

# PowerPlex® 35GY System for Use on the Spectrum Compact CE System Technical Manual

Instructions for Use of Product DC3520



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All technical literature is available at: [www.promega.com/protocols/](http://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](mailto:genetic@promega.com)

# 1 Description

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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 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® 35GY System<sup>(a,b,c)</sup> is a 35-locus multiplex for human identification applications including forensic analysis, relationship testing and research use. This eight-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, Penta E, and SE33 loci are also included to increase discrimination and allow searching of databases that include profiles with these loci. Finally, ten additional Y-STR loci, DYS385ab, DYS390, DYS438, DYS448, DYS456, DYS458, DYS481, DYS635 and DYS643 are included in the multiplex. This extended panel of STR markers is intended to satisfy both CODIS and ESS recommendations.

The PowerPlex® 35GY 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® 35GY 5X Master Mix.

This manual contains protocols for use of the PowerPlex® 35GY System with the ProFlex® PCR System in addition to protocols to separate amplified products and detect separated material on the Spectrum Compact CE System. Amplification and detection instrumentation may vary. You may need to optimize protocols, including the amount of template DNA, cycle number, loading volume and injection conditions for your laboratory instrumentation. In-house validation should be performed. A protocol to operate the Spectrum Compact CE System is available separately. See the *Spectrum Compact CE System Operating Manual #TMD058*.

Information about other Promega fluorescent STR systems is available upon request from Promega or online at: [www.promega.com](http://www.promega.com)

# 2 Product Components and Storage Conditions

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PRODUCT	SIZE	CAT.#
PowerPlex® 35GY System	200 (or 400 direct-amp) reactions	DC3520

Not For Medical Diagnostic Use. This system contains sufficient reagents for 200 reactions of 25µl each or 400 direct-amplification reactions of 12.5µl each. Includes:

### Pre-Amplification Components Box

- 1,000µl PowerPlex® 35GY 5X Master Mix
- 1,000µl PowerPlex® 35GY 5X Primer Pair Mix
- 25µl 2800M Control DNA, 10ng/µl
- 5 × 1,250µl Water, Amplification Grade

### Post-Amplification Components Box

- 100µl PowerPlex® 35GY Allelic Ladder Mix
- 250µl CCO Internal Lane Standard 500



The PowerPlex® 35GY 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 +2°C to +10°C. The PowerPlex® 35GY 5X Primer Pair Mix, PowerPlex® 35GY Allelic Ladder Mix and CCO Internal Lane Standard 500 (CCO 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.



Do not freeze reagents.

## Available Separately

PRODUCT	SIZE	CAT.#
PowerPlex® 35GY QI Reagent	800µl	DM1551
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
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 storage card punches prior to direct amplification. The SwabSolution™ Kit is required to process swabs prior to direct amplification.

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](http://www.promega.com/PowerPlexPanels)**

The PowerPlex® 8C Matrix Standard (Cat.# DG5010), available separately, is required for initial setup of the color separation matrix (see Section 3.2).

# 3 Before You Begin

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## 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 Compact 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 Compact CE System Technical Manual* #TMD062. This manual is available online at: [www.promega.com/protocols/](http://www.promega.com/protocols/)

# 4 Protocols for DNA Amplification Using the PowerPlex® 35GY System

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The PowerPlex® 35GY System is designed 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) and a 12.5µl reaction volume using 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 25µl reaction volume using lytic and nonlytic storage card punches and swabs are included in Sections 10.3 and 10.4, respectively.

The PowerPlex® 35GY System is compatible with the ProFlex® PCR System.

**Note:** It may be possible to use thermal cyclers other than the one listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory.

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 8.

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

### Materials to be Supplied by the User

- 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

We routinely amplify 1ng of template DNA in a 25µl reaction volume using the protocol detailed below.

### Amplification Setup

1. Upon receipt, store the PowerPlex® 35GY 5X Master Mix, PowerPlex® 35GY 5X Primer Pair Mix and Water, Amplification Grade, at +2°C to +10°C.

**Note:** Centrifuge tubes briefly to move 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 new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new, 0.2ml reaction tubes required, and label them appropriately.

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

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

PCR Amplification Mix Component <sup>1</sup>	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25µl	×		=	
PowerPlex® 35GY 5X Master Mix	5µl	×		=	
PowerPlex® 35GY 5X Primer Pair Mix	5µl	×		=	
template DNA (1ng) <sup>2,3</sup>	up to 15µl				
<b>total reaction volume</b>	<b>25µl</b>				

<sup>1</sup>Add Water, Amplification Grade, to the tube first, and then add PowerPlex® 35GY 5X Master Mix and PowerPlex® 35GY 5X Primer Pair Mix. The template DNA will be added at Step 6.

<sup>2</sup>Store DNA templates in TE<sup>-4</sup> buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE<sup>-4</sup> buffer with 20µg/ml glycogen. If the DNA template is stored in TE buffer that is not at 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.

<sup>3</sup>Apparent 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.

- 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.

- Add the template DNA (1ng) for each sample to the respective well containing PCR amplification mix.

**Note:** The PowerPlex® 35GY System is optimized and balanced using 1ng 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.

- For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 1ng in the desired template DNA volume. Add 1ng of diluted DNA to a reaction well containing PCR amplification mix.
- For the negative amplification control, pipet Water, Amplification Grade or TE<sup>-4</sup> buffer instead of template DNA into a reaction well containing PCR amplification mix.

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

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

## Thermal Cycling

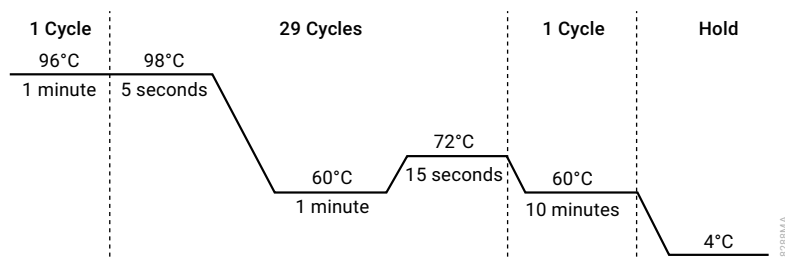
Amplification and detection instrumentation may vary. You may need to optimize protocols including the amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 29 cycles work well for 1ng of purified DNA templates. In-house validation should be performed.

- Place the reaction plate or tubes in the thermal cycler.
- Select and run the recommended protocol, which is provided below and in Figure 1. The total cycling time is approximately 1 hour and 5 minutes.

**Note:** Confirm that the ramp speed is set to 6°C/s and the reaction volume to 25µl.

### Thermal Cycling Protocol

96°C for 1 minute, then:  
 98°C for 5 seconds  
 60°C for 1 minute  
 72°C for 15 seconds for  
 29 cycles, then:  
 60°C for 10 minutes  
 4°C soak



**Figure 1. The thermal cycling protocol for the 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 12.5µl Reaction Volume

### Materials to be Supplied by the User

- 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
- PowerPlex® 35GY QI Reagent (Cat.# DM1551)
- 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 12.5µl reaction volume using the PowerPlex® 35GY System and the ProFlex® PCR System. A protocol for direct amplification of DNA from storage card punches in a 25µl reaction volume is provided in Section 10.3.

For 12.5µl reactions, we recommend amplifying one 1.2mm punch of a lytic or nonlytic storage card containing buccal cells or whole blood.

#### **Lytic storage 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 storage 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. Upon receipt, store the PowerPlex® 35GY 5X Master Mix, PowerPlex® 35GY 5X Primer Pair Mix, PowerPlex® 35GY QI Reagent, and Water, Amplification Grade, at +2°C to +10°C.

**Note:** Centrifuge tubes briefly to move 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 new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new, 0.2ml reaction tubes required, and label them appropriately.
4. Add the final volume of each reagent listed in Table 2 to a new tube. The amount of PowerPlex® 35GY QI Reagent will depend on the cycle number:
  - For 23 cycles, use 4µl of PowerPlex® 35GY QI Reagent
  - For 24 cycles, use 2µl of PowerPlex® 35GY QI Reagent
  - For 25 cycles, use 1µl of PowerPlex® 35GY QI Reagent

**Table 2. PCR Amplification Mix for Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume.**

<b>PCR Amplification Mix Component<sup>1</sup></b>	<b>Volume per Reaction</b>	<b>×</b>	<b>Number of Reactions</b>	<b>=</b>	<b>Final Volume</b>
Water, Amplification Grade	to a final volume of 12.5µl	×		=	
PowerPlex® 35GY 5X Master Mix	2.5µl	×		=	
PowerPlex® 35GY 5X Primer Pair Mix	2.5µl	×		=	
PowerPlex® 35GY QI Reagent	up to 4.0µl				
<b>total reaction volume</b>	<b>12.5µl</b>				

<sup>1</sup>Add Water, Amplification Grade, to the tube first, and then add PowerPlex® 35GY 5X Master Mix, PowerPlex® 35GY 5X Primer Pair Mix, PowerPlex® 35GY QI Reagent. For lytic card punches, the template DNA will be added at Step 6.

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

- 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 punch.

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

- For the positive amplification control, vortex the tube of 2800M Control DNA, and then add 1.6µl (16ng) of the 2800M Control DNA (10ng/µl) to a reaction well containing 12.5µl of PCR amplification mix.

**Notes:**

- Optimization of the amount of control DNA may be required, depending on cycling conditions and laboratory preferences.
  - When performing more than 24 cycles with 12.5µl volume reactions, you may need to dilute the 2800M Control DNA to 8ng/µl prior to adding 1µl (8ng) to positive control reactions.
  - Do not include blank storage card punches in the positive control reactions.
- 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.



- Seal or cap the plate, or close the tubes. Briefly centrifuge the reactions to move 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 (23–25 cycles), injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 24 cycles work well for a variety of sample types. In-house validation should be performed.

- Place the reaction plate or tubes in the thermal cycler.
- Select and run the recommended protocol, which is provided below and in Figure 2. The total cycling time is approximately 55 minutes.

**Note:** Confirm that the ramp speed is set to 6°C/s and the reaction volume to 13µl.

### Thermal Cycling Protocol

96°C for 1 minute, then:

98°C for 5 seconds

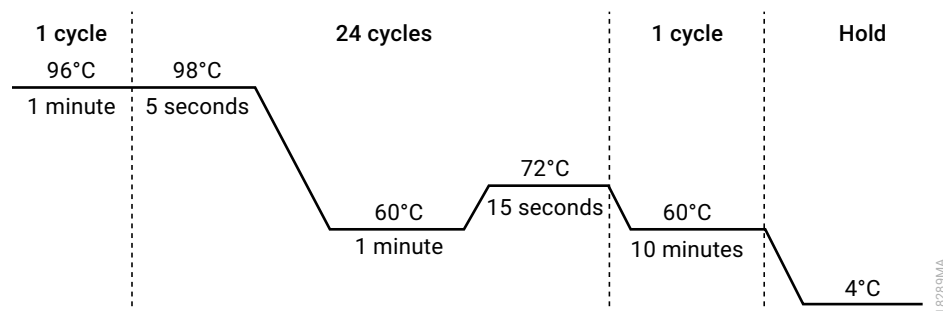
60°C for 1 minute

72°C for 15 seconds

for 24 cycles, then:

60°C for 10 minutes

4°C soak

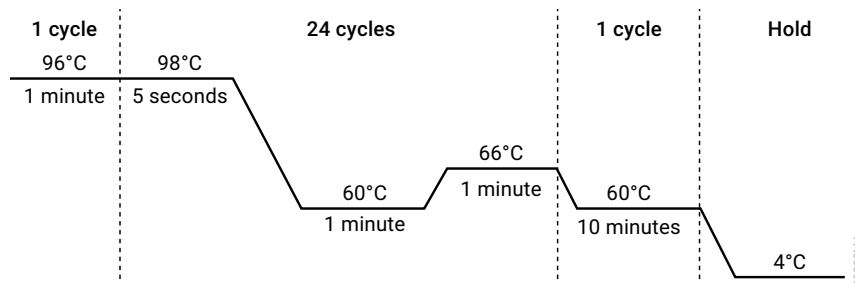


**Figure 2. Thermal cycling protocol for the ProFlex® PCR System.**

**Note:** For challenging sample types, an adjusted thermal cycling protocol can be used as shown below and in Figure 3.

**Thermal Cycling Protocol  
for Challenging Samples**

96°C for 1 minute, then:  
98°C for 5 seconds  
60°C for 1 minute  
66°C for 1 minute  
for 24 cycles, then:  
60°C for 10 minutes  
4°C soak



**Figure 3. Thermal cycling protocol for challenging samples for the ProFlex® PCR System.**

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at  $-20^{\circ}\text{C}$  protected from light.

**Note:** Long-term storage of amplified samples at  $4^{\circ}\text{C}$  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 (23–25 cycles).
5. Following amplification, use your laboratory’s validated separation and detection protocols to determine the optimal cycle number for the sample type.

## 4.3 Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume

### Materials to be Supplied by the User

- 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
- PowerPlex® 35GY QI Reagent (Cat.# DM1551)
- SwabSolution™ Kit (Cat.# DC8271)

This section contains a protocol for amplifying DNA from swab extracts in a 12.5µl reaction volume using the PowerPlex® 35GY System and the ProFlex® PCR System. A protocol for direct amplification of DNA from swab extracts in a 25µl reaction volume is provided in Section 10.4.

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

### Amplification Setup

1. Upon receipt, store the PowerPlex® 35GY 5X Master Mix, PowerPlex® 35GY 5X Primer Pair Mix, PowerPlex® 35GY QI Reagent, and Water, Amplification Grade, at +2°C to +10°C.
 

**Note:** Centrifuge tubes briefly to move 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 new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new, 0.2ml reaction tubes required, and label them appropriately.
4. Add the final volume of each reagent listed in Table 3 to a new tube. The amount of PowerPlex® 35GY QI Reagent will depend on the cycle number:
  - For 23 cycles, use 4µl of PowerPlex® 35GY QI Reagent
  - For 24 cycles, use 2µl of PowerPlex® 35GY QI Reagent
  - For 25 cycles, use 1µl of PowerPlex® 35GY QI Reagent

**Table 3. PCR Amplification Mix for Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume.**

PCR Amplification Mix Component <sup>1</sup>	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 12.5µl	×		=	
PowerPlex® 35GY 5X Master Mix	2.5µl	×		=	
PowerPlex® 35GY 5X Primer Pair Mix	2.5µl	×		=	
PowerPlex® 35GY QI Reagent	up to 4.0µl				
swab extract	2.0µl				
<b>total reaction volume</b>	<b>12.5µl</b>				

<sup>1</sup>Add Water, Amplification Grade, to the tube first, and then add PowerPlex® 35GY 5X Master Mix and PowerPlex® 35GY 5X Primer Pair Mix, PowerPlex® 35GY QI Reagent. The swab extract will be added at Step 6.

