

PowerPlex® Y23 System for Use on the Spectrum CE System Technical Manual

Instructions for Use of Products DC2305 and DC2320



Table of Contents

1	Description.....	5
2	Product Components and Storage Conditions.....	6
3	Before You Begin	9
	3.1 Precautions	9
	3.2 Spectral Calibration	10
4	Protocols for DNA Amplification Using the PowerPlex® Y23 System.....	11
	4.1 Amplification of Extracted DNA in a 25µl Reaction Volume	12
	4.2 Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume	15
	4.3 Direct Amplification of DNA from Swabs in a 25µl Reaction Volume ..	20
5	Detection of Amplified Fragments Using the Spectrum CE System	24
	5.1 Sample Preparation	25
	5.2 Instrument Preparation	26
	5.3 Create a Plate Record	28
	5.4 Assembling the Plate Base Retainer and Loading the Plate	34
	5.5 Linking a Plate for a Run	35
	5.6 Monitoring a Run	36
	5.7 Exporting Data	36

6	Data Analysis Using GeneMapper® <i>ID-X</i> Software	37
6.1	Importing PowerPlex® Y23 Panels, Bins and Stutter Text Files into GeneMapper® <i>ID-X</i> Software	37
6.2	Importing the WEN ILS 500 Size Standard into GeneMapper® <i>ID-X</i> Software	38
6.3	Creating a Size Standard with GeneMapper® <i>ID-X</i> Software	39
6.4	Creating a Casework Analysis Method with GeneMapper® <i>ID-X</i> Software	40
6.5	Creating a Databasing or Paternity Analysis Method with GeneMapper® <i>ID-X</i> Software	43
6.6	Controls in GeneMapper® <i>ID-X</i> Software	45
7	Data Analysis Using GeneMarker® HID Software for Spectrum CE Systems	46
7.1	Creating an Analysis Method with GeneMarker® HID Software for Spectrum CE Systems	46
7.2	Controls in GeneMarker® HID Software for Spectrum CE Systems	51
8	Results.....	52
9	Troubleshooting.....	55
9.1	Amplification and Fragment Detection	55
9.2	Amplification of Extracted DNA	57
9.3	Direct Amplification of DNA from Storage Card Punches	59
9.4	Direct Amplification of DNA from Swabs	62
9.5	GeneMapper® <i>ID-X</i> Software	65
9.6	GeneMarker® HID Software for Spectrum CE Systems	67

10	References.....	71
11	Appendix	74
11.1	Advantages of Using the Loci in the PowerPlex® Y23 System	74
11.2	The WEN Internal Lane Standard 500 Y23	78
11.3	Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume	79
11.4	Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume	84
11.5	Composition of Buffers and Solutions	88
11.6	Related Products	89

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 that can be detected using the polymerase chain reaction (5–9). Alleles of STR loci are differentiated by the number of copies of the repeat sequence contained within the amplified region and are distinguished from one another using fluorescence detection following electrophoretic separation.

STR markers on the Y chromosome (Y-STR) have qualities that are distinct from autosomal markers and are useful for human identification (10–16). Y-STR markers are found on the nonrecombining region of the Y chromosome (NRY) and produce a haploid profile when amplified from male DNA. This quality simplifies male/female mixture interpretation by removing the female contribution from an amplification profile (17,18). Strict paternal inheritance of these markers makes them useful for paternity and kinship studies.

The PowerPlex® Y23 System^(a) allows co-amplification and four-color fluorescent detection of 23 loci, including DYS576, DYS389I, DYS448, DYS389II, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643, DYS393, DYS458, DYS385a/b, DYS456 and Y-GATA-H4.

The PowerPlex® Y23 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® Y23 Master Mix. This manual contains a protocol for use of the PowerPlex® Y23 System with the GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler and ProFlex® PCR System, in addition to a protocol to separate amplified products and detect separated material on the Spectrum CE System. A protocol to operate the Spectrum Compact CE System is available separately: see the *Spectrum CE System Operating Manual #TMD052*. Amplification and detection instrumentation may vary. You may need to optimize protocols, including amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. In-house validation should be performed.

