

PowerPlex® Fusion System for Use on the Spectrum CE System Technical Manual

Instructions for Use of Products DC2402 and DC2408



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All technical literature is available at: www.promega.com/protocols/

Visit the web site to verify that you are using the most current version of this Technical Manual.

E-mail Promega Technical Services if you have questions on use of this system: genetic@promega.com

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® Fusion System^(a-b) is a 24-locus multiplex for human identification applications including forensic analysis, relationship testing and research use. This five-color system allows co-amplification and fluorescent detection of the 20 autosomal loci in the expanded CODIS core loci (CSF1PO, FGA, TH01, TPOX, vWA, D1S1656, D2S1338, D2S441, D3S1358, D5S818, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11 and D22S1045) as well as Amelogenin and DYS391 for gender determination. The Penta D and Penta E loci are also included to increase discrimination and allow searching of databases that include profiles with these loci. This extended panel of STR markers is intended to satisfy both CODIS and ESS recommendations.

The PowerPlex® Fusion 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® Fusion 5X Master Mix.

This manual contains protocols for use of the PowerPlex® Fusion System with the GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler and ProFlex® PCR System, in addition to a protocol to separate amplified products and detect separated material on the Spectrum CE System. 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. A protocol to operate the Spectrum CE System is available separately. See the *Spectrum CE System Operating Manual #TMD052*.

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

Product Components and Storage Conditions

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PRODUCT	SIZE	CAT.#
PowerPlex® Fusion System	200 reactions	DC2402

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

Pre-Amplification Components Box

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

Post-Amplification Components Box

- 100µl PowerPlex® Fusion Allelic Ladder Mix
- 200µl WEN Internal Lane Standard 500

PRODUCT	SIZE	CAT.#
PowerPlex® Fusion System	800 reactions	DC2408

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

Pre-Amplification Components Box

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

Post-Amplification Components Box

- 4 × 100µl PowerPlex® Fusion Allelic Ladder Mix
- 4 × 200µl WEN Internal Lane Standard 500



The PowerPlex® Fusion 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. Store this component with the pre-amplification components after opening.

Storage Conditions

Upon receipt, store all components at -30°C to -10°C in a nonfrost-free freezer. Make sure that the 2800M Control DNA is stored at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$ for **at least 24 hours** before use. After the first use, store the WEN Internal Lane Standard 500 (WEN ILS 500) at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$, protected from light; do not refreeze. The PowerPlex® Fusion 5X Primer Pair Mix, PowerPlex® Fusion 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® Fusion System components may be stored for up to 1 year at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$ without loss of activity.

Available Separately

PRODUCT	SIZE	CAT.#
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
5X AmpSolution™ Reagent	500µl	DM1231
GeneMarker®HID Software for Spectrum CE Systems, Local	1 seat	CE3001
GeneMarker®HID Software for Spectrum CE Systems, Network	1 seat	CE3010
GeneMarker®HID Software for Spectrum CE Systems, Client	1 seat	CE3011

The PunchSolution™ Kit is required to process nonlytic card punches prior to direct amplification. The SwabSolution™ Kit is required to process swabs prior to direct amplification. The 5X AmpSolution™ Reagent is required for direct amplification of DNA from storage card punches in a 12.5µl reaction volume. Both the PunchSolution™ Kit and SwabSolution™ Kit include the 5X AmpSolution™ Reagent.

The proper panels, bins and stutter text files for use with GeneMapper® *ID-X* software are available for download at: www.promega.com/PowerPlexPanels.

The proper panel .xml file for use with GeneMarker®HID Software for Spectrum CE Systems (GMHID-Spectrum) is preloaded in GMHID-Spectrum or downloadable at: www.promega.com/PowerPlexPanels.

The PowerPlex® 5C Matrix Standard (Cat.# DG4850), available separately, is required for initial setup of the color separation matrix (see Section 3.2).

Protocols for DNA Amplification Using the PowerPlex® Fusion System

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The PowerPlex® Fusion System was developed for amplification of extracted DNA and direct-amplification samples. Slight protocol variations are recommended for optimal performance with each template source. Protocols for amplification in a 25µl reaction volume using extracted DNA (Section 4.1), lytic and nonlytic storage card punches (Section 4.2) and swabs (Section 4.3) are included in the following amplification sections. Protocols for amplification in a 12.5µl reaction volume using lytic and nonlytic storage card punches and swabs are included in Sections 11.3 and 11.4, respectively.

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

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

The use of gloves and aerosol-resistant pipette tips is highly recommended to prevent cross-contamination. Keep all pre- 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 9.

4.1. Amplification of Extracted DNA in a 25µl Reaction Volume

The following protocol has been routinely used to amplify 0.25–0.5ng of template DNA in a 25µl reaction volume using the PowerPlex® Fusion System.

Materials to be Supplied by the User

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

Amplification Setup

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

Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 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 it appropriately. Alternatively, determine the number of clean 0.2ml reaction tubes required, and label them appropriately.
4. Add the final volume of each reagent listed in Table 1 to a clean tube.

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

PCR Amplification Mix Component ¹	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25µl	×		=	
PowerPlex® Fusion 5X Master Mix	5µl	×		=	
PowerPlex® Fusion 5X Primer Pair Mix	5µl	×		=	
template DNA (0.25–0.5ng) ^{2,3,4}	up to 15µl				
total reaction volume	25µl				

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

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

³Apparent DNA concentrations can differ, depending on the DNA quantification method used (13). We strongly recommend that you perform experiments to determine the optimal DNA amount based on your DNA quantification method.

⁴The PowerPlex® Fusion System was optimized and balanced for 0.25–0.5ng of DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different.

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



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

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

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

Note: The PowerPlex® Fusion System is optimized and balanced using 0.25–0.5ng of DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 0.5ng in the desired template volume. Add 0.5ng of diluted DNA to a reaction well containing PCR amplification mix.
8. For the negative amplification control, pipet Water, Amplification Grade, or TE⁻⁴ buffer instead of template DNA into a reaction well containing PCR amplification mix.
9. Seal or cap the plate, or close the tubes.
Optional: Briefly centrifuge the plate to bring contents to the bottom of the wells and remove any air bubbles.

Thermal Cycling

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

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 1.

Notes:

1. When using the ProFlex® PCR System, use the 9700 Simulation Mode as the ramp speed.
2. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
3. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol

96°C for 1 minute, then:

94°C for 10 seconds

59°C for 1 minute

72°C for 30 seconds

for 30 cycles, then:

60°C for 10 minutes

4°C soak

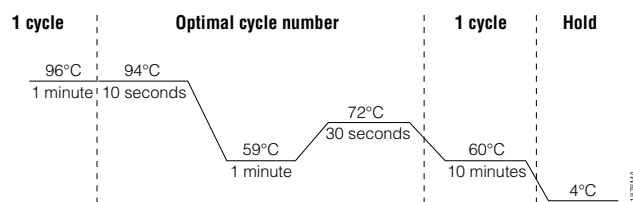


Figure 1. Thermal cycling protocol for the GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler and ProFlex® PCR System.

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

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

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

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block, Veriti® 96-well Thermal Cycler or ProFlex® PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plates or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- PunchSolution™ Kit (Cat.# DC9271) for nonlytic 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 25 μl reaction volume using the PowerPlex® Fusion System and GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler or ProFlex® PCR System. A protocol for direct amplification of DNA from storage card punches in a 12.5 μl reaction volume is provided in Section 11.3.

We recommend amplifying one or two 1.2mm punches of a storage card containing buccal cells or one 1.2mm punch of a storage card containing whole blood in a 25 μl reaction using the protocols detailed below.

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

Lytic card sample types include:

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

Nonlytic card sample types include:

- Buccal samples on Bode Buccal DNA Collector™ devices
- Blood and buccal samples on nonlytic cards (e.g., S&S 903)

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

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

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

Note: Static may be problematic when adding a punch to a well. For lytic card punches, adding PCR amplification mix to the well before adding the punch may help alleviate static problems. For nonlytic 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® Fusion 5X Master Mix, PowerPlex® Fusion 5X Primer Pair Mix and Water, Amplification Grade, completely.

Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 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 it appropriately. Alternatively, determine the number of clean 0.2ml reaction tubes required and label them 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 Storage Card Punches in a 25µl Reaction Volume.

PCR Amplification Mix Component ¹	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	15µl	×		=	
PowerPlex® Fusion 5X Master Mix	5µl	×		=	
PowerPlex® Fusion 5X Primer Pair Mix	5µl	×		=	
total reaction volume	25µl				

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

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



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

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

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

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

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

Notes:

- Do not include blank storage card punches in the positive control reactions.
 - Optimization of the amount of 2800M Control DNA may be required depending on thermal cycling conditions and laboratory preferences.
8. Reserve a well containing PCR amplification mix as a negative amplification control.
- Note:** An additional negative control with a blank punch may be performed to detect contamination from the storage card or punch device.
9. Seal or cap the plate, or close the tubes. Briefly centrifuge the reactions to bring storage card punches to the bottom of the wells and remove any air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols, including the number of storage card punches, cycle number (25–28 cycles), injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 27 cycles works well for a variety of sample types. Cycle number should be optimized in each laboratory for each sample type.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 2.

Notes:

1. When using the ProFlex® PCR System, use the 9700 Simulation Mode as the ramp speed.
2. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
3. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.
4. The final extension for direct amplification was extended to 20 minutes compared to 10 minutes for the extracted DNA protocol to allow sufficient time for adenylation of large amounts of amplicon.

Thermal Cycling Protocol

96°C for 1 minute, then:

94°C for 10 seconds

59°C for 1 minute

72°C for 30 seconds

for 27 cycles, then:

60°C for 20 minutes

4°C soak

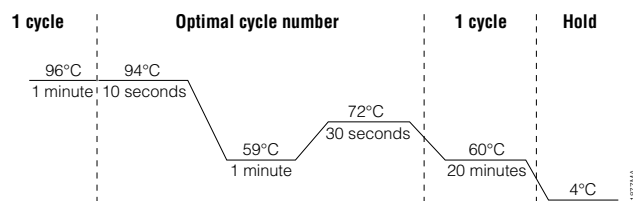


Figure 2. Thermal cycling protocol for the GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler and ProFlex® PCR System.

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

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

PCR Optimization

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

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Depending on your preferred protocol, place one or two 1.2mm storage card punches containing buccal cells or one 1.2mm punch of a storage card containing whole blood into each well of a reaction plate. Be sure to pretreat nonlytic samples with the PunchSolution™ Kit (Cat.# DC9271).
3. Prepare four 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 (25–28 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.

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

Materials to Be Supplied by the User

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

This section contains a protocol for amplifying DNA from swab extracts in a 25µl reaction volume using the PowerPlex® Fusion System and GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler or ProFlex® PCR System. A protocol for direct amplification from swab extracts in a 12.5µl reaction volume is provided in Section 11.4.

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

Amplification Setup

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

Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 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 it appropriately. Alternatively, determine the number of clean 0.2ml reaction tubes required, and label them appropriately.
4. Add the final volume of each reagent listed in Table 3 to a clean tube.

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

PCR Amplification Mix Component ¹	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	13µl	×		=	
PowerPlex® Fusion 5X Master Mix	5µl	×		=	
PowerPlex® Fusion 5X Primer Pair Mix	5µl	×		=	
swab extract	2µl				
total reaction volume	25µl				

¹Add Water, Amplification Grade, to the tube first, and then add PowerPlex® Fusion 5X Master Mix and PowerPlex® Fusion 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 23µl of PCR amplification mix into each reaction well.



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

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

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

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

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

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

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

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

Optional: Briefly centrifuge the reactions 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 (25–28 cycles), injection conditions and loading volume for your laboratory instrumentation. 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.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 3.

Notes:

1. When using the ProFlex® PCR System, use the 9700 Simulation Mode as the ramp speed.
2. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
3. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a silver or gold-plated sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.
4. The final extension for direct amplification was extended to 20 minutes compared to 10 minutes for the extracted DNA protocol to allow sufficient time for adenylation of large amounts of amplicon.

Thermal Cycling Protocol

96°C for 1 minute, then:

94°C for 10 seconds

59°C for 1 minute

72°C for 30 seconds

for 27 cycles, then:

60°C for 20 minutes

4°C soak

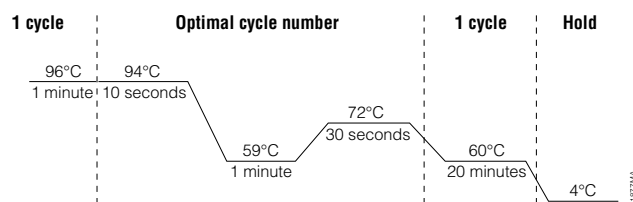


Figure 3. Thermal cycling protocol for the GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler and ProFlex® PCR System.

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

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

PCR Optimization

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

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Prepare four 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 (25–28 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.

Before You Begin

3.1. Precautions

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

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

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

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.

3.2. Spectral Calibration

Proper spectral calibration is critical to evaluate multicolor systems with the Spectrum CE System. A matrix must be generated for each individual instrument.

For protocol and additional information about matrix generation and spectral calibration, see the *PowerPlex® Matrix Standards for Use on the Spectrum CE System Technical Manual #TMD068*. This manual is available online at: www.promega.com/protocols/

Detection of Amplified Fragments Using the Spectrum CE System

5

Materials to be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice, ice-water bath or freezer plate block
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- Spectrum Capillary Array, 8-Capillary (Cat.# CE2008)
- Spectrum Polymer4, 384 Wells (Cat.# CE2048) or Spectrum Polymer4, 960 Wells (Cat.# CE2040)
- Spectrum Buffer (Cat.# CE2001)
- Spectrum Cathode Septa Mat (Cat.# CE2002)
- Septa Mat, 96-Well (Cat.# CE2696) or equivalent Applied Biosystems septa mat
- Spectrum Plate Base & Retainer, 96-Well (Cat.# CE5004)
- MicroAmp® optical 96-well plate (or equivalent; 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.

Note: Wear gloves when handling consumables and sample cartridges.

