

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

Instructions for Use of Product DC3520



Table of Contents

1	Description.....	5
2	Product Components and Storage Conditions.....	6
3	Before You Begin	8
	3.1 Precautions	8
	3.2 Spectral Calibration	9
4	Protocols for DNA Amplification Using the PowerPlex® 35GY System	11
	4.1 Amplification of Extracted DNA in a 25µl Reaction Volume	11
	4.2 Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume	14
	4.3 Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume	19

5	Detection of Amplified Fragments Using the Spectrum CE System.....	23
5.1	Sample Preparation	24
5.2	Instrument Preparation	24
5.3	Create a Plate Record	27
5.4	Preparing and Loading the Plate Assembly	33
5.5	Linking a Plate for a Run	34
5.6	Monitoring a Run	35
5.7	Exporting Data	35
6	Data Analysis Using GeneMarker®HID Software.....	36
6.1	Creating an Analysis Method with GeneMarker®HID Software for Spectrum CE Systems	36
6.2	Controls in GeneMarker®HID Software for Spectrum CE Systems	42
6.3	Quality Indicators Small (QIS) and Large (QIL)	42
7	Results.....	43
8	Troubleshooting.....	47
8.1	Amplification and Fragment Detection	47
8.2	Amplification of Extracted DNA	50
8.3	Direct Amplification of DNA from Storage Card Punches	52
8.4	Direct Amplification of DNA from Swabs	56
8.5	GeneMarker® HID Software for Spectrum CE Systems	59
9	References.....	62

10 Appendix 64

- 10.1 Advantages of Using the Loci in the PowerPlex® 35GY System 64
- 10.2 The CCO Internal Lane Standard 500 69
- 10.3 Direct Amplification of DNA from Storage Card Punches
in a 25µl Reaction Volume 69
- 10.4 Direct Amplification of DNA from Swabs
in a 25µl Reaction Volume 75
- 10.5 Composition of Buffers and Solutions 78
- 10.6 Related Products 79

11 Summary of Changes..... 81

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

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

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

1 Description

STR (short tandem repeat) loci consist of short, repetitive sequence elements 3–7 base pairs in length (1–4). These repeats are well distributed throughout the human genome and are a rich source of highly polymorphic markers, which may be detected using 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^(a,b) 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 CE System. Amplification and detection instrumentation may vary. You may need to optimize protocols, including 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 CE System is available separately. See the *Spectrum CE System Operating Manual #TMD052*.

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

2 Product Components and Storage Conditions

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

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

3.1 Precautions

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

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

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

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

3.2 Spectral Calibration

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

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

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

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 ¹	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) ^{2,3}	up to 15µl				
total reaction volume	25µl				

¹Add 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.

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

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

- 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⁻⁴ 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

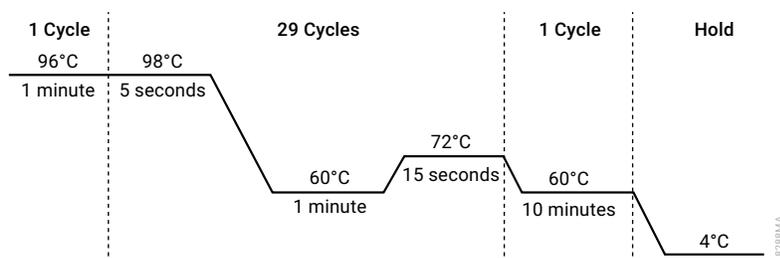


Figure 1. The thermal cycling protocol for the ProFlex® PCR System.

- 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.

PCR Amplification Mix Component ¹	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				
total reaction volume	12.5µl				

¹Add 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

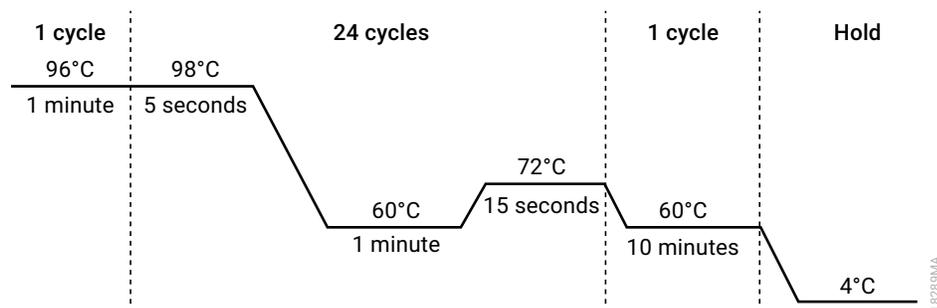


Figure 2. Thermal cycling protocol for the ProFlex® PCR System.

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

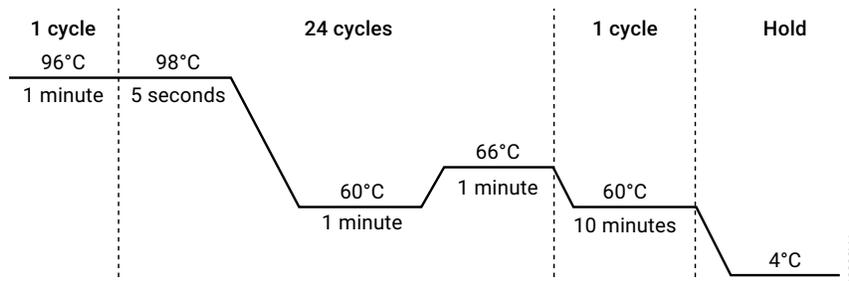


Figure 3. Thermal cycling protocol for challenging samples for the ProFlex® PCR System.

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

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

PCR Optimization

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

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Place one 1.2mm storage card punch containing buccal cells or whole blood in each well of a reaction plate. Be sure to pretreat nonlytic samples with the PunchSolution™ Kit (Cat.# DC9271).
3. Prepare three identical reaction plates with punches from the same samples.
4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (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 ¹	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				
total reaction volume	12.5µl				

¹Add 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⁻⁴ buffer instead of swab extract into a reaction well containing PCR amplification mix.

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

- 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 works well for a variety of sample types. Cycle number will need to be optimized in each laboratory for each sample type that is amplified.

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

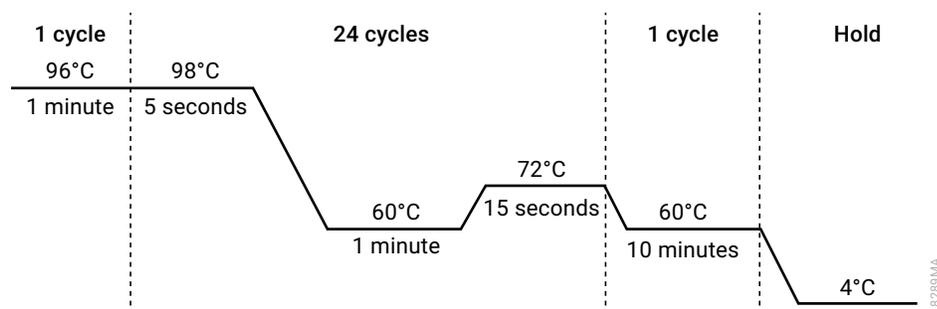


Figure 4. Thermal cycling protocol for the ProFlex® PCR System.