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



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

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

- Pipet 2µl of swab extract for each sample into the appropriate well of the reaction plate.
- For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 8ng/µl. Add 2µl (16ng) of the 2800M Control DNA to a reaction well containing 10.5µl of PCR amplification mix.

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

- For the negative amplification control, pipet 2µl of Water, Amplification Grade or TE<sup>-4</sup> 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.

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

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

## Thermal Cycling

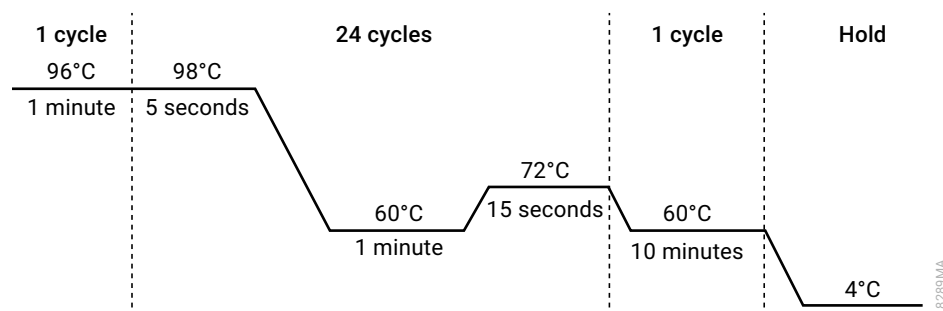
Amplification and detection instrumentation may vary. You will need to optimize protocols including the amount of template DNA, cycle number (23–25 cycles), injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 24 cycles work well for a variety of sample types. Cycle number will need to be optimized in each laboratory for each sample type that is amplified.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 4. The total cycling time is approximately 55 minutes.

**Note:** Confirm that the ramp speed is set to 6°C/s and the reaction volume to 13µl.

### Thermal Cycling Protocol

96°C for 1 minute, then:  
 98°C for 5 seconds  
 60°C for 1 minute  
 72°C for 15 seconds  
 for 24 cycles, then:  
 60°C for 10 minutes  
 4°C soak



**Figure 4. Thermal cycling protocol for the 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 (23–25 cycles).

**Note:** This recommendation is for 2µl of swab extract. Additional cycle number testing may be required.

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

# 5 Detection of Amplified Fragments Using the Spectrum Compact CE System

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Instructions for detecting amplified fragments using the Spectrum Compact CE System can be used with the HITACHI DS3000 Compact CE Sequencer.

## 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 8-tube strips
- aerosol-resistant pipette tips
- Spectrum Compact Capillary Cartridge, 4-Capillary 36cm (Cat.# CE2340)
- Spectrum Compact Polymer4 (Cat.# CE2304, CE2404)
- Spectrum Compact Buffer (Cat.# CE2300)
- Spectrum Compact Cathode Septa Mat (Cat.# CE2301)
- Spectrum Compact Cathode Retainer (Cat.# CE2302)
- Spectrum Compact Strip Base & Retainer, 32-Well (Cat.# CE2332)
- MicroAmp® Optical 8-Tube Strip, 0.2ml (Applied Biosystems Cat.# 4316567)
- Strip Septa Mat, 8-Well (Cat.# CE2308)
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)



Formamide quality 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 can 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.

**Notes:**

- a. Only use MicroAmp™ Optical 8-Tube Strips (0.2ml) (Applied Biosystems Cat.# 4316567) as a source of 8-well strip tubes. Use of other 8-well strip tubes may affect performance or damage the Spectrum Compact CE System.
- b. Wear gloves when handling consumables and sample cartridges.

**Sample Layout**

Samples prepared in 8-well strip tubes are assembled into the strip base and retainer (Figure 5) to form the sample cartridge (Figure 6) which can be loaded onto the Spectrum Compact CE System. Samples are injected in groups of four, such that one 8-well strip tube can be used for two injections. Each 8-well strip tube corresponds to one lane (A, B, C or D) of the sample cartridge. Samples are injected in groups of four across a lane (not by column). For example, samples in well positions A1 through A4 are injected together, followed by samples in well positions A5 through A8 for the second injection. This pattern is then repeated for samples in lanes B, C and D. Refer to the *Spectrum Compact CE System Operating Manual #TMD058* for information on changing the order of injections during run setup.

		Wells							
		1	2	3	4	5	6	7	8
Lane	A	Injection 1				Injection 2			
	B	Injection 3				Injection 4			
	C	Injection 5				Injection 6			
	D	Injection 7				Injection 8			

**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 10µl of formamide/internal lane standard mix into each well of the 8-well strip tube(s).



4. Add 1 µl of amplified sample (or 1 µl of Allelic Ladder Mix) to each well. Cover wells with the Strip Septa Mat, 8-Well.

**Notes:**

- a. To prevent cross-contamination, do not reuse Strip Septa mat, 8-Well. Always use a new Strip Septa Mat, 8-Well for each 8-well strip tube.
  - b. 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 Compact CE System Operating Manual #TMD058*. 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.
  - c. Use an allelic ladder volume that results in peak heights that are all consistently above the peak amplitude threshold determined as part of your internal validation.
  - d. Include an allelic ladder injection every 4 injections (16 samples).
5. Centrifuge the 8-well strip tube(s) 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.

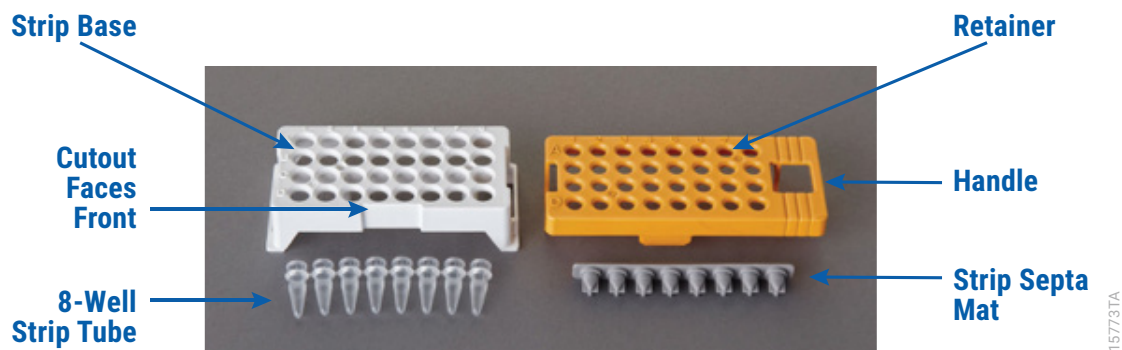
## 5.2 Assembling Sample Cartridge

1. Place the 8-well strip tube(s) with the Strip Septa Mat, 8-well, into the strip base (Figure 5). When using fewer than 4 strips in the run, you can place the strips in any lane.

**Note:** Lanes A to D and wells 1 to 8 are embossed on the strip base. Be sure to check the lane when placing a sample 8-well strip tube into the strip base to make certain that the correct 8-well strip tube is in the correct lane.

Additionally, wells 1–8 are embossed on each 8-well strip tube. When placing a sample 8-well strip tube into the strip base, be sure that the well numbers on each sample 8-well strip tube match those on the strip base.

2. To complete the assembly, place the retainer over the strip(s) in the strip base, aligning the lanes A to D and wells 1 to 8 on the retainer to those on the strip base and pressing until the retainer clicks into the strip base (Figure 6).



**Figure 5. Assembling the Spectrum Compact Strip Base and Retainer.**



**Figure 6. Assembled Spectrum Compact Sample Cartridge.**

### 5.3 Instrument Preparation

These instructions are intended as a guide for running samples on the Spectrum Compact CE System (after successful spatial and spectral calibration with the PowerPlex® 8C Matrix Standard). They are not intended as comprehensive instructions for using the Spectrum Compact CE System. Refer to the *Spectrum Compact CE System Operating Manual #TMD058* for instrument maintenance, instructions for installing consumables, performing a spatial calibration and managing protocols (Assay, Instrument, and Sizecalling protocols). Refer to *PowerPlex® Matrix Standards for the Spectrum Compact CE System Technical Manual #TMD062* for instructions on performing a spectral calibration.

1. Before starting a run on the Spectrum Compact CE System, ensure all consumables are installed and in sufficient supply. For best quality results, use unexpired reagents that are within the recommended use range (Figure 8 and Table 4). Refer to the 'Consumables' screen to determine if any consumables need to be replaced. To access the 'Consumables' screen, select the **Consumables** icon in the Header on the 'Main Menu' screen (Figure 7). The 'Consumables' screen displays information for the four consumables on the instrument: Polymer, Capillary Cartridge and Anode and Cathode buffers.



Figure 7. Spectrum Compact CE System Software 'Main Menu' screen.

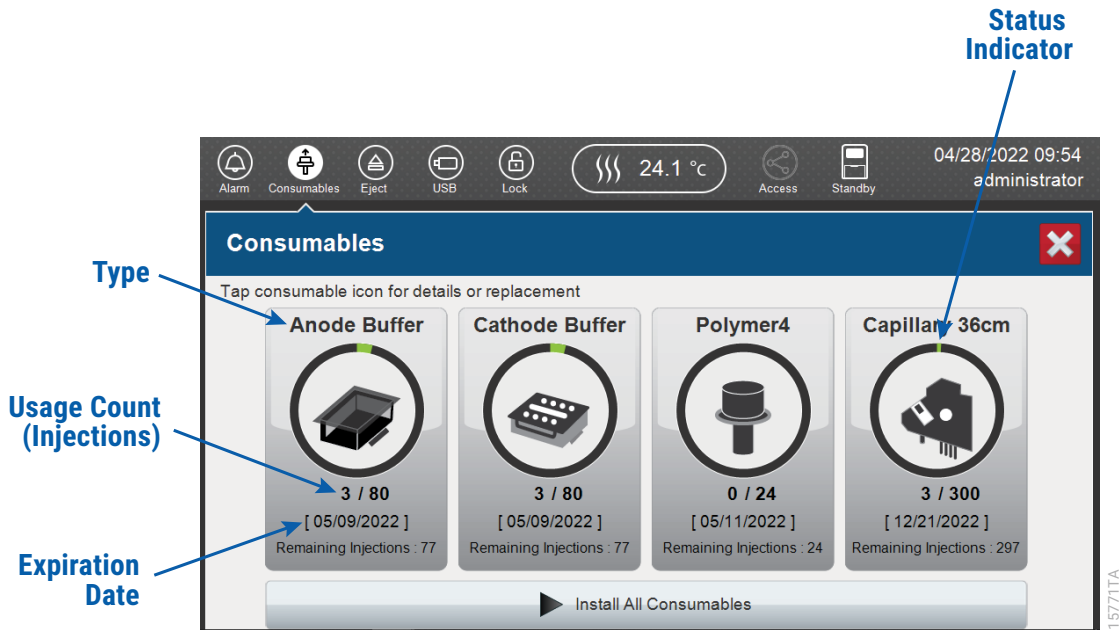





Figure 8. Spectrum Compact CE System 'Consumables' screen. Usage count (number of injections), on-instrument expiration date and remaining injections are displayed on the 'Consumables' screen for each consumable as well as polymer type.

A consumable status indicator will appear on the icons of consumables that need attention. There are three indicators (Table 4).

**Table 4. Consumable Warning Symbols**

Symbol	Description
	Reaching consumable expiration date, on-instrument expiration date or injection limit for consumable.
	Consumable expiration date, on-instrument expiration date or injection limit for consumable has passed.
	Cannot perform a run because maximum injection count was reached for the polymer.

2. Select the **Oven Temperature** icon in the Header on the 'Main Menu' screen as shown in Figure 9 to start preheating the oven temperature to 60°C. The temperature displayed will change as the temperature of the oven increases. When 60°C is reached a check mark will appear adjacent to the temperature.

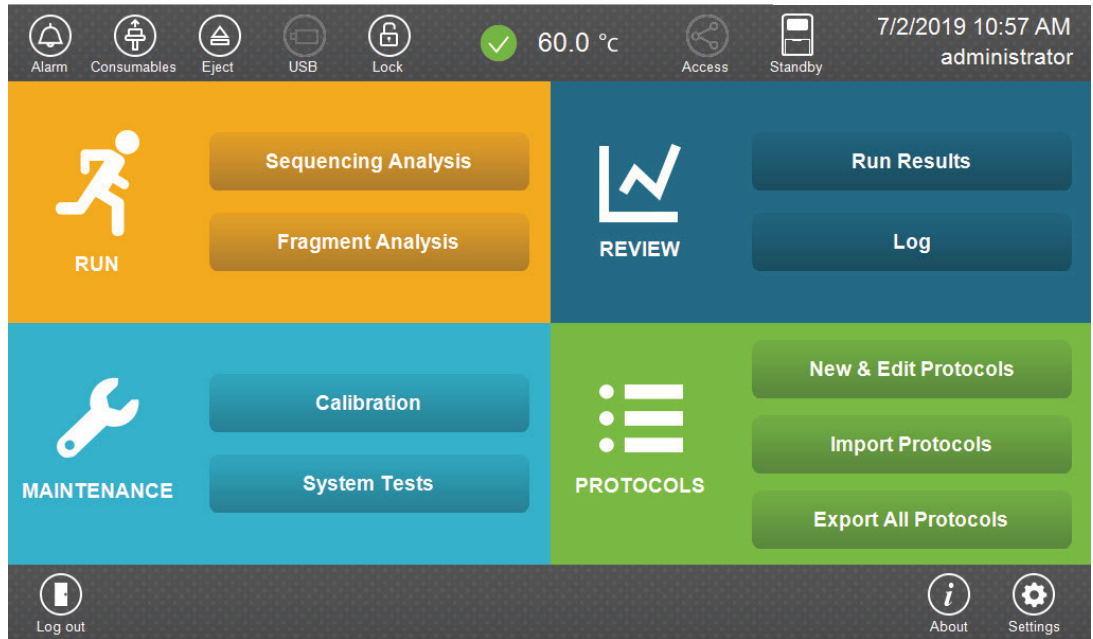
**Note:** We recommend that you preheat the oven for at least 30 minutes prior to starting a run. The oven will automatically turn off after 2 hours if a run is not started.