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

2 Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
PowerPlex® Y23 System	50 reactions	DC2305

Not For Medical Diagnostic Use. This system contains sufficient reagents for 50 reactions of 25µl each. Includes:

Pre-Amplification Components Box

- 250µl PowerPlex® Y23 5X Master Mix
- 125µl PowerPlex® Y23 10X Primer Pair Mix
- 25µl 2800M Control DNA, 10ng/µl
- 1,250µl Water, Amplification Grade

Post-Amplification Components Box

- 25µl PowerPlex® Y23 Allelic Ladder Mix
- 200µl WEN Internal Lane Standard 500 Y23

PRODUCT	SIZE	CAT.#
PowerPlex® Y23 System	200 reactions	DC2320

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

Pre-Amplification Components Box

- 4 × 250µl PowerPlex® Y23 5X Master Mix
- 4 × 125µl PowerPlex® Y23 10X Primer Pair Mix
- 25µl 2800M Control DNA, 10ng/µl
- 5 × 1,250µl Water, Amplification Grade

Post-Amplification Components Box

- 4 × 25µl PowerPlex® Y23 Allelic Ladder Mix
- 200µl WEN Internal Lane Standard 500 Y23



The PowerPlex® Y23 Allelic Ladder Mix is provided in a separate, sealed bag for shipping. After opening, move the PowerPlex® Y23 Allelic Ladder Mix to the post-amplification box. Water, Amplification Grade, is provided in a separate, sealed bag for shipping. After opening, move the Water, Amplification Grade, to the pre-amplification box.

Storage Conditions

Upon receipt, store all components except the 2800M Control DNA at -30°C to -10°C in a nonfrost-free freezer. Store the 2800M Control DNA at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$. Make sure that the 2800M Control DNA is stored at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$ for **at least 24 hours** before use. After the first use, store the WEN Internal Lane Standard (WEN ILS) 500 Y23 at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$, protected from light; do not refreeze. The PowerPlex® Y23 10X Primer Pair Mix, PowerPlex® Y23 Allelic Ladder Mix and WEN ILS 500 Y23 are light-sensitive and must be stored in the dark. We strongly recommend that pre-amplification and post-amplification reagents be stored and used separately with different pipettes, tube racks, etc.

Optional: The PowerPlex® Y23 System components can be stored for up to 1 year at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$ without loss of activity.

Available Separately

PRODUCT	SIZE	CAT. #
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
5X AmpSolution™ Reagent	500µl	DM1231
Stabilizer Reagent	500 preps	DM6571
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 punches prior to direct amplification. The SwabSolution™ Kit is required to process swabs prior to direct amplification. The 5X AmpSolution™ Reagent is required for direct amplification of DNA from storage card punches in a 12.5µl reaction volume. Both the PunchSolution™ Kit and SwabSolution™ Kit contain the 5X AmpSolution™ Reagent.

If samples in loading cocktail will be stored in the injection plate for up to 48 hours prior to injection, we strongly recommend including Stabilizer Reagent in the loading cocktail. Omitting Stabilizer Reagent from loading cocktails where the injection plate is stored for up to 48 hours prior to injection may result in loss of signal in amplified samples.

The proper panels, bins and stutter text files and size standard .xml files for use with GeneMapper® ID-X software can be downloaded at: www.promega.com/PowerPlexPanels/

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

The PowerPlex® 5C Matrix Standard (Cat.# DG4850) 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 (19,20). Guidelines for the validation process are published in the *Internal Validation Guide of Y-STR Systems for Forensic Laboratories* (21).

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 at: **www.promega.com/protocols/**


4 Protocols for DNA Amplification Using the PowerPlex® Y23 System

The PowerPlex® Y23 System was developed for amplification of extracted DNA and direct-amplification samples. Slight protocol variations are recommended for optimal performance with each template source. Protocols for amplification in a 25µl reaction volume using extracted DNA (Section 4.1), lytic and nonlytic storage card punches (Section 4.2) and swabs (Section 4.3) are included in the following amplification sections. Protocols for amplification in a 12.5µl reaction volume using lytic and nonlytic storage card punches and swabs are included in Sections 11.3 and 11.4, respectively.

