The expected 2800M Control DNA allele designations for each locus are listed in Table 6 (Section 9.A).

6.L. Results

Representative results of the PowerPlex® 18D System are shown in Figure 21. The PowerPlex® 18D Allelic Ladder Mix is shown in Figure 22.
Figure 21. The PowerPlex® 18D System. Two 1.2mm punches were taken from a buccal sample transferred to an FTA® card and amplified for 27 cycles using the PowerPlex® 18D System. Amplification products were mixed with WEN Internal Lane Standard 500 and analyzed with an Applied Biosystems® 3500xL Genetic Analyzer using a 1.2kV, 24-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-ET-labeled loci: Amelogenin, vWA, D8S1179, TPOX and FGA. Panel D. An electropherogram showing the peaks of the CXR-ET-labeled loci: D19S433 and D2S1338. Panel E. An electropherogram showing the 60bp to 500bp fragments of the WEN Internal Lane Standard 500.
Figure 22. The PowerPlex® 18D Allelic Ladder Mix. The PowerPlex® 18D Allelic Ladder Mix was analyzed with an Applied Biosystems® 3500xL Genetic Analyzer using a 1.2kV, 24-second injection. The sample file was analyzed with the GeneMapper® ID-X software, version 1.4, and PowerPlex® 18D 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-ET-labeled allelic ladder components and their allele designations. Panel D. The CXR-ET-labeled allelic ladder components and their allele designations.
Artifacts and Stutter

Stutter products are a common amplification artifact associated with STR analysis (13,14). Stutter products are often observed one repeat unit below the true allele peak and, occasionally, two repeat units smaller or one repeat unit larger than the true allele peak. Frequently, alleles with a greater number of repeat units will exhibit a higher percent stutter. The pattern and intensity of stutter may differ slightly between primer sets for the same loci.

The mean stutter plus three standard deviations at each locus is used in the PowerPlex® 18D panels text files for locus-specific filtering in the GeneMapper® ID software, version 3.2, and GeneMapper® ID-X software. In addition to stutter peaks, other artifact peaks can be observed at some of the PowerPlex® 18D System loci (Table 3). These extra peaks occur when the amplified peaks are particularly intense; formamide, polymer or capillary was of poor quality; or denaturation was ineffective. See Section 7 for more information on how to minimize these artifacts.

Table 3. Artifact Peaks Observed with the PowerPlex® 18D System.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Artifact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein</td>
<td>D3S1358 increased stutter&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>D21S11 n–2, n+2&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>D5S818 n–1, n–2&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>One or more peaks between 138 and 150 bases&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>214 bases</td>
</tr>
<tr>
<td></td>
<td>247 bases&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>D16S539 n–20&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>CXR</td>
<td>D2S1338 n–20&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Artifact has been observed in direct amplifications with large amounts of template DNA.

<sup>2</sup>Two bases above and below the true allele peak.

<sup>3</sup>One or two bases below the true allele peak.

<sup>4</sup>Artifact is due to secondary structure, which can occur in particularly intense or poorly denatured samples.
7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: [www.promega.com](http://www.promega.com). E-mail: [genetic@promega.com](mailto:genetic@promega.com)