5.1. Sample Preparation

1. Prepare a loading cocktail by combining and mixing internal lane standard (ILS) and Hi-Di™ formamide as follows: $[(0.5\mu\text{l ILS}) \times (\# \text{ samples})] + [(9.5\mu\text{l formamide}) \times (\# \text{ samples})]$

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 10 μl of formamide/internal lane standard mix into each well of the 96-well plate.
4. Add 1 μl of amplified sample (or 1 μl of Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

Notes:

1. Instrument detection limits vary; therefore, injection time or the amount of sample mixed with loading cocktail may need to be increased or decreased. To modify the injection time, refer to the *Spectrum CE System Operating Manual #TMD052*. If peak heights are higher than desired, use less DNA template in the amplification reactions or reduce the number of cycles in the amplification program to achieve the desired signal intensity.
2. Use a volume of allelic ladder that results in peak heights that are all consistently above the peak amplitude threshold determined as part of your internal validation.
3. Include an injection of allelic ladder every 2 injections (16 samples).
5. Centrifuge the plate briefly to remove air bubbles from the wells.
6. Denature samples at 95°C for 3 minutes, and then immediately chill on crushed ice or a freezer plate block or in an ice-water bath for 3 minutes. Denature samples just prior to loading the instrument.

5.2. Instrument Preparation

Refer to the *Spectrum CE System Operating Manual #TMD052* for the instrument maintenance schedule and instructions to install the capillary array, anode buffer, cathode buffer and polymer pouch. The appropriate spectral calibration must be performed and accepted on the instrument prior to use for samples. Refer to the *PowerPlex® Matrix Standards for Use on the Spectrum CE System Technical Manual #TMD068* for instructions on how to perform a spectral calibration.

Open the Spectrum Control Software (SCS). Upon launching the SCS, the 'Home' screen will be displayed (Figure 4). This screen provides access to the four workflow menus (Home, Review Plates, Maintenance and Settings; see Figure 5) as well as status indicators. The screen is divided into three sections: Header, Task Pane and Status Bar.

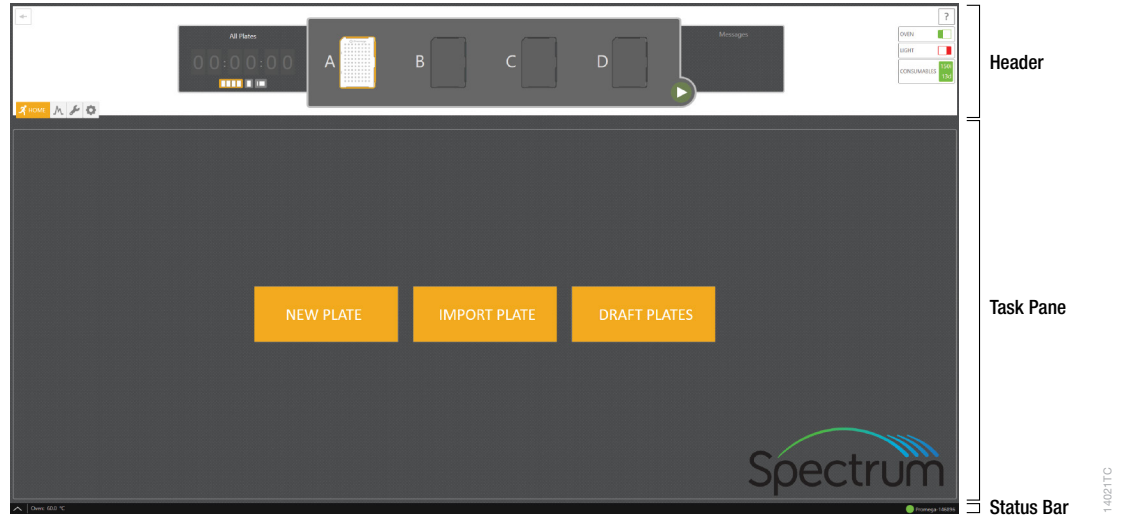


Figure 4. Spectrum Control Software 'Home' screen.

The header ribbon (Figure 5) is fixed and remains available to the user throughout all navigation processes. This area contains Status Indicators, Message Center, Run Time Indicator, Start Arrow and Workflow Menu. The header also shows the status of the plate positions within the Plate Drawer.

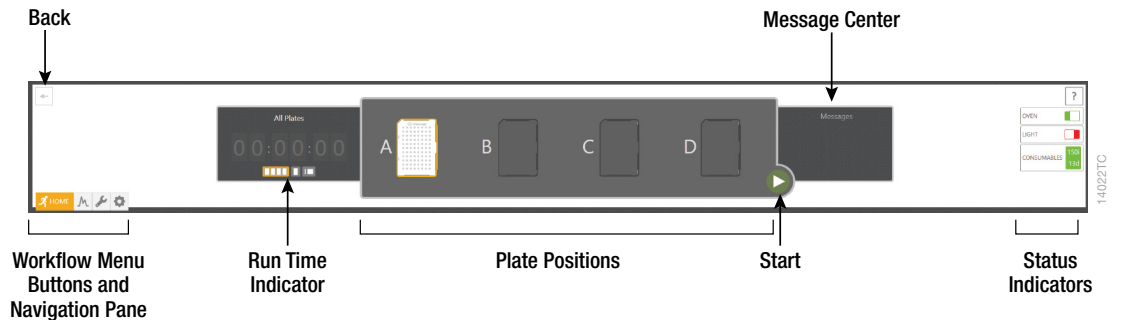


Figure 5. Header ribbon.

Each button in the Workflow Menu provides access to a list of workflow-specific functions. Workflow Buttons and the Navigation Pane are highlighted to indicate the user's current location within the SCS.

Icon	Button Name	Function
	Back	Navigates to the previous screen
	Start/stop	Starts or stops all currently scheduled plates (enabled when plates are linked for run and sufficient consumables are available)
	Home	Displays Home Menu
	Review Plates	Displays Review Plates Menu
	Maintenance	Displays Maintenance Menu
	Settings	Displays Settings Menu

The SCS contains several indicators in the header. Each indicator provides information about a specific function or component. Refer to the *Spectrum CE System Operating Manual #TMD052* for more information.

Before starting a run, ensure all consumables are installed and in sufficient supply. For best results, use unexpired reagents that are within the recommended use range. Refer to the 'Consumables' screen (Figure 6) to determine if any consumables need to be replaced. To access the 'Consumables' screen, select the **Consumables** status indicator in the header on the SCS screen (Figure 5). The 'Consumables' screen (Figure 6) displays information for the four RFID-tagged consumables on the instrument: Polymer, Capillary Array and Anode and Cathode Buffers.

The screenshot shows the 'Consumables' screen with four panels, each representing a different consumable. Each panel includes a status bar, RFID information table, and a 'REPLACE...' button. Brackets on the right side of the image label these elements as 'Status Bars', 'RFID Info', and 'Replace Wizards'.

Consumable	Remaining Injections	Days Remaining	Part Number	Lot Number	Initial Installation Date	Expiration Date	Injections	Installation Count
POLYMER	164/154	11/14	CE2048	0000487193	2/27/2022	7/21/2024	0	1
CAPILLARY ARRAY	285/300		CE2008	0421-19	2/27/2022	7/21/2024	12	1
ANODE BUFFER	228/240	11/14	CE200A	0000480162	2/27/2022	7/21/2024	12	1
CATHODE BUFFER	228/240	11/14	CE200B	0000480163	2/27/2022	7/21/2024	12	1

Figure 6. 'Consumables' screen.

The following information is tracked through these tags:

- Part Number
- Lot Number
- Initial Installation Date
- Expiration Date
- Injections
- Installation Count

Refer to the *Spectrum CE System Operating Manual* #TMD052 for more details and information on installing consumables.

Select the **Oven Heater Indicator** in the header (Figure 5) to start preheating the oven to 60°C. The oven temperature will be displayed in the status bar at the bottom of the screen and the indicator will change from red to green when the oven preheating is complete.

Note: We recommend you preheat the oven for at least 30 minutes prior to starting a run. The oven will automatically turn off after 2 hours if a run is not started.

5.3 Create a Plate Record

On the 'Home' screen, there are three options displayed for plate setup: New Plate, Import Plate and Draft Plates (Figure 4).

Create a plate record for each plate run. This record defines the Sample IDs of each sample on a plate, the protocol to apply to each injection and other user-defined items. There are four main methods for preparing a new plate record:

- Manually entering a new plate record
- Manually importing a plate record document
- Automatically importing a plate record document through the barcoding process
- Duplicating information from a completed plate

The following instructions are for manually entering a new plate record. For the other methods, refer to the *Spectrum CE System Operating Manual* #TMD052.

1. Select **New Plate** from the 'Home' screen. This will open a blank 'Plate' screen that is divided into three sections: Plate Information, Sample Information and Injection Information (Figure 7).

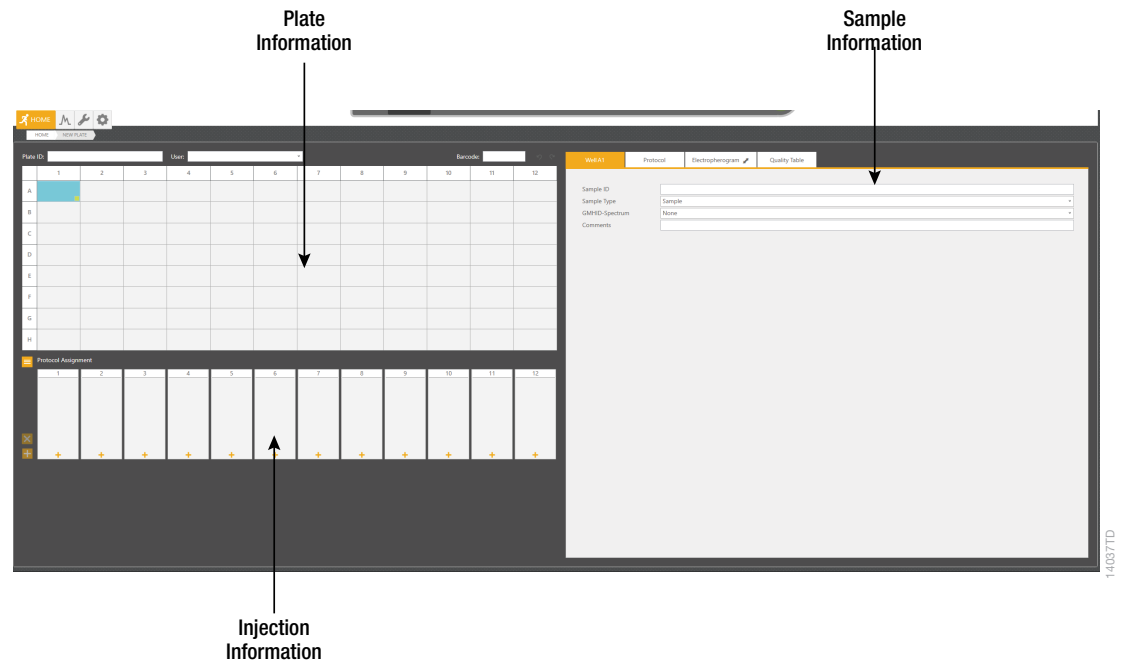


Figure 7. 'Plate' screen.

2. Use the default Plate ID that includes date and time or enter information into the 'Plate ID' field (Figure 8).
3. If desired, enter user information into the 'User' field (Figure 8).

Note: The 'User' field is a user-defined section that can be used to search or filter previously created or completed plates (refer to the *Spectrum CE System Operating Manual #TMD052* for information on reviewing plate records). Previously entered user information is available via the drop-down option on the right side of the field (Figure 8).

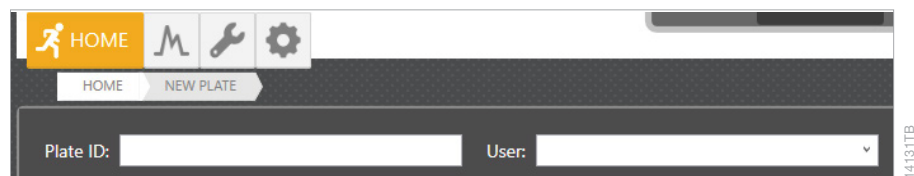


Figure 8. 'Plate ID' and 'User' fields of the plate screen.

4. Enter Sample IDs (sample names) for each sample on the plate.

Enter a Sample ID directly in the cells of the plate map area of the Plate Information section by selecting the appropriate well(s) and entering the ID information. Right clicking a sample well provides editing options: Cut, Copy, Paste and Undo. There are also **Undo** and **Redo** shortcut buttons to the right of the bar code field.

Alternatively, enter Sample IDs in the 'Sample ID' field of the 'Sample Information' section (Figure 7).

The green box in the lower right corner of a sample well (Figure 9) can be used to fill other wells with the same information by left clicking and dragging the green box to the other wells.

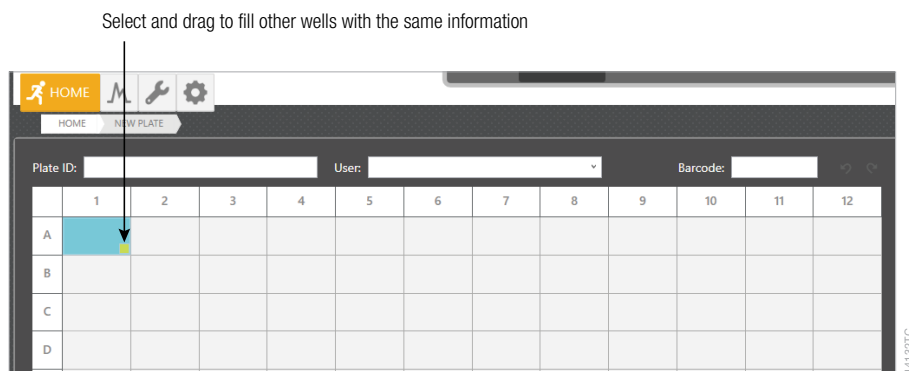


Figure 9. Filling information across multiple sample wells.