The PowerPlex® Y23 System is compatible with the GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block, the Veriti® 96-Well Thermal Cycler and the ProFlex® PCR System.

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is **not** recommended with the PowerPlex® Y23 System.

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

 Meticulous care must be taken to ensure successful amplification. A guide to amplification troubleshooting is provided in Section 9.

4.1 Amplification of Extracted DNA in a 25µl Reaction Volume

Materials to be Supplied by the User

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

We routinely amplify 0.5ng of template DNA in a 25µl reaction volume using the protocol detailed below.

Amplification Setup

1. Thaw the PowerPlex® Y23 5X Master Mix, PowerPlex® Y23 10X Primer Pair Mix and Water, Amplification Grade, completely.

Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex reagents for 15 seconds before each use. Do not centrifuge the 10X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
3. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.

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

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

PCR Amplification Mix Component ¹	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25.0µl	×		=	
PowerPlex® Y23 5X Master Mix	5.0µl	×		=	
PowerPlex® Y23 10X Primer Pair Mix	2.5µl	×		=	
template DNA (0.5ng) ^{2,3,4}	up to 17.5µl				
total reaction volume	25.0µl				

¹Add Water, Amplification Grade, to the tube first, then add PowerPlex® Y23 5X Master Mix and PowerPlex® Y23 10X Primer Pair Mix. The template DNA will be added at Step 6.

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

³Apparent DNA concentrations can differ, depending on the DNA quantification method used (22). We strongly recommend that you perform experiments to determine the optimal DNA amount based on your DNA quantification method.

⁴The PowerPlex® Y23 System is optimized and balanced for 0.5ng of DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different.

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



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

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

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

Note: The PowerPlex® Y23 System is optimized and balanced for 0.5ng of DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different.

- For the positive amplification control, vortex the tube of 2800M Control DNA, then dilute an aliquot to 0.5ng in the desired template volume. Add 0.5ng of diluted DNA to a reaction well containing PCR amplification mix.

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

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

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

Thermal Cycling

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

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

Notes:

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

Thermal Cycling Protocol

96°C for 2 minutes, then:

94°C for 10 seconds

61°C for 1 minute

72°C for 30 seconds

for 30 cycles, then:

60°C for 20 minutes

4°C soak

3. 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 can produce artifacts.

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

Materials to be Supplied by the User

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

This section contains a protocol for direct amplification of DNA from storage card punches in a 25µl reaction volume using the PowerPlex® Y23 System and GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler or ProFlex® PCR System. A protocol for direct amplification of DNA from storage card punches in a 12.5µl reaction volume is provided in Section 11.3.

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

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

Lytic card sample types include:

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

Nonlytic card sample types include:

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

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

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

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

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

For nonlytic card punches, adding PunchSolution™ Reagent to the well before adding the punch during pretreatment may help alleviate static problems.

Amplification Setup

1. Thaw the PowerPlex® Y23 5X Master Mix, PowerPlex® Y23 10X Primer Pair Mix and Water, Amplification Grade, completely.

Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex reagents for 15 seconds before each use. Do not centrifuge the 10X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
3. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.

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

Table 2. PCR Amplification Mix for Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume.

PCR Amplification Mix Component ¹	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	17.5µl	×		=	
PowerPlex® Y23 5X Master Mix	5.0µl	×		=	
PowerPlex® Y23 10X Primer Pair Mix	2.5µl	×		=	
total reaction volume	25.0µl				

¹Add Water, Amplification Grade, to the tube first, and then add PowerPlex® Y23 5X Master Mix and PowerPlex® Y23 10X Primer Pair Mix. For lytic card punches, the template DNA will be added at Step 6.

5. Vortex the PCR amplification mix for 5–10 seconds, 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 a buccal sample 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 also is acceptable to add the lytic card punch first, then add the PCR amplification mix.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, dilute an aliquot to 5.0ng/µl and add 1µl to a reaction well containing 25µl of PCR amplification mix.

Notes:

- Optimization of the amount of 2800M Control DNA may be required, depending on thermal 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 air bubbles.