7.A. Amplification and Fragment Detection

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faint or absent allele peaks</td>
<td>The PowerPlex® D 5X Master Mix was not vortexed well before use. Vortex the 5X Master Mix for 5–10 seconds before dispensing into the PCR amplification mix.</td>
</tr>
<tr>
<td></td>
<td>Thermal cycler or plate problems. Review the thermal cycling protocol in Section 4 or 9. We have not tested other reaction plates or thermal cyclers. Calibrate the thermal cycler heating block if necessary.</td>
</tr>
<tr>
<td></td>
<td>Primer concentration was too low. Use the recommended primer concentration. Vortex the PowerPlex® 18D 5X Primer Pair for 15 seconds before use.</td>
</tr>
<tr>
<td></td>
<td>Positive control did not amplify. Do not include a blank punch in the positive control reaction. Check to be sure that the 2800M Control DNA was diluted correctly; prepare a new dilution if necessary. Be sure to store the 2800M Control DNA correctly (see Section 4 or 9).</td>
</tr>
<tr>
<td></td>
<td>Improper storage of the 2800M Control DNA.</td>
</tr>
<tr>
<td></td>
<td>Poor capillary electrophoresis injection (WEN ILS 500 peaks also affected). Re-inject the sample. Check the laser power.</td>
</tr>
<tr>
<td></td>
<td>Samples were not denatured completely. Heat-denature samples for the recommended time, then cool on crushed ice or in an ice-water bath immediately prior to capillary electrophoresis. Do not cool samples in a thermal cycler set at 4°C, as this may lead to artifacts due to DNA re-annealing.</td>
</tr>
<tr>
<td></td>
<td>Poor-quality formamide was used. Use only the recommended formamide when analyzing samples.</td>
</tr>
<tr>
<td>Symptoms</td>
<td>Causes and Comments</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Faint or absent allele peaks (continued)</td>
<td>Instrument run ended before all WEN ILS 500 fragments or sample fragments were detected. Lengthen the run time. For the Applied Biosystems® 3130 and 3130xl Genetic Analyzers, lengthen the run time in the Run Module in the Module Manager (Step 5.B, Step 1). For the 3500 and 3500xL Genetic Analyzers, lengthen the run time in the Instrument Protocol window (Step 5.A, Step 2). Otherwise, changes to the Instrument Protocol, such as lengthening the run time, made in the Assay window may not be incorporated into the instrument run.</td>
</tr>
</tbody>
</table>
| Extra peaks visible in one or all color channels | Contamination with another template DNA or previously amplified DNA. Cross-contamination can be a problem. Use aerosol-resistant pipette tips, and change gloves regularly. Samples were not denatured completely. Heat-denature samples for the recommended time, and then cool on crushed ice or in an ice-water bath immediately prior to capillary electrophoresis. Do not cool samples in a thermal cycler set at 4°C, as this may lead to artifacts due to DNA re-annealing. Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the punches as soon as possible to each well and follow immediately by thermal cycling. 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.  
- Decrease the number of cycles.  
- Increase the final extension time.  
- Decrease the amount of template DNA. Using more than the recommended amount can result in incomplete adenylation.  
<p>| Artifacts. The signal strength of certain artifacts increases with storage of the amplification plate at 4°C (see Table 5), some times 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. |</p>
<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extra peaks visible in one or all color channels</td>
<td>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.</td>
</tr>
<tr>
<td>(continued)</td>
<td>CE-related artifacts (&quot;spikes&quot;). 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.</td>
</tr>
<tr>
<td></td>
<td>Incorrect G5 spectral was active. Re-run samples, and confirm that the PowerPlex® 5-dye G5 spectral is set for G5. See instrument preparation instructions in Section 5.A, Step 2, and Section 5.B, Step 6.</td>
</tr>
<tr>
<td></td>
<td>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.</td>
</tr>
<tr>
<td></td>
<td>• Perform a new spectral calibration, and re-run the samples.</td>
</tr>
<tr>
<td></td>
<td>• Instrument sensitivities can vary. Optimize the injection conditions. See Section 5.</td>
</tr>
<tr>
<td></td>
<td>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 water; change vials and wash buffer reservoir.</td>
</tr>
<tr>
<td></td>
<td>Repeat sample preparation using fresh formamide. Long-term storage of amplified sample in formamide can result in degradation.</td>
</tr>
<tr>
<td></td>
<td>The CE polymer was beyond its expiration date, or polymer was stored at room temperature for more than one week.</td>
</tr>
<tr>
<td></td>
<td>Maintain instrumentation on a daily or weekly basis, as recommended by the manufacturer.</td>
</tr>
<tr>
<td>Symptoms</td>
<td>Causes and Comments</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>---------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Allelic ladder not running the same as samples</td>
<td>Allelic ladder and primer pair mix were not compatible. Ensure that the allelic ladder is from the same kit as the primer pair mix.</td>
</tr>
<tr>
<td></td>
<td>Poor-quality formamide. Use only the recommended formamide when analyzing samples.</td>
</tr>
<tr>
<td></td>
<td>Be sure the allelic ladder and samples are from the same instrument run.</td>
</tr>
<tr>
<td></td>
<td>Migration of samples changed slightly over the course of a CE run with many samples. This may be due to changes in temperature or the CE column over time. Use a different injection of allelic ladder to determine sizes.</td>
</tr>
<tr>
<td></td>
<td>Poor injection of allelic ladder. Include more than one ladder per instrument run.</td>
</tr>
<tr>
<td>Peak height imbalance</td>
<td>Excessive amount of DNA. Amplification of &gt;20ng of template can result in an imbalance, with smaller loci showing more product than larger loci. Use less template DNA, or decrease number of cycles.</td>
</tr>
<tr>
<td></td>
<td>Insufficient template DNA. Use the recommended amount of template DNA if available. Stochastic effects can occur when amplifying low amounts of template.</td>
</tr>
<tr>
<td></td>
<td>Miscellaneous balance problems. Thaw the 5X Primer Pair Mix and 5X Master Mix completely, and vortex for 15 seconds before use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after mixing. Calibrate thermal cyclers and pipettes routinely.</td>
</tr>
<tr>
<td></td>
<td>PCR amplification mix prepared in Section 4 or 9 was not mixed well. Vortex the PCR amplification mix for 5–10 seconds before dispensing into the reaction plate.</td>
</tr>
<tr>
<td></td>
<td>Impure template DNA. Inhibitors that may be present in forensic samples can lead to allele dropout or imbalance.</td>
</tr>
</tbody>
</table>
7.B. Direct Amplification of DNA from Storage Card Punches

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

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faint or absent allele peaks</td>
<td>DNA was not accessible on nonlytic material. Pretreat nonFTA materials with PunchSolution™ Reagent to ensure that DNA is liberated from cellular proteins.</td>
</tr>
<tr>
<td></td>
<td>Poor sample deposition. Shedding and collection of donor cells was variable. Increase cycle number.</td>
</tr>
<tr>
<td></td>
<td>Poor sample transfer to storage card or variable sampling from the storage card. Take punches from a different portion of the card. Increasing cycle number also can improve low peak heights.</td>
</tr>
<tr>
<td></td>
<td>Too much sample in the reaction. Use the recommended number of punches. Follow the manufacturer’s recommendations when depositing sample onto the storage card.</td>
</tr>
<tr>
<td></td>
<td>Amplification was inhibited when using more than one storage card punch with blood. Use only one 1.2mm storage card punch with blood.</td>
</tr>
<tr>
<td></td>
<td>Active PunchSolution™ Reagent carried over into the amplification reaction when using nonFTA card punches. Ensure that the heat block reached 70°C and samples were incubated for 30 minutes or until wells were dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. We have not tested longer incubation times.</td>
</tr>
<tr>
<td></td>
<td>Inactive PunchSolution™ Reagent. Thaw the PunchSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze; avoid multiple freeze-thaw cycles, as this may reduce activity.</td>
</tr>
<tr>
<td>Symptoms</td>
<td>Causes and Comments</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| Faint or absent peaks for the positive control reaction | If the positive control reaction failed to amplify, check positive control reaction make sure that the correct amount of 2800M Control DNA was added to the reaction. We recommend 5ng of 2800M Control DNA per amplification reaction.  
  • Do not include a blank punch in the positive control reaction. Presence of a blank punch may inhibit amplification of 2800M Control DNA.  
  • Optimize the amount of 2800M Control DNA for your thermal cycling conditions and laboratory preferences.  
  Improper storage of the 2800M Control DNA. |
| Extra peaks visible in one or all color channels | Punch was contaminated. Take punches from blank paper samples, and include a reaction with one or two blank punches as a negative control.  
  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. 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.  
  Artifacts of STR amplification. Direct amplification of >20ng of template can result in a higher number of artifact peaks. Use the recommended punch size and number of punches. Optimize the cycle number. See Section 6.I for additional information on stutter and artifacts. |
### 7.B. Direct Amplification of DNA from Storage Card Punches (continued)