5. Select sample types for each sample or group of samples on the plate. Select the sample(s) then use the drop-down menu (Figure 10) to designate the well as Sample, Ladder, Positive Control or Negative Control. These designations will be included in the output file for downstream analysis.

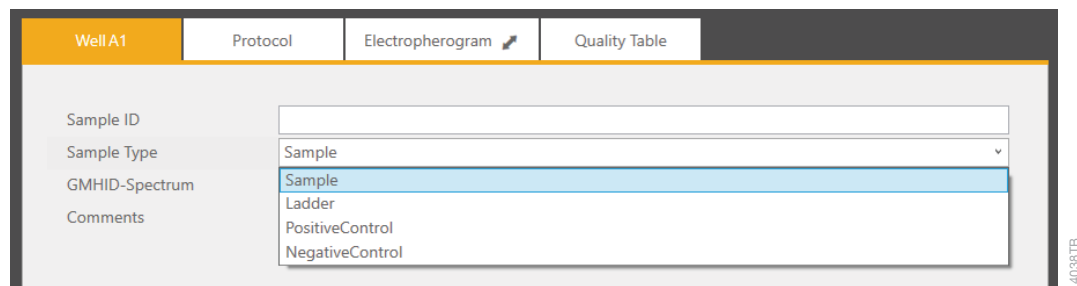


Figure 10. Drop-down options for sample type.

6. **Optional:** Select **PowerPlex_Fusion** in the GMHID-Spectrum drop-down menu (Figure 11).

Notes:

- a. GMHID-Spectrum will generate a Quality Table populated with quality metrics based on the data generated and the template selected. These templates are preloaded and edited within the GeneMarker®HID Software for Spectrum CE Systems (GMHID-Spectrum). Select the appropriate sample type for each well to ensure GMHID-Spectrum functionality. Refer to the *Spectrum CE System Operating Manual #TMD052* and *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555* for more information.
- b. Selecting the GMHID-Spectrum template does not affect downstream analysis.

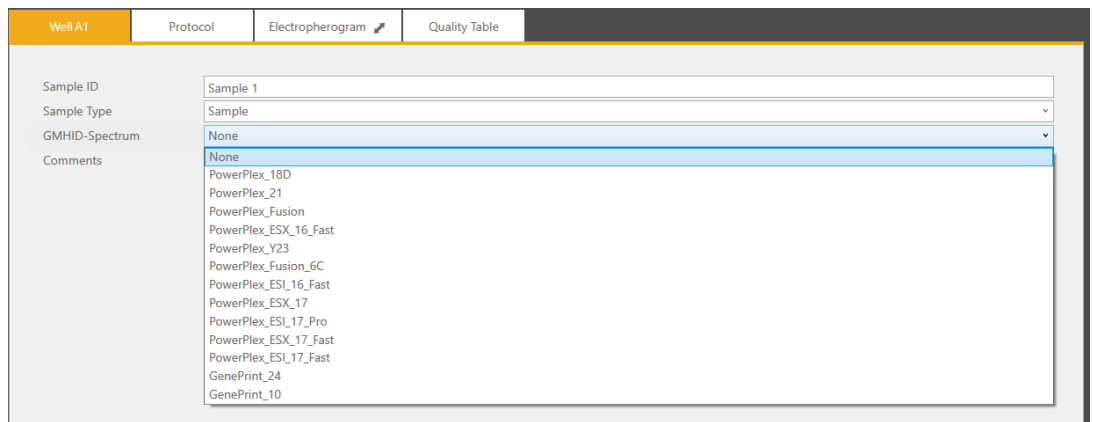


Figure 11. Drop-down options for GMHID-Spectrum run wizard templates.

7. There are three methods to assign run protocol(s) to samples:

Note: When a run protocol has been assigned to an injection set, an injection number will be assigned and displayed in a circle within the injection set box.

- To add a run protocol to selected samples, highlight the desired wells (Ctrl + right-click or drag with the mouse). Then right-click, mouse over **Add Injection To Selected Wells** then select the **Promega 5-Dye (5C)** protocol from the pop-up menu.
- To add a run protocol to individual injections, select the **+** button under the box for that injection set then select the **Promega 5-Dye (5C)** protocol from the pop-up menu.
- To add a run protocol to multiple injections, highlight the desired injections in the plate map (click-drag, Ctrl + click or click the upper left cell). Then select the **+** button on the left side of the injection information section and choose **Promega 5-Dye (5C)** (Figure 12) from the pop-up menu.

Notes:

- a. A sample ID must be present in at least one well of an injection set for a run protocol to be assigned to the set.
- b. Protocols can be assigned to selected samples, individual injection sets or multiple injection sets.
- c. The Spectrum Control Software includes preloaded run protocols for use with Promega chemistries. Refer to the *Spectrum CE System Operating Manual #TMD052* for instructions on creating a new protocol or modifying an existing protocol.

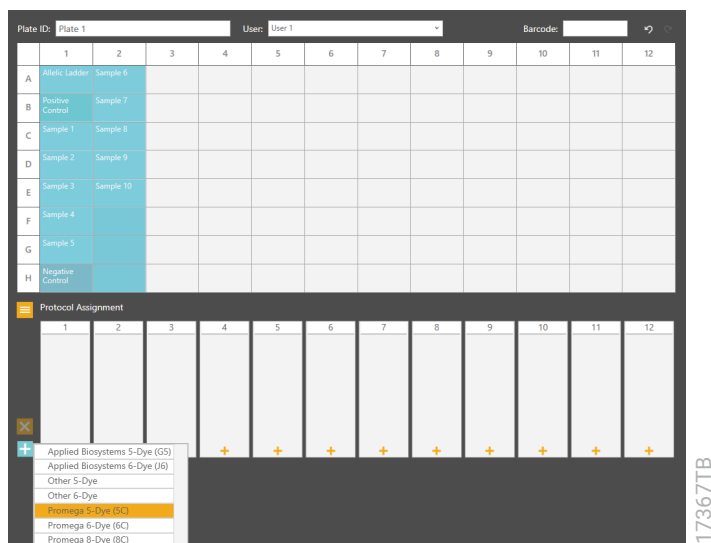


Figure 12. Assigning run protocol to multiple injections.

8. Verify the assigned run protocol(s).

To verify the parameters of the assigned run protocol(s), select the injection number or a sample well in the plate map then select the 'Protocol' tab of the Sample Information section (Figure 13), which now displays the name of the previously selected protocol. The information in this tab is read-only.

Note: Refer to the *Spectrum CE System Operating Manual #TMD052* for instructions on creating or modifying protocols.

Well A1	Promega 5-Dye (5C)	Electropherogram	Quality Table
Conditions			
Oven Temperature (°C)	60		
Array Heater (°C)	45		
Tertiary Heater (°C)	45		
Pre-Electrophoresis			
Pre-E. Voltage (kV)	17		
Pre-E. Duration (sec)	120		
Sample Injection			
Injection Voltage (kV)	2		
Injection Duration (sec)	15		
Electrophoresis			
Voltage (kV)	13		
Collection Duration (sec)	1550		
Data Delay (sec)	90		
Spectral Calibration			
Dye Set	Promega 5-Dye (5C)		
Properties			
Description	Preset Run Protocol		
Created By	Promega		
Date Modified	6/29/2022 10:13:56 AM		
Name			
Calibration			

Figure 13. The 'Protocol' tab of the Sample Information section.

9. The plate record is automatically saved and in Draft status until it is linked to a plate position. To access a list of all plates in the Draft status, select the **Draft Plates** button from the 'Home' screen.

5.4 Creating and Loading the Plate Assembly

1. Place the 96-well plate created in Section 5.1 into the Spectrum plate base, lining up the notch above well A12 with the notch on the base.
2. To complete the plate assembly, place the Spectrum plate retainer over the plate/base assembly, lining up the notch on the retainer with the notch on the plate and base. Verify that the retainer is locked in place on both sides of the plate, sitting evenly on top of the base (Figure 14).



Figure 14. The Spectrum plate assembly.

3. Confirm the drawer handle light is illuminated, indicating that the drawer is unlocked and ready for plate loading. Alternatively, check the Message Center to verify that the drawer is unlocked. The Drawer Status indicator is in the Message Center located in the header (Figure 5).
4. Check the Plate Position Status indicator to note which of the four plate positions (A, B, C or D) is open and does not already contain a plate.
5. Open the plate drawer and place the plate assembly in an open plate position, verify that the notch on the plate base retainer aligns with the notch in the plate drawer then close the drawer.

Notes:

- a. When the plate is seated properly, the Plate Positions indicator will indicate that there is a plate present in the corresponding plate position.
- b. An error message will be displayed if the plate drawer is occupied by a plate being processed.

5.5 Linking a Plate for a Run

1. A plate record must be linked to the plate before adding it to the run queue.
2. Open the draft plate record, then select **Link** in the Plate Position indicator under the position where the plate was placed. Once the plate is successfully linked, the text under the position will change status to “Unlink” and the Plate ID will appear above the position (Figure 15).
3. Select the **Start** arrow to begin the run.

Notes:

- a. If a plate is linked to a plate position while the instrument is processing another plate, the newly linked plate will be entered automatically into the run queue.
- b. The status of all linked plates will update in the Plate Positions indicator throughout the run.
- c. To access the plate screen for linked plates, select the plate image in the Plate Positions indicator.

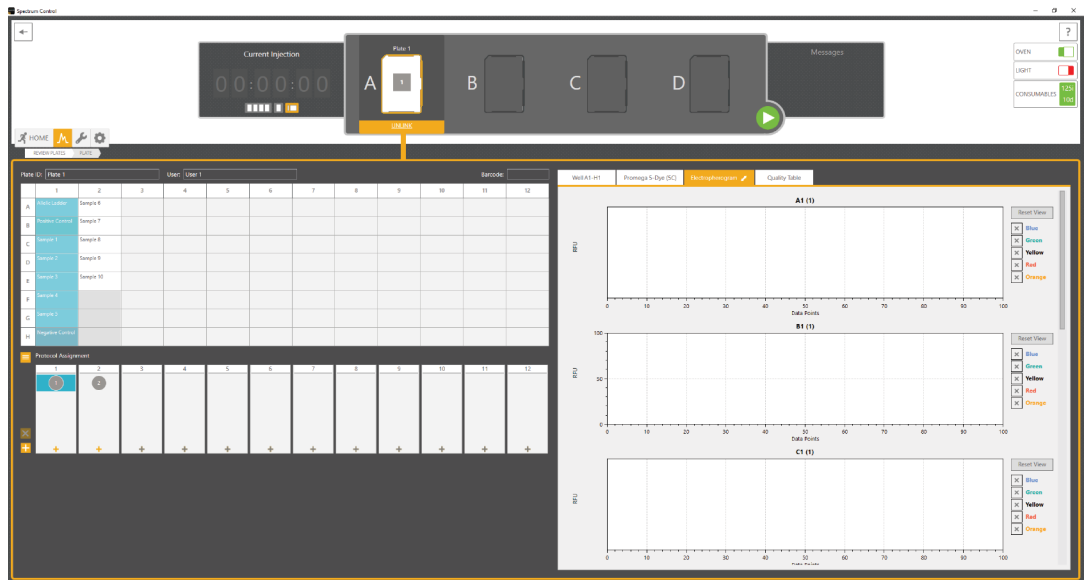


Figure 15. Linked plate in the Spectrum Control Software.

5.6 Monitoring a Run

1. The Plate Status is indicated by the icon in the Plate Positions indicator section of the header.
2. To access the injection status and view data, select the plate image in the header.
 - a. The injection status will be displayed in the plate record.
 - b. To view electropherograms, select a well or column and then select the 'Electropherogram' tab in the Sample Information section.
3. Refer to the *Spectrum CE System Operating Manual #TMD052* for more information on plate and injection status indicators as well as reordering or removing injections and plates in the run queue.

5.7 Exporting Data

Data from injections are automatically exported into a defined location on the instrument hard drive. File formats for GeneMapper® ID-X (.fsa) and GeneMarker®HID Software for Spectrum CE Systems (.promega) are available.

Refer to the *Spectrum CE System Operating Manual #TMD052* for more information on file formats, file location and file naming conventions.

Data Analysis Using GeneMapper® *ID-X* Software

6

GeneMapper® *ID-X* Version 1.4 or greater is required for analysis of PowerPlex® Fusion data from the Spectrum CE System (.fsa files).

Note: GeneMapper® *ID-X* software cannot analyze .promega files.

6.1. Importing PowerPlex® Fusion Panels, Bins and Stutter Text Files into GeneMapper® *ID-X* Software

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

Getting Started

1. To obtain the proper panels, bins and stutter text files and WEN_ILS_500_IDX.xml file for the PowerPlex® Fusion System, go to: **www.promega.com/PowerPlexPanels**
2. Select the PowerPlex® System that you are using, and then select 'GeneMapper ID-X.' Enter your contact information, and then select **Submit**.
3. Save the PowerPlex_Fusion_Panels_IDX_vX.x.txt, PowerPlex_Fusion_Bins_IDX_vX.x.txt and PowerPlex_Fusion_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 Files

1. Open the GeneMapper® ID-X software.
2. Select **Tools**, and then **Panel Manager**.
3. Highlight the 'Panel Manager' icon in the upper left navigation pane.
4. Select **File**, and then **Import Panels**.
5. Navigate to the panels text file downloaded in the Getting Started section above. Select the file, and then **Import**.
6. Select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.
7. In the navigation pane, highlight the PowerPlex Fusion panels folder that you just imported in Step 5.
8. Select **File**, and then **Import Bin Set**.
9. Navigate to the bins text file downloaded in the Getting Started section above. Select the file, and then **Import**.
10. In the navigation pane, highlight the PowerPlex Fusion panels folder that you just imported in Step 5.
11. Select **File**, and then **Import Marker Stutter**. A warning box will appear asking if you want to overwrite current values. Select **Yes**.
12. Navigate to the stutter text file downloaded in the Getting Started section above. Select the file, and then **Import**.
13. At the bottom of the Panel Manager window, select **OK**. This will save the panels, bins and stutter text files, and then close the window.