Thermal Cycling

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

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

Notes:

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

Thermal Cycling Protocol

96°C for 2 minutes, then:

94°C for 10 seconds

61°C for 1 minute

72°C for 30 seconds

for 26 cycles, then:

60°C for 20 minutes

4°C soak

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

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

For initial testing, amplify using the following cycle numbers. Additional testing may be required:

Blood sample on one 1.2mm lytic or pretreated nonlytic punch: 25, 26 and 27 cycles

Buccal cells on two 1.2mm lytic punches: 26, 27 and 28 cycles

Buccal cells on one 1.2mm lytic punch: 27, 28 and 29 cycles

Buccal cells on one 1.2mm pretreated nonlytic punch: 25, 26 and 27 cycles

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

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

Materials to be Supplied by the User

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

This section contains a protocol for amplifying DNA from swab extracts in a 25µl reaction volume using the PowerPlex® Y23 System and GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler or ProFlex® PCR System. A protocol for direct amplification of DNA from swab extracts in a 12.5µl reaction volume is provided in Section 11.4.

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. Thaw the PowerPlex® Y23 5X Master Mix, PowerPlex® Y23 10X Primer Pair Mix and Water, Amplification Grade, completely.

Note: Centrifuge tubes briefly to bring contents to the bottom, then vortex reagents for 15 seconds before each use. Do not centrifuge the 10X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.
2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
3. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.

- Add the final volume of each reagent listed in Table 3 to a clean tube.

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

PCR Amplification Mix Component ¹	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	15.5µl	×		=	
PowerPlex® Y23 5X Master Mix	5.0µl	×		=	
PowerPlex® Y23 10X Primer Pair Mix	2.5µl	×		=	
swab extract	2.0µl				
total reaction volume	25.0µl				

¹Add Water, Amplification Grade, to the tube first, and then add PowerPlex® Y23 5X Master Mix and PowerPlex® Y23 10X Primer Pair Mix. The swab extract will be added at Step 6.

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



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, dilute an aliquot to 2.5ng/µl, and add 2µl to a reaction well or tube containing 23µl of PCR amplification mix.

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

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

Thermal Cycling

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

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

Notes:

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

Thermal Cycling Protocol

96°C for 2 minutes, then:

94°C for 10 seconds

61°C for 1 minute

72°C for 30 seconds

for 26 cycles, then:

60°C for 20 minutes

4°C soak

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

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

PCR Optimization

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

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

Note: This recommendation is for 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)
- Stabilizer Reagent (Cat.# DM6571)



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.



If samples in an injection plate will not be injected immediately, we strongly recommend including Stabilizer Reagent in the loading cocktail, and injecting the samples within 48 hours. Omitting Stabilizer Reagent from the loading cocktail may result in loss of signal in the amplified samples.

Note: Wear gloves when handling consumables and sample cartridges.

5.1 Sample Preparation

1. Prepare a loading cocktail by combining and mixing internal lane standard (ILS) and formamide as follows: $[(0.5\mu\text{l ILS}) \times (\# \text{ samples})] + [(9.5\mu\text{l formamide}) \times (\# \text{ samples})]$
2. If samples in injection plate will not be injected immediately or if there is the possibility that the samples may be reinjected within 48 hours of setting up the plate, prepare a loading cocktail combining and mixing WEN ILS 500 Y23, Stabilizer Reagent and formamide as follows:

$[(0.5\mu\text{l WEN ILS 500 Y23}) \times (\# \text{ samples})] + [(0.5\mu\text{l Stabilizer Reagent}) \times (\# \text{ samples})] + [(9.5\mu\text{l formamide}) \times (\# \text{ samples})]$



Be sure to use the WEN ILS 500 Y23 as the size standard when using the PowerPlex® Y23 System. Do not use the WEN ILS 500 (Cat.# DG5001). The WEN_ILS_500.xml file can be used to assign fragment sizes for the WEN ILS 500 Y23.

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.



If samples in an injection plate will not be injected immediately, we strongly recommend including Stabilizer Reagent in the loading cocktail, and injecting the samples within 48 hours.