**Oven Temperature Icon**



**Figure 9. Preheating Oven.**

3. Select **Fragment Analysis** on the 'Main Menu' screen (Figure 10).



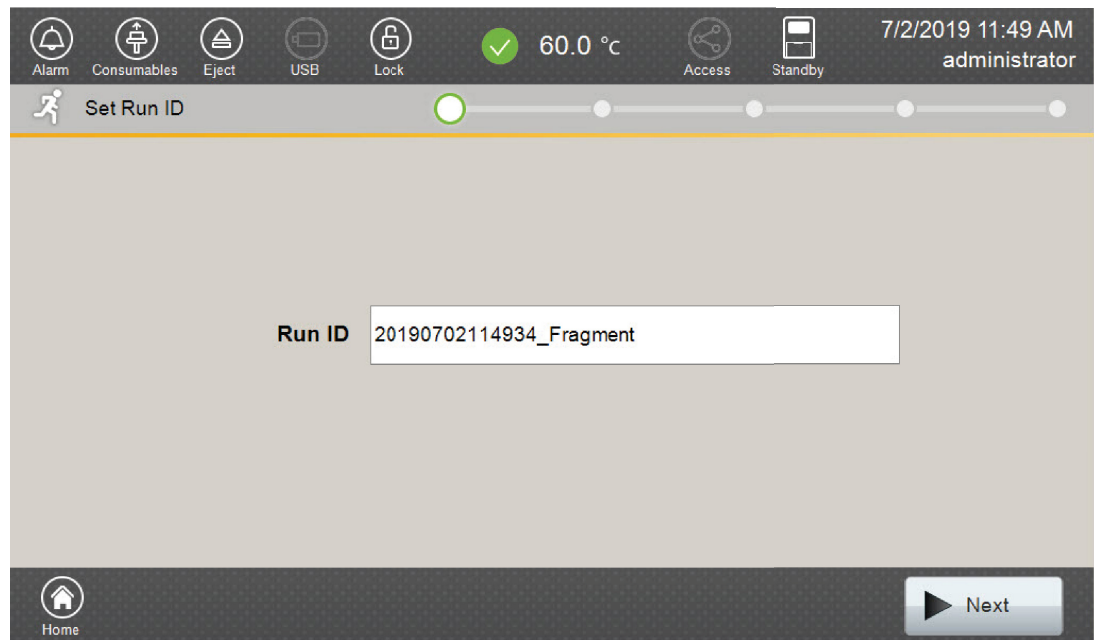
**Figure 10. Spectrum Compact CE System Software 'Main Menu' screen.**

4. Use the default name that includes date, time and "Fragment" or enter a Run ID on the 'Set Run ID' screen (Figure 11). Select the Run ID box to open the 'Run ID' window and a keypad will become active on the touch screen. Alternatively, the Run ID can be entered using a traditional keyboard if one is connected to the Spectrum Compact CE System.

**Notes:**

- a. The Run ID will be included in the file names for the samples.
- b. The following table lists rules for characters that can be used for a Run ID, Strip ID and sample name.

Acceptable Characters	1 to 30 characters for Strip IDs
	1 to 50 characters for Run IDs and sample names
	Upper and lowercase alphabetic characters
	Numbers
	Symbols unless listed below
Unacceptable Characters	#%&{\}<>*?/\$!":@+' = and spaces

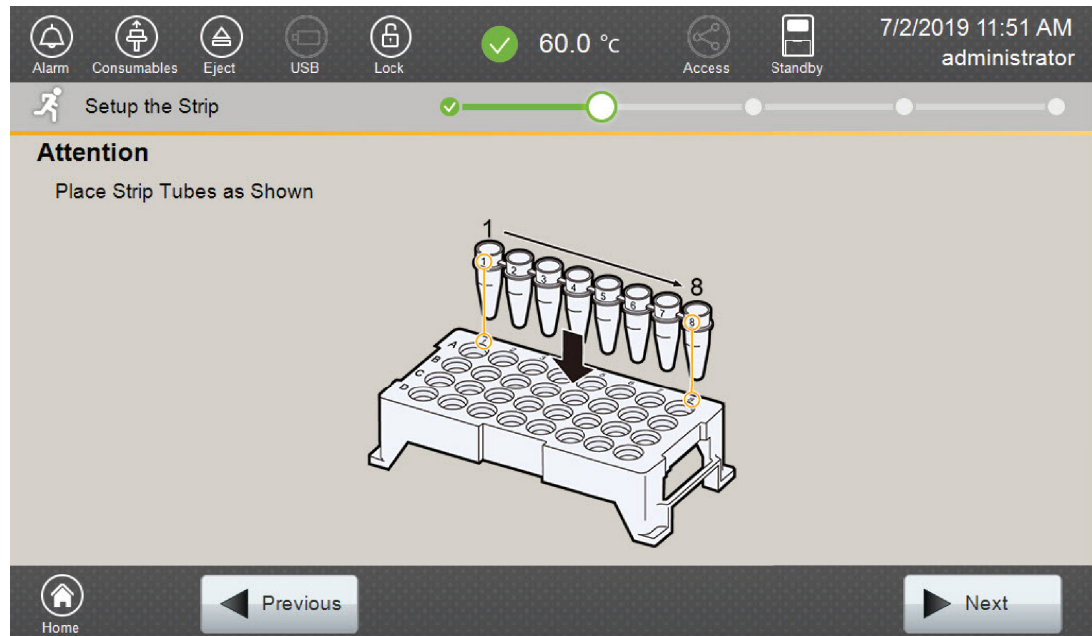


**Figure 11. 'Set Run ID' screen.**

5. Select **Next** to proceed to the message screen for placement of strips into the sample cartridge.

- Follow the message screen for placement of strips into the sample cartridge (Figure 12).

**Note:** Ensure that the correct strip tube is placed into the correct lane (A through D) on the strip base and that wells 1 to 8 of the strip tube are correctly aligned with well positions 1 to 8 on the strip base.



**Figure 12.** 'Setup the Strip' screen.

## 5.4 Assigning Run Information to Samples

There are four methods to assign run information to samples:

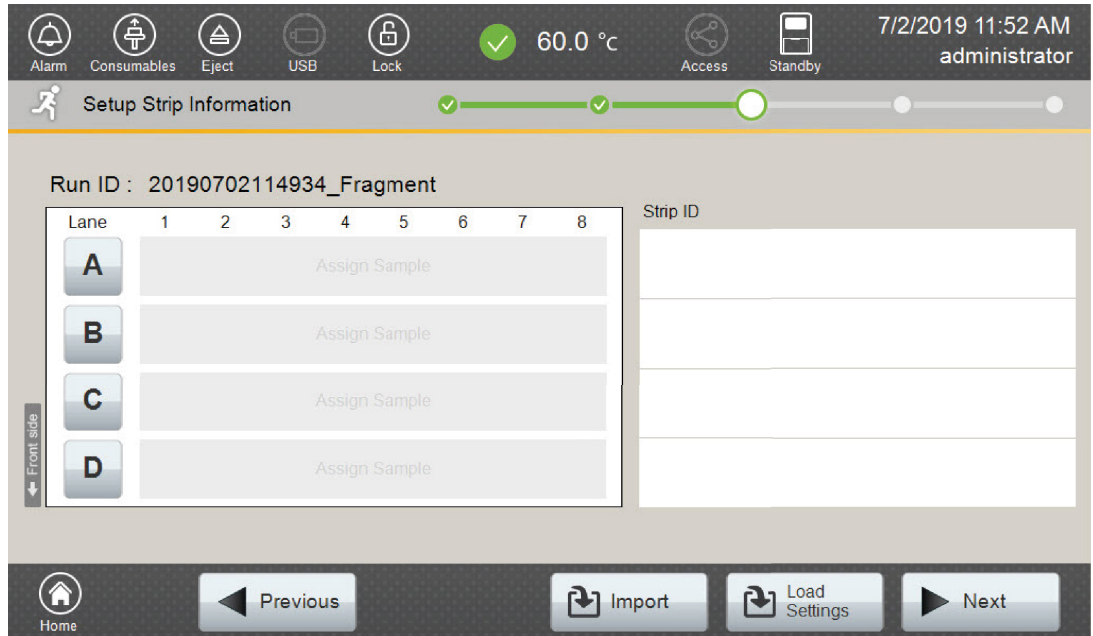
- Creating new strip information,
- Reusing run information from a list of completed runs,
- Loading saved strip information and
- Importing strip information.

The following instructions are for creating new strip information only. For details on the remaining three methods, please see the *Spectrum Compact CE System Operating Manual* #TMD058.

**Note:** The Spectrum Compact CE System includes software for remote access to the instrument. This software allows a user to create and setup sample strips by remotely accessing the Spectrum Compact CE System software using a PC connected directly or via a lab network to the instrument. This software also allows a user to edit protocols and assays, monitor runs and export data. Refer to the *Spectrum Compact CE System Remote Access Software Manual* #TMD064.

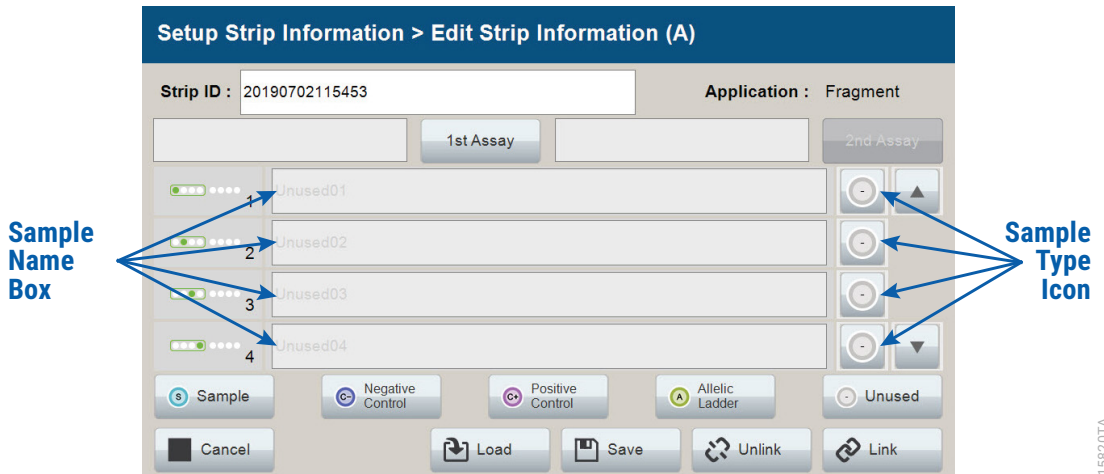
1. Select **Next** on the 'Setup the Strip' screen shown in Figure 12. This will take you to the 'Setup Strip Information' screen shown in Figure 13.

**Note:** The 'Setup Strip Information' screen is divided into two sections: Sample information and Strip ID.



**Figure 13. 'Setup Strip Information' screen.**

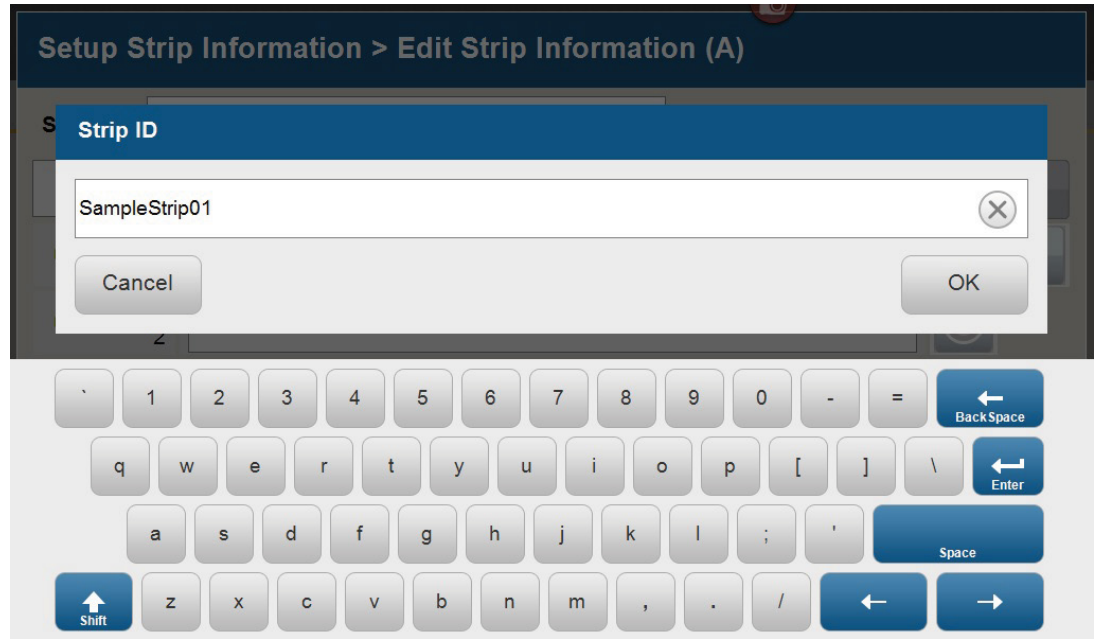
2. Select a lane icon (A, B, C, or D) to enter information for that 8-well strip tube. This opens the 'Edit Strip Information' screen for that lane (Figure 14). This screen displays fields for defining the Strip ID, run assay, sample name and sample type for that lane. Each well in the strip is represented along the left side of the screen. The first injection set of the strip (Wells 1–4) is displayed on the screen. You can use the arrows on the right side of the screen to scroll to the second injection set (Wells 5–8).



**Figure 14. 'Edit Strip Information' screen.**








- The default Strip ID is date and time. It can be modified by selecting the Strip ID box. This opens the 'Set Strip ID' window and a keypad will become active on the touch screen (Figure 15). Alternatively, the Strip ID can be entered using a traditional keyboard if one is connected to the Spectrum Compact CE System. Enter the appropriate Strip ID, then select **OK** to exit and return to the 'Edit Strip Information' screen.



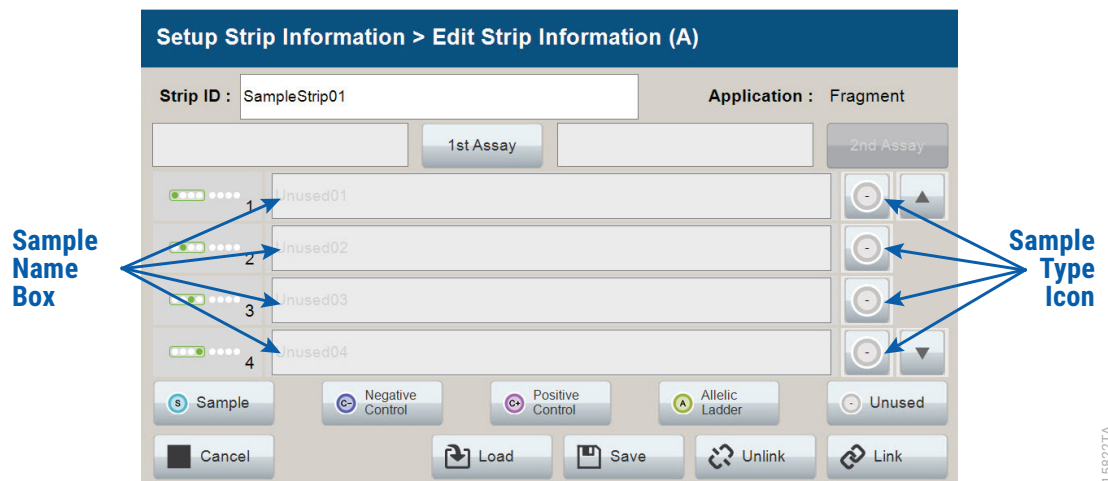
**Figure 15. 'Set Strip ID' window.**

- Select a sample type on the 'Edit Strip Information' screen (Figure 16). Sample types must be selected for each well position before the Sample Name box becomes active for entry of sample name. Sample types are as follows.