Note: If you are using GeneMapper® ID-X software, version 1.4 or higher, before closing the Panel Manager, check the boxes to indicate DYS391 is a Y-marker. See Figure 16.

Marker Name	Dye Color	Min Size	Max Size	Control Alleles	Marker	Comments	Y Marker	Ladder Alleles
1 AMEL	Blue	80.0	92.0	X,Y	9	none	<input type="checkbox"/>	X,Y
2 DSS1398	Blue	93.0	150.0	17,18	4	none	<input type="checkbox"/>	9,10,11,12,13,14,15,16,17,18,19,20
3 D1S1656	Blue	151.0	207.0	12,13	4	none	<input type="checkbox"/>	9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27
4 D2S441	Blue	207.5	247.5	10,14	4	none	<input type="checkbox"/>	8,9,10,11,12,13,14,15,16,17
5 D10S1248	Blue	248.0	295.0	13,15	4	none	<input type="checkbox"/>	8,9,10,11,12,13,14,15,16,17,18,19
6 D13S317	Blue	295.2	350.0	9,11	4	none	<input type="checkbox"/>	5,6,7,8,9,10,11,12,13,14,15,16,17
7 Penta E	Blue	354.5	474.9	7,14	5	none	<input type="checkbox"/>	5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24
8 D16S539	Green	73.0	129.0	9,13	4	none	<input type="checkbox"/>	4,5,6,7,8,9,10,11,12,13,14,15,16
9 D18S51	Green	129.1	215.5	16,18	4	none	<input type="checkbox"/>	7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27
10 D2S1338	Green	218.0	299.0	22,25	4	none	<input type="checkbox"/>	10,12,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28
11 CSF1PO	Green	309.0	363.0	12	4	none	<input type="checkbox"/>	5,6,7,8,9,10,11,12,13,14,15,16
12 Penta D	Green	370.0	461.0	12,13	5	none	<input type="checkbox"/>	2,2,3,2,5,6,7,8,9,10,11,12,13,14,15,16,17
13 TH01	Yellow	69.0	118.0	6,9,3	4	none	<input type="checkbox"/>	3,4,5,6,7,8,9,9,3,10,11,13,3
14 YWA	Yellow	122.0	192.0	16,19	4	none	<input type="checkbox"/>	10,11,12,13,14,15,16,17,18,19,20,21,22,23,24
15 D21S11	Yellow	198.0	266.5	29,31,2	4	none	<input type="checkbox"/>	24,24,2,25,25,2,26,27,28,28,2,29,29,2,30,30,2,31,31,2,32,32,2,33,33,2,34,34,2,35,35,2,36,37,38
16 D7S820	Yellow	267.0	316.0	8,11	4	none	<input type="checkbox"/>	5,6,7,8,9,10,11,12,13,14,15,16
17 D5S918	Yellow	316.5	379.0	12	4	none	<input type="checkbox"/>	6,7,8,9,10,11,12,13,14,15,16,17,18
18 TPOX	Yellow	387.0	443.5	11	4	none	<input type="checkbox"/>	4,5,6,7,8,9,10,11,12,13,14,15,16
19 DYS391	Yellow	443.6	495.0	10	4	none	<input checked="" type="checkbox"/>	5,6,7,8,9,10,11,12,13,14,15,16
20 D8S1179	Red	72.0	131.2	14,15	4	none	<input type="checkbox"/>	7,8,9,10,11,12,13,14,15,16,17,18,19
21 D12S391	Red	131.4	191.0	18,23	4	none	<input type="checkbox"/>	14,15,16,17,17,3,18,18,3,19,20,21,22,23,24,25,26,27
22 D19S433	Red	191.5	256.5	13,14	4	none	<input type="checkbox"/>	5,2,6,2,8,9,10,11,12,12,13,13,2,14,14,2,15,15,2,16,16,2,17,17,2,18,18,2
23 FGA	Red	257.0	415.0	20,23	4	none	<input type="checkbox"/>	14,15,16,17,18,18,2,19,19,2,20,20,2,21,21,2,22,22,2,23,23,2,24,24,2,25,25,2,26,27,28,29,30,31,2,32,2,33,2,42,2,43,2,44,2,45,2,46,2,48,2,50,2
24 D22S1045	Red	420.0	472.0	16	3	none	<input type="checkbox"/>	7,8,9,10,11,12,13,14,15,16,17,18,19,20

Figure 16. The GeneMapper® ID-X Y-Marker check box.

6.2. Importing the WEN ILS 500 Size Standard into GeneMapper® ID-X Software

There are two options when creating a size standard. Use this protocol or the alternative protocol in Section 6.3.

1. Select **Tools**, and then **GeneMapper ID-X Manager**.
2. Select the 'Size Standard' tab.
3. Select **Import**.
4. Navigate to the location of the WEN_ILS_500_IDX.xml file downloaded in Section 6.1, Getting Started.
5. Highlight the file, and then select **Import**.
6. Select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.
7. Select **Done** to save the changes and close the GeneMapper® ID-X Manager.

6.3. Creating a Size Standard with GeneMapper® ID-X Software

1. Select **Tools**, and then **GeneMapper ID-X Manager**.
2. Select the 'Size Standard' tab.
3. Select **New**.
4. In the 'Size Standard Editor' window (Figure 17), 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.

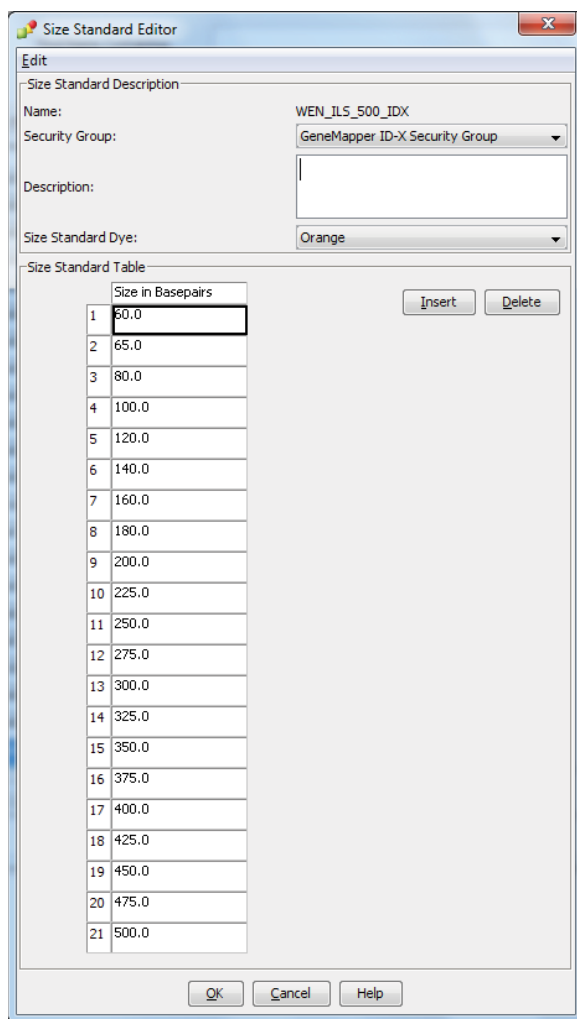


Figure 17. The GeneMapper® ID-X Size Standard Editor.

5. Enter a detailed name, such as “WEN_ILS_500_IDX.”
6. Choose **Orange** for the Size Standard Dye.
7. Enter the sizes of the internal lane standard fragments (60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases). See Section 11.2, Figure 29.
8. Select **OK**.

6.4. Creating a Casework Analysis Method with GeneMapper® ID-X Software

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

1. Select **Tools**, and then **GeneMapper ID-X Manager**.
2. Select the 'Analysis Methods' tab.
3. Select **New**, and a new analysis method dialog box will open.
4. In the 'Analysis Method Editor' window, select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.
5. Enter a descriptive name for the analysis method, such as "PowerPlex Fusion".
6. Select the 'Allele' tab (Figure 18).

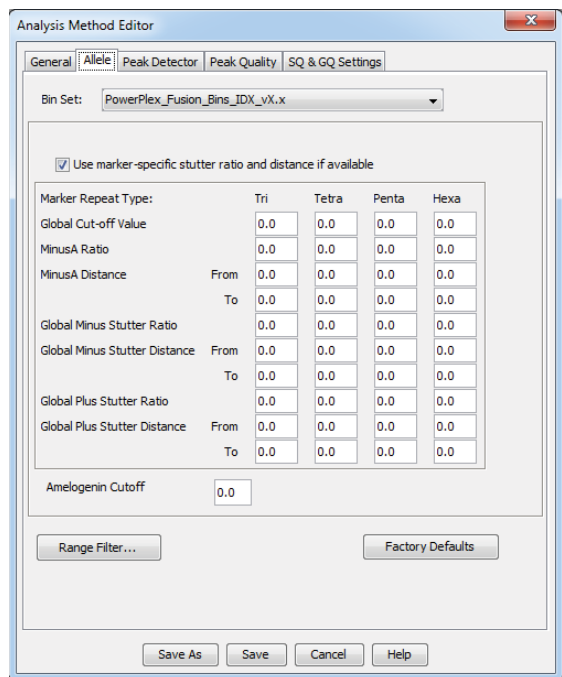


Figure 18. The GeneMapper® ID-X 'Allele' tab.

7. Select the bins text file that was imported in Section 6.1.
8. Ensure that the "Use marker-specific stutter ratio and distance if available" box is checked.

9. Select the 'Peak Detector' tab (Figure 19). 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 or unchecked.

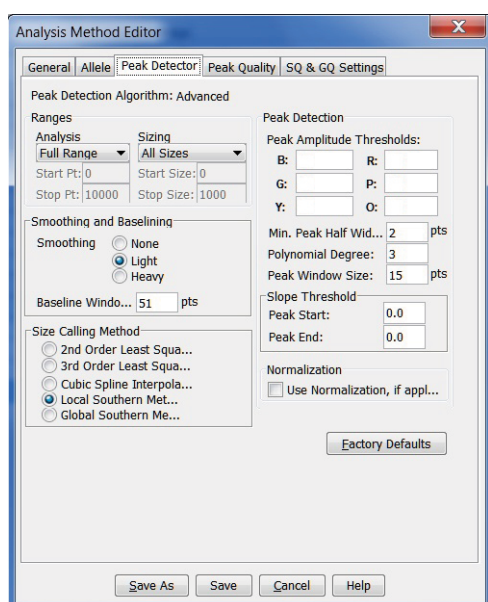


Figure 19. The GeneMapper® ID-X 'Peak Detector' tab.

10. Select the 'Peak Quality' tab. You can change the settings for peak quality.
11. Select the 'SQ & GQ Settings' tab. You can change these settings.

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

12. Select **Save** to save the new analysis method.
13. Select **Done** to exit the GeneMapper® ID-X Manager.

Processing Data for Casework Samples

1. Select **File**, and then **New Project**.
2. Select **Edit**, and then **Add Samples to Project**.
3. Browse to the location of the run files. Highlight desired files, and then select **Add to List**, followed by **Add**.

Note: The Spectrum CE System generates .fsa and .promega files for each sample run. The .promega files are not compatible with GeneMapper® ID-X Software

4. The sample types assigned in Section 5.3 will be included in the 'Sample Type' column. If needed, the drop-down menu in the 'Sample Type' column can be used to select **Allelic Ladder**, **Sample**, **Positive Control** or **Negative Control** as appropriate for the sample. Every run folder in the project must contain at least one allelic ladder injection that is designated as **Allelic Ladder** in the 'Sample Type' column for proper genotyping.
5. In the 'Analysis Method' column, select the analysis method created previously in this section.
6. In the 'Panel' column, select the panels text file that was imported in Section 6.1.
7. In the 'Size Standard' column, select the size standard that was imported in Section 6.2 or created in Section 6.3.
8. Select **Analyze** (green arrow button) to start data analysis.

Note: By default, the software is set to display the 'Analysis Requirements Summary' window if an analysis requirement is not met.

9. If all analysis requirements are met, the 'Save Project' window will open (Figure 20).

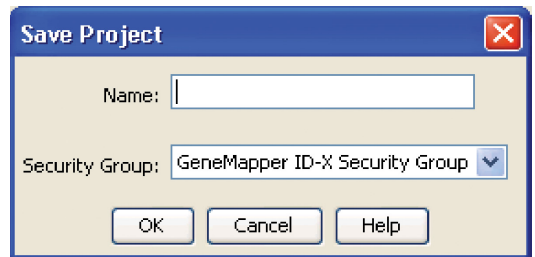


Figure 20. 'Save Project' window.

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

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

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

6.5. Creating a Databasing or Paternity Analysis Method with GeneMapper® ID-X Software

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

1. Select **Tools**, and then **GeneMapper ID-X Manager**.
2. Select the 'Analysis Methods' tab.
3. Select **New**, and a new analysis method dialog box will open.
4. In the 'Analysis Method Editor' window, select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.
5. Enter a descriptive name for the analysis method, such as "PowerPlex Fusion 20% Filter".
6. Select the 'Allele' tab (Figure 21).
7. Select the bins text file that was imported in Section 6.1.
8. Ensure that the "Use marker-specific stutter ratio and distance if available" box is checked. Doing this will assign locus-specific stutter files and distances from the imported stutter file. Ensure that the appropriate global filter is applied to this analysis method. For example, for a 20% filter, enter "0.20" for the Global Cut-off Value for Tri, Tetra and Penta repeats (Figure 21).