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 CE System

Materials to be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice, ice-water bath or freezer plate block
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- Spectrum Capillary Array, 8-capillary (Cat.# CE2008)
- Spectrum Polymer4, 384 Wells (Cat.# CE2048) or Spectrum Polymer4, 960 Wells (Cat.# CE2040)
- Spectrum Buffer (Cat.# CE2001)
- Spectrum Cathode Septa Mat (Cat.# CE2002)
- Septa Mat, 96-Well (Cat.# CE2696) or equivalent Applied Biosystems septa mat
- Spectrum Plate Base & Retainer, 96-Well (Cat.# CE5004)
- MicroAmp® optical 96-well plate (or equivalent; Applied Biosystems)
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)

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

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

Note: Wear gloves when handling consumables.

5.1 Sample Preparation

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

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

2. Vortex for 10–15 seconds to mix.
3. Pipet 10 μl of formamide/internal lane standard mix into each well of the 96-well plate.
4. Add 1 μl of amplified sample (or 1 μl of Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

Notes:

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

5.2 Instrument Preparation

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

Open the Spectrum Control Software (SCS). Upon launching the SCS, the 'Home' screen will be displayed (Figure 5). This screen provides access to the four workflow menus (Home, Review Plates, Maintenance and Settings; see Figure 6) as well as status indicators. The screen is divided into three sections: Header, Task Pane and Status Bar.



Figure 5. Spectrum Control Software 'Home' screen.

The Header (Figure 6) is fixed and remains available to the user throughout all navigation processes. This area contains Status Indicators, Message Center, Start Button, Run Time Indicator and Workflow Menu. The Header also shows the status of the plate positions within the Plate Drawer.

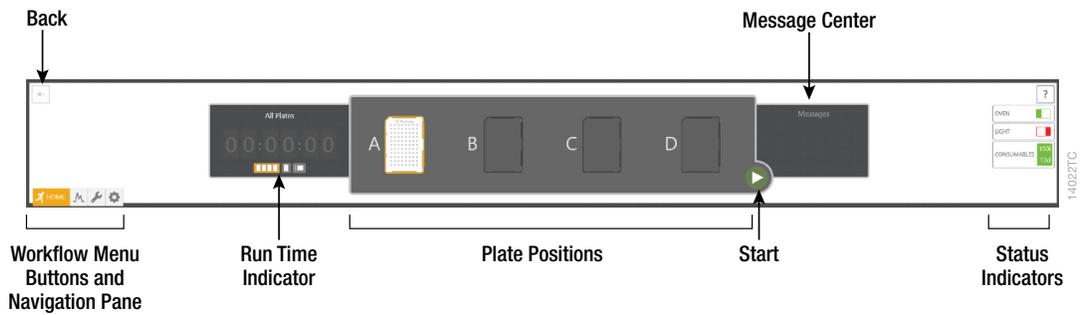


Figure 6. Header.

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

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

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

Before starting a run, ensure all consumables are installed and in sufficient supply. For best results, use unexpired reagents that are within the recommended use range. Refer to the 'Consumables' screen (Figure 7) to determine if any consumables need to be replaced. To access the 'Consumables' screen, select the Consumables status indicator in the Header on the SCS screen (Figure 6). The 'Consumables' screen (Figure 7) displays information for the four RFID-tagged consumables on the instrument: Polymer, Capillary Array and Anode and Cathode Buffers.

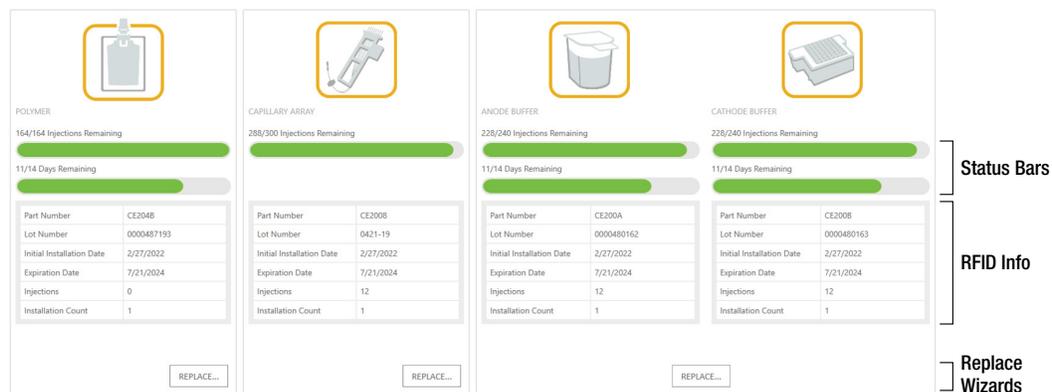


Figure 7. 'Consumables' screen.

The following information is tracked through these tags:

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

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

Select **Oven Heater Indicator** in the Header (Figure 6) to start preheating the oven to 60°C. The oven temperature will be displayed in the status bar at the bottom of the screen and the indicator will change from red to green when the oven preheating is complete.

Note: We recommend 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. Inspect the fluid block, tubing, polymer syringe and polymer pouch for bubbles, and perform a bubble purge if necessary.

5.3 Create a Plate Record

On the 'Home' screen, there are three options displayed for plate setup: New Plate, Import Plate and Draft Plates (Figure 5).

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

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

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

1. Select **New Plate** from the 'Home' screen. This will open a blank 'Plate' screen that is divided into three sections: Plate Information, Sample Information and Injection Information (Figure 8).

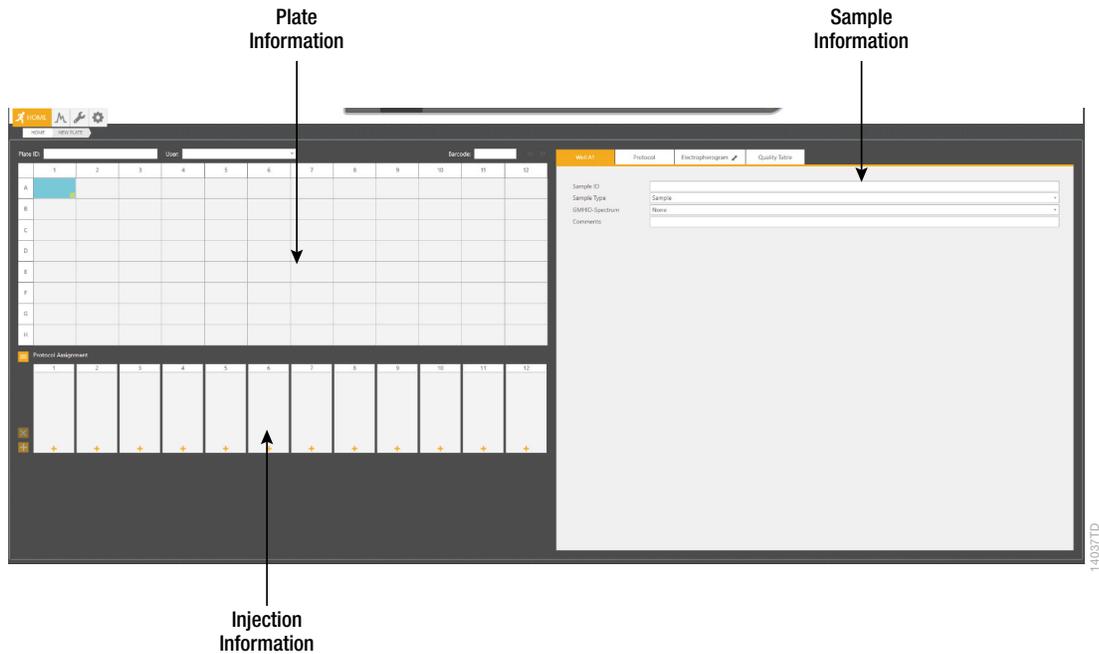


Figure 8. 'Plate' screen.