Symbol	Sample Type
	Sample
	Negative Control
	Positive Control
	Allelic Ladder
	Unused

- To assign a sample type to a well, select the appropriate sample type along the bottom of the 'Edit Strip Information' screen (Figure 16), then select the **Sample Type** icon to the right of the sample name field for the desired well. This icon will then display the sample type selected for that well (Figure 17).

**Note:** A sample type other than "Unused" must be assigned to at least one well in each injection set. If all of the four wells in an injection set are assigned as "Unused", the injection set will not be run. If all eight wells in a strip are assigned as "Unused", a warning message will be displayed, and no strip information will be assigned. Unused wells for any set of four wells being injected should contain formamide alone. Do not leave uninjected wells empty.



**Figure 16. Sample Type and Sample Name on 'Edit Strip Information' screen.**

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- Enter a sample name for each well position by selecting the Sample Name box adjacent to the well number on the 'Edit Strip Information' screen (Figure 17). This opens the Set Sample Name window and a keypad will become active on the touch screen (Figure 18). Alternatively, the Sample Name can be entered using a traditional keyboard if one is connected to the Spectrum Compact CE System. Enter the appropriate sample name, then select **OK** to exit and return to the 'Edit Strip Information' screen.

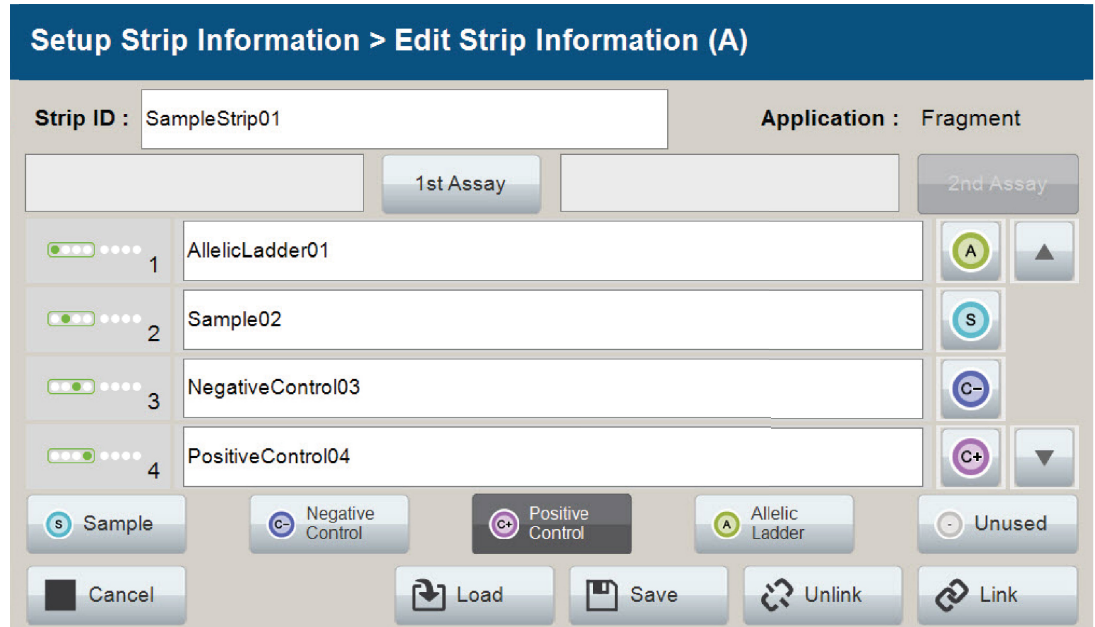


Figure 17. Sample Name Entry on 'Edit Strip Information' screen.

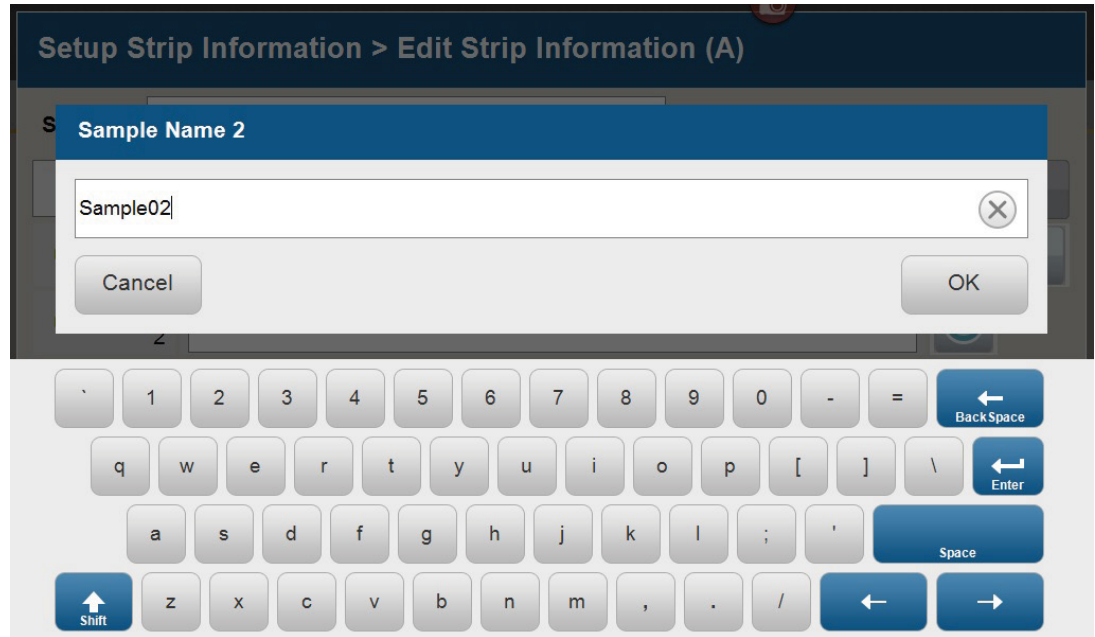


Figure 18. Set Sample Name window.

- The Spectrum Compact Control Software includes preloaded run assays for use with chemistries available from Promega. Refer to the *Spectrum Compact CE System Operating Manual #TMD058* for information on creating or modifying assays. To assign a run assay to an injection set, select **1st Assay** on the right side of the 1st Assay field (Figure 17). This opens the 'Select Assay' window (Figure 19). Select **Promega\_8Dye\_CCOILS\_36\_P4** from the drop-down list (Figure 19) and then select **Apply**.

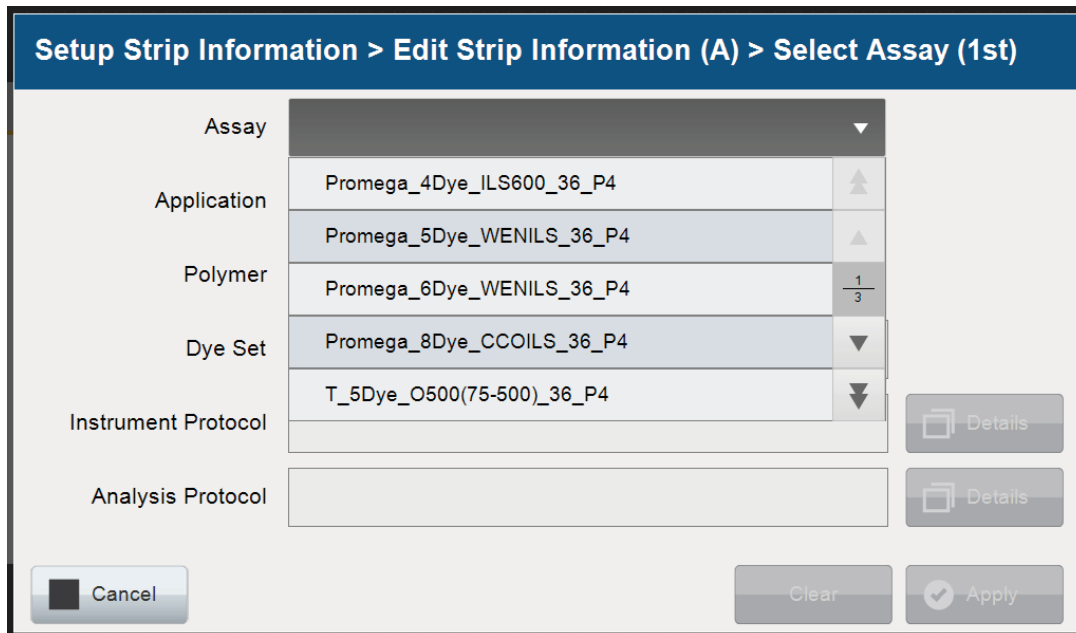


Figure 19. 'Select Assay' window.

8. You can verify the settings of the Instrument and Analysis Protocols associated with the assay chosen by selecting **Details** next to these fields (Figure 20). This will display a window showing the settings in these protocols but will not allow you to edit these settings. Refer to the *Spectrum Compact CE System Operating Manual #TMD058* to edit the Instrument Protocol or Analysis Protocol. When the assay information is confirmed, select **Apply** to return to the 'Edit Strip Information' screen.

The screenshot shows a software interface window titled "Setup Strip Information > Edit Strip Information (A) > Select Assay (1st)". The window contains the following fields and controls:

- Assay:** A dropdown menu showing "Promega\_8Dye\_CCOILS\_36\_P4".
- Application:** A text input field containing "Fragment".
- Polymer:** A text input field containing "Polymer4".
- Dye Set:** A text input field containing "Promega 8-dye".
- Instrument Protocol:** A text input field containing "Fragment\_Analysis36\_Polymer4" with a "Details" button to its right.
- Analysis Protocol:** A text input field containing "Sizecalling\_CCOILS500" with a "Details" button to its right.
- Buttons:** At the bottom, there are three buttons: "Cancel" (with a square icon), "Clear", and "Apply" (with a checkmark icon).

**Figure 20. Accessing Instrument and Analysis Protocols on 'Select Assay' window.**

9. Repeat these steps for the 2nd Assay field if a second assay will be run for the strip. A second assay might include a longer or shorter injection time.
10. Use the down arrow on the right side of the 'Edit Strip Information' screen to enter sample information and assign an assay for samples in wells 5–8 of the strip.
11. When all information is entered and verified for the strip, select **Link** on the lower right corner of the 'Edit Strip Information' screen (Figure 21). This will link the strip to the run. If you want to save the strip information to use in future runs, you need to save the information by selecting **Save** at the bottom of the 'Edit Strip Information' screen (Figure 21). This will save the strip information so that it can be loaded later into another run, as well as link the strip to the run. Refer to the *Spectrum Compact CE System Operating Manual #TMD058* for use of saved strip information.

**Notes:**

- If you select **Unlink** without having previously selected **Save** at the bottom of the 'Edit Strip Information' screen, you will lose the strip information.
- If you forget to assign an assay in the 'Edit Strip Information' screen, a warning window stating "Invalid Data Entered" will appear. Close this window and assign an assay on the 'Edit Strip Information' screen before continuing.

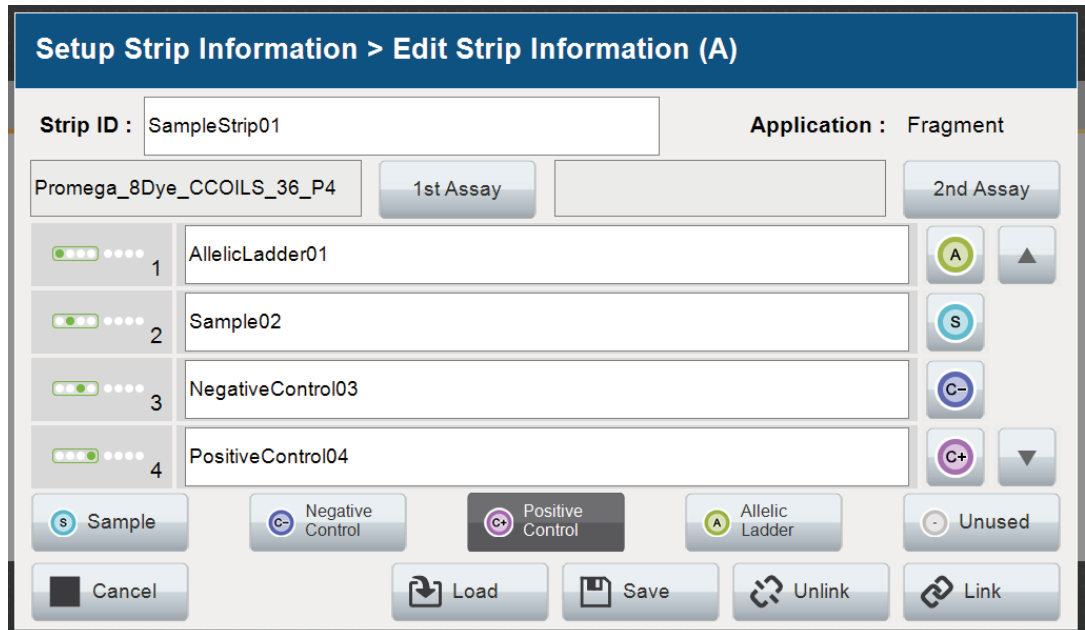


Figure 21. Completed 'Edit Strip Information' screen.

12. Repeat Steps 2–11 for additional lanes as necessary.
13. After all the required lanes have been set up, select **Next** on the 'Setup Strip Information' screen to load the sample cartridge (Figure 22).