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extra peaks visible in one or all color channels (continued)</td>
<td>Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´ A residue. Be sure to perform a 20-minute extension step at 60°C after thermal cycling (Section 4 or 9.C).</td>
</tr>
</tbody>
</table>
| Peak height imbalance | Excessive amount of DNA. Amplification of >20ng of template can result in an imbalance with smaller loci showing more product than larger loci.  
  - Use the recommended number of punches. Follow the manufacturer’s recommendations when depositing sample onto the storage card.  
  - Decrease number of cycles. |
| | Amplification was inhibited when using more than one storage card punch with blood. Use only one 1.2mm storage card punch with blood. |
| | DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat nonFTA materials with PunchSolution™ Reagent to ensure that DNA is liberated from cellular proteins. |
| | Active PunchSolution™ Reagent carried over into the amplification reaction. Larger loci are most susceptible to carryover and will drop out before the smaller loci.  
  - Ensure that the heat block reached 70°C and samples were incubated for 30 minutes or until wells are dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent.  
  - Reducing the PunchSolution™ Reagent volume may improve results for reactions with reduced amplification volumes. Optimization and validation are required. |
| | Inactive PunchSolution™ Reagent. Thaw the PunchSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity. |
### Symptoms  
Peak height imbalance (continued)

<table>
<thead>
<tr>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carryover of excess PunchSolution™ Reagent into amplification reaction. We recommend treating one 1.2mm nonFTA card punch with 10µl of PunchSolution™ Reagent, and using one punch per 25µl amplification reaction. Use of a smaller amplification reaction volume may compromise performance if using 10µl of PunchSolution™ Reagent. Reducing the PunchSolution™ Reagent volume may improve results when using a reduced amplification reaction volume. Laboratory optimization and validation are required.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Extreme variability in sample-to-sample peak heights</th>
</tr>
</thead>
<tbody>
<tr>
<td>There can be significant individual-to-individual variability in sample-to-sample peak heights due to variability in the deposition of cells onto a punch, resulting in peak height variability between samples. The PunchSolution™ Kit increases the recovery of amplifiable DNA from samples but does not normalize the amount of DNA present.</td>
</tr>
</tbody>
</table>

### 7.C. Direct Amplification of DNA from Swabs

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

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faint or absent allele peaks</td>
<td>Poor sample deposition. Shedding and collection of donor cells was variable. Increase cycle number.</td>
</tr>
</tbody>
</table>

**Inactive SwabSolution™ Reagent.** Thaw the SwabSolution™ Reagent completely in a 37°C water bath, and mix by gentle inversion. Store the SwabSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.

**Active SwabSolution™ Reagent carried over into the amplification reaction.** Ensure that the heat block reached 70°C (90°C if using a 2.2ml, Square-Well Deep Well Plate) and samples were incubated for the full 30 minutes. Incubation for shorter time periods may result in incomplete reagent inactivation. Do not use an incubator to incubate tubes or plates: Heat transfer is inefficient and will result in poor performance. Only use a heat block to maintain efficient heat transfer. We have tested 60-minute incubation times and observed no difference in performance compared to a 30-minute incubation.
### 7.C. Direct Amplification of DNA from Swabs (continued)

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faint or absent peaks for the positive control reaction</td>
<td>If the positive control reaction failed to amplify, check to make sure that the correct amount of 2800M Control DNA was added to the reaction. Due to the reduced cycle numbers used with swab extracts, it is necessary to increase the mass of 2800M Control DNA to obtain a profile. We recommend 5ng of 2800M Control DNA per amplification reaction. This mass of DNA should be reduced if the cycle number used is increased and decreased if the cycle number is increased. Increase or decrease by twofold the mass of 2800M Control DNA for every one-cycle decrease or increase, respectively.</td>
</tr>
<tr>
<td>Extra peaks visible in one or all color channels</td>
<td>Swab extract was contaminated. Include a blank swab as a negative control when processing samples. Artifacts of STR amplification. Amplification of swab extracts with high concentrations of DNA can result in artifact peaks due to overamplification, resulting in saturated signal on the CE instrument. If signal is saturated, repeat the amplification with less swab extract or a reduced cycle number. Amplification of excess template for a given cycle number resulted in overloading of the capillary upon electrokinetic injection. In addition to signal saturation, excess DNA in the capillary is difficult to maintain in a denatured single-stranded state. Some single-stranded DNA renatures and becomes double-stranded. Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis and appears as “shadow” peaks migrating in front of the main peaks. If this occurs at a heterozygous locus it is possible to observe the presence of two “shadow” peaks that differ in size by approximately the same distance as the single-stranded alleles.</td>
</tr>
<tr>
<td>Peak height imbalance</td>
<td>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.</td>
</tr>
</tbody>
</table>
### Symptoms

<table>
<thead>
<tr>
<th>Peak height imbalance (continued)</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active SwabSolution carried over from swab extracts into the amplification reaction. Larger loci are most susceptible to reagent carryover and will drop out before 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. Only use a heat block to maintain efficient heat transfer.</td>
<td></td>
</tr>
<tr>
<td>Inactive SwabSolution™ Reagent. Thaw the SwabSolution™ Reagent completely in a 37°C water bath, and mix by gentle inversion. Store the SwabSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not re-freeze, as this may reduce activity.</td>
<td></td>
</tr>
<tr>
<td>Extreme variability in sample-to-sample peak heights</td>
<td>There can be significant individual-to-individual variability in cell deposition onto buccal swabs. This will appear as variability in peak heights between swab extracts. The extraction process maximizes recovery of amplifiable DNA from buccal swabs but does not normalize the amount of DNA present. If variability is extreme, quantitate the DNA using a fluorescence-based double-stranded DNA quantitation method or qPCR-based quantitation method. The quantitation values can be used to normalize input template amounts to minimize variation in signal intensity.</td>
</tr>
</tbody>
</table>