2. Use the default Plate ID that includes date and time or enter information into the 'Plate ID' field (Figure 9).
3. If desired, enter user information into the 'User' field (Figure 9).

Note: The 'User' field is a user-defined section that can be used to search or filter previously created or completed plates (refer to the *Spectrum CE System Operating Manual #TMD052* for information on reviewing plate records). Previously entered user information is available via the drop-down option on the right side of the field (Figure 9).

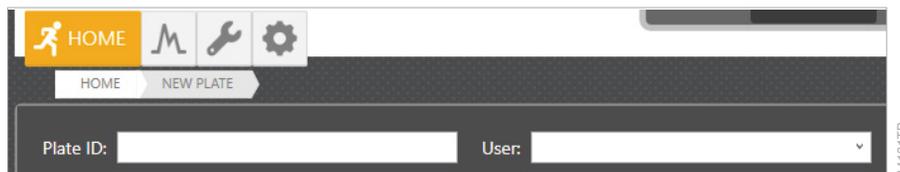


Figure 9. 'Plate ID' and 'User' fields of the plate screen.

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

Alternatively, enter Sample IDs in the 'Sample ID' field of the Sample Information section (Figure 8).

The green box in the lower right corner of a sample well (Figure 10) can be used to fill other wells with the same information by left clicking and dragging the green box to the other wells.

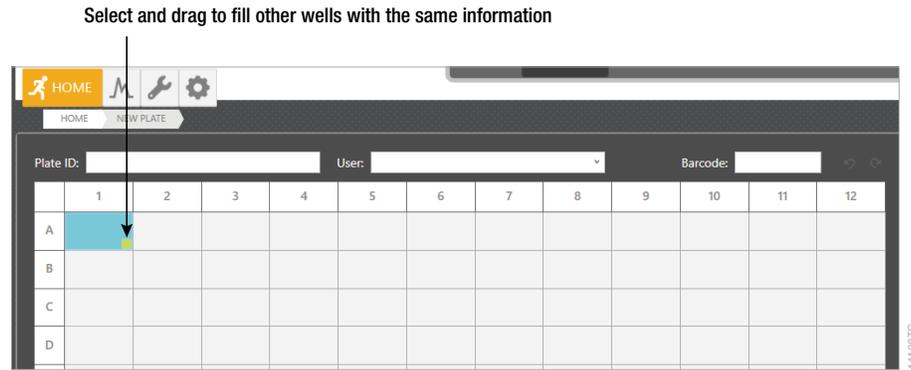


Figure 10. Filling information across multiple sample wells.

5. Select sample types for each sample or group of samples on the plate. Select the sample(s) then use the drop-down menu (Figure 11) to designate the well(s) as Sample, Ladder, Positive Control or Negative Control. These designations will be included in the output file for downstream analysis.

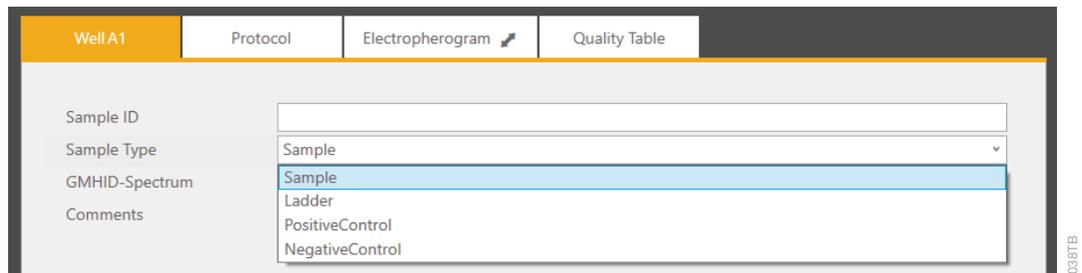


Figure 11. Drop-down options for sample type.

6. **Optional:** Select PowerPlex_35GY in the GMHID-Spectrum drop-down menu (Figure 12).

Notes:

- The appropriate GMHID-Spectrum Run Wizard template must be selected for use of the Quality Table. These templates are preloaded and edited within the Spectrum CE System workstation's copy of GMHID- Spectrum. Select the appropriate sample type for each well to ensure complete GMHID-Spectrum analysis functionality. Refer to the *Spectrum CE System Operating Manual #TMD052* and *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555* for more information.
- Selecting the GMHID-Spectrum template does not affect downstream analysis.

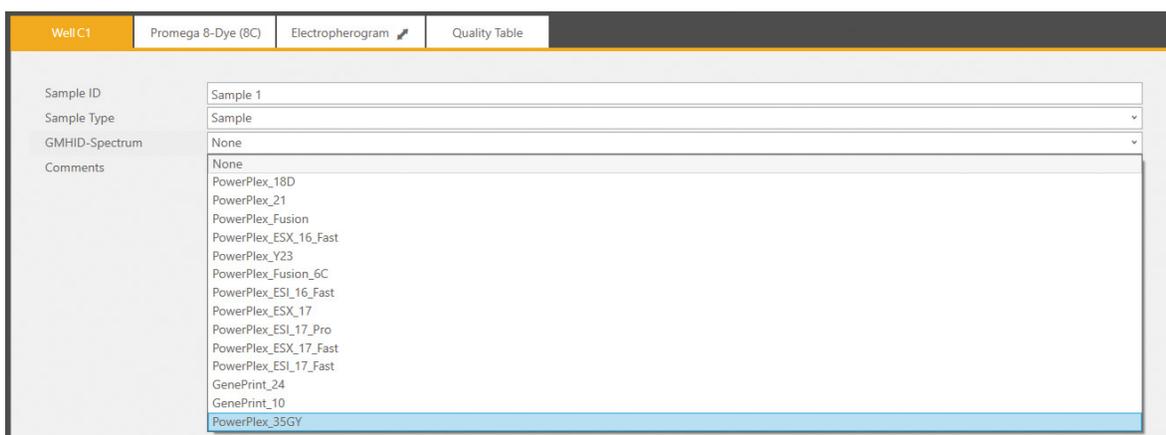


Figure 12. Drop-down options for GeneMarker®HID Software for Spectrum CE Systems run wizard templates.

- When a run protocol has been assigned to an injection set, an injection number will be assigned and displayed in a circle within the injection set box. There are three methods to assign run protocol(s) to samples:
 - To add a run protocol to selected samples, highlight the desired wells (Ctrl + right-click or drag with the mouse). Then right-click, mouse over **Add Injection To Selected Wells** then select the **Promega 8-Dye (8C)** protocol from the pop-out menu.
 - To add a run protocol to individual injections, select the **+** button under the box for that injection set then select the **Promega 8-Dye (8C)** protocol from the pop-out menu.
 - To add a run protocol to multiple injections, highlight the desired injections in the plate map (click-drag, Ctrl + left-click or click the upper left cell). Then select the **+** button on the left side of the injection information section and select **Promega 8-Dye (8C)** (Figure 13) from the pop-out menu.