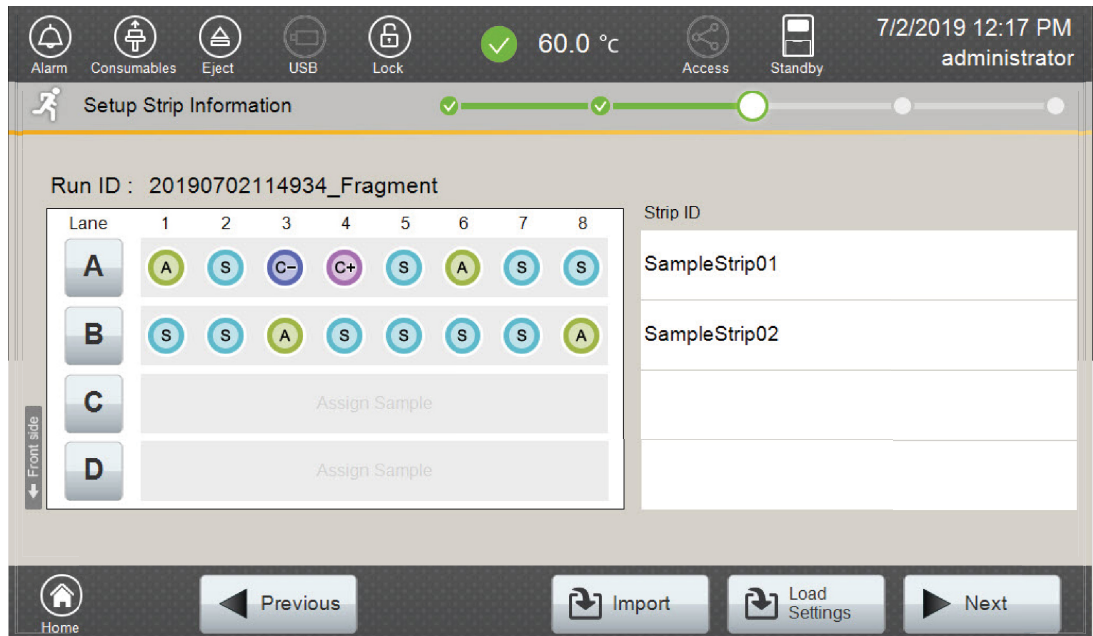


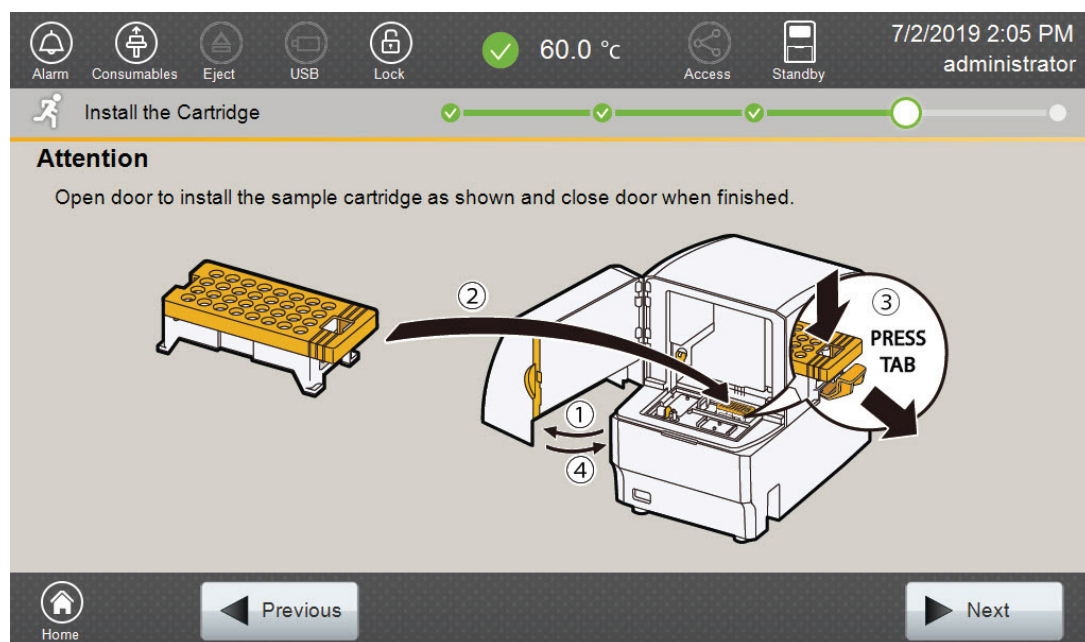
Figure 22. Completed 'Setup Strip Information' screen.

## 5.5. Loading the Sample Cartridge

1. After selecting **Next** on the 'Setup Strip Information' screen, a message window will open, indicating that the autosampler is moving and telling the user to not open the door. In addition, the status indicator flashes green while the autosampler is moving. After autosampler movement is complete, the message window closes and the status indicator returns to a steady green.

**!** **Note:** Do not open the door while the autosampler is in motion.

2. Open the instrument door, then place the sample cartridge on the autosampler following the instructions displayed on the 'Install the Cartridge' screen (Figure 23). Press down on the yellow tab on the autosampler deck that locks the sample cartridge in place before placing sample cartridge into position. Release the tab to lock the sample cartridge in place on the autosampler deck.



**Figure 23. 'Install the Cartridge' screen.**

3. When the sample cartridge is locked into place on the autosampler, close the instrument door and wait for the status indicator to stop flashing amber and turn steady green.

**!** **Note:** Do not open the door while the autosampler is in motion. Follow the instructions displayed on the screen.

4. After the autosampler has returned to its home position, the 'Edit Injection List' screen will be displayed (Figure 24).
5. Select **Run** to start the run.

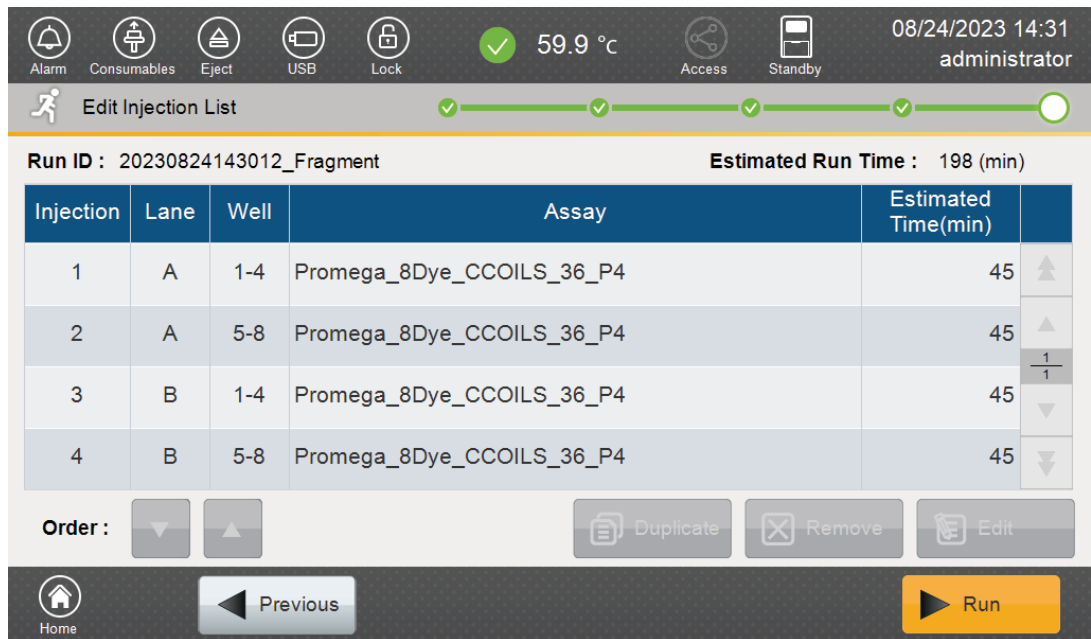


Figure 24. 'Edit Injection List' screen.

6. Refer to the *Spectrum Compact CE System Operating Manual #TMD058* for instructions on editing injection information, monitoring a run, viewing run results and exporting run data.



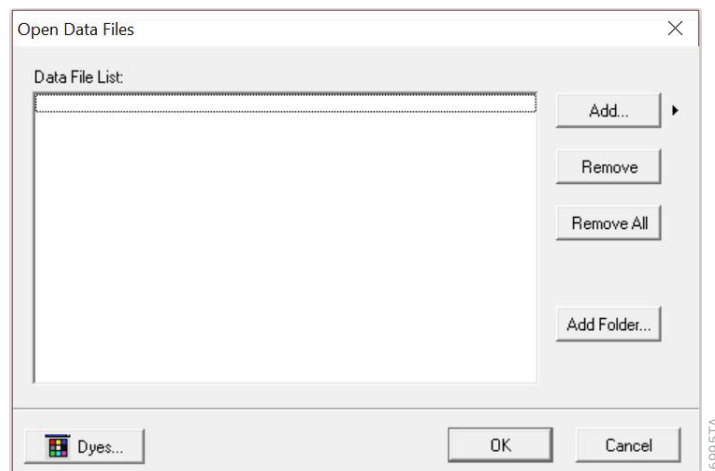
# 6 Data Analysis Using GeneMarker®HID Software

## 6.1 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](http://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 .promega files.
4. Select **Open**, and the selected files will appear in the Data File List (Figure 25).



**Figure 25. The GMHID-Spectrum Data File List.**

5. Select **OK** in the 'Open Data Files' window, and the data will be imported into GMHID-Spectrum. In the 'Raw Data' folder in the File Navigator, verify that the sample types (ladder sample, positive control and negative control) assigned in Section 5 are designated correctly. If sample types are not correct, designate sample types by right-clicking on the file name and selecting **Set Sample Type**.
6. Use the Panel Editor in the Tools menu to select the PowerPlex\_35GY panel from the Panel Template list. Click on the plus symbol to expand the list, select a marker name, then right-click and 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 26). This will set values for peaks within the marker range. 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. There are two options for setting stutter filters: marker-specific and allele-specific. To use marker-specific stutter filters, select **Use Marker-Specific Values**. Marker-specific stutter filters are preloaded in the PowerPlex\_35GY panel file. Stutter filters for one marker can be applied to all markers in a kit by checking the box "Apply Stutter Settings to All Markers". To use allele-specific values, select the radio button next to Use Allele-Specific Values (From Panel) in the 'Edit Marker' window, then enter the appropriate stutter filters in the Panel Table of the 'Panel Editor' window. Refer to *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555* for more information.

**Note:** Individual laboratories should determine their peak amplitude thresholds from internal validation studies.
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.

Edit Marker ×

Marker Parameters

Marker Name:

Nucleotide Repeats (x):

Boundary:  To

Min Homozygote Intensity:

< = Inconclusive <=

Min Heterozygote Intensity:

< = Inconclusive <=

Max Heterozygote Imbalance(%):

Min Heterozygote Imbalance(%):

Apply Homo/Hetero Settings to All Markers

Apply Homo/Hetero Settings to Markers in this dye

Stutter Filter:

Use Marker-Specific Values

N - 2x %

N + x %  N - x %

N+0.5x %  N-0.5x %

Use Allele-Specific Values(From Panel)

Apply Stutter Settings to All Markers

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Figure 26. The 'Edit Marker' window for the D3S1358 marker.

- Choose **Run** in the Magic Wizard or select the **Run Project** icon (green arrow) in the toolbar. The 'Template Selection' screen will appear (Figure 27). Select the PowerPlex\_35GY template and the settings shown in Figure 27. Verify that the panel that you created in Step 6 and 7 is selected in the panel drop-down field. The Size Standard must be ILS500 and the Standard Color must be Brown. Select **Next**.

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

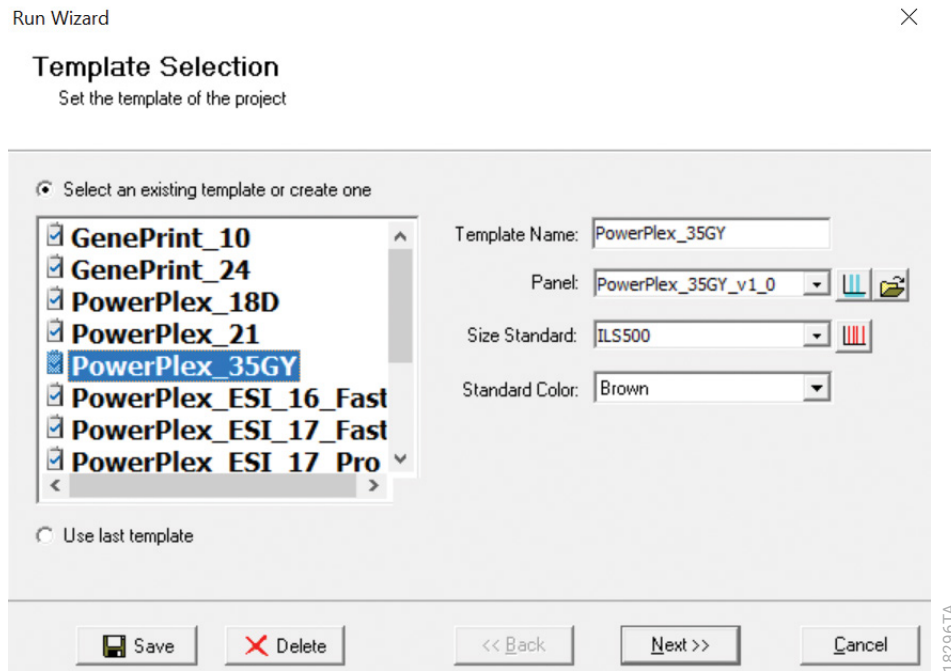
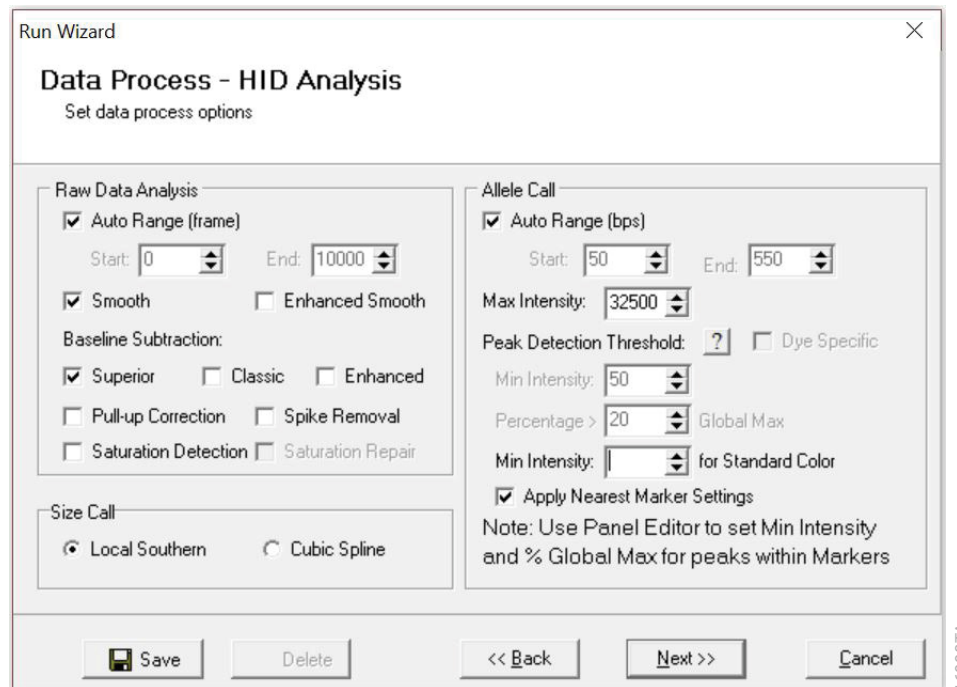


Figure 27. The 'Template Selection' window.