### 7.D. GeneMapper® ID-X Software

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stutter peaks not filtered</td>
<td>Stutter file was not imported into the Panel Manager when the panels and bins text files were imported.</td>
</tr>
<tr>
<td></td>
<td>Stutter distance was not defined in the Analysis Method Allele tab.</td>
</tr>
<tr>
<td>Samples in the project not analyzed</td>
<td>The Analysis Requirement Summary window was not active, and there was an analysis requirement that was not met. Turn on Analysis Requirement Summary in the Options menu, and correct the necessary analysis requirements to continue analysis.</td>
</tr>
</tbody>
</table>
### 7D. GeneMapper® ID-X Software (continued)

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edits in label edit viewer cannot be viewed</td>
<td>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.</td>
</tr>
<tr>
<td>Marker header bars for some loci are gray</td>
<td>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.</td>
</tr>
</tbody>
</table>
| Alleles not called                            | To analyze samples with GeneMapper® ID-X software, at least one allelic ladder must be defined. Peaks in ILS were not captured. Not all WEN ILS 500 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. |
| Off-ladder alleles                            | An allelic ladder from a different run than the samples was used. Re-analyze samples with an allelic ladder from the same run. The GeneMapper® ID-X software requires that the allelic ladder be imported from the same folder as the sample. Be sure that the allelic ladder is in the same folder as the sample. Create a new project and re-analyze, as described in Section 6.D or 6.E.  
  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. |
| Size standard not called correctly           | Starting data point was incorrect for the partial range chosen in 6.E. Adjust the starting data point in the analysis method. Alternatively, use a full range for the analysis. |
### Symptoms

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size standard not called correctly (continued)</td>
<td>Extra peaks in size standard. Open the Size Match Editor. Highlight the extra peak, select “Edit” and select “delete size label”. Select “auto adjust sizes”. Peaks in ILS were not captured. Not all WEN ILS 500 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.</td>
</tr>
<tr>
<td>Peaks in size standard missing</td>
<td>If peaks are low-quality, redefine the size standard for the sample to skip these peaks.</td>
</tr>
<tr>
<td>Significantly raised baseline</td>
<td>Poor spectral calibration. Perform a new spectral calibration, and re-run the samples. Incorrect G5 spectral was active. Re-run samples, and confirm that the PowerPlex® 5-dye G5 spectral is set for G5. See instructions for instrument preparation in Section 5.</td>
</tr>
</tbody>
</table>

### 7.E. GeneMapper® ID Software

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alleles not called</td>
<td>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. Run was too short, and larger peaks in ILS were not captured. Not all WEN ILS 500 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.</td>
</tr>
<tr>
<td>Off-ladder alleles</td>
<td>An allelic ladder from a different run than the samples was used. Re-analyze samples with an allelic ladder from the same run. The GeneMapper® ID software requires that the allelic ladder be imported from the same folder as the sample. Be sure that the allelic ladder is in the same folder as the sample. Create a new project and re-analyze as described in Section 6.I or 6.J. Panels text file selected for analysis was incorrect for the STR system used. Assign correct panels text file that corresponds to the STR system used for amplification.</td>
</tr>
</tbody>
</table>
### 7.E. GeneMapper® ID Software (continued)

#### Symptoms | Causes and Comments
---|---
Off-ladder alleles (continued) | The allelic ladder was not identified as an allelic ladder in the Sample Type column.
The wrong analysis type was chosen for the analysis method. Be sure to use the HID analysis type.
The internal lane standard was not properly identified in the sample. Manually redefine the sizes of the size standard fragments in the sample.

Size standard not called correctly | Starting data point was incorrect for the partial range chosen in Section 6.I. 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”.
Peaks in ILS were not captured. Not all WEN ILS 500 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 | An incorrect internal lane standard was used.
If peaks are of low quality, redefine the size standard for the sample to skip these peaks.

Error message: “Either panel, size standard, or analysis method is invalid” | The size standard and analysis method were not in the same mode (“Classic” vs. “Basic or Advanced”). Be sure both files are set to the same mode, either Classic or Basic or Advanced mode.

No alleles called, but no error 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.
<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Causes and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Error message: “Both the Bin Set used in the Analysis Method and the Panel must belong to the same Chemistry Kit”</td>
<td>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.</td>
</tr>
<tr>
<td>Significantly raised baseline</td>
<td>Poor spectral calibration for the ABI PRISM® 3100 and 3100-Avant Genetic Analyzers and Applied Biosystems® 3130 and 3130xl Genetic Analyzers. Perform a new spectral calibration, and re-run the samples. Use of Classic mode analysis method. Use of Classic mode analysis on samples can result in baselines with more noise than those analyzed using the Basic or Advanced mode analysis method. Advanced mode analysis methods and size standards are recommended. Incorrect G5 spectral was active. Re-run samples, and confirm that the PowerPlex® 5-dye G5 spectral is set for G5. See instrument preparation instructions in Section 5.B, Step 6.</td>
</tr>
<tr>
<td>Error message after attempting to import panel and bin files: “Unable to save panel data: java.SQLEEException: ORA-00001: unique constraint (IFA.CKP_NNN) violated”</td>
<td>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.</td>
</tr>
<tr>
<td>Allelic ladder peaks labeled off-ladder</td>
<td>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.</td>
</tr>
</tbody>
</table>
8. References


9. Appendix

9.A. Advantages of Using the Loci in the PowerPlex® 18D System

The loci included in the PowerPlex® 18D System (Tables 4 and 5) were selected because they satisfy the needs of several major standardization bodies throughout the world. The PowerPlex® 18D System 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® 18D System a single-amplification system with a power of exclusion sufficient to resolve paternity disputes definitively. The Amelogenin locus is included in the PowerPlex® 18D System to allow gender identification of each sample. Finally, D2S1338 and D19S433 were added because of their popularity and inclusion in a number of state databases within the U.S. Table 6 lists the PowerPlex® 18D System alleles amplified from the 2800M Control DNA.

Terminal nucleotide addition (15,16) occurs when nonproofreading thermostable 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 20 minutes (17) to the amplification protocols to provide conditions for essentially complete terminal nucleotide addition when recommended amounts of template DNA are used.
9.A. Advantages of Using the Loci in the PowerPlex® 18D System (continued)

Table 4. The PowerPlex® 18D System Locus-Specific Information.