Notes:

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

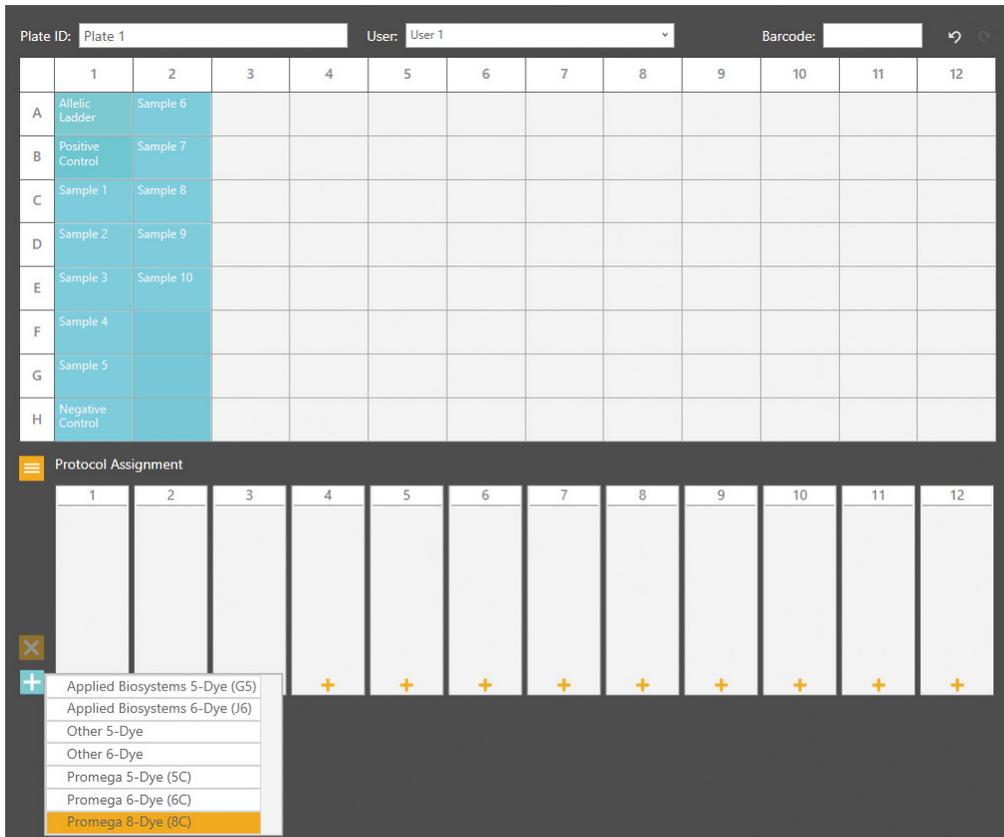


Figure 13. Assigning run protocol to multiple injections.

8. Verify the assigned run protocol(s).

To verify the parameters of the assigned run protocol(s), select the injection number or a sample well in the plate map then select the 'Protocol' tab of the Sample Information section (Figure 14), which now displays the name of the assigned protocol. The information in this tab is read-only.

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

Well A1	Promega 8-Dye (8C)	Electropherogram	Quality Table
Conditions			
Oven Temperature (°C)	60		
Array Heater (°C)	45		
Tertiary Heater (°C)	45		
Pre-Electrophoresis			
Pre-E. Voltage (kV)	17		
Pre-E. Duration (sec)	120		
Sample Injection			
Injection Voltage (kV)	2		
Injection Duration (sec)	15		
Electrophoresis			
Voltage (kV)	13		
Collection Duration (sec)	1550		
Data Delay (sec)	90		
Spectral Calibration			
Dye Set	Promega 8-Dye (8C)		
Properties			
Description	Preset Run Protocol		
Created By	Promega		
Date Modified	6/27/2022 1:55:42 PM		

Figure 14. The 'Protocol' tab of the Sample Information section.

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

5.4 Preparing and Loading the Plate Assembly

1. Place the 96-well plate created in Section 5.1 into the Spectrum plate base, lining up the notch above well A12 with the notch on the base.
2. To complete the plate assembly, place the Spectrum plate retainer over the plate/base assembly, lining up the notch on the retainer with the notch on the plate and base. Verify that the retainer is locked in place on both sides of the plate, sitting evenly on top of the base (Figure 15).



Figure 15. The Spectrum plate assembly.

3. Confirm the drawer handle light is illuminated, indicating that the drawer is unlocked and ready for plate loading. Alternatively, check the Message Center to verify that the drawer is unlocked. The Drawer Status indicator is in the Message Center located in the Header (Figure 6).
4. Check the Plate Position Status indicator to note which of the four plate positions (A, B, C or D) is open and does not already contain a plate.
5. Open the plate drawer and place the plate assembly in an open plate position, verify that the notch on the plate base retainer aligns with the notch in the plate drawer then close the drawer.

Notes:

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

5.5 Linking a Plate for a Run

1. A plate record must be linked to the plate before adding it to the run queue.
2. Open the draft plate record, then select **Link** in the Plate Position indicator under the position where the plate was placed. Once the plate is successfully linked, the text under the position will change status to **Unlink** and the Plate ID will appear above the position (Figure 16).
3. Select the **Start** button to begin the run.

Notes:

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

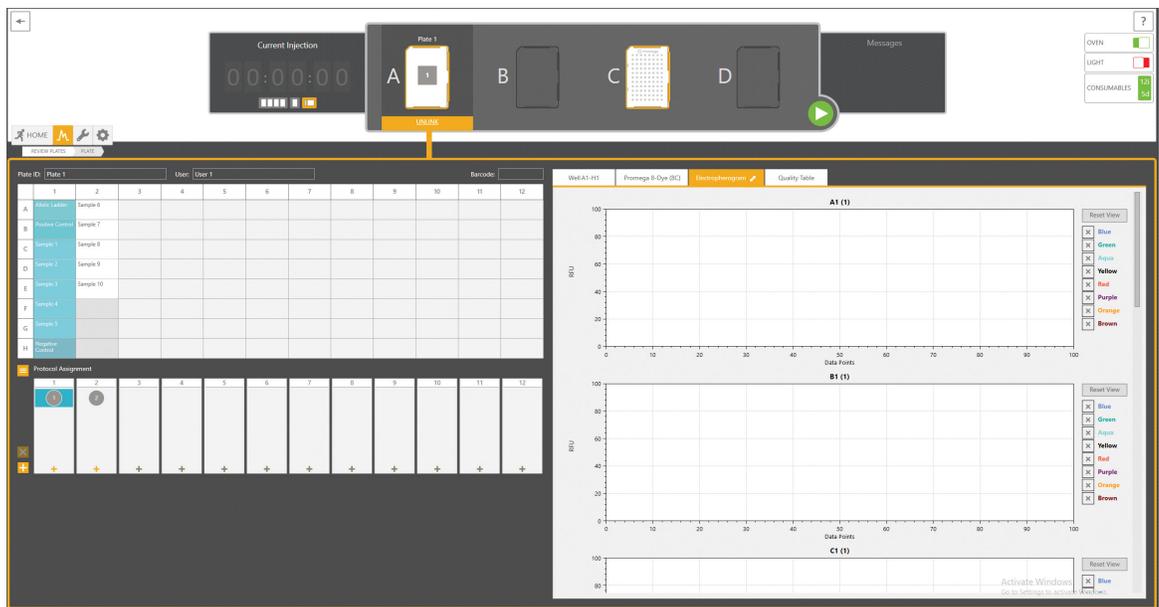


Figure 16. Linked plate in the Spectrum Control Software.