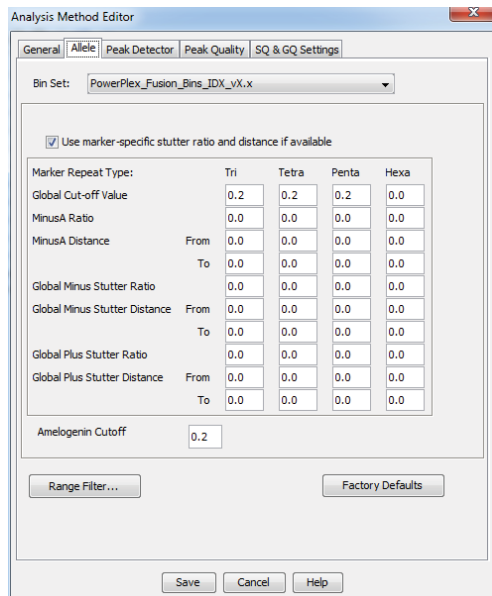


Figure 21. The GeneMapper® ID-X 'Allele' tab with settings for using a 20% peak filter.

9. Select the 'Peak Detector' tab (Figure 19). 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 or unchecked.
10. Select the 'Peak Quality' tab. You can change the settings for peak quality.
 11. Select the 'SQ & GQ Settings' tab. You can change these settings.

Note: For Steps 10 and 11, see the GeneMapper® ID-X user's manual for more information. The settings in Steps 10 and 11 should be based on the results of your internal validation.
 12. Select **Save** to save the new analysis method.
 13. Select **Done** to exit the GeneMapper® ID-X Manager.

Processing Data for Databasing or Paternity Samples

1. Select **File**, and then **New Project**.
2. Select **Edit**, and then **Add Samples to Project**.
3. Browse to the location of the run files. Highlight the desired files, and then select **Add to List**, followed by **Add**.

Note: The Spectrum CE System generates .fsa and .promega files for each sample run. The .promega files are not compatible with GeneMapper® ID-X Software

4. The sample types assigned in Section 5.3 will be included in the 'Sample Type' column. If needed, the drop-down menu in the 'Sample Type' column can be used to select **Allelic Ladder**, **Sample**, **Positive Control** or **Negative Control** as appropriate for the sample. Every run folder in the project must contain at least one allelic ladder injection that is designated as **Allelic Ladder** in the 'Sample Type' column for proper genotyping.
5. In the 'Analysis Method' column, select the analysis method created previously in this section.
6. In the 'Panel' column, select the panels text file that was imported in Section 6.1.
7. In the 'Size Standard' column, select the size standard that was imported in Section 6.2 or created in Section 6.3.
8. Select **Analyze** (green arrow button) to start data analysis.

Note: By default, the software is set to display the 'Analysis Requirements Summary' window if an analysis requirement is not met.

9. If all analysis requirements are met, the 'Save Project' window will open (Figure 20).
10. Enter the project name.
11. Choose the applicable security group from the drop-down menu, and then select **OK**.

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

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

6.6. Controls in GeneMapper® ID-X Software

1. Observe the results for the negative control. Using the protocols defined in this manual, the negative control should be devoid of amplification products.
2. Observe the results for the 2800M Control DNA. The expected 2800M DNA allele designations for each locus are listed in Table 8 (Section 11.1).

Data Analysis Using GeneMarker®HID Software for Spectrum CE Systems

7

7.1. Creating an Analysis Method with GeneMarker®HID Software for Spectrum CE Systems

These instructions are intended as a guide to start analyzing data in GeneMarker®HID Software for Spectrum CE Systems (GMHID-Spectrum). They are not intended as comprehensive instructions for using GMHID-Spectrum. More detailed instructions can be found in the *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555*.

The current PowerPlex.xml panel files are preloaded in the software or available at www.promega.com/PowerPlexPanels.

1. Open GMHID-Spectrum.
2. To access your data files, select **Open Data** in the Magic Wizard or under the File menu.
3. Select **Add**, then navigate to the directory containing your raw data files and select the desired files.

Note: The Spectrum CE System generates .fsa and .promega files for each sample run. We recommend using the .promega files with GMHID-Spectrum.

4. Select **Open**, and the selected files will appear in the Data File List (Figure 22).

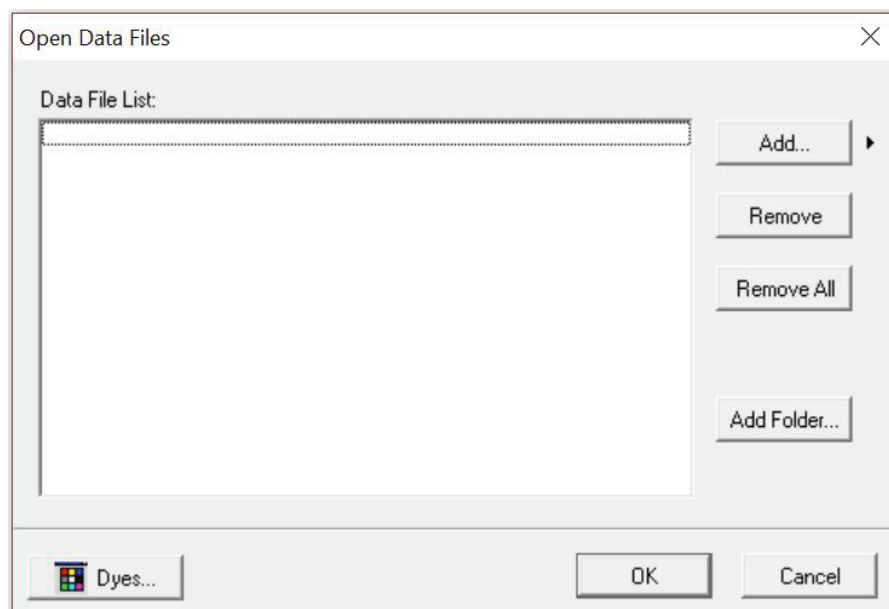


Figure 22. The GMHID-Spectrum Data File List.

5. Select **OK** in the 'Open Data Files' window, and the data will be uploaded into GMHID-Spectrum. In the Raw Data Tree, verify that the sample types (allelic ladder, positive control and negative control) are designated correctly. If sample types are not correct, designate sample types by right-clicking on the file name and selecting **Set Sample Type**.

Notes:

1. For .promega files, the sample types assigned in Section 5.3 will be included.
 2. For .fsa files, sample types are designated in the file name using preferences. In the View menu, select the 'Forensic' tab under Preferences, then enter sample name identifiers for ladder, positive and negative controls.
6. Use the Panel Editor in the Tools menu to select the PowerPlex_Fusion panel from the Panel Template list. Click on the plus symbol to expand the list, right-click on a marker, and then select **Edit**. Enter laboratory-specified values for Min Homozygote Intensity, Homozygote Inconclusive Range, Min Heterozygote Intensity, Heterozygote Inconclusive Range, Min Heterozygote Imbalance and Max Heterozygote Imbalance (Figure 23). This will set values for peaks within the marker range. Refer to *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555* for more information.

Notes:

1. Individual laboratories should determine their peak amplitude thresholds from internal validation studies.
 2. These settings can be applied to all markers by checking the "Apply Homo/Hetero Settings to All Markers" check box or by dye channel by checking the "Apply Homo/Hetero Settings to Markers in this dye" check box.
7. Select **OK** and close the 'Panel Editor' window. Select **Save Changes** or **Save as New Panel** under the File menu then close the window.

The 'Edit Marker' window for the D3S1358 marker displays the following settings:

- Marker Name:** D3S1358
- Nucleotide Repeats (x):** 4
- Boundary:** 89.6 To 146.5
- Min Homozygote Intensity:** [Empty field]
- < = Inconclusive <=** [Empty field]
- Min Heterozygote Intensity:** [Empty field]
- < = Inconclusive <=** [Empty field]
- Max Heterozygote Imbalance(%):** [Empty field]
- Min Heterozygote Imbalance(%):** [Empty field]
- Apply Homo/Hetero Settings to All Markers
- Apply Homo/Hetero Settings to Markers in this dye
- Stutter Filter:**
 - Use Marker-Specific Values
 - N - 2x: 0 %
 - N + x: 0 %
 - N - x: 11.9 %
 - N+0.5x: 0 %
 - N-0.5x: 0 %
 - Use Allele-Specific Values(From Panel)
 - Apply Stutter Settings to All Markers

Buttons: OK, Cancel

17063TA

Figure 23. The 'Edit Marker' window for the D3S1358 marker.

- Click on **Run** in the Magic Wizard or click the **Run Project** icon (green arrow) in the toolbar. The 'Template Selection' screen will appear (Figure 24). Select the PowerPlex_Fusion template and the settings shown in Figure 24. Verify the panel that you created in Step 6 is selected in the panel drop-down field. The Size Standard must be ILS500 and the Standard Color must be Orange. Select **Next**.

Note: Changes to the template can be saved at each step during the Run Project process: 'Template Selection', 'Data Process' and 'Additional Settings' screens.

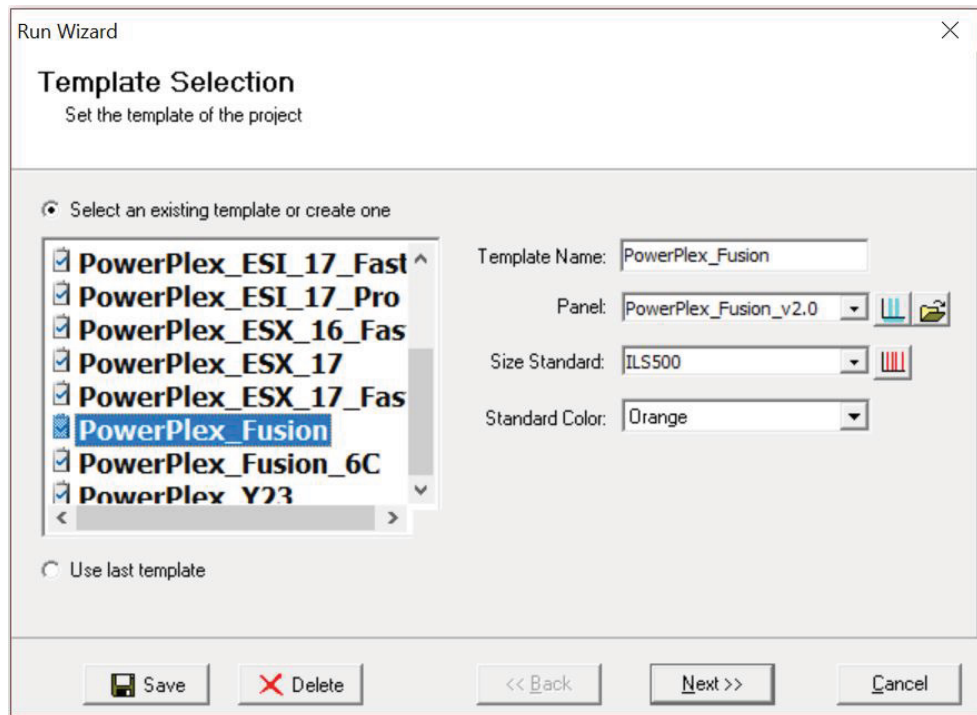


Figure 24. The 'Template Selection' window.

9. The 'Data Process' window will appear (Figure 25). For the Raw Data Analysis, we recommend using Auto Range, Smooth, Superior Baseline Subtraction and Local Southern for the Size Call.

For the Allele Call, we recommend using Auto Range. Setting of the Max Intensity (peaks above which will be flagged) and the Min Intensity for Standard Color should be determined by your laboratory. For peaks outside of the panel range, the settings from the panel can be applied by checking the "Apply Nearest Marker Setting" box. Refer to *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555* for more information on use of the Peak Detection Threshold. Select **Next**.

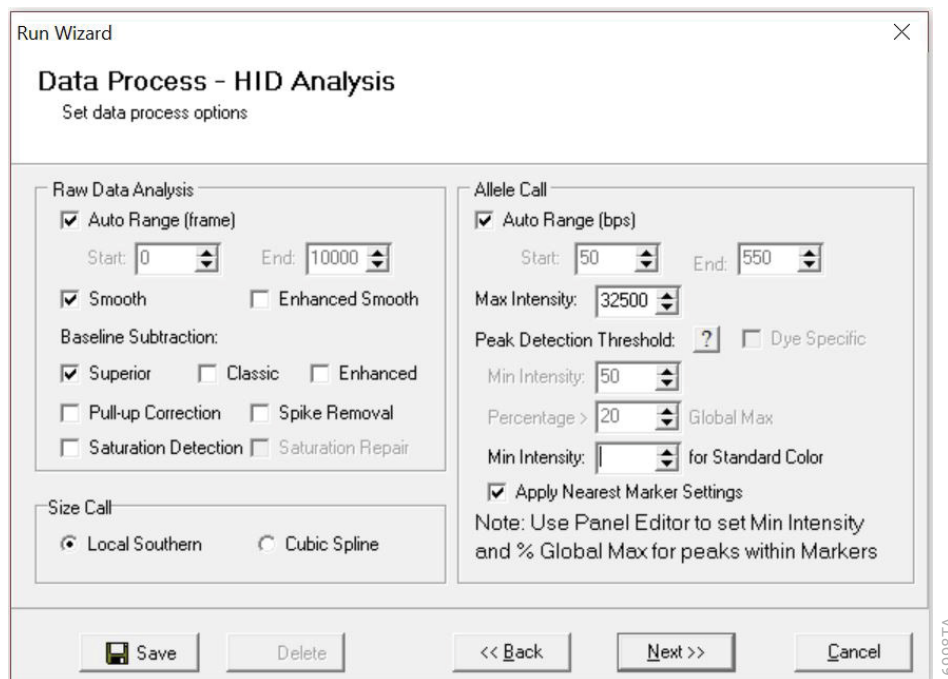


Figure 25. The 'Data Process' window for an analysis method.

- The 'Additional Settings' window will appear (Figure 26). Select **Fusion_2800M** as the P.C. Template 1 and verify that the boxes are checked for "Auto Select Best Ladder" and "Auto Panel Adjust". The values displayed in the 'Allele Evaluation' dialogue box are defaults and will affect the quality values displayed in the plot settings. For more information on quality values, use of a second positive control and mixture evaluation, refer to the *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555*. Select **OK** to analyze the raw data.

Figure 26. The 'Additional Settings' window.

- When the analysis is complete, the 'Main Analysis' window will appear. We recommend that you review any yellow or red flagged markers and handle them according to your laboratory's standard operating procedures.