<table>
<thead>
<tr>
<th>STR Locus</th>
<th>Label</th>
<th>Chromosomal Location(^1)</th>
<th>GenBank® Locus and Locus Definition</th>
<th>Repeat Sequence(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penta E</td>
<td>FL</td>
<td>15q</td>
<td>NA</td>
<td>AAAGA</td>
</tr>
<tr>
<td>D18S51</td>
<td>FL</td>
<td>18q21.3</td>
<td>HUMUT574</td>
<td>AGAA (18)</td>
</tr>
<tr>
<td>D21S11</td>
<td>FL</td>
<td>21q11–21q21</td>
<td>HUMD21LOC</td>
<td>TCTA Complex (18)</td>
</tr>
<tr>
<td>TH01</td>
<td>FL</td>
<td>11p15.5</td>
<td>HUMTH01, human tyrosine hydroxylase gene</td>
<td>AATG (18)</td>
</tr>
<tr>
<td>D3S1358</td>
<td>FL</td>
<td>3p</td>
<td>NA</td>
<td>TCTA Complex</td>
</tr>
<tr>
<td>FGA</td>
<td>TMR-ET</td>
<td>4q28</td>
<td>HUMFIBRA, human fibrinogen alpha chain gene</td>
<td>TTTC Complex (18)</td>
</tr>
<tr>
<td>TPOX</td>
<td>TMR-ET</td>
<td>2p24–2pter</td>
<td>HUMTPOX, human thyroid peroxidase gene</td>
<td>AATG</td>
</tr>
<tr>
<td>D8S1179</td>
<td>TMR-ET</td>
<td>8q24.13</td>
<td>NA</td>
<td>TCTA Complex (18)</td>
</tr>
<tr>
<td>vWA</td>
<td>TMR-ET</td>
<td>12p13.31</td>
<td>HUMVWFA31, human von Willebrand factor gene</td>
<td>TCTA Complex (18)</td>
</tr>
<tr>
<td>Amelogenin(^3)</td>
<td>TMR-ET</td>
<td>Xp22.1–22.3 and Y</td>
<td>HUMAMEL, human Y chromosomal gene for Amelogenin-like protein</td>
<td>NA</td>
</tr>
<tr>
<td>Penta D</td>
<td>JOE</td>
<td>21q</td>
<td>NA</td>
<td>AAAGA</td>
</tr>
<tr>
<td>CSF1PO</td>
<td>JOE</td>
<td>5q33.3–34</td>
<td>HUMCSF1PO, human c-fms proto-oncogene for CSF-1 receptor gene</td>
<td>AGAT</td>
</tr>
<tr>
<td>D16S539</td>
<td>JOE</td>
<td>16q24–qter</td>
<td>NA</td>
<td>GATA</td>
</tr>
<tr>
<td>D7S820</td>
<td>JOE</td>
<td>7q11.21–22</td>
<td>NA</td>
<td>GATA</td>
</tr>
<tr>
<td>D13S317</td>
<td>JOE</td>
<td>13q22–q31</td>
<td>NA</td>
<td>TATC</td>
</tr>
<tr>
<td>D5S818</td>
<td>JOE</td>
<td>5q23.3–32</td>
<td>NA</td>
<td>AGAT</td>
</tr>
<tr>
<td>D2S1338</td>
<td>CXR-ET</td>
<td>2q35</td>
<td>TGCC/TTCC</td>
<td></td>
</tr>
<tr>
<td>D19S433</td>
<td>CXR-ET</td>
<td>19q12</td>
<td>AAGG Complex</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Information about chromosomal location of these loci can be found in references 19 and 20 and at:
www.cstl.nist.gov/biotech/strbase/chrom.htm

\(^2\)The August 1997 report (21,22) of the DNA Commission of the International Society for Forensic Haemogenetics (ISFH)
states, “1) for STR loci within coding genes, the coding strand shall be used and the repeat sequence motif defined using the first possible 5’ nucleotide of a repeat motif; and 2) for STR loci not associated with a coding gene, the first database entry or original literature description shall be used”.

\(^3\)Amelogenin is not an STR.

NA = not applicable
**Table 5. The PowerPlex® 18D System Allelic Ladder Information.**

<table>
<thead>
<tr>
<th>STR Locus</th>
<th>Label</th>
<th>Size Range of Allelic Ladder Components(^1,2) (bases)</th>
<th>Repeat Numbers of Allelic Ladder Components(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penta E</td>
<td>FL</td>
<td>379–474</td>
<td>5–24</td>
</tr>
<tr>
<td>TH01</td>
<td>FL</td>
<td>152–195</td>
<td>3–9, 9.3, 10–11, 13.3</td>
</tr>
<tr>
<td>D3S1358</td>
<td>FL</td>
<td>103–147</td>
<td>9–20</td>
</tr>
<tr>
<td>TPOX</td>
<td>TMR-ET</td>
<td>265–293</td>
<td>6–13</td>
</tr>
<tr>
<td>D8S1179</td>
<td>TMR-ET</td>
<td>203–251</td>
<td>7–19</td>
</tr>
<tr>
<td>vWA</td>
<td>TMR-ET</td>
<td>127–183</td>
<td>10–24</td>
</tr>
<tr>
<td>Amelogenin(^4)</td>
<td>TMR-ET</td>
<td>109, 115</td>
<td>X, Y</td>
</tr>
<tr>
<td>Penta D</td>
<td>JOE</td>
<td>376–449</td>
<td>2.2, 3.2, 5–17</td>
</tr>
<tr>
<td>CSF1PO</td>
<td>JOE</td>
<td>321–357</td>
<td>6–15</td>
</tr>
<tr>
<td>D16S539</td>
<td>JOE</td>
<td>264–304</td>
<td>5, 8–15</td>
</tr>
<tr>
<td>D7S820</td>
<td>JOE</td>
<td>218–250</td>
<td>6–14</td>
</tr>
<tr>
<td>D13S317</td>
<td>JOE</td>
<td>176–208</td>
<td>7–15</td>
</tr>
<tr>
<td>D5S818</td>
<td>JOE</td>
<td>122–158</td>
<td>7–16</td>
</tr>
<tr>
<td>D2S1338</td>
<td>CXR-ET</td>
<td>223–295</td>
<td>10, 12, 14–28</td>
</tr>
</tbody>
</table>

\(^1\)The length of each allele in the allelic ladder has been confirmed by sequence analyses.