5.6 Monitoring a Run

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

5.7 Exporting Data

Data from completed injections can be automatically exported as .promega files into a defined location on the instrument hard drive. Please confirm that the 'Activate Auto Export' check box has been selected in the 'Data Export' tab of the Preferences section. Exported .promega files can be analyzed using GeneMarker®HID Software for Spectrum CE Systems.

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

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

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 17).

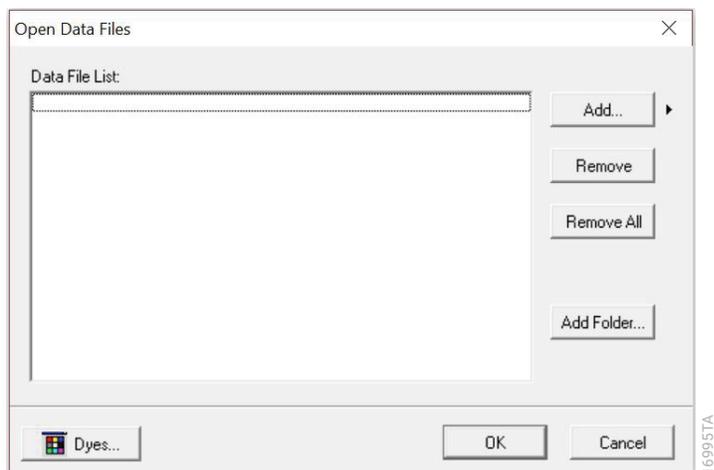


Figure 17. The GMHID-Spectrum Data File List.

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 18). 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 18. The 'Edit Marker' window for the D3S1358 marker.

8. Click on **Run** in the Magic Wizard or click the **Run Project** icon (green arrow) in the toolbar. The 'Template Selection' screen will appear (Figure 19). Select the PowerPlex_35GY template and the settings shown in Figure 19. 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.

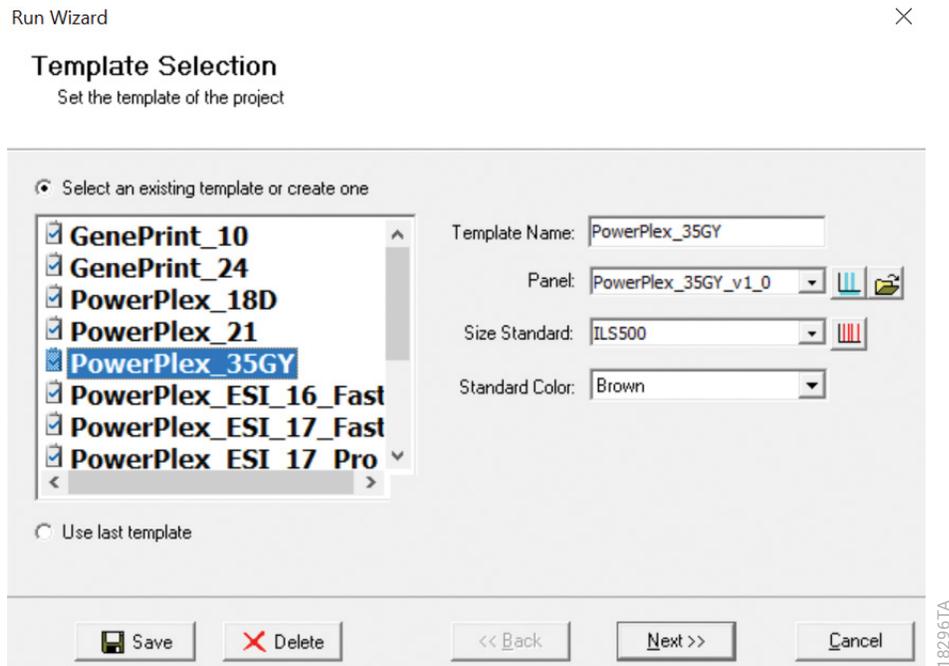


Figure 19. The 'Template Selection' window.

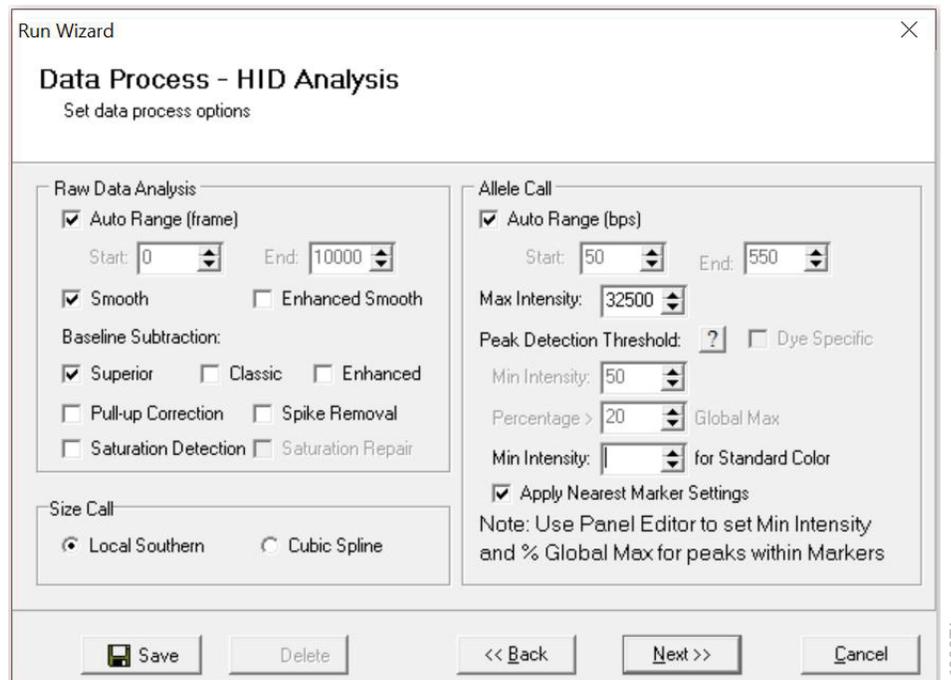


Figure 20. The 'Data Process' window for an analysis method.

- The 'Data Process – HID Analysis' window will appear (Figure 20). 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

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 21. The 'Additional Settings' window for an analysis method.

- The 'Additional Settings – HID Analysis' window will appear (Figure 21). 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.
- 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 10 (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 4 provides additional information to assist with the interpretation of QIS and QIL data.

Table 4. 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 22. The PowerPlex® 35GY Allelic Ladder Mix is shown in Figure 23.