3. Vortex for 10–15 seconds to mix.
4. Pipet 10 μl of formamide/internal lane standard mix (or 10.5 μl for formamide/Stabilizer Reagent/internal lane standard mix if Stabilizer Reagent is used) into each well of the 96-well plate.
5. 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. We recommend including an injection of allelic ladder every 2 injections (16 samples).
6. Centrifuge the plate briefly to remove air bubbles from the wells.
 7. Denature samples at 95°C for 3 minutes, and then immediately chill on crushed ice or a freezer plate block or in an ice-water bath for 3 minutes. Denature samples just prior to loading the instrument.

5.2 Instrument Preparation

Refer to the *Spectrum CE System Operating Manual #TMD052* for the instrument maintenance schedule and instructions to install the capillary array, anode buffer, cathode buffer and polymer pouch. The appropriate spectral calibration must be performed and accepted on the instrument prior to use for samples. Refer to the *Spectrum Calibration 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 1). This screen provides access to the four workflow menus (Home, Review Plates, Maintenance and Settings) as well as status indicators. The screen is divided into three sections: Header, Task Pane and Status Bar.



Figure 1. Spectrum Control Software 'Home' Screen.

The Header (Figure 2) 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.

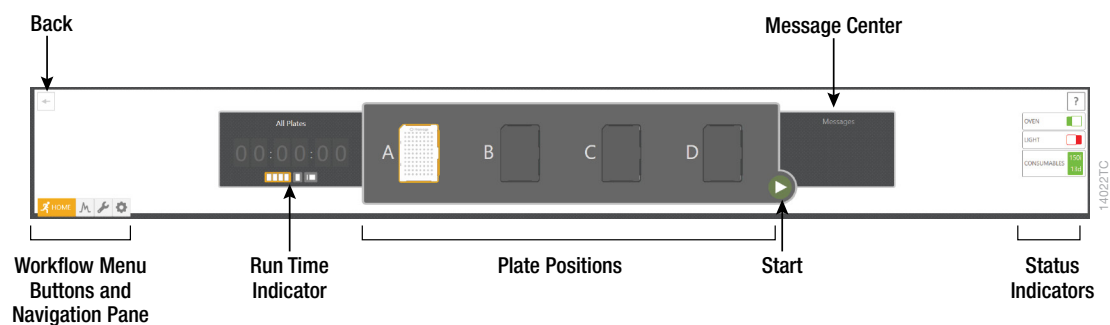









Figure 2. Header.

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 the PDF of the <i>Spectrum CE System Operating Manual</i>
	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 Calibration Technical Manual #TMD068* 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 3) 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 2). The 'Consumables' screen (Figure 3) displays information for the four RFID-tagged consumables on the instrument: Polymer, Capillary Array and Anode and Cathode Buffers.

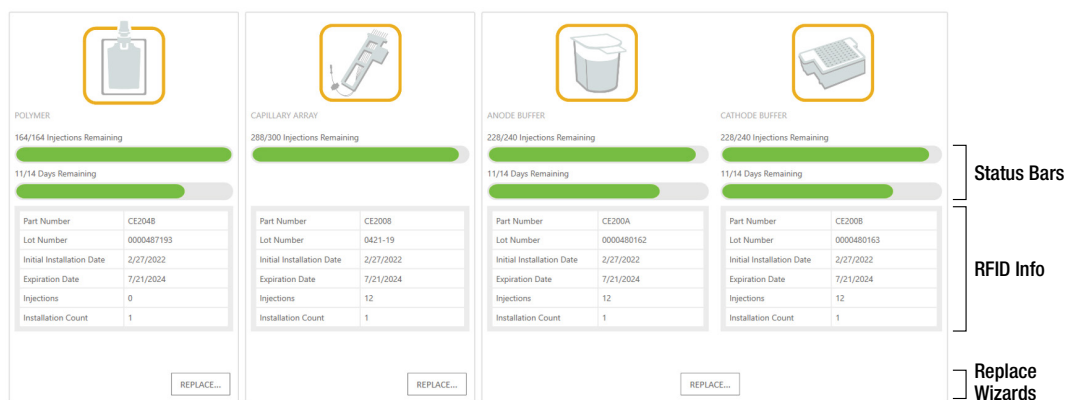


Figure 3. 'Consumables' screen.