**Figure 28. The 'Data Process' window for an analysis method.**

- The 'Data Process – HID Analysis' window will appear (Figure 28). 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**.

Run Wizard X

### Additional Settings - HID Analysis

Set additional options related to the different analysis type

Allelic Ladder:

P.C. Template 1:

P.C. Template 2:

Allele Evaluation

Peak Score:

Reject <  Check  < Pass

Mixture Evaluation

Valid Mixture Peak Percentage:  %

Min Mixture Marker Number:

Auto Select Best Ladder

Allow Match # Variance:

Max Average Size Diff:

Use Ladder Library

Min Heterozygosity:

Auto Panel Adjustment

Sample Quality Peak(s)

Small Allele: [QIS:QIS](#)

Large Allele: [QIL:QIL](#)

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**Figure 29. The 'Additional Settings' window for an analysis method.**

10. The 'Additional Settings – HID Analysis' window will appear (Figure 29). Select **35GY\_2800M** as the P.C. Template 1 and verify that the boxes are checked for "Auto Select Best Ladder", "Auto Panel Adjustment" and "Sample Quality Peak(s)". Ensure that "QIS:QIS" is selected for the Small Allele and "QIL:QIL" for the Large Allele in the Sample Quality Peak(s) Section. 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.
11. When the analysis is complete, the 'Main Analysis' window will appear, displaying the processed data. We recommend that you review any yellow or red flagged peaks 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.

## 6.2 Controls in GeneMarker®HID Software for Spectrum CE Systems

1. Observe the results for the negative control. Using the protocols defined in this manual, the negative controls should be devoid of amplification products.
2. Observe the results for the 2800M Control DNA. The expected 2800M allele designations for each locus are listed in Table 11 (Section 10.1).

## 6.3 Quality Indicators Small (QIS) and Large (QIL)

The PowerPlex® 35GY System includes PCR primers and synthetic DNA templates used to amplify two Quality Indicators, assisting in the analysis and interpretation of STR data. The Quality Indicator Small (QIS) migrates at approximately 75bp while the Quality Indicator Large (QIL) migrates at approximately 435bp. QIS and QIL cover the lower and higher base pair range of the amplification product. While the presence or absence of QIS and QIL can be used to assess the overall success of the PCR reaction, their relative peak heights are also useful in determining sample inhibition or degradation. Table 5 provides additional information to assist with the interpretation of QIS and QIL data.

**Table 5. Interpretation of QIS and QIL Peaks.**

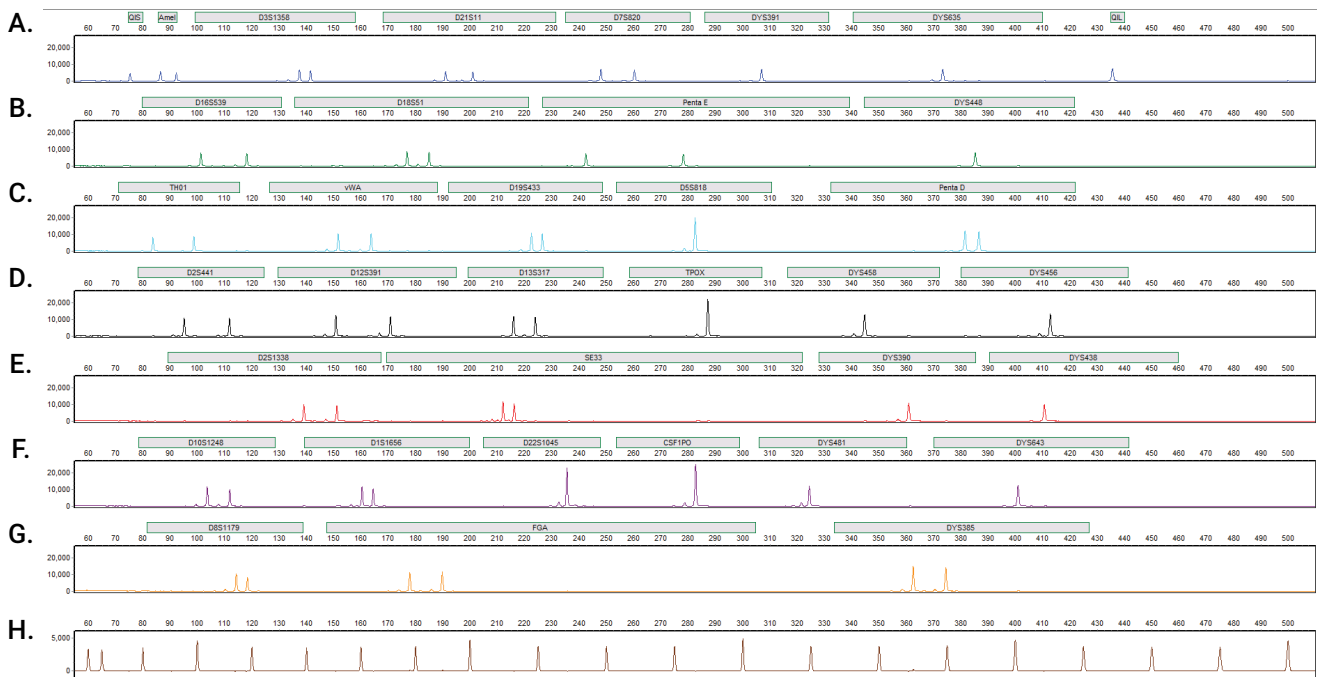
Allele Peaks	QIS Peak	QIL Peak	Amplification Interpretation
High	Present	Present	PCR successful
Absent	Present	Present	No or low DNA
Absent	Absent	Absent	PCR failure
Absent	Present	Absent	PCR inhibition
Ski-Slope	Present	Absent	PCR inhibition
Ski-Slope	Present	Present	DNA degradation

GeneMarker®HID Software for Spectrum CE Systems can automatically flag samples if the ratio of QIL/QIS falls below a user-defined threshold. This threshold can be set using the 'Quality Indicator Flag 'Q' in the 'Sample Quality' tab of the 'Preferences' window. In-house validation should be performed to define the QIL/QIS threshold for each laboratory. Refer to *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555* for more information.

**Note:** Only one peak should be present for QIS and QIL in successful amplification reactions, even though the allelic ladder shows the presence of two alleles each. GeneMarker®HID Software for Spectrum CE Systems requires the presence of two alleles per marker in the allelic ladder for successful analysis.

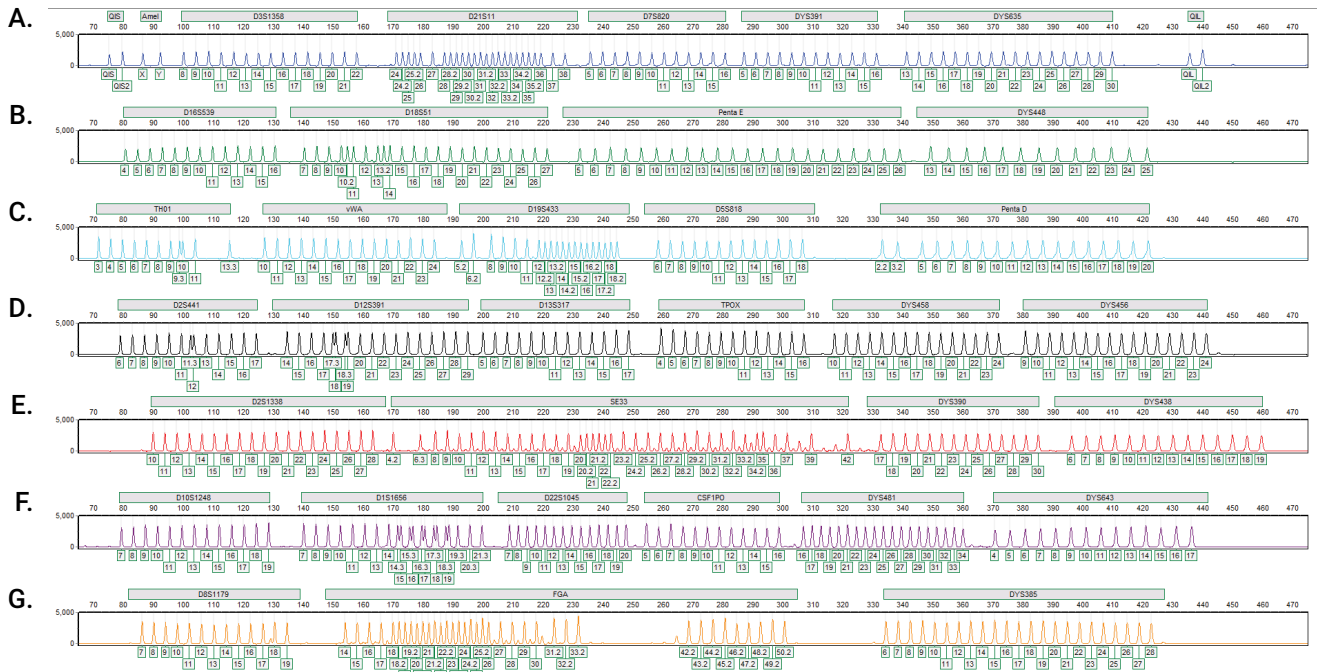
# 7 Results

Representative results of the PowerPlex® 35GY System are shown in Figure 30. The PowerPlex® 35GY Allelic Ladder Mix is shown in Figure 31.



**Figure 30. The PowerPlex® 35GY System.** The 2800M Control DNA (1.0ng) was amplified using the PowerPlex® 35GY System. Amplification products were mixed with CCO Internal Lane Standard 500 and analyzed with a Spectrum Compact CE System and a 1.6kV, 9-second injection. Results were analyzed using GeneMarker® HID Software for Spectrum CE Systems, version 3.2.0 and PowerPlex® 35GY panel file. **Panel A.** An electropherogram showing the peaks of the FL-8C-labeled loci: QIS, Amelogenin, D3S1358, D21S11, D7S820, DY391, DY635 and QIL. **Panel B.** An electropherogram showing the peaks of the JOE-8C-labeled loci: D16S539, D18S51, Penta E and DY448. **Panel C.** An electropherogram showing the peaks of the AQA-8C-labeled loci: TH01, vWA, D19S433, D5S818 and Penta D. **Panel D.** An electropherogram showing the peaks of the TMR-8C-labeled loci: D2S441, D12S391, D13S317, TPOX, DY458 and DY456. **Panel E.** An electropherogram showing the peaks of the CXR-8C-labeled loci: D2S1338, SE33, DY390 and DY438. **Panel F.** An electropherogram showing the peaks of the TOM-8C-labeled loci: D10S1248, D1S1656, D22S1045, CSF1PO, DY481 and DY643. **Panel G.** An electropherogram showing the peaks of the WEN-8C-labeled loci: D8S1179, FGA and DY385a/b. **Panel H.** An electropherogram showing the 60bp to 500bp fragments of the CCO Internal Lane Standard 500.





**Figure 31. The PowerPlex® 35GY Allelic Ladder Mix.** The PowerPlex® 35GY Allelic Ladder Mix was analyzed with a Spectrum CE Compact System and a 1.6kV, 9-second injection. The sample file was analyzed with the GeneMarker® HID Software for Spectrum CE Systems, version 3.2.0, and PowerPlex® 35GY panel file. **Panel A.** The FL-8C-labeled allelic ladder components and their allele designations. **Panel B.** The JOE-8C-labeled allelic ladder components and their allele designations. **Panel C.** The AQA-8C-labeled allelic ladder components and their allele designations. **Panel D.** The TMR-8C-labeled allelic ladder components and their allele designations. **Panel E.** The CXR-8C-labeled allelic ladder components and their allele designations. **Panel F.** The TOM-8C-labeled allelic ladder components and their allele designations. **Panel G.** The WEN-8C-labeled allelic ladder components and their allele designations.

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## 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. Trinucleotide repeat loci, like DYS481 and 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\_35GY panel file for marker-specific filtering and is preloaded in GMHID-Spectrum.

In addition to stutter peaks, you may observe the following low-level DNA-dependent artifact peaks (Table 6) and DNA-independent (with or without human genomic DNA) artifact peaks (Table 7) with the PowerPlex® 35GY System.

**Table 6. DNA-Dependent Artifacts Observed in Amplification Reactions with Human Genomic DNA.**

<b>Locus</b>	<b>Artifact Size</b>
Amelogenin	$n-1, n+1$
D18S51	$n-2$
D2S441	$n-2$
TPOX	$n-12$ to $n-18$
SE33	$n-2$
D1S1656	$n-2$
FGA	$n-2$

**Note:** Tri-allelic patterns may occasionally be seen in SE33, coincident with sister allele imbalance of the two major alleles. Signal intensity of the third allele, which migrates 11 repeat units downstream of the imbalanced major allele, is much lower than the two major alleles.

**Table 7. DNA-Independent Artifacts Observed in Amplification Reactions with and without Human Genomic DNA.**

<b>Dye Label</b>	<b>Artifact Size<sup>1</sup></b>
FL-8C	~58–73 bases
	~120–126 bases
	~144–151 bases
JOE-8C	~58–65 bases
	~70–76 bases
AQA-8C	~58–63 bases
	~398–400 bases
TMR-8C	~58–67 bases
	~70–80 bases
CXR-8C	~58–67 bases
	~72–79 bases
TOM-8C	~58–77 bases
	~119–121 bases <sup>2</sup>
WEN-8C	~58–83 bases

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

<sup>2</sup>This artifact is occasionally seen when amplifying DNA from swab extracts and might be called as a 17 allele in D10S1248.

Testing was performed with a variety of nonhuman DNA templates from bacteria, yeast, mammals and primates to characterize known artifacts with the PowerPlex® 35GY System. The artifacts listed in Table 8 were noted above a 150RFU threshold with 10ng of template DNA using the Spectrum CE System. Partial profiles were obtained with all primate species tested, but these profiles can be distinguished from a human profile because most of the alleles were called as off-ladder or were outside of the locus panels.

**Table 8. Nonhuman DNA Cross-Reactivity.**

<b>DNA Source</b>	<b>Artifact Size</b>	<b>Dye Label</b>
Chicken	~289 bases	TOM-8C
Pig	~370–372 bases	JOE-8C
	~373 bases	TMR-8C

# 8 Troubleshooting

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

Refer to the *Spectrum Compact CE System Operating Manual #TMD058* for instrument troubleshooting. For troubleshooting GMHID-Spectrum, refer to the *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555*.

## 8.1 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.
	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. Vortex before use.

Symptoms	Causes and Comments
Faint or absent allele peaks for the positive control reaction (continued)	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.
	Poor-quality formamide was used. Use only Hi-Di™ formamide when analyzing samples.
Extra peaks visible in one or all color channels	Contamination with another template DNA or previously amplified DNA. Cross-contamination can be a problem. Use aerosol-resistant pipette tips and change gloves regularly.
	Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the DNA source as soon as possible to each well and follow immediately by thermal cycling.
	Samples were not denatured completely. Heat-denature samples for the recommended time, and 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.  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 reannealing.
	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. Ensure no urea crystals are present in the polymer cartridge before installing on the CE.