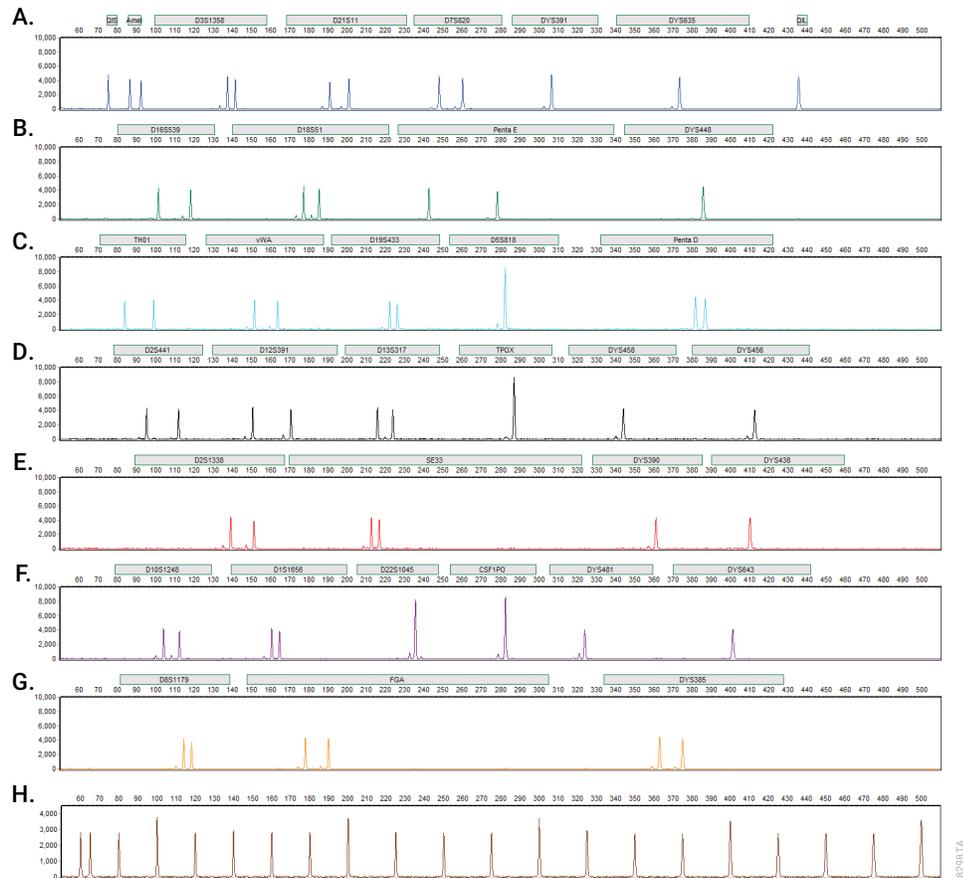


Figure 22. The PowerPlex® 35GY System. The 2800M Control DNA (1.0ng) was amplified using the PowerPlex® 35GY System. Amplification products were mixed with CCO Internal Lane Standard 500 and analyzed with a Spectrum CE System and a 2kV, 15-second injection. Results were analyzed using GeneMarker® HID Software for Spectrum CE Systems, version 3.2 and PowerPlex® 35GY panel files. **Panel A.** An electropherogram showing the peaks of the FL-8C-labeled loci: QIS, Amelogenin, D3S1358, D21S11, D7S820, DYS391, DYS635 and QIL. **Panel B.** An electropherogram showing the peaks of the JOE-8C-labeled loci: D16S539, D18S51, Penta E and DYS448. **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, DYS458 and DYS456. **Panel E.** An electropherogram showing the peaks of the CXR-8C-labeled loci: D2S1338, SE33, DYS390 and DYS438. **Panel F.** An electropherogram showing the peaks of the TOM-8C-labeled loci: D10S1248, D1S1656, D22S1045, CSF1PO, DYS481 and DYS643. **Panel G.** An electropherogram showing the peaks of the WEN-8C-labeled loci: D8S1179, FGA and DYS385a/b. **Panel H.** An electropherogram showing the 60bp to 500bp fragments of the CCO Internal Lane Standard 500.

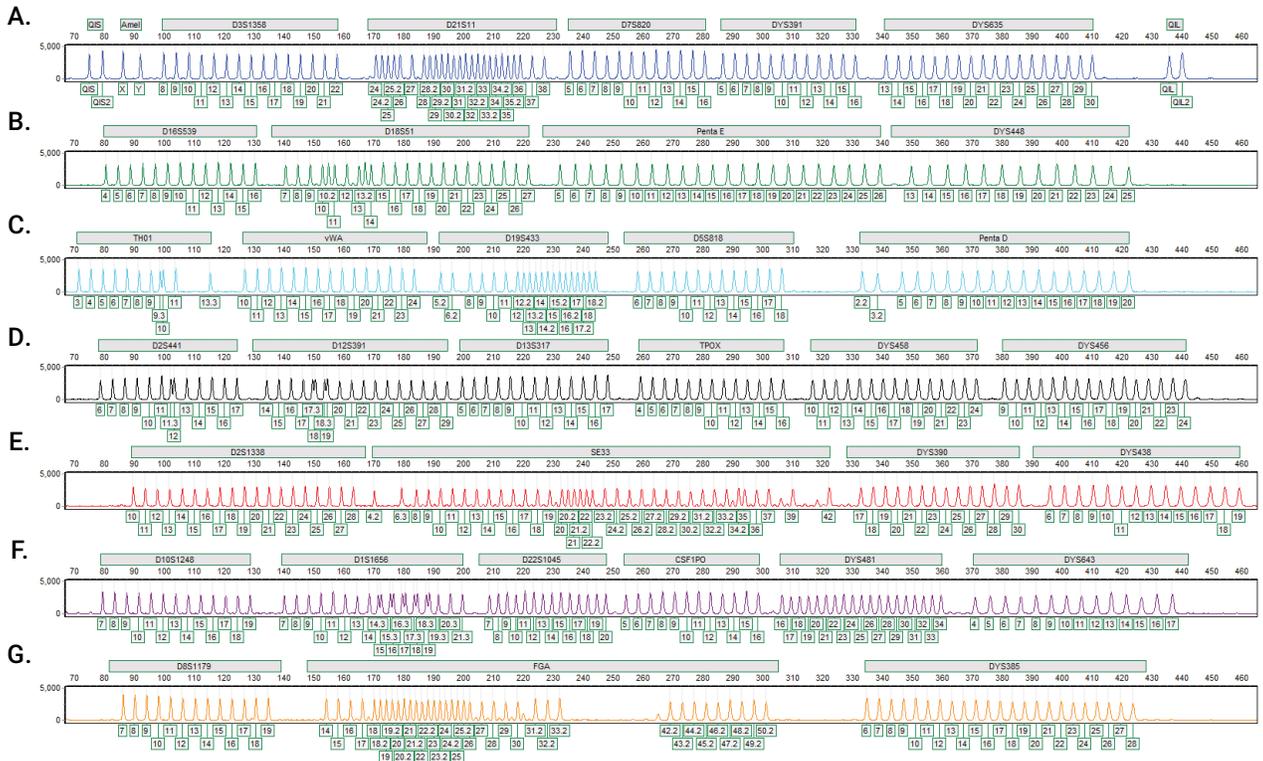


Figure 23. The PowerPlex® 35GY Allelic Ladder Mix. The PowerPlex® 35GY Allelic Ladder Mix was analyzed with a Spectrum CE System and a 2kV, 15-second injection. The sample file was analyzed with the GeneMarker® HID Software for Spectrum CE Systems, version 3.2.0, and PowerPlex® 35GY panel files. **Panel 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.