\(^2\)When using an internal lane standard, such as the WEN ILS 500, the calculated sizes of allelic ladder components may differ from those listed. This occurs because different sequences in allelic ladder and ILS components may cause differences in migration. The dye label also affects migration of alleles.

\(^3\)For a current list of microvariants, see the Variant Allele Report published at the U.S. National Institute of Standards and Technology (NIST) web site at: [www.cstl.nist.gov/div831/strbase/](http://www.cstl.nist.gov/div831/strbase/)

\(^4\)Amelogenin is not an STR.
9.A. Advantages of Using the Loci in the PowerPlex® 18D System (continued)

Table 6. The PowerPlex® 18D System Allele Determinations for the 2800M Control DNA.

<table>
<thead>
<tr>
<th>STR Locus</th>
<th>2800M</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3S1358</td>
<td>17, 18</td>
</tr>
<tr>
<td>TH01</td>
<td>6, 9.3</td>
</tr>
<tr>
<td>D21S11</td>
<td>29, 31.2</td>
</tr>
<tr>
<td>D18S51</td>
<td>16, 18</td>
</tr>
<tr>
<td>Penta E</td>
<td>7, 14</td>
</tr>
<tr>
<td>D5S818</td>
<td>12, 12</td>
</tr>
<tr>
<td>D13S317</td>
<td>9, 11</td>
</tr>
<tr>
<td>D7S820</td>
<td>8, 11</td>
</tr>
<tr>
<td>D16S539</td>
<td>9, 13</td>
</tr>
<tr>
<td>CSF1PO</td>
<td>12, 12</td>
</tr>
<tr>
<td>Penta D</td>
<td>12, 13</td>
</tr>
<tr>
<td>Amelogenin</td>
<td>X, Y</td>
</tr>
<tr>
<td>vWA</td>
<td>16, 19</td>
</tr>
<tr>
<td>D8S1179</td>
<td>14, 15</td>
</tr>
<tr>
<td>TPOX</td>
<td>11, 11</td>
</tr>
<tr>
<td>FGA</td>
<td>20, 23</td>
</tr>
<tr>
<td>D19S433</td>
<td>13, 14</td>
</tr>
<tr>
<td>D2S1338</td>
<td>22, 25</td>
</tr>
</tbody>
</table>
9.B. The WEN Internal Lane Standard 500

The WEN Internal Lane Standard 500 contains 21 DNA fragments of 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases in length (Figure 23). Each fragment is labeled with WEN dye and can be detected separately (as a fifth color) in the presence of PowerPlex® 18D-amplified material. The WEN ILS 500 is designed for use in each CE injection to increase precision in analyses when using the PowerPlex® 18D System. Protocols to prepare and use this internal lane standard are provided in Section 5.

Low-level artifact peaks at approximately 132 and 176 bases may be observed with the WEN ILS 500 in the orange channel. The peak height of these artifacts may vary from lot to lot and may be labeled by the software. These peaks are not used during sizing of the peaks present in the sample.

![Figure 23. WEN Internal Lane Standard 500](image-url). An electropherogram showing the WEN Internal Lane Standard 500 fragments.
9.C. Protocol for Amplification of Extracted DNA

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block or Veriti® 96-Well 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

Testing at Promega has shown successful amplification of 1ng of template DNA in a 25µl reaction volume using the protocols detailed below. Alternatively, 5ng of template DNA can be used, but the number of cycles should be decreased to 27 cycles. Cycle number and quantity of input DNA can be optimized to achieve the desired sensitivity and signal. Protocols for direct amplification using the PowerPlex® 18D System are provided in Section 4.

The locus-to-locus balance for the PowerPlex® 18D System is optimized for direct-amplification methods. Amplification of extracted DNA may result in locus-to-locus imbalance.

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

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

Amplification Setup

1. Thaw the PowerPlex® D 5X Master Mix, PowerPlex® 18D 5X Primer Pair Mix and Amplification-Grade Water completely.
   
   Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

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

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

4. Add the final volume of each reagent listed in Table 7 to a sterile tube.
Table 7. PCR Amplification Mix for Amplification of Extracted DNA.

<table>
<thead>
<tr>
<th>PCR Amplification Mix Component¹</th>
<th>Volume Per Reaction</th>
<th>×</th>
<th>Number of Reactions</th>
<th>=</th>
<th>Final Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, Amplification Grade</td>
<td>to a final volume of 25.0µl</td>
<td>×</td>
<td>=</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PowerPlex® D 5X Master Mix</td>
<td>5.0µl</td>
<td>×</td>
<td>=</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PowerPlex® 18D 5X Primer Pair Mix</td>
<td>5.0µl</td>
<td>×</td>
<td>=</td>
<td></td>
<td></td>
</tr>
<tr>
<td>template DNA (1.0ng)²,⁴</td>
<td>up to 15.0µl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>total reaction volume</strong></td>
<td><strong>25µl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Add Water, Amplification Grade, to the tube first, then add PowerPlex® D 5X Master Mix and PowerPlex® 18D 5X Primer Pair Mix. The template DNA will be added at Step 6.

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

³Apparent DNA concentrations can differ, depending on the DNA quantification method used (23). The amount of DNA template recommended here is based on DNA concentrations determined by measuring absorbance at 260nm. We strongly recommend that you perform experiments to determine the optimal DNA amount based on your DNA quantification method.

⁴Alternatively, 5ng of DNA template can be used, but the number of cycles should be decreased to 27 cycles.