Note: Quality and display settings are set using Preferences under the View menu. Refer to *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555* for more information.

7.2. Controls in GeneMarker®HID Software for Spectrum CE Systems

- Observe the results for the negative control. Using the protocols defined in this manual, the negative controls should be devoid of amplification products.
- Observe the results for the 2800M Control DNA. The expected 2800M allele designations for each locus are listed in Table 8 (Section 11.1).

Representative results of the PowerPlex® Fusion System are shown in Figure 27. The PowerPlex® Fusion Allelic Ladder Mix is shown in Figure 28.

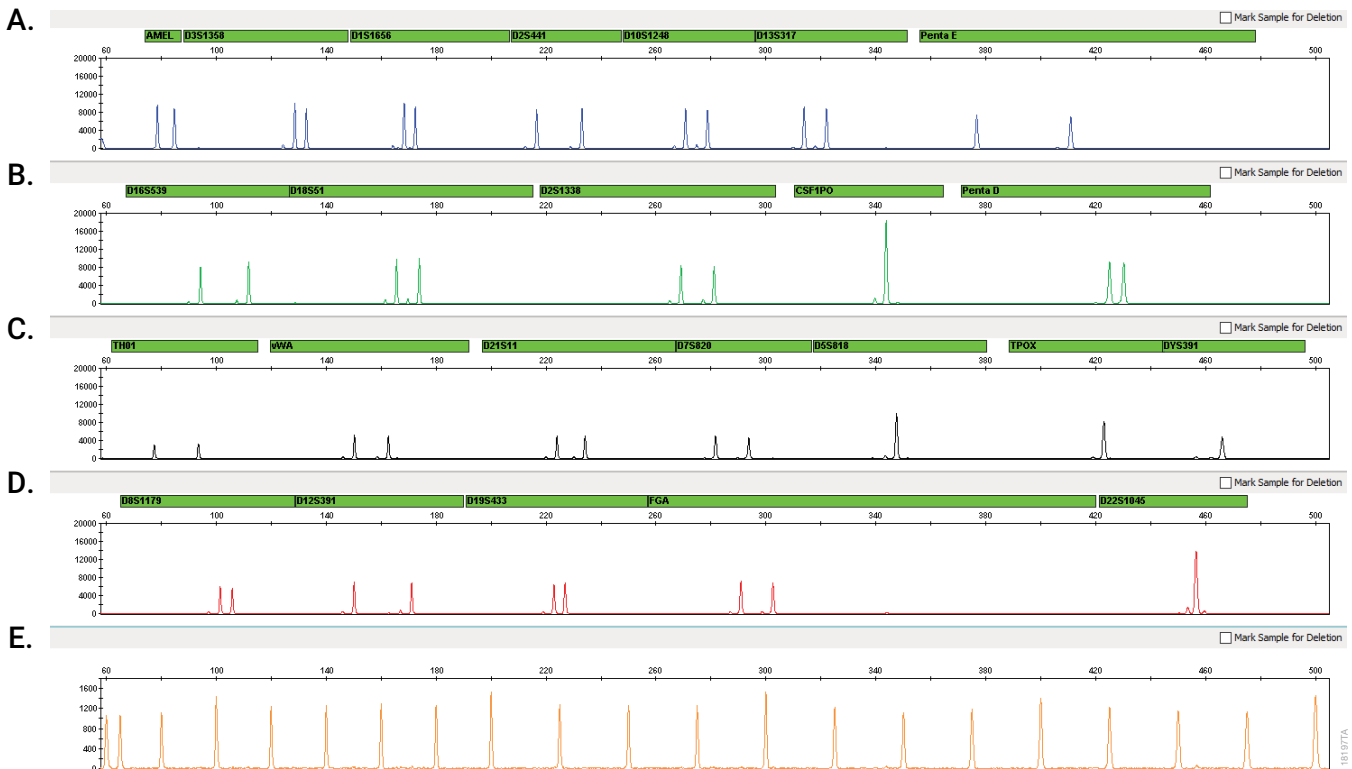


Figure 27. The PowerPlex® Fusion System. The 2800M Control DNA (0.5ng) was amplified using the PowerPlex® Fusion System. Amplification products were mixed with WEN Internal Lane Standard 500 and analyzed with a Spectrum CE System using a 2kV, 15-second injection. Results were analyzed using GeneMapper® *ID-X* software, version 1.4, and PowerPlex® Fusion panels and bins text files. **Panel A.** An electropherogram showing the peaks of the fluorescein-labeled loci: Amelogenin, D3S1358, D1S1656, D2S441, D10S1248, D13S317 and Penta E. **Panel B.** An electropherogram showing the peaks of the JOE-labeled loci: D16S539, D18S51, D2S1338, CSF1PO and Penta D. **Panel C.** An electropherogram showing the peaks of the TMR-ET-labeled loci: TH01, vWA, D21S11, D7S820, D5S818, TPOX and DYS391. **Panel D.** An electropherogram showing the peaks of the CXR-ET-labeled loci: D8S1179, D12S391, D19S433, FGA and D22S1045. **Panel E.** An electropherogram showing the 60bp to 500bp fragments of the WEN Internal Lane Standard 500.

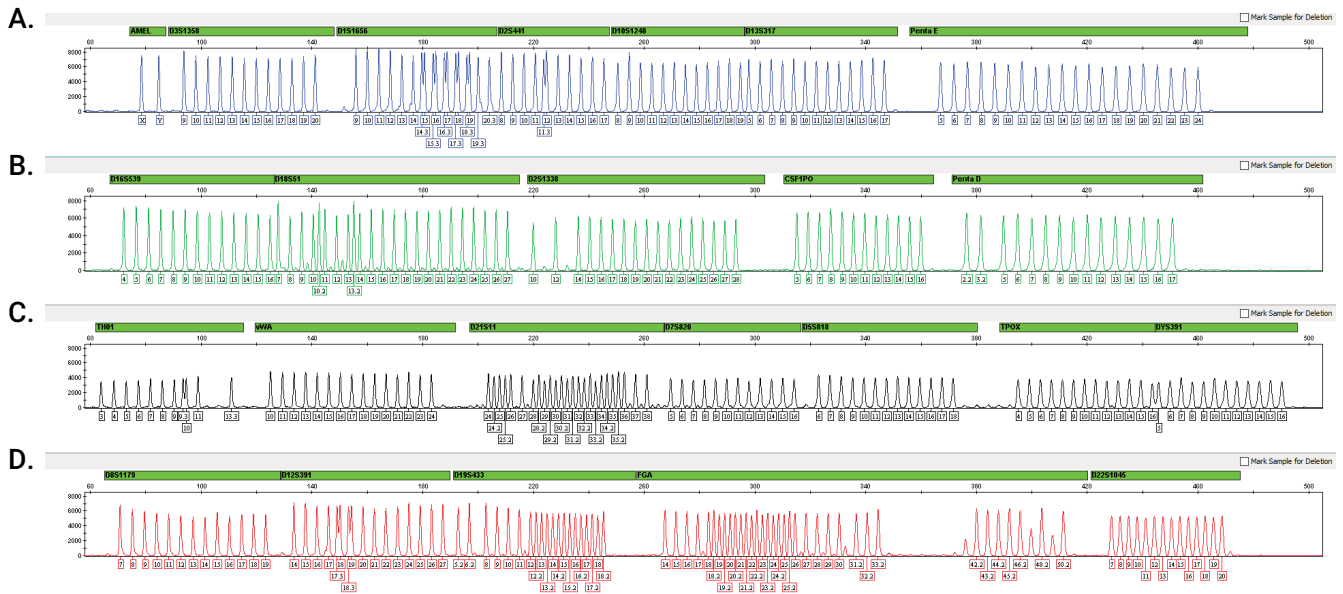


Figure 28. The PowerPlex® Fusion Allelic Ladder Mix. The PowerPlex® Fusion Allelic Ladder Mix was analyzed with a Spectrum CE System Instrument using a 2kV, 15-second injection. The sample file was analyzed with the GeneMapper® *ID-X* software, version 1.4, and PowerPlex® Fusion panels and bins text 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. 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. A trinucleotide repeat locus, like D22S1045, will have more pronounced stutter in both $n-3$ and $n+3$ positions than a typical tetranucleotide repeat locus. The pattern and intensity of stutter may differ slightly between primer sets for the same loci.

The mean stutter plus three standard deviations observed at each locus is used in the PowerPlex® Fusion stutter text file for locus-specific filtering in GeneMapper® *ID-X* software and in the panel file included in GMHID-Spectrum.

In addition to stutter peaks, you may observe the following low-level DNA-dependent artifact peaks (Table 4) and DNA-independent (with or without human genomic DNA) artifact peaks (Table 5) with the PowerPlex® Fusion System.

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

Locus or Dye Label	Artifact Size
JOE	~77–80 bases ¹ ~214 bases ¹ ~247 bases ¹
Fluorescein	~88–112 ²
CXR	~175–183
TMR-ET	~69–72 bases ¹
Amelogenin	n–1
TH01	n–2, n+2 ³
D1S1656	n–2, n+2
D2S441	n–1, n–2, n+2
D13S317	n–2, n+2
D18S51	n–2, n+2
D21S11	n–2, n+2
D7S820	n–2, n+2
D5S818	n–2, n+2 n–6 to n–7 ⁴ n–8 to n–9 ⁴
D12S391	n–2, n+2, n–3
D19S433	n–2, n+2

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

²For artifacts in this size range, rfu approximately 1.5% or less of the main peaks may be observed at the D3S1358 locus.

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

⁴Low intensity peaks (50–150rfu) that migrate approximately 6–7 bases and 8–9 bases in front of the main allele may represent DNA secondary structure.

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

Dye Label	Artifact Size ¹
Fluorescein	~61–63 bases ~64–66 bases ~83–86 bases
JOE	~65–68 bases
TMR-ET	~58–61 bases ~64–67 bases

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

Troubleshooting

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

Refer to the *Spectrum CE System Operating Manual #TMD052* for instrument troubleshooting. For troubleshooting GMHID-Spectrum, refer to the *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555*.

9.1. Amplification and Fragment Detection

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

Symptoms	Causes and Comments
<p>Extra peaks visible in one or all color channels</p>	<p>Contamination with another template DNA or previously amplified DNA. Cross-contamination can be a problem. Use aerosol-resistant pipette tips and change gloves regularly.</p>
	<p>Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the DNA source as soon as possible to each well and follow immediately by thermal cycling.</p>
	<p>Samples were not denatured completely. Heat-denature samples for the recommended time, and cool on crushed ice or a freezer plate block 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.</p>
	<p>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.</p>
	<p>CE-related artifacts (‘spikes’). Minor voltage changes or urea crystals passing by the laser can cause ‘spikes’ or unexpected peaks. Spikes sometimes appear in one color but often are easily identified by their presence in more than one color. Re-inject samples to confirm.</p>
	<p>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.</p> <ul style="list-style-type: none"> • Perform a new spectral calibration and rerun the samples. • Confirm that the correct spectral was used for the sample run. • Instrument sensitivities can vary. Optimize the injection conditions. Refer to the instrument user manual.
	<p>Dye blob artifacts. The signal strength of certain dye blob artifacts increases with storage of the amplification plate at 4°C, sometimes in as short a time period as overnight but more commonly when left at 4°C for a few days. We recommend storing amplification products at –20°C.</p>
	<p>An incorrect internal lane standard was used. Use the size standard provided in the kit.</p>

Symptoms	Causes and Comments
Allelic ladder not running the same as samples	Be sure the allelic ladder and samples are from the same instrument run.
	Allelic ladder and primer pair mix were not compatible. Ensure that the allelic ladder is from the same kit as the primer pair mix.
	Migration of samples changed slightly over the course of a CE run with many samples. This may be due to changes in temperature over time. Use a different injection of allelic ladder to determine sizes.
	Poor injection of allelic ladder. Include more than one ladder per instrument run. Include one allelic ladder for every 16 samples.
	Internal size standard was not assigned correctly. Evaluate the sizing labels on the ILS and correct if necessary.
Peak height imbalance	Miscellaneous balance problems. At the first use, thaw the Primer Pair Mix and Master Mix completely. Vortex the Primer Pair Mix and Master Mix for 15 seconds before use; do not centrifuge the Primer Pair Mix or Master Mix after mixing. Calibrate thermal cyclers and pipettes routinely.
	PCR amplification mix was not mixed well. Vortex the PCR amplification mix for 5–10 seconds before dispensing into the reaction tubes or plate.

9.2. Amplification of Extracted DNA

The following information is specific to amplification of purified DNA. For information about general amplification and detection, see Section 9.1.

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

Symptoms	Causes and Comments
<p>Extra peaks visible in one or all color channels</p>	<p>Amplification of more than the recommended amount of purified DNA can result in a higher number of artifact peaks due to overamplification, resulting in saturating signal. Use the recommended amount of template DNA. See Results section for additional information about stutter and artifacts. The amount of template will need to be optimized if you are using reduced reaction volumes.</p>
	<p>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.</p> <ul style="list-style-type: none"> • Be sure to perform the recommended extension step at 60°C after thermal cycling. • Decrease the amount of template DNA. Using more than the recommended amount of template DNA can result in incomplete adenylation. • Decrease cycle number. • Increase the final extension time.
<p>Peak height imbalance</p>	<p>Amplification of greater than the recommended amount of template can result in an imbalance, with smaller loci showing more product than larger loci. Use less template or fewer cycles. The amount of template will need to be optimized if you are using reduced reaction volumes.</p>
	<p>Degraded DNA sample. DNA template was degraded, and larger loci showed diminished yield.</p>
	<p>Insufficient template DNA. Use the recommended amount of template DNA if available. Stochastic effects can occur when amplifying low amounts of template.</p>
	<p>Impure template DNA. Inhibitors that may be present in forensic samples can lead to allele dropout or imbalance.</p>
	<p>Imbalance may be seen more often when using the maximum template volume or a reduced amplification reaction volume.</p>
	<p>The reaction volume was too low. This system is optimized for a final reaction volume of 25µl. Decreasing the reaction volume may result in suboptimal performance.</p>

9.3. Direct Amplification of DNA from Storage Card Punches

The following information is specific to direct amplification of DNA from storage card punches. For additional information about general amplification and detection, see Section 9.1.