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 5) and DNA-independent (with or without human genomic DNA) artifact peaks (Table 6) with the PowerPlex® 35GY System.

Table 5. DNA-Dependent Artifacts Observed in Amplification Reactions with Human Genomic DNA.

Locus	Artifact Size
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 6. DNA-Independent Artifacts Observed in Amplification Reactions with and without Human Genomic DNA.

Dye Label	Artifact Size¹
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 ²
WEN-8C	~58–83 bases

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

²This 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 7 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 7. Nonhuman DNA Cross-Reactivity.

DNA Source	Artifact Size	Dye Label
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. E-mail: genetic@promega.com

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

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 re-annealing.
	CE-related artifacts ('spikes'). Minor voltage changes or urea crystals passing by the laser can cause 'spikes' or unexpected peaks. Spikes sometimes appear in one color but often are easily identified by their presence in more than one color. Re-inject samples to confirm. Ensure no urea crystals are present in the polymer pouch 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"> • Perform a new spectral calibration and rerun the samples. • Confirm that the correct spectral was used for the sample run. • Instrument sensitivities can vary. Optimize the injection conditions. Refer to the instrument user manual.
	<p>Dye blob artifacts. The signal strength of certain dye blob artifacts increases with storage of the amplification plate at 4°C, sometimes in as short a time period as overnight but more commonly when left at 4°C for a few days. We recommend storing amplification products at –20°C.</p>
	<p>An incorrect internal lane standard was used. Use the size standard provided in the kit.</p>
<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.

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

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

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>

Symptoms	Causes and Comments
<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"> • Be sure to perform the recommended extension step at 60°C after thermal cycling. • Decrease cycle number. • Increase the final extension time.
<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"> • Be sure to use the recommended number of punches. Follow the manufacturer's recommendations when depositing sample onto the card. • Decrease cycle number.
	<p>The cycle number was too high. Decrease the cycle number by one cycle and repeat the amplification.</p>
	<p>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>

Symptoms	Causes and Comments
Peak height imbalance (continued)	<p>Active PunchSolution™ Reagent carried over into amplification reactions with nonlytic card punches. Larger loci are most susceptible to carryover and will drop out before the smaller loci.</p> <ul style="list-style-type: none"> • Ensure that the heat block reached 70°C and samples were incubated for 30 minutes or until wells are dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. • We recommend treating one 1.2mm nonlytic card punch with 10µl of PunchSolution™ Reagent and using one punch per 12.5µl or 25µl amplification reaction. Reducing the PunchSolution™ Reagent volume may improve results for reactions with reduced amplification volumes. Optimization and validation are required.
	<p>Inactive PunchSolution™ Reagent was used to pretreat nonlytic punches. Thaw the PunchSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.</p>
Extreme variability in sample-to- sample peak heights	<p>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.

Symptoms	Causes and Comments
Faint or absent allele peaks	Poor sample deposition. Shedding and collection of donor cells was variable. Increase cycle number.
	Inactive SwabSolution™ Reagent. Thaw the SwabSolution™ Reagent completely in a 37°C water bath and mix by gentle inversion. Store the SwabSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
	Active SwabSolution™ Reagent carried over into the amplification reaction. Ensure that the heat block reached 70°C (90°C if using a 2.2ml, Square- Well Deep Well Plate) and samples were incubated for the full 30 minutes. Incubation for shorter time periods may result in incomplete reagent inactivation. Do not use an incubator for tubes or plates; heat transfer is inefficient and will result in poor performance. Use only a heat block to maintain efficient heat transfer. We have tested 60-minute incubation times and observed no difference in performance compared to a 30-minute incubation.
	DNA was not accessible on nonlytic material. Pretreat swabs with SwabSolution™ Reagent to ensure that DNA is released from cellular proteins.
Faint or absent peaks for the positive control reaction	If the positive control reaction failed to amplify, check to make sure that the correct amount of 2800M Control DNA was added to the reaction. Due to the reduced cycle numbers used with swab extracts, it is necessary to increase the mass of 2800M Control DNA to obtain a profile. Use the recommended amount of 2800M Control DNA per amplification reaction. This mass of DNA should be reduced if cycle number is increased and increased if the cycle number is decreased. Increase or decrease by twofold the mass of 2800M Control DNA for every one-cycle decrease or increase, respectively.

Symptoms	Causes and Comments
<p>Extra peaks visible in one or all color channels</p>	<p>Swab extract was contaminated. Assemble a reaction containing the swab extract prepared from a blank swab or assemble a reaction where the SwabSolution™ Reagent is processed and incubated as a blank without a swab.</p>
	<p>Artifacts of STR amplification. Amplification of swab extracts with high DNA concentrations can result in artifact peaks due to overamplification, resulting in saturated signal on the CE instrument. We recommend 2µl of swab extract per reaction.</p> <p>Using more than 2µl may result in overamplification and signal saturation. If signal is saturated, repeat amplification with less swab extract or reduced cycle number.</p>
	<p>Amplification of excess template for a given cycle number resulted in overloading of the capillary upon electrokinetic injection. Excess DNA in the capillary is difficult to maintain in a denatured single-stranded state. Some single-stranded DNA renatures and becomes double-stranded. Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis and appears as 'shadow' peaks migrating in front of the main peaks. If this occurs at a heterozygous locus it is possible to observe the presence of two 'shadow' peaks that differ in size by approximately the same distance as the single-stranded alleles.</p>
	<p>Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3' A residue.</p> <ul style="list-style-type: none"> • Be sure to perform the recommended extension step at 60°C after thermal cycling. • Use 2µl of swab extract in an amplification reaction. A larger volume of swab extract may contain more than the recommended amount of DNA template, resulting in incomplete adenylation. • Decrease cycle number. • Increase the final extension time.

Symptom	Causes and Comments
Peak height imbalance	<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>
Extreme variability in sample-to- sample peak heights	<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"> • Create a new size standard using the internal lane standard fragments present in the sample. • Rerun samples using a longer run time.
	A low-quality allelic ladder was used during analysis. Ensure that only high- quality allelic ladders are used for analysis. 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"> • There is an error in bin shifting • Peaks in virtual bins have greater than expected peak heights • The expected peaks cannot be identified in a marker. <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"> • The sample size standard does not contain all peaks that are marked as "Enabled" in the chosen size standard template. • 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 Update Calibration. • 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.
	An incorrect size standard was used.
	Run was too short, and larger peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run. <ul style="list-style-type: none"> • Create a new size standard using the internal lane standard fragments present in the sample. • Rerun samples using a longer run time.
Peaks in size standard missing	If peaks are low-quality, redefine the size standard for the sample to skip these peaks.
	An incorrect size standard was used.
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

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10 Appendix

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 (Tables 8 and 9). 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 10 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 8. The PowerPlex® 35GY 8C System Locus-Specific Information.