Symptoms	Causes and Comments
<p>Extra peaks visible in one or all color channels (continued)</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"> <li>• Perform a new spectral calibration and rerun the samples.</li> <li>• Confirm that the correct spectral was used for the sample run.</li> <li>• Instrument sensitivities can vary. Optimize the injection conditions. Refer to the instrument user manual.</li> </ul>
	<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>
<p>Allelic ladder not running the same as samples</p>	<p>Be sure the allelic ladder and samples are from the same instrument run.</p>
	<p>Allelic ladder and primer pair mix were not compatible. Ensure that the allelic ladder is from the same kit as the primer pair mix.</p>
	<p>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.</p>
	<p>Poor injection of allelic ladder. Include more than one ladder per instrument run. Include one allelic ladder for every 16 samples.</p>
	<p>Internal size standard was not assigned correctly. Evaluate the sizing labels on the ILS and correct if necessary.</p>
<p>Peak height imbalance</p>	<p>Miscellaneous balance problems. 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.</p>
	<p>PCR amplification mix was not mixed well. Vortex the PCR amplification mix for 5–10 seconds before dispensing into the reaction tubes or plate.</p>

## 8.2 Amplification of Extracted DNA

The following information is specific to amplification of purified DNA. For information about general amplification and detection, see Section 8.1.

<b>Symptoms</b>	<b>Causes and Comments</b>
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 <sup>+</sup> , Na <sup>+</sup> , Mg <sup>2+</sup> or EDTA from the DNA sample can negatively affect PCR. A change in pH also may affect PCR. Store DNA in TE <sup>-4</sup> buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE <sup>-4</sup> 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 adenylation.</p> <ul style="list-style-type: none"> <li>• Be sure to perform the recommended extension step at 60°C after thermal cycling.</li> <li>• Decrease the amount of template DNA. Using more than the recommended amount of template DNA can result in incomplete adenylation.</li> <li>• Decrease cycle number.</li> <li>• Increase the final extension time.</li> </ul>
<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>



### 8.3 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 8.1.

Symptoms	Causes and Comments
Faint or absent allele peaks	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 excess 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>

<b>Symptoms</b>	<b>Causes and Comments</b>
<p>Extra peaks visible in one or all color channels (continued)</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 7) 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 adenylation.</p> <ul style="list-style-type: none"> <li>• Be sure to perform the recommended extension step at 60°C after thermal cycling.</li> <li>• Decrease cycle number.</li> <li>• Increase the final extension time.</li> </ul>
<p>Peak height imbalance</p>	<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"> <li>• Be sure to use the recommended number of punches. Follow the manufacturer's recommendations when depositing sample onto the card.</li> <li>• Decrease cycle number.</li> </ul>
	<p>The cycle number was too high. Decrease the cycle number by one cycle and repeat the amplification.</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>

<b>Symptoms</b>	<b>Causes and Comments</b>
Peak height imbalance (continued)	<p data-bbox="748 306 1421 447">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 data-bbox="748 468 1421 919" style="list-style-type: none"> <li data-bbox="748 468 1421 646">• 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.</li> <li data-bbox="748 667 1421 919">• 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.</li> </ul> <p data-bbox="748 940 1421 1119">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>
Extreme variability in sample-to- sample peak heights	<p data-bbox="748 1140 1421 1348">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.</p>

## 8.4 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 8.1.

<b>Symptoms</b>	<b>Causes and Comments</b>
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"> <li>• Be sure to perform the recommended extension step at 60°C after thermal cycling.</li> <li>• 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.</li> <li>• Decrease cycle number.</li> <li>• Increase the final extension time.</li> </ul>

Symptom	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>

## 8.5 GeneMarker®HID Software for Spectrum CE Systems

Symptoms	Causes and Comments
Stutter peaks not filtered	Be sure the 'Use Marker-Specific Values' box in the 'Panel Editor' is checked. Alternatively, select the 'Use Allele-Specific Values (From Panel)' box in the 'Edit Marker' window and enter the appropriate stutter filters in the Panel Table of the 'Panel Editor' window.
Alleles not called	To analyze samples with GMHID-Spectrum, at least one allelic ladder must be present in the data set. Ensure that the allelic ladder is designated as such in Section 5.3 or 6.1.
	<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"> <li>• Create a new size standard using the internal lane standard fragments present in the sample.</li> <li>• Rerun samples using a longer run time.</li> </ul>
	A low-quality allelic ladder was used during analysis. Ensure that only high- quality allelic ladders are used for analysis. See the 'Off-ladder or off-bin alleles' column below for more information.
Off-ladder or off-bin 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 panel file selected for analysis was incorrect for the STR system used. Assign a correct panel file that corresponds to the STR system used for amplification.
	The allelic ladder was not designated as an allelic ladder in Section 5.3 or 6.1.
	The internal lane standard was not properly identified in the sample. Manually redefine the sizes of the size standard fragments in the sample.



Symptoms	Causes and Comments
<p>Off-ladder or off-bin alleles (continued)</p>	<p>A low-quality allelic ladder was used during analysis. Ensure that only high- quality allelic ladders are used for analysis.</p> <p>Allelic ladders are flagged for Analysis Quality when:</p> <ul style="list-style-type: none"> <li>• There is an error in bin shifting.</li> <li>• Peaks in virtual bins have greater than expected peak heights.</li> <li>• The expected peaks cannot be identified in a marker.</li> </ul> <p>When expected peaks cannot be identified in a marker, the marker bar will be shaded dark green, indicating the marker failed analysis.</p> <p>When errors in bin shifting occur or when peaks in virtual bins have higher than expected peak heights, the marker bars will be outlined in red. Any peaks that cause an 'Analysis Quality' flag are indicated in the electropherogram and Peak Table.</p> <p>When Flag Variant Alleles in Ladder is selected in the 'Display' tab of the 'Preferences' window, peaks in virtual bins with higher than expected peak heights are indicated by yellow vertical bars and green allele labels. You can set your preferences to automatically delete these peaks by selecting Auto-Delete Alleles in Virtual Bins in Allelic Ladder in the 'Forensics' tab of the 'Preferences' window.</p> <p>Peaks that are not present in the expected bins are marked with red vertical bars and red allele labels.</p>
<p>Size standard not called or poor quality</p>	<p>If a partial range was chosen for the Allele Call analysis in the Data Process - HID Analysis window of the Run Wizard, the chosen starting or end point was incorrect. Adjust the starting point or end point or use the Auto Range setting for the analysis.</p> <p>The incorrect Standard Color was selected in the Template Selection window of the Run Wizard. The Standard Color should be Brown.</p>

Symptoms	Causes and Comments
Size standard not called or poor quality (continued)	If the quality of the size standard peaks is not sufficient (and the 'Low ILS Quality Flag 'SQ"' section in the 'Sample Quality' tab of the 'Preferences' window is selected), the size standard is marked with a red 'SQ' flag in the Sample File Tree of the 'Main Analysis' window and in the upper left corner of the sample electropherogram. There are three criteria that determine whether a size standard fails the size quality requirements: <ul style="list-style-type: none"> <li>• The sample size standard does not contain all peaks that are marked as "Enabled" in the chosen size standard template.</li> <li>• Not all of the expected size standard peaks are called in the 'Main Analysis' window. If the expected size standard peaks are present but are not labeled, open the Calibration Charts and select the affected sample from the Sample List. Edit the size calls of the sample size standard to remove the extra peak(s) and define the correct peak(s). Right-click anywhere in the Sample Size Standard Chart and select <b>Update Calibration</b>.</li> <li>• Not all of the expected size standard peaks are of high quality such that the absolute difference in size between the actual size standard peaks and the expected, Enabled size standard peaks is less than 0.2bp.</li> </ul>
	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"> <li>• Create a new size standard using the internal lane standard fragments present in the sample.</li> <li>• Rerun samples using a longer run time.</li> </ul>
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 rerun the samples.
	Confirm that the correct spectral was used to run the samples.

# 9

## References

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# 10 Appendix

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## 10.1 Advantages of Using the Loci in the PowerPlex® 35GY System

A single PowerPlex® 35GY System reaction amplifies all core loci required for US expanded CODIS and European databases (Table 9 and Table 10). The male-specific DYS391 locus is included to identify null Y results for Amelogenin. Additionally, ten Y-STR loci are included in the system. Table 11 lists the PowerPlex® 35GY System alleles amplified from the 2800M standard DNA template.

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 9. The PowerPlex® 35GY System Locus-Specific Information.**

<b>STR Locus</b>	<b>Label</b>	<b>Chromosomal Location<sup>1</sup></b>	<b>Repeat Sequence<sup>2</sup> 5' -&gt;3'</b>
Amelogenin <sup>3</sup>	FL-8C	Xp22.1–22.3 and Y	NA
D3S1358	FL-8C	3p21.31 (45.557Mb)	TCTA Complex
D21S11	FL-8C	21q21.1 (19.476Mb)	TCTA Complex (20)
D7S820	FL-8C	7q21.11 (83.433Mb)	GATA
DYS391	FL-8C	Y	TCTA
DYS635	FL-8C	Y	TSTA compound
D16S539	JOE-8C	16q24.1 (84.944Mb)	GATA
D18S51	JOE-8C	18q21.33 (59.1Mb)	AGAA (20)
Penta E	JOE-8C	15q26.2 (95.175Mb)	AAAGA
DYS448	JOE-8C	Y	AGAGAT
TH01	AQA-8C	11p15.5 (2.149Mb)	AATG (20)
vWA	AQA-8C	12p13.31 (5.963Mb)	TCTA Complex (20)
D19S433	AQA-8C	19q12 (35.109Mb)	AAGG Complex
D5S818	AQA-8C	5q23.2 (123.139Mb)	AGAT
Penta D	AQA-8C	21q22.3 (43.88Mb)	AAAGA
D2S441	TMR-8C	2p14 (68.214Mb)	TCTA
D12S391	TMR-8C	12p12 (12.341Mb)	AGAT/AGAC Complex
D13S317	TMR-8C	13q31.1 (81.62Mb)	TATC
TPOX	TMR-8C	2p25.3 (1.472Mb)	AATG
DYS458	TMR-8C	Y	GAAA
DYS456	TMR-8C	Y	AGAT
D2S1338	CXR-8C	2q35 (218.705Mb)	TGCC/TTCC
SE33	CXR-8C	6q14 (89.043Mb)	AAAG Complex
DYS390	CXR-8C	Y	(TCTA) (TCTG)
DYS438	CXR-8C	Y	TTTTTC
D10S1248	TOM-8C	10q26.3 (130.567Mb)	GGAA
D1S1656	TOM-8C	1q42 (228.972Mb)	TAGA Complex
D22S1045	TOM-8C	22q12.3 (35.779Mb)	ATT
CSF1PO	TOM-8C	5q33.1 (149.436Mb)	AGAT
DYS481	TOM-8C	Y	CTT

**Table 9. The PowerPlex® 35GY System Locus-Specific Information (continued).**

STR Locus	Label	Chromosomal Location <sup>1</sup>	Repeat Sequence <sup>2</sup> 5'→3'
DYS643	TOM-8C	Y	CTTTT
D8S1179	WEN-8C	8q24.13 (125.976Mb)	TCTA Complex (20)
FGA	WEN-8C	4q28 (155.866Mb)	TTTC Complex (20)
DYS385a/b	WEN-8C	Y	GAAA

<sup>1</sup>Information about chromosomal location of these loci can be found in references 19, 20 and 21 and at:

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

<sup>2</sup>The 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."

<sup>3</sup>Amelogenin is not an STR

NA = Not Applicable

**Table 10. The PowerPlex® 35GY System Allelic Ladder Information.**

STR Locus	Label	Size Range of Allelic Ladder Components <sup>1,2</sup> (bases)	Repeat Numbers of Allelic Ladder Components <sup>3</sup>
QIS	FL-8C	81, 85	QIS, QIS2
Amelogenin	FL-8C	90, 96	X, Y
D3S1358	FL-8C	103–159	8–22
D21S11	FL-8C	172–228	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	FL-8C	238–282	5–16
DYS391	FL-8C	287–331	5–16
DYS635	FL-8C	341–409	13–30
QIL	FL-8C	438, 442	QIL, QIL2
D16S539	JOE-8C	84–132	4–16
D18S51	JOE-8C	141–221	7-10, 10.2, 11–13, 13.2, 14–27
Penta E	JOE-8C	233–338	5–26
DYS448	JOE-8C	348–420	13–25
TH01	AQA-8C	73–116	3–9, 9.3, 10–11, 13.3
vWA	AQA-8C	127–183	10–24
D19S433	AQA-8C	194–246	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

**Table 10. The PowerPlex® 35GY System Allelic Ladder Information (continued).**

STR Locus	Label	Size Range of Allelic Ladder Components <sup>1,2</sup> (bases)	Repeat Numbers of Allelic Ladder Components <sup>3</sup>
D5S818	AQA-8C	257–305	6–18
Penta D	AQA-8C	330–418	2.2, 3.2, 5–20
D2S441	TMR-8C	81–125	6–11, 11.3, 12–17
D12S391	TMR-8C	134–194	14–17, 17.3, 18, 18.3, 19–29
D13S317	TMR-8C	200–248	5–17
TPOX	TMR-8C	258–306	4–16
DYS458	TMR-8C	317–373	10–24
DYS456	TMR-8C	379–439	9–24
D2S1338	CXR-8C	91–163	10–28
SE33	CXR-8C	169–319	4.2, 6.3, 8–20, 20.2, 21, 21.2, 22, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 31.2, 32.2, 33.2, 34.2, 35–37, 39, 42
DYS390	CXR-8C	334–386	17–30
DYS438	CXR-8C	396–461	6–19
D10S1248	TOM-8C	80–128	7–19
D1S1656	TOM-8C	140–199	7–14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18, 18.3, 19, 19.3, 20.3, 21.3
D22S1045	TOM-8C	207–246	7–20
CSF1PO	TOM-8C	256–300	5–16
DYS481	TOM-8C	307–361	16–34
DYS643	TOM-8C	372–437	4–17
D8S1179	WEN-8C	76–124	7–19
FGA	WEN-8C	144–290	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, 47.2, 48.2, 49.2, 50.2
DYS385a/b	WEN-8C	324–412	6–28

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

<sup>2</sup>When using an internal lane standard, such as the CCO 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.