Symptoms	Causes and Comments
Faint or absent allele peaks	For direct amplification reactions that require the addition of AmpSolution™ Reagent, its omission can result in inhibition of the amplification reaction. Review the amplification setup and confirm whether or not AmpSolution™ Reagent is required for your direct amplification reactions.
	DNA was not accessible on nonlytic material. Pretreat nonlytic materials with PunchSolution™ Reagent to ensure that DNA is released from cellular proteins.
	Poor sample deposition. Shedding and collection of donor cells was variable. Increase cycle number.
	Poor sample transfer to storage card or variable sampling from storage card. Take punches from a different portion of the card. Increasing cycle number can increase peak heights.
	Too much sample in the reaction can result in inhibition, decreasing intensity of peaks (especially larger amplicons). Be sure to use the recommended number of punches. Follow the manufacturer's recommendations when depositing sample onto the storage card.
	Amplification was inhibited when using more than one storage card punch with blood. Use only one 1.2mm storage card punch with blood.
	The reaction volume was too low. Decreasing the reaction volume from that recommended in the protocol may result in suboptimal performance. Use the recommended number of punches for the reaction volume used.
	Active PunchSolution™ Reagent carried over into amplification reactions with nonlytic card punches. Ensure that the heat block reached 70°C and samples were incubated for 30 minutes or until wells are dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. We have not tested longer incubation times.
	Inactive PunchSolution™ Reagent was used to pretreat nonlytic punches. Thaw the PunchSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.

Symptoms	Causes and Comments
Faint or absent allele peaks in the positive control reaction	<p>If the positive control reaction failed to amplify, check to make sure that the correct amount of 2800M Control DNA was added to the reaction. Due to the reduced cycle number, it is necessary to increase the mass of 2800M Control DNA to obtain a profile. Use the recommended amount of 2800M Control DNA per amplification reaction. This mass of DNA should be reduced if cycle number is increased and increased if the cycle number is decreased. Increase or decrease by twofold the mass of 2800M Control DNA for every one-cycle decrease or increase, respectively. We do not recommend including blank punches in the 2800M Control DNA reaction.</p>
Extra peaks visible in one or all color channels	<p>Punch contaminated with DNA from another sample. Perform punches on a blank card between samples to minimize potential for carryover between samples.</p>
	<p>Amplification of processed punches with high amounts of DNA can result in artifact peaks due to overamplification, resulting in saturating signal on the CE instrument. Be sure to use the recommended number of punches. Use of a larger punch size or a smaller reaction volume may result in overamplification and signal saturation. If the signal is saturated, repeat the amplification with a smaller punch, a larger reaction volume or reduced cycle number.</p>
	<p>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.</p>
	<p>Artifacts of STR amplification. Direct amplification of high amounts of template can result in a higher number of artifact peaks. Use the recommended punch size and number of punches. Optimize the cycle number. See Results section for additional information on stutter and artifacts.</p>
	<p>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.</p> <ul style="list-style-type: none"> • Be sure to perform the recommended extension step at 60°C after thermal cycling. • Decrease cycle number. • Increase the final extension time.

Symptoms	Causes and Comments
Peak height imbalance	<p>Excessive amount of DNA. Amplification of high amounts of template can result in an imbalance, with smaller loci showing more product than larger loci.</p> <ul style="list-style-type: none"> • Be sure to use the recommended number of punches. Follow the manufacturer’s recommendations when depositing sample onto the card. • Decrease cycle number.
	<p>The cycle number was too high. Decrease the cycle number by one cycle and repeat the amplification.</p>
	<p>For direct amplification reactions that require the addition of AmpSolution™ Reagent, its omission can result in inhibition of the amplification reaction. Review the amplification setup and confirm whether or not AmpSolution™ Reagent is required for your direct amplification reactions.</p>
	<p>Amplification was inhibited when using more than one storage card punch with blood. Use only one 1.2mm storage card punch with blood.</p>
	<p>The reaction volume was too low. Decreasing the reaction volume can result in suboptimal performance due to inhibitors present in lytic cards and PunchSolution™ Reagent.</p>
	<p>DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat nonlytic materials with PunchSolution™ Reagent to ensure that DNA is liberated from cellular proteins.</p>
	<p>Active PunchSolution™ Reagent carried over into amplification reactions with nonlytic card punches. Larger loci are most susceptible to carryover and will drop out before the smaller loci.</p> <ul style="list-style-type: none"> • Ensure that the heat block reached 70°C and samples were incubated for 30 minutes or until wells are dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. • We recommend treating one 1.2mm nonlytic card punch with 10µl of PunchSolution™ Reagent and using one punch per 12.5µl or 25µl amplification reaction. Reducing the PunchSolution™ Reagent volume may improve results for reactions with reduced amplification volumes. Optimization and validation are required.
	<p>Inactive PunchSolution™ Reagent was used to pretreat nonlytic punches. Thaw the PunchSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.</p>

Symptoms	Causes and Comments
Extreme variability in sample-to-sample peak heights	There can be significant individual-to-individual variability in the number of cells on a card, resulting in peak height variability between samples. The PunchSolution™ Kit maximizes the recovery of amplifiable DNA from nonlytic punches but does not normalize the amount of DNA present.

9.4. Direct Amplification of DNA from Swabs

The following information is specific to direct amplification of DNA from swabs after pretreatment using the SwabSolution™ Kit. For additional information about general amplification and detection, see Section 9.1.

Symptoms	Causes and Comments
Faint or absent allele peaks	Poor sample deposition. Shedding and collection of donor cells was variable. Increase cycle number.
	Inactive SwabSolution™ Reagent. Thaw the SwabSolution™ Reagent completely in a 37°C water bath and mix by gentle inversion. Store the SwabSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
	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 for tubes or plates; heat transfer is inefficient and will result in poor performance. Use only a heat block to maintain efficient heat transfer. We have tested 60-minute incubation times and observed no difference in performance compared to a 30-minute incubation.
	DNA was not accessible on nonlytic material. Pretreat swabs with SwabSolution™ Reagent to ensure that DNA is released from cellular proteins.
Faint or absent peaks for the positive control reaction	If the positive control reaction failed to amplify, check to make sure that the correct amount of 2800M Control DNA was added to the reaction. Due to the reduced cycle numbers used with swab extracts, it is necessary to increase the mass of 2800M Control DNA to obtain a profile. Use the recommended amount of 2800M Control DNA per amplification reaction. This mass of DNA should be reduced if cycle number is increased and increased if the cycle number is decreased. Increase or decrease by twofold the mass of 2800M Control DNA for every one-cycle decrease or increase, respectively.

Symptoms	Causes and Comments
<p>Extra peaks visible in one or all color channels</p>	<p>Swab extract was contaminated. Assemble a reaction containing the swab extract prepared from a blank swab or assemble a reaction where the SwabSolution™ Reagent is processed and incubated as a blank without a swab.</p>
	<p>Artifacts of STR amplification. Amplification of swab extracts with high DNA concentrations can result in artifact peaks due to overamplification, resulting in saturated signal on the CE instrument. We recommend 2µl of swab extract per reaction.</p> <p>Using more than 2µl may result in overamplification and signal saturation. If signal is saturated, repeat amplification with less swab extract or reduced cycle number.</p>
	<p>Amplification of excess template for a given cycle number resulted in overloading of the capillary upon electrokinetic injection. Excess DNA in the capillary is difficult to maintain in a denatured single-stranded state. Some single-stranded DNA renatures and becomes double-stranded. Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis and appears as ‘shadow’ peaks migrating in front of the main peaks. If this occurs at a heterozygous locus it is possible to observe the presence of two ‘shadow’ peaks that differ in size by approximately the same distance as the single-stranded alleles.</p>
	<p>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.</p> <ul style="list-style-type: none"> • Be sure to perform the recommended extension step at 60°C after thermal cycling. • Use 2µl of swab extract in an amplification reaction. A larger volume of swab extract may contain more than the recommended amount of DNA template, resulting in incomplete adenylation. • Decrease cycle number. • Increase the final extension time.

Symptoms	Causes and Comments
<p>Peak height imbalance</p>	<p>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 cycle number.</p>
	<p>Active SwabSolution™ Reagent carried over from swab extracts into the amplification reaction. Larger loci are most susceptible to reagent carryover and will drop out before the smaller loci. Ensure that the heat block reached 70°C (90°C if using 2.2ml, Square-Well Deep Well Plates) and samples were incubated for the full 30 minutes. Shorter incubations may result in incomplete reagent inactivation. Do not use an incubator for tubes or plates; heat transfer is inefficient and will result in poor performance. Use only a heat block to maintain efficient heat transfer.</p>
	<p>Inactive SwabSolution™ Reagent. Thaw the SwabSolution™ Reagent completely in a 37°C water bath and mix by gentle inversion. Store the SwabSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.</p>
	<p>DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat swabs with SwabSolution™ Reagent to ensure that DNA is released from cellular proteins.</p>
<p>Extreme variability in sample-to-sample peak heights</p>	<p>There can be significant individual-to-individual variability in cell deposition onto buccal swabs. This can 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.</p>

9.5. GeneMapper® ID-X Software

Symptoms	Causes and Comments
Stutter peaks not filtered	Stutter text file was not imported into the Panel Manager when the panels and bins text files were imported.
	Be sure that the 'Use marker-specific stutter ratio and distance if available' box is checked. If the 'Use marker-specific stutter ratio and distance if available' box is not checked, stutter distance must be defined in the Analysis Method 'Allele' tab.
Samples in the project not analyzed	The 'Analysis Requirement Summary' window was not active, and there was an analysis requirement that was not met. Turn on 'Analysis Requirement Summary' in the 'Project Options' menu under File , and correct the necessary analysis requirements to continue analysis.
Edits in label edit viewer cannot be viewed	To view edits made to a project, the project must first be saved. Close the plot view window, return to the main GeneMapper® ID-X page and save the project. Display the plot window again, and then view the label edit table.
Marker header bar for some loci are gray	When an edit is made to a locus, the quality flags and marker header bar automatically changes to gray. To change the GQ and marker header bar for a locus to green, override the GQ in the plot window.
Alleles not called	To analyze samples with GeneMapper® ID-X software, at least one allelic ladder must be defined per folder of sample files being analyzed in the project.
	<p>Run was too short, and larger peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run.</p> <ul style="list-style-type: none"> • Create a new size standard using the internal lane standard fragments present in the sample. • Re-run samples using a longer run time.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.

Symptoms	Causes and Comments
Off-ladder alleles	An allelic ladder from a different run than the samples was used. Re-analyze samples with an allelic ladder from the same run.
	The GeneMapper® <i>ID-X</i> 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 the section 'Processing Data for Casework Samples' or 'Processing Data for Databasing or Paternity Samples.'
	Panels text file selected for analysis was incorrect for the STR system used. Assign correct panels text file that corresponds to the STR system used for amplification.
	The allelic ladder was not identified as an allelic ladder in the 'Sample Type' column.
	The internal lane standard was not properly identified in the sample. Manually redefine the sizes of the size standard fragments in the sample.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.
	Incorrect polymer was used. Use of a polymer other than Polymer4 can change migration of the fragments, causing alleles to migrate outside of the panel range.
Size standard not called correctly	Starting data point was incorrect for the partial range chosen for the analysis in the GeneMapper® <i>ID-X</i> Peak Detector tab as described in sections 'Creating a Casework Analysis Method' or 'Creating a Databasing or Paternity Analysis Method.' Adjust the starting data point or use a full range for the analysis.
	Extra peaks in size standard. Open the Size Match Editor. Highlight the extra peak, select Edit and select Delete Size Label . Select Auto-Adjust Sizes .
	An incorrect size standard was used.
	Run was too short, and larger peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run. <ul style="list-style-type: none"> • Create a new size standard using the internal lane standard fragments present in the sample. • Re-run samples using a longer run time.
Peaks in size standard missing	If peaks are low-quality, redefine the size standard for the sample to skip these peaks.
	An incorrect size standard was used.
Significantly raised baseline	Poor spectral calibration. Perform a new spectral calibration and re-run the samples.
	Confirm that the correct spectral was used to run the samples.

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11.1. Advantages of Using the Loci in the PowerPlex® Fusion System

A single PowerPlex® Fusion System reaction amplifies all core loci required for US expanded CODIS and European databases (Table 6 and Table 7). The male-specific DYS391 locus is included to identify null Y results for Amelogenin. Table 8 lists the PowerPlex® Fusion System alleles amplified from the 2800M Control DNA.

We have carefully selected primers to avoid or minimize artifacts, including those associated with DNA polymerases, such as repeat slippage and terminal nucleotide addition (14,15). Repeat slippage, sometimes called “n-4 peaks”, “stutter” or “shadow peaks”, is due to the loss of a repeat unit during DNA amplification, somatic variation within the DNA or both. The amount of this artifact observed depends primarily on the locus and the DNA sequence being amplified.

Terminal nucleotide addition (16,17) occurs when a thermostable nonproofreading DNA polymerase adds a nucleotide, generally adenine, to the 3' ends of amplified DNA fragments in a template-independent manner. The efficiency with which this occurs varies with different primer sequences. Thus, an artifact peak one base shorter than expected (i.e., missing the terminal addition) is sometimes seen. We have modified primer sequences and added a final extension step at 60°C (18) to the amplification protocols to provide conditions for essentially complete terminal nucleotide addition when recommended amounts of template DNA are used.

Table 6. The PowerPlex® Fusion System Locus-Specific Information.