STR Locus	Label	Chromosomal Location¹	Repeat Sequence² 5' ->3'
Amelogenin ³	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 8. The PowerPlex® 35GY 8C System Locus-Specific Information (continued).

STR Locus	Label	Chromosomal Location ¹	Repeat Sequence ² 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

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

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

²The August 1997 report (22,23) of the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) states, "1) for STR loci within coding genes, the coding strand shall be used and the repeat sequence motif defined using the first possible 5' nucleotide of a repeat motif; and 2) for STR loci not associated with a coding gene, the first database entry or original literature description shall be used."

³Amelogenin is not an STR

NA = Not Applicable

Table 9. The PowerPlex® 35GY 8C System Allelic Ladder Information.

STR Locus	Label	Size Range of Allelic Ladder Components ^{1,2} (bases)	Repeat Numbers of Allelic Ladder Components ³
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 9. The PowerPlex® 35GY 8C System Allelic Ladder Information (continued).

STR Locus	Label	Size Range of Allelic Ladder Components ^{1,2} (bases)	Repeat Numbers of Allelic Ladder Components ³
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

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

²When 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.

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

Table 10. The PowerPlex® 35GY 8C System Allele Determinations for the 2800M Control DNA.

STR Locus	2800M
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 24). 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.

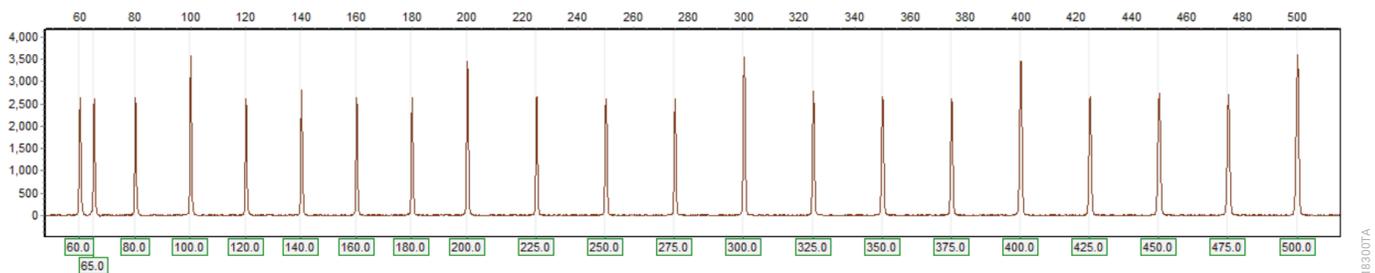


Figure 24. CCO Internal Lane Standard 500. An electropherogram showing the CCO Internal Lane Standard 500 fragments.

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

PCR Amplification Mix Component ¹	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	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				
total reaction volume	25µl				

¹Add 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 25. 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

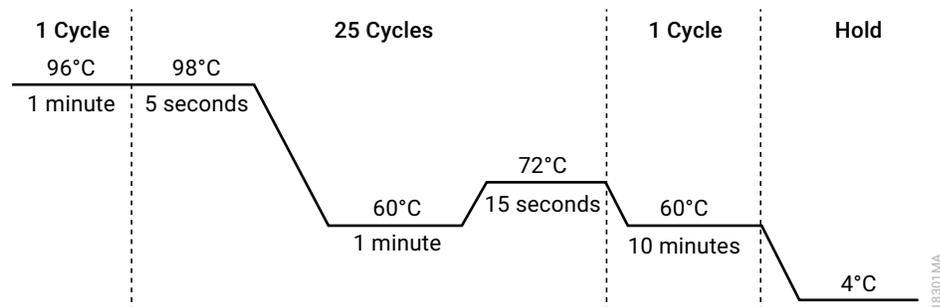


Figure 25. Thermal cycling protocol for ProFlex® PCR System.

Note: For challenging sample types, an adjusted thermal cycling protocol can be used as provided below and in Figure 26.

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

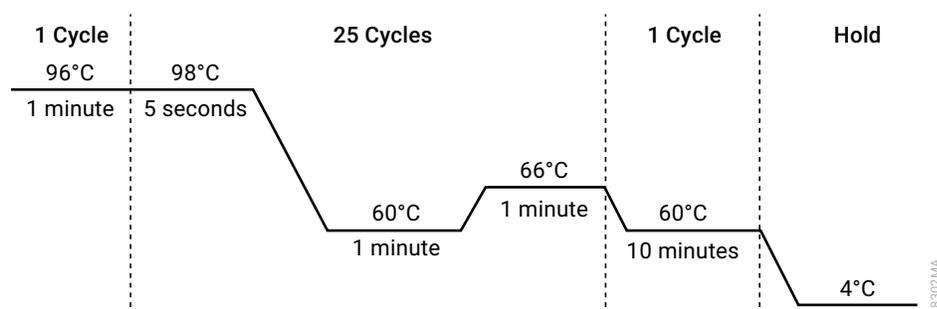


Figure 26. Thermal cycling protocol for challenging samples for the ProFlex® PCR System.

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

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

PCR Optimization

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

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Depending on your preferred protocol, place one or two 1.2mm storage card punches containing buccal cells or one 1.2mm punch of a storage card containing whole blood into each well of a reaction plate. Be sure to pretreat nonlytic samples with the PunchSolution™ Kit (Cat.# DC9271).
3. Prepare 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 25µl 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 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
- PowerPlex® 35GY QI Reagent (Cat.# DM1551)
- SwabSolution™ Kit (Cat.# DC8271)

This section contains a protocol for direct amplification of DNA from swab extracts 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.

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

Amplification Setup

1. 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 Swabs in a 25µl Reaction Volume.

PCR Amplification Mix Component ¹	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				
total reaction volume	25µl				

¹Add 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.

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



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

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

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

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including the amount of template DNA, cycle number (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.

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 27. 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

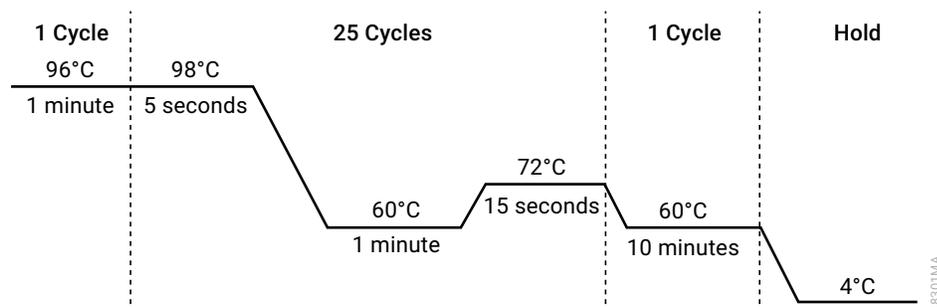


Figure 27. Thermal cycling protocol for the ProFlex® PCR System.

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 (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⁻⁴ 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⁻⁴ 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 CE System Accessories and Consumables

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

Not for Medical Diagnostic Use.

11 Summary of Changes

The following changes were been made to the 7/23 revision of this document:

Updated Tables 5, 6 and 9.

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

^(b)AQA-8C, TMR-8C, CXR-8C, TOM-8C, WEN-8C and CCO-8C dyes are proprietary

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Class 1 Laser Product.