The following information is tracked through these tags:

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

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

Select the **Oven Heater Indicator** in the Header (Figure 2) 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 1).

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 4).

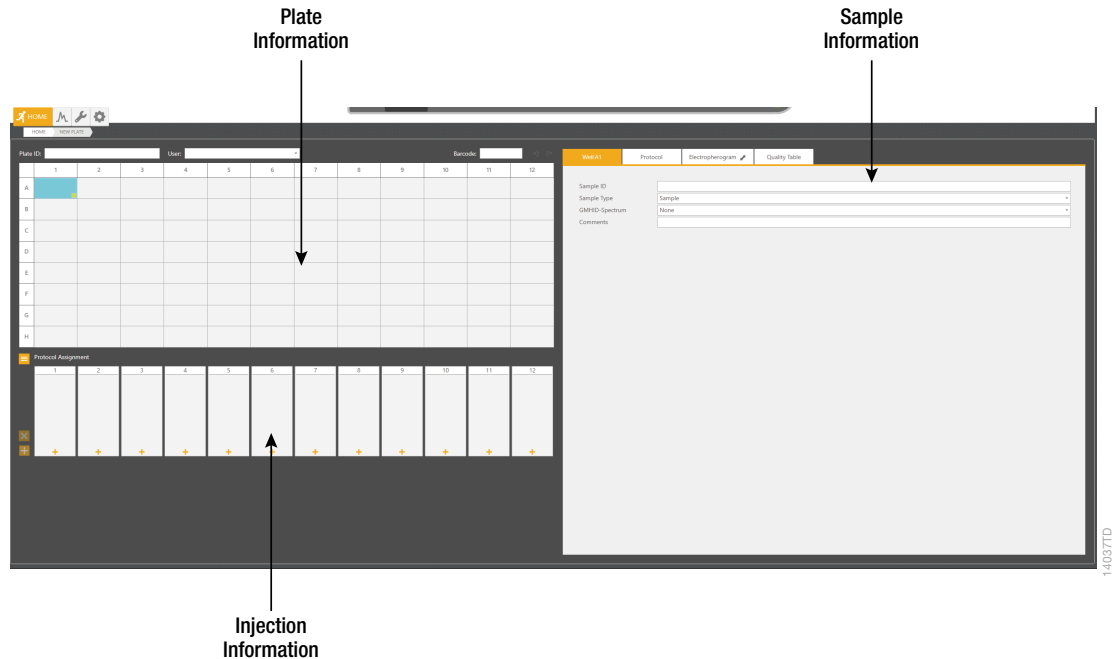


Figure 4. 'Plate' screen.

2. Use the default Plate ID that includes date and time or enter information into the 'Plate ID' field (Figure 5).
3. If desired, enter user information into the 'User' field (Figure 5).

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 5).

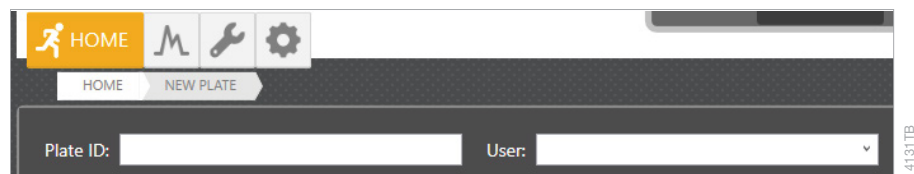


Figure 5. 'Plate ID' and 'User' fields of the plate screen.

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 4).

The green box in the lower right corner of a sample well (Figure 6) can be used to fill other wells with the same information by left clicking and dragging the green box to the other wells.