STR Locus	Label	Chromosomal Location¹	Repeat Sequence² 5'→3'
Amelogenin ³	Fluorescein	Xp22.1–22.3 and Y	NA
D3S1358	Fluorescein	3p21.31 (45.557Mb)	TCTA Complex
D1S1656	Fluorescein	1q42 (228.972Mb)	TAGA Complex
D2S441	Fluorescein	2p14 (68.214Mb)	TCTA
D10S1248	Fluorescein	10q26.3 (130.567Mb)	GGAA
D13S317	Fluorescein	13q31.1 (81.62Mb)	TATC
Penta E	Fluorescein	15q26.2 (95.175Mb)	AAAGA
D16S539	JOE	16q24.1 (84.944Mb)	GATA
D18S51	JOE	18q21.33 (59.1Mb)	AGAA (19)
D2S1338	JOE	2q35 (218.705Mb)	TGCC/TTCC
CSF1PO	JOE	5q33.1 (149.436Mb)	AGAT
Penta D	JOE	21q22.3 (43.88Mb)	AAAGA
TH01	TMR-ET	11p15.5 (2.149Mb)	AATG (19)
vWA	TMR-ET	12p13.31 (5.963Mb)	TCTA Complex (19)
D21S11	TMR-ET	21q21.1 (19.476Mb)	TCTA Complex (19)
D7S820	TMR-ET	7q21.11 (83.433Mb)	GATA
D5S818	TMR-ET	5q23.2 (123.139Mb)	AGAT
TPOX	TMR-ET	2p25.3 (1.472Mb)	AATG
DYS391	TMR-ET	Y	TCTA
D8S1179	CXR-ET	8q24.13 (125.976Mb)	TCTA Complex (19)
D12S391	CXR-ET	12p12 (12.341Mb)	AGAT/AGAC Complex
D19S433	CXR-ET	19q12 (35.109Mb)	AAGG Complex
FGA	CXR-ET	4q28 (155.866Mb)	TTTC Complex (19)
D22S1045	CXR-ET	22q12.3 (35.779Mb)	ATT

¹Information about chromosomal location of these loci can be found in references 20 and 21 and at:

<https://strbase.nist.gov/chrom.htm>

²The August 1997 report (22,23) 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.”

³Amelogenin is not an STR.

NA = Not Applicable

Table 7. The PowerPlex® Fusion System Allelic Ladder Information.

STR Locus	Label	Size Range of Allelic Ladder Components^{1,2} (bases)	Repeat Numbers of Allelic Ladder Components³
Amelogenin ⁴	Fluorescein	89, 95	X, Y
D3S1358	Fluorescein	103–147	9–20
D1S1656	Fluorescein	161–208	9–14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18, 18.3, 19, 19.3, 20.3
D2S441	Fluorescein	214–250	8–11, 11.3, 12–17
D10S1248	Fluorescein	255–299	8–19
D13S317	Fluorescein	302–350	5–17
Penta E	Fluorescein	371–466	5–24
D16S539	JOE	84–132	4–16
D18S51	JOE	134–214	7–10, 10.2, 11–13, 13.2, 14–27
D2S1338	JOE	224–296	10, 12, 14–28
CSF1PO	JOE	318–362	5–16
Penta D	JOE	377–450	2.2, 3.2, 5–17
TH01	TMR-ET	72–115	3–9, 9.3, 10–11, 13.3
vWA	TMR-ET	127–183	10–24
D21S11	TMR-ET	203–259	24, 24.2, 25, 25.2, 26–28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36–38
D7S820	TMR-ET	269–313	5–16
D5S818	TMR-ET	321–369	6–18
TPOX	TMR-ET	393–441	4–16
DYS391	TMR-ET	442–486	5–16
D8S1179	CXR-ET	76–124	7–19
D12S391	CXR-ET	133–185	14–17, 17.3, 18, 18.3, 19–27
D19S433	CXR-ET	193–245	5.2, 6.2, 8–12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2, 18, 18.2
FGA	CXR-ET	265–411	14–18, 18.2, 19, 19.2, 20, 20.2, 21, 21.2, 22, 22.2, 23, 23.2, 24, 24.2, 25, 25.2, 26–30, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 48.2, 50.2
D22S1045	CXR-ET	425–464	7–20

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

²When using an internal lane standard, such as the WEN Internal Lane Standard 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 and linker also affect migration of alleles.

³For a current list of microvariants, see the Variant Allele Report published at the U.S. National Institute of Standards and Technology (NIST) web site at:

https://strbase.nist.gov/var_tab.htm

⁴Amelogenin is not an STR.

Table 8. The PowerPlex® Fusion System Allele Determinations for the 2800M Control DNA.

STR Locus	2800M
Amelogenin	X, Y
D3S1358	17, 18
D1S1656	12, 13
D2S441	10, 14
D10S1248	13, 15
D13S317	9, 11
Penta E	7, 14
D16S539	9, 13
D18S51	16, 18
D2S1338	22, 25
CSF1PO	12, 12
Penta D	12, 13
TH01	6, 9.3
vWA	16, 19
D21S11	29, 31.2
D7S820	8, 11
D5S818	12, 12
TPOX	11, 11
DYS391	10
D8S1179	14, 15
D12S391	18, 23
D19S433	13, 14
FGA	20, 23
D22S1045	16, 16

11.2. 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 29). Each fragment is labeled with WEN dye and can be detected separately (as a fifth color) in the presence of PowerPlex® Fusion-amplified material. The WEN ILS 500 is designed for use in each CE injection to increase precision in analyses when using the PowerPlex® Fusion System. Protocols to prepare and use this internal lane standard are provided in Section 5.

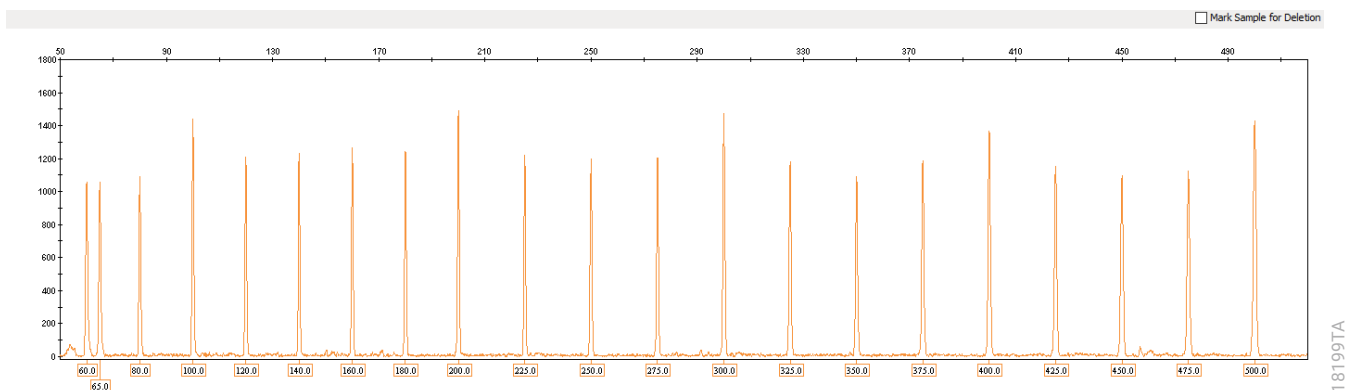


Figure 29. WEN Internal Lane Standard 500. An electropherogram showing the WEN Internal Lane Standard 500 fragments.

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

Testing at Promega has shown successful direct amplification from storage card punches in a 12.5µl reaction volume (24). This section contains a protocol for direct amplification of DNA from storage card punches in a 12.5µl reaction volume using the PowerPlex® Fusion System and GeneAmp® PCR System 9700 or ProFlex® PCR System. When using the protocol detailed below, add only one 1.2mm storage card punch to each 12.5µl amplification reaction.

Materials to be Supplied by the User

- GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block or ProFlex® PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plates or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- PunchSolution™ Kit (Cat.# DC9271) for nonlytic 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® Fusion 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.

Lytic card sample types include:

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

Nonlytic card sample types include:

- Buccal samples on Bode Buccal DNA Collector™ devices
- Blood and buccal samples on nonlytic cards (e.g., S&S 903)

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

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

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

Note: Static may be problematic when adding a punch to a well. For lytic card punches, adding PCR amplification mix to the well before adding the punch may help alleviate static problems. For nonlytic 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® Fusion 5X Master Mix, PowerPlex® Fusion 5X Primer Pair Mix and Water, Amplification Grade, completely.

Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 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°C to +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 it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.
5. Add the final volume of each reagent listed in Table 9 to a clean tube.

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

PCR Amplification Mix Component ¹	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	5.0µl	×		=	
PowerPlex® Fusion 5X Master Mix	2.5µl	×		=	
PowerPlex® Fusion 5X Primer Pair Mix	2.5µl	×		=	
5X AmpSolution™ Reagent	2.5µl	×		=	
total reaction volume	12.5µl				

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

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



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

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

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

Note: It also is acceptable to add the lytic 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 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. Reserve a well containing PCR amplification mix as a negative amplification control.

Note: An additional negative control with a blank punch may be performed to detect contamination from the storage card or punch device.
 10. Seal or cap the plate, or close the tubes. Briefly centrifuge the plate to bring storage card punches to the bottom of the wells and remove any air bubbles.

Thermal Cycling

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

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 30.

Notes:

1. When using the ProFlex® PCR System, use the 9700 Simulation Mode as the ramp speed.
2. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.
3. The final extension for direct amplification was extended to 20 minutes compared to 10 minutes for the extracted DNA protocol to allow sufficient time for adenylation of large amounts of amplicon.

Thermal Cycling Protocol

96°C for 1 minute, then:

94°C for 10 seconds

59°C for 1 minute

72°C for 30 seconds

for 26 cycles, then:

60°C for 20 minutes

4°C soak

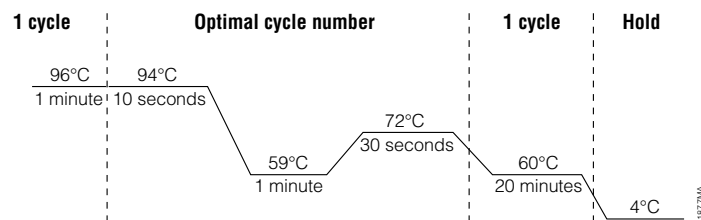


Figure 30. Thermal cycling protocol for the GeneAmp® PCR System 9700 and ProFlex® PCR System.

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

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

PCR Optimization

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

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Place one 1.2mm storage card punch containing buccal cells or whole blood in each well of a reaction plate. Be sure to pretreat nonlytic 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.

11.4. 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 (24). This section contains a protocol for amplifying DNA from swab extracts in a 12.5µl reaction volume using the PowerPlex® Fusion System and GeneAmp® PCR System 9700 or ProFlex® PCR System.

Materials to be Supplied by the User

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

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

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

Amplification Setup

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

Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 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 it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.
4. Add the final volume of each reagent listed in Table 10 to a clean tube.

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

PCR Amplification Mix Component ¹	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	5.5µl	×		=	
PowerPlex® Fusion 5X Master Mix	2.5µl	×		=	
PowerPlex® Fusion 5X Primer Pair Mix	2.5µl	×		=	
swab extract	2.0µl				
total reaction volume	12.5µl				

¹Add Water, Amplification Grade, to the tube first, and then add PowerPlex® Fusion 5X Master Mix and PowerPlex® Fusion 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.



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

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

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

7. For the positive amplification control, vortex the tube of 2800M Control DNA, 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 Water, Amplification Grade or TE⁻⁴ buffer instead of swab extract into a reaction well containing PCR amplification mix.

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

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

Optional: Briefly centrifuge 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 that is amplified.

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® Fusion System.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 31.

Notes:

1. When using the ProFlex® PCR System, use the 9700 Simulation Mode as the ramp speed.
2. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the Select Method Options screen appears, select **Max** for the ramp speed and enter the reaction volume.
3. The final extension for direct amplification was extended to 20 minutes compared to 10 minutes for the extracted DNA protocol to allow sufficient time for adenylation of large amounts of amplicon.

Thermal Cycling Protocol

96°C for 1 minute, then:

94°C for 10 seconds

59°C for 1 minute

72°C for 30 seconds

for 26 cycles, then:

60°C for 20 minutes

4°C soak

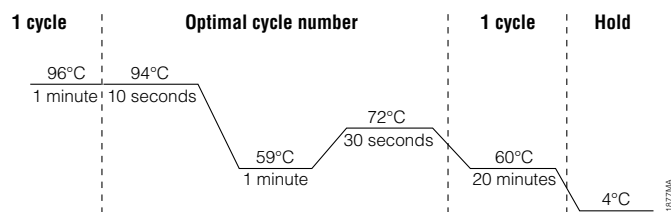


Figure 31. Thermal cycling protocol for the GeneAmp® PCR System 9700 and ProFlex® PCR System.

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

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

PCR Optimization

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

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

Note: This recommendation is for $2\mu\text{l}$ 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.

11.5. 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.

11.6. Related Products

PRODUCT	SIZE	CAT.#
PowerPlex® 5C Matrix Standard	5 preps	DG4850
WEN Internal Lane Standard 500	200µl	DG5001
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
2800M Control DNA (10ng/µl)	25µl	DD7101
2800M Control DNA (0.25ng/µl)	500µl	DD7251
Water, Amplification Grade	6,250µl (5 × 1,250µl)	DW0991
5X AmpSolution™ Reagent	500µl	DM1231

Not for Medical Diagnostic Use.

Spectrum CE System Accessories and Consumables

PRODUCT	SIZE	CAT.#
Spectrum Capillary Array, 8-Capillary	1 each	CE2008
Spectrum Polymer4	384 wells	CE2048
	960 wells	CE2040
Spectrum Buffer	2 pair	CE2001
Spectrum Cathode Septa Mat	10 each	CE2002
Spectrum Buffer and Cathode Septa Mat Bundle	1 each	CE2012
Septa Mat, 96-Well	10 each	CE2696
Spectrum Plate Base & Retainer, 96-Well	4 each	CE5004
Spectrum Wash Solution	1 each	CE2099

Not for Medical Diagnostic Use.

^(a)U.S. Pat. No. 9,139,868, European Pat. No. 2972229, Japanese Pat. No. 6367307 and other patents pending.

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

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Class 1 Laser Product.