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

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

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

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

7. For the positive amplification control, vortex the tube of 2800M Control DNA, then dilute an aliquot to 1.0ng in the desired template DNA volume. Add 1.0ng of the diluted DNA to a reaction well containing PCR amplification mix.

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

9. Seal 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.
9.C. Protocol for Amplification of Extracted DNA (continued)

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including cycle number (26–30 cycles) and injection conditions (or loading volume) for each laboratory instrument. Testing at Promega shows that 30 cycles works well for 1ng of DNA. You will need to optimize cycle number. In-house validation should be done.

1. Place the MicroAmp® plate in the thermal cycler.
2. Select and run the recommended protocol.

Notes:

1. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
2. When using the GeneAmp® PCR System 9700, the program must be run with 9600 as the ramp speed. 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.

<table>
<thead>
<tr>
<th>Thermal Cycling Protocol</th>
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<tbody>
<tr>
<td>96°C for 2 minutes, then:</td>
</tr>
<tr>
<td>94°C for 10 seconds</td>
</tr>
<tr>
<td>60°C for 1 minute</td>
</tr>
<tr>
<td>for 30 cycles, then:</td>
</tr>
<tr>
<td>60°C for 20 minutes</td>
</tr>
<tr>
<td>4°C soak</td>
</tr>
</tbody>
</table>

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

Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.
9.D. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume

Testing at Promega has shown successful direct amplification of DNA from storage card punches in a 12.5µl reaction volume. This section contains a protocol for direct amplification of DNA from storage card punches in a 12.5µl reaction volume using the PowerPlex® 18D System and GeneAmp® PCR System 9700. When using the protocol detailed below, add only one 1.2mm storage card punch to each 12.5µl amplification reaction.

Materials to Be Supplied by the User

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

Notes:

1. It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is NOT recommended with the PowerPlex® 18D System.
2. You will need to optimize and validate the number of storage card punches per reaction in your laboratory. See the PCR Optimization recommendations at the end of this section.

FTA®-based sample types include:

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

NonFTA sample types include:

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

Pretreat nonFTA sample types with the PunchSolution™ Kit (Cat.# DC9271) to lyse nonFTA samples before adding the PCR amplification mix. For more information, see the PunchSolution™ Kit Technical Manual #TMD038. Failure to pretreat these samples may result in incomplete profiles.
9.D. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume (continued)

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

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

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

**Amplification Setup**

1. Thaw the PowerPlex® D 5X Master Mix, PowerPlex® 18D 5X Primer Pair Mix and Amplification-Grade Water completely.

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

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

   **Note:** The 5X AmpSolution™ Reagent should be thawed completely, mixed by vortexing and stored at 2–10°C. The reagent may be turbid after thawing or storage at 4°C. If this occurs, warm the buffer briefly at 37°C, and then vortex until clear. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Storing reagents in the refrigerator door can compromise stability.

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

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

5. Add the final volume of each reagent listed in Table 8 to a clean tube.
Table 8. PCR Amplification Mix for Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume.

<table>
<thead>
<tr>
<th>PCR Amplification Mix Component¹</th>
<th>Volume Per Reaction (µl)</th>
<th>Number of Reactions</th>
<th>Final Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, Amplification Grade</td>
<td>5.0</td>
<td>×</td>
<td>=</td>
</tr>
<tr>
<td>PowerPlex® D 5X Master Mix</td>
<td>2.5</td>
<td>×</td>
<td>=</td>
</tr>
<tr>
<td>PowerPlex® 18D 5X Primer Pair Mix</td>
<td>2.5</td>
<td>×</td>
<td>=</td>
</tr>
<tr>
<td>5X AmpSolution™ Reagent</td>
<td>2.5</td>
<td>×</td>
<td>=</td>
</tr>
<tr>
<td><strong>total reaction volume</strong></td>
<td><strong>12.5</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Add Water, Amplification Grade, to the tube first, and then add PowerPlex® D 5X Master Mix, PowerPlex® 18D 5X Primer Pair Mix and 5X AmpSolution™ Reagent. For FTA® card punches, the template DNA will be added at Step 7.

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

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

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

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

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

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

**Notes:**

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

2. Optimization of the amount of 2800M Control DNA may be required depending on thermal cycling conditions and laboratory preferences. Typically, 5ng of 2800M Control DNA is sufficient to provide a robust profile using the cycle numbers recommended here. A one-cycle reduction in cycle number will require a twofold increase in mass of DNA template to generate similar signal intensity. Similarly, a one-cycle increase in cycle number will require a twofold reduction in the amount of 2800M Control DNA to avoid signal saturation.
9.D. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume (continued)

9. Reserve a well containing PCR amplification mix as a negative amplification control.
   **Note:** An additional negative control with a blank punch may be performed to detect contamination from the storage card or punch device.

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

**Thermal Cycling**

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

**Note:** It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is NOT recommended with the PowerPlex® 18D System.

1. Place the reaction plate or tubes in the thermal cycler. Select and run the recommended protocol.
   **Note:** When using the GeneAmp® PCR System 9700, the program must be run with 9600 as the ramp speed. 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.

<table>
<thead>
<tr>
<th><strong>Thermal Cycling Protocol</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>96°C for 2 minutes, then:</td>
</tr>
<tr>
<td>94°C for 10 seconds</td>
</tr>
<tr>
<td>60°C for 1 minute</td>
</tr>
<tr>
<td>for 26 cycles, then:</td>
</tr>
<tr>
<td>60°C for 20 minutes</td>
</tr>
<tr>
<td>4°C soak</td>
</tr>
</tbody>
</table>

2. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at −20°C in a light-protected box.
   **Note:** Long-term storage of amplified samples at 4°C or higher may produce artifacts.
PCR Optimization

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

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

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

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

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

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

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

Testing at Promega has shown successful direct amplification of DNA from swabs in a 12.5µl reaction volume. This section contains a protocol for amplifying DNA from swab extracts in a 12.5µl reaction volume using the PowerPlex® 18D System and GeneAmp® PCR System 9700.