Select and drag to fill other wells with the same information

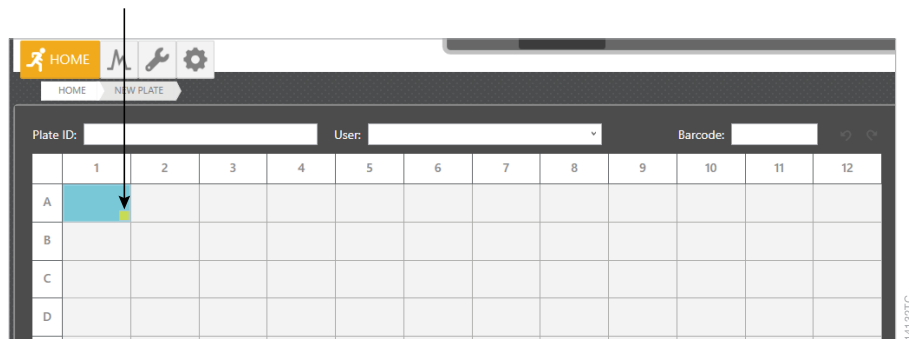


Figure 6. Filling information across multiple sample wells.

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 7) to designate the well as Sample, Ladder, Positive Control or Negative Control. These designations will be included in the output file for downstream analysis.

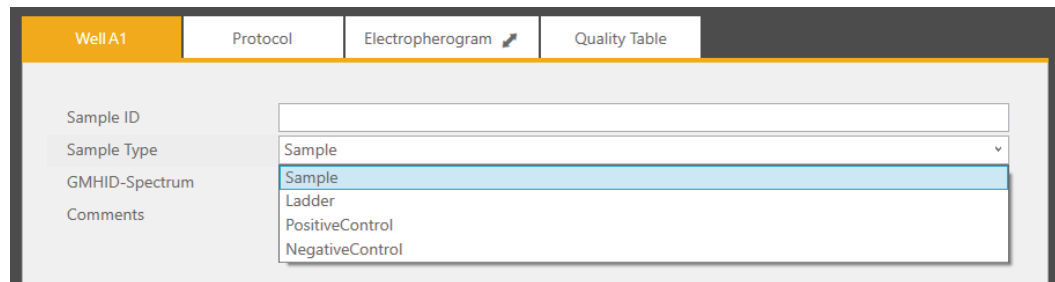


Figure 7. Drop-down options for sample type.

6. **Optional:** Select **PowerPlex_Y23** in the GeneMarker®HID drop-down menu (Figure 8).

Notes:

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

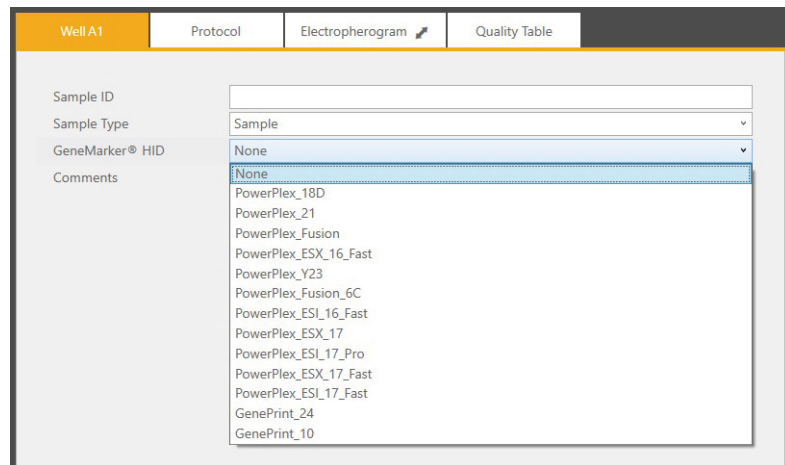


Figure 8. Drop-down options for GeneMarker®HID Software for Spectrum CE Systems run wizard templates.

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

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

- To add a run protocol to selected samples, highlight the desired wells (Ctrl + right-click or drag with the mouse). Then right-click, mouse over “Add Injection To Selected Wells” then select the **Promega 5-Dye (5C)** protocol from the pop-up menu.
- To add a run protocol to individual injections, select the **+** button under the box for that injection set then select the **Promega 5-Dye (5C)** protocol from the pop-up menu.
- To add a run protocol to multiple injections, highlight the desired injections in the plate map (click-drag, Ctrl + click or click the upper left cell). Then select the **+** button on the left side of the injection information section and choose **Promega 5-Dye (5C)** (Figure 9) from the pop-up menu.