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

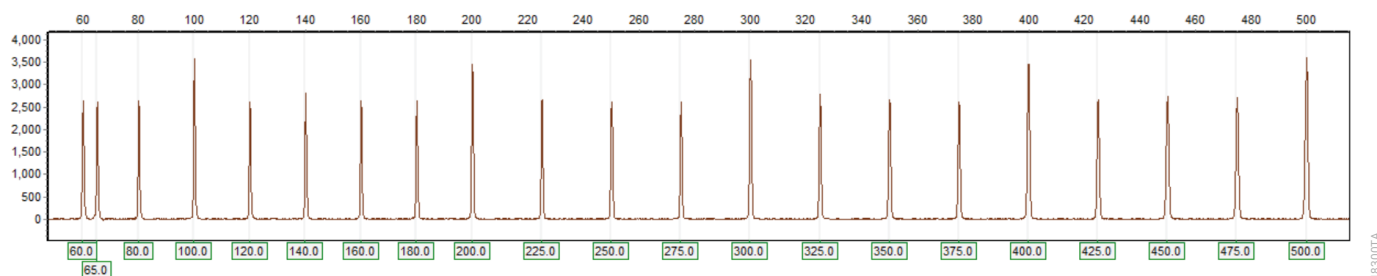


**Table 11. The PowerPlex® 35GY System Allele Determinations for the 2800M Control DNA.**

<b>STR Locus</b>	<b>2800M</b>
Amelogenin	X, Y
D3S1358	17, 18
D21S11	29, 31.2
D7S820	8, 11
DYS391	10
DYS635	21
D16S539	9, 13
D18S51	16, 18
Penta E	7, 14
DYS448	19
TH01	6, 9.3
vWA	16, 19
D19S433	13, 14
D5S818	12, 12
Penta D	12, 13
D2S441	10, 14
D12S391	18, 23
D13S317	9, 11
TPOX	11, 11
DYS458	17
DYS456	17
D2S1338	22, 25
SE33	15, 16
DYS390	24
DYS438	9
D10S1248	13, 15
D1S1656	12, 13
D22S1045	16, 16
CSF1PO	12, 12
DYS481	22
DYS643	10
D8S1179	14, 15
FGA	20, 23
DYS385	13, 16

## 10.2 The CCO Internal Lane Standard 500

The CCO 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 32). Each fragment is labeled with CCO-8C dye and can be detected separately (as an eighth color) in the presence of PowerPlex® 35GY-amplified material. The CCO ILS 500 is designed for use in each CE injection to increase precision in analysis when using the PowerPlex® 35GY System. Protocols to prepare and use this internal lane standard are provided in Section 5.



**Figure 32. CCO Internal Lane Standard 500.** An electropherogram showing the CCO Internal Lane Standard 500 fragments.

## 10.3 Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume

Depending on the procedure used to prepare storage cards, high amounts of cells may be present in each punch. In these cases, performing direct amplification in 25µl reaction volumes may provide better results.

### Materials to be Supplied by the User

- 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
- PowerPlex® 35GY QI Reagent (Cat.# DM1551)
- 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 25µl reaction volumes using the PowerPlex® 35GY System and the ProFlex® PCR System. The PowerPlex® 35GY System contains sufficient reagents for 400 direct-amplification reactions of 12.5µl each; when performing 25µl reactions, the system contains sufficient reagents for 200 reactions.

**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.

For 25µl amplification reactions with lytic cards, 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. For nonlytic cards, we recommend amplifying one 1.2mm punch of a storage card containing buccal cells or whole blood.

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

**Lytic storage card sample types include:**

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

**Nonlytic storage card sample types include:**

- Buccal samples on Bode Buccal DNA Collector™ devices
- Blood and buccal samples on nonlytic card punches (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. Upon receipt, store the PowerPlex® 35GY 5X Master Mix, PowerPlex® 35GY 5X Primer Pair Mix, PowerPlex® 35GY QI Reagent, and Water, Amplification Grade, at +2°C to +10°C.

**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 new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new 0.2ml reaction tubes required and label them appropriately.
4. Add the final volume of each reagent listed in Table 12 to a new tube. The amount of PowerPlex® 35GY QI Reagent will depend on the cycle number:
  - For 24 cycles, use 4µl of PowerPlex® 35GY QI Reagent
  - For 25 cycles, use 2µl of PowerPlex® 35GY QI Reagent
  - For 26 cycles, use 1µl of PowerPlex® 35GY QI Reagent

**Table 12. PCR Amplification Mix for Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume**

PCR Amplification Mix Component <sup>1</sup>	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25µl	×		=	
PowerPlex® 35GY 5X Master Mix	5µl	×		=	
PowerPlex® 35GY 5X Primer Pair Mix	5µl	×		=	
PowerPlex® 35GY QI Reagent	up to 4µl				
<b>total reaction volume</b>	<b>25µl</b>				

<sup>1</sup>Add Water, Amplification Grade, to the tube first, and then add PowerPlex® 35GY 5X Master Mix, PowerPlex® 35GY 5X Primer Pair Mix, PowerPlex® 35GY QI Reagent. For lytic card punches, the template DNA will be added at Step 6.

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

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


**Note:** Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add 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 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 1.6µl (16ng) of the 2800M Control DNA (10ng/µl) to a reaction well containing 25µl of PCR amplification mix.

**Notes:**

-  Optimization of the amount of control DNA may be required, depending on cycling conditions and laboratory preferences.
- Do not include blank storage card punches in the positive control reactions.

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 cycle number (24–26 cycles), injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 25 cycles works well for a variety of sample types. 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 33. The total cycling time is approximately 1 hour.

**Note:** Confirm that the ramp speed is set to 6°C/s and the reaction volume to 25µl.

### Thermal Cycling Protocol

96°C for 1 minute, then:

98°C for 5 seconds

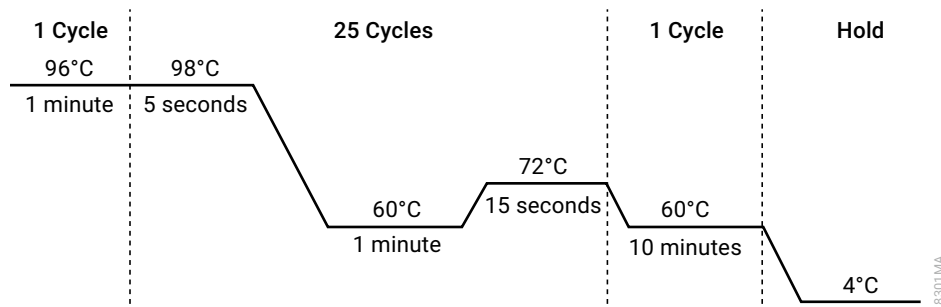
60°C for 1 minute

72°C for 15 seconds

for 25 cycles, then:

60°C for 10 minutes

4°C soak

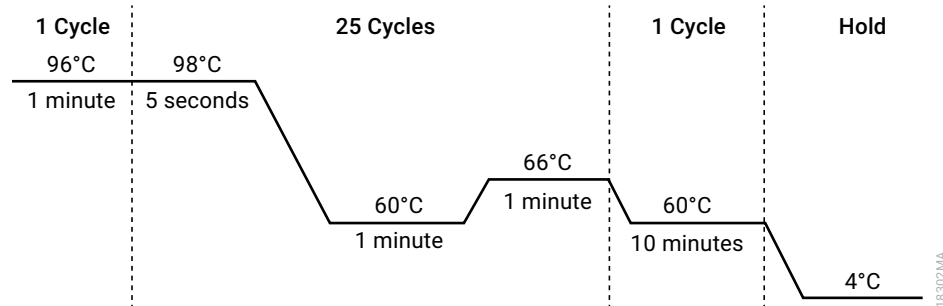


**Figure 33. Thermal cycling protocol for ProFlex® PCR System.**

**Note:** For challenging sample types, an adjusted thermal cycling protocol can be used as provided below and in Figure 34.

### Thermal Cycling Protocol for Challenging Samples.

96°C for 1 minute, then:  
 98°C for 5 seconds  
 60°C for 1 minute  
 66°C for 1 minute  
 for 25 cycles, then:  
 60°C for 10 minutes  
 4°C soak



**Figure 34. Thermal cycling protocol for challenging samples for the ProFlex® PCR System.**

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at  $-20^{\circ}\text{C}$  protected from light.

**Note:** Long-term storage of amplified samples at  $4^{\circ}\text{C}$  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 three identical reaction plates with punches from the same samples.
4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (24–26 cycles).
5. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type.

## 10.4 Direct Amplification of DNA from Swabs in a 25 $\mu$ l Reaction Volume

Depending on the procedure used to prepare the swabs, high amounts of cells may be present on each swab. In these cases, performing direct amplification in 25 $\mu$ l reaction volumes may provide better results.

### Materials to be Supplied by the User

- ProFlex<sup>®</sup> PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp<sup>®</sup> optical 96-well reaction plates or 0.2ml MicroAmp<sup>®</sup> reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- PowerPlex<sup>®</sup> 35GY QI Reagent (Cat.# DM1551)
- SwabSolution<sup>™</sup> Kit (Cat.# DC8271)

This section contains a protocol for direct amplification of DNA from swab extracts in 25 $\mu$ l reaction volumes using the PowerPlex<sup>®</sup> 35GY System and the ProFlex<sup>®</sup> PCR System. The PowerPlex<sup>®</sup> 35GY System contains sufficient reagents for 400 direct-amplification reactions of 12.5 $\mu$ l each; when performing 25 $\mu$ l reactions, the system contains sufficient reagents for 200 reactions.

**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.

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

### Amplification Setup

1. Upon receipt, store the PowerPlex<sup>®</sup> 35GY 5X Master Mix, PowerPlex<sup>®</sup> 35GY 5X Primer Pair Mix, PowerPlex<sup>®</sup> 35GY QI Reagent, and Water, Amplification Grade, at +2°C to +10°C.

**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 new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new 0.2ml reaction tubes required and label them appropriately.
4. Add the final volume of each reagent listed in Table 13 to a new tube. The amount of PowerPlex® 35GY QI Reagent will depend on the cycle number:
  - For 24 cycles, use 4µl of PowerPlex® 35GY QI Reagent
  - For 25 cycles, use 2µl of PowerPlex® 35GY QI Reagent
  - For 26 cycles, use 1µl of PowerPlex® 35GY QI Reagent

**Table 13. PCR Amplification Mix for Direct Amplification of DNA from Swabs in a 25µl Reaction Volume.**

PCR Amplification Mix Component <sup>1</sup>	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25µl	×		=	
PowerPlex® 35GY 5X Master Mix	5µl	×		=	
PowerPlex® 35GY 5X Primer Pair Mix	5µl	×		=	
PowerPlex® 35GY QI Reagent	up to 4µl				
swab extract	2µl				
<b>total reaction volume</b>	<b>25µl</b>				

<sup>1</sup>Add Water, Amplification Grade, to the tube first, and then add PowerPlex® 35GY 5X Master Mix and PowerPlex® 35GY 5X Primer Pair Mix, PowerPlex® 35GY QI Reagent. 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 8ng/µl. Add 2µl (16ng) of the 2800M Control DNA to a reaction well containing 23µl of PCR amplification mix.

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

- For the negative amplification control, pipet 2µl of Water, Amplification Grade or TE<sup>-4</sup> 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.

- 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

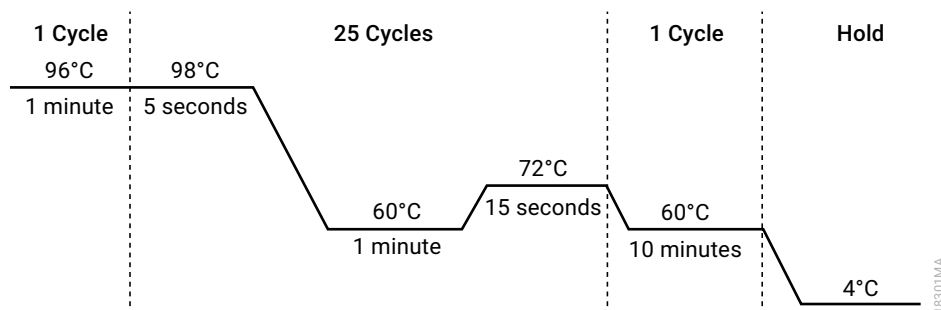
Amplification and detection instrumentation may vary. You will need to optimize protocols including the amount of template DNA, cycle number (24–26 cycles), injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 25 cycles works well for a variety of sample types. Cycle number will need to be optimized in each laboratory for each sample type that is amplified.

- Place the reaction plate or tubes in the thermal cycler.
- Select and run the recommended protocol, which is provided below and in Figure 35. The total cycling time is approximately 1 hour.

**Note:** Confirm that the ramp speed is set to 6°C/s and the reaction volume to 25µl.

### Thermal Cycling Protocol

96°C for 1 minute, then:  
 98°C for 5 seconds  
 60°C for 1 minute  
 72°C for 15 seconds  
 for 25 cycles, then:  
 60°C for 10 minutes  
 4°C soak



**Figure 35. Thermal cycling protocol for the ProFlex® PCR System.**

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at  $-20^{\circ}\text{C}$  protected from light.

**Note:** Long-term storage of amplified samples at  $4^{\circ}\text{C}$  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 (24–26 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.

## 10.5 Composition of Buffers and Solutions

### **TE<sup>-4</sup> buffer (10mM Tris-HCl, 0.1mM EDTA [pH 8.0])**

1.21g Tris base  
0.037g EDTA ( $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ )

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

### **TE<sup>-4</sup> buffer with 20 $\mu\text{g}/\text{ml}$ glycogen**

1.21g Tris base  
0.037g EDTA ( $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ )  
20 $\mu\text{g}/\text{ml}$  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.

## 10.6 Related Products

PRODUCT	SIZE	CAT.#
PowerPlex® 8C Matrix Standard	5 preps	DG5010
CCO Internal Lane Standard 500	200µl	DG8571
PowerPlex® 35GY QI Reagent	800µl	DM1551
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

Not for Medical Diagnostic Use.

### Spectrum Compact CE System Accessories and Consumables

PRODUCT	SIZE	CAT.#
Spectrum Compact Capillary Cartridge, 4-Capillary, 36cm	1 each	CE2340
Spectrum Compact Polymer4	4 × 64 wells	CE2304
	4 × 96 wells	CE2404
Spectrum Compact Buffer	2 pairs	CE2300
Spectrum Compact Cathode Septa Mat	10 each	CE2301
Spectrum Compact Cathode Retainer	4 each	CE2302
Spectrum Compact Strip Base & Retainer, 32-Well	4 each	CE2332
Strip Septa Mat, 8-Well	24 each	CE2308

Not for Medical Diagnostic Use.

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

<sup>(b)</sup> U.S. Pat. Nos. 9,056,885 and 10,319,400; European Pat. No. 2782916; Japanese Pat. Nos. 6522339, 6574821, and 7227741; and other patents pending.

<sup>(c)</sup>AQA-8C, TMR-8C, CXR-8C, TOM-8C, WEN-8C and CCO-8C dyes are proprietary

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