Materials to Be Supplied by the User

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

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

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is NOT recommended with the PowerPlex® 18D System.
9.E. Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume (continued)

Amplification Setup

1. Thaw the PowerPlex® D 5X Master Mix, PowerPlex® 18D 5X Primer Pair Mix and Amplification-Grade Water completely.
   
   **Note:** Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

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

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

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

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

<table>
<thead>
<tr>
<th>PCR Amplification Mix Component¹</th>
<th>Volume Per Reaction</th>
<th>Number of Reactions</th>
<th>Final Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, Amplification Grade</td>
<td>5.5µl</td>
<td>×</td>
<td>=</td>
</tr>
<tr>
<td>PowerPlex® D 5X Master Mix</td>
<td>2.5µl</td>
<td>×</td>
<td>=</td>
</tr>
<tr>
<td>PowerPlex® 18D 5X Primer Pair Mix</td>
<td>2.5µl</td>
<td>×</td>
<td>=</td>
</tr>
<tr>
<td>swab extract</td>
<td>2.0µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>total reaction volume</strong></td>
<td><strong>12.5µl</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Add Water, Amplification Grade, to the tube first, and then add PowerPlex® D 5X Master Mix and PowerPlex® 18D 5X Primer Pair Mix. The swab extract will be added at Step 6.

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

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

6. Pipet 2.0µl of swab extract for each sample into the appropriate well of the reaction plate.
7. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 2.5ng/μl. Add 2μl (5ng) to a reaction well containing 10.5μl of PCR amplification mix.

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

8. For the negative amplification control, pipet 2μl of Amplification-Grade Water 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 the plate to bring contents to the bottom of the wells and remove any air bubbles.

### Thermal Cycling

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

**Note:** It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is NOT recommended with the PowerPlex® 18D System.

1. Place the reaction plate or tubes in the thermal cycler. Select and run the recommended protocol.

**Note:** When using the GeneAmp® PCR System, the program must be run with 9600 as the ramp speed. 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

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>96°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>94°C</td>
<td>10 seconds</td>
</tr>
<tr>
<td>60°C</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td>for 26 cycles, then:</td>
</tr>
<tr>
<td>60°C</td>
<td>20 minutes</td>
</tr>
<tr>
<td>4°C</td>
<td>soak</td>
</tr>
</tbody>
</table>

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

**Note:** Long-term storage of amplified samples at 4°C or higher may produce artifacts.
9.E. Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume (continued)

PCR Optimization

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

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Prepare three identical reaction plates with aliquots of the same swab extracts.
3. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (25, 26 and 27 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.

9.F. Composition of Buffers and Solutions

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

1.21g Tris base
0.037g EDTA (Na₂EDTA • 2H₂O)

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 (Na₂EDTA • 2H₂O)
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.G. Related Products

Fluorescent STR Systems

<table>
<thead>
<tr>
<th>Product</th>
<th>Size</th>
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<tbody>
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<td></td>
<td>400 reactions</td>
<td>DC1610</td>
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<tr>
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<td>DC6591</td>
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<td>PowerPlex® 16 Monoplex System, Penta D (JOE)*</td>
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<td>DC6651</td>
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<td>PowerPlex® ES Monoplex System, SE33 (JOE)*</td>
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<td>PowerPlex® Y23 System</td>
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<td>200 reactions</td>
<td>DC2320</td>
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</table>

Not for Medical Diagnostic Use.

*Additional monoplexes are available.

Accessory Components

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</tr>
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<td>10 each</td>
<td>CE2696</td>
</tr>
<tr>
<td>PowerPlex® 5C Matrix Standard</td>
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<tr>
<td>SwabSolution™ Kit</td>
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<td>5X AmpSolution™ Reagent</td>
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Not for Medical Diagnostic Use.
Sample Preparation and DNA Quantification Systems

<table>
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<tr>
<th>Product</th>
<th>Size</th>
<th>Cat. #</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA IQ™ System</td>
<td>100 reactions</td>
<td>DC6701</td>
</tr>
<tr>
<td></td>
<td>400 reactions</td>
<td>DC6700</td>
</tr>
<tr>
<td>Differex™ System*</td>
<td>50 samples</td>
<td>DC6801</td>
</tr>
<tr>
<td></td>
<td>200 samples</td>
<td>DC6800</td>
</tr>
<tr>
<td>Tissue and Hair Extraction Kit (for use with DNA IQ™)</td>
<td>100 reactions</td>
<td>DC6740</td>
</tr>
<tr>
<td>Maxwell® FSC Instrument</td>
<td>1 each</td>
<td>AS4600</td>
</tr>
<tr>
<td>Maxwell® FSC DNA IQ™ Casework Kit</td>
<td>48 preps</td>
<td>AS1550</td>
</tr>
<tr>
<td>Casework Extraction Kit</td>
<td>100 preps</td>
<td>DC6745</td>
</tr>
<tr>
<td>PowerQuant® System*</td>
<td>200 reactions</td>
<td>PQ5002</td>
</tr>
<tr>
<td></td>
<td>800 reactions</td>
<td>PQ5008</td>
</tr>
<tr>
<td>Plexor® HY System*</td>
<td>200 reactions</td>
<td>DC1001</td>
</tr>
<tr>
<td></td>
<td>800 reactions</td>
<td>DC1000</td>
</tr>
<tr>
<td>Slicprep™ 96 Device</td>
<td>10 pack</td>
<td>V1391</td>
</tr>
</tbody>
</table>

*Not for Medical Diagnostic Use.

9.H. Summary of Changes

The following changes were made to the 4/17 revision of this document:

1. Added support for half-volume direct-amplification reactions and use of the Veriti® 96-Well Thermal Cycler.
2. Added caution against storing PCR amplification mix prior to thermal cycling.
3. Revised document name to specify that this manual is for use on Applied Biosystems instruments.
4. Incorporated other general updates.


(c) U.S. Pat. No. 6,238,863, Chinese Pat. No. ZL99802696.4, European Pat. No. 1058727, Japanese Pat. No. 4494630 and other patents pending.

(d) U.S. Pat. No. 9,139,868 and other patents pending.

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

(f) TMR-ET, CXR-ET and WEN dyes are proprietary.

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