Notes:

- 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.
- 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.

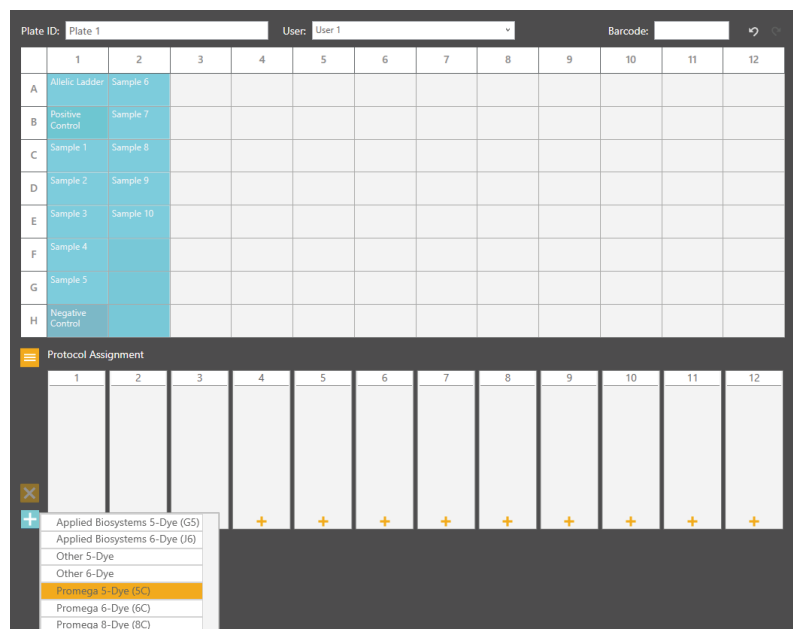


Figure 9. Assigning run protocol to multiple injections.

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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 10), which now displays the name of the previously selected protocol. The information in this tab is read-only.

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

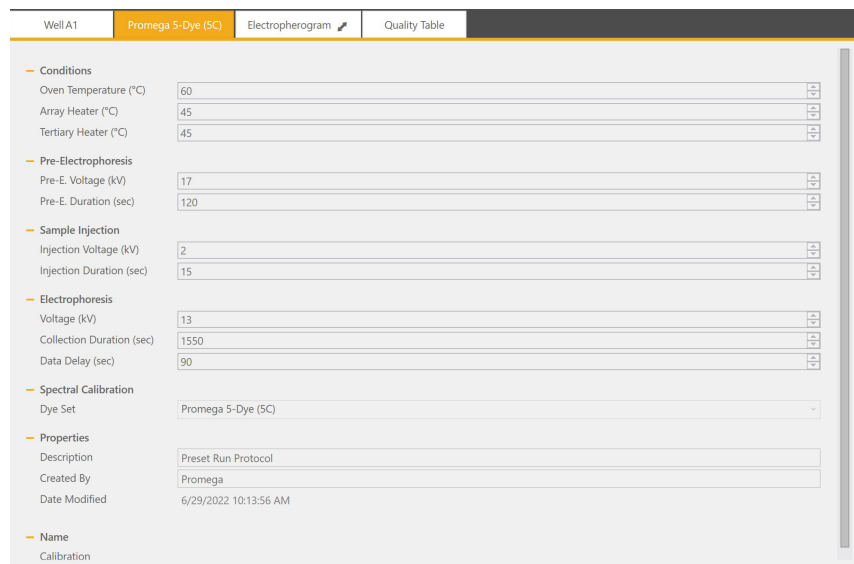


Figure 10. The 'Protocol' tab of the Sample Information section.

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

5.4 Assembling the Plate Base Retainer and Loading the Plate

1. Place the 96-well plate created in Section 5.1 into the Spectrum 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 11. The Spectrum plate assembly.

3. Confirm the drawer handle light is illuminated, indicating that the drawer is unlocked and ready for plate loading. The Drawer Status indicator is in the Message Center located in the Header (Figure 2)
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 icon on the screen will indicate that there is a plate present.
- 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 12).
3. Select the **Start** arrow to begin the run.

Notes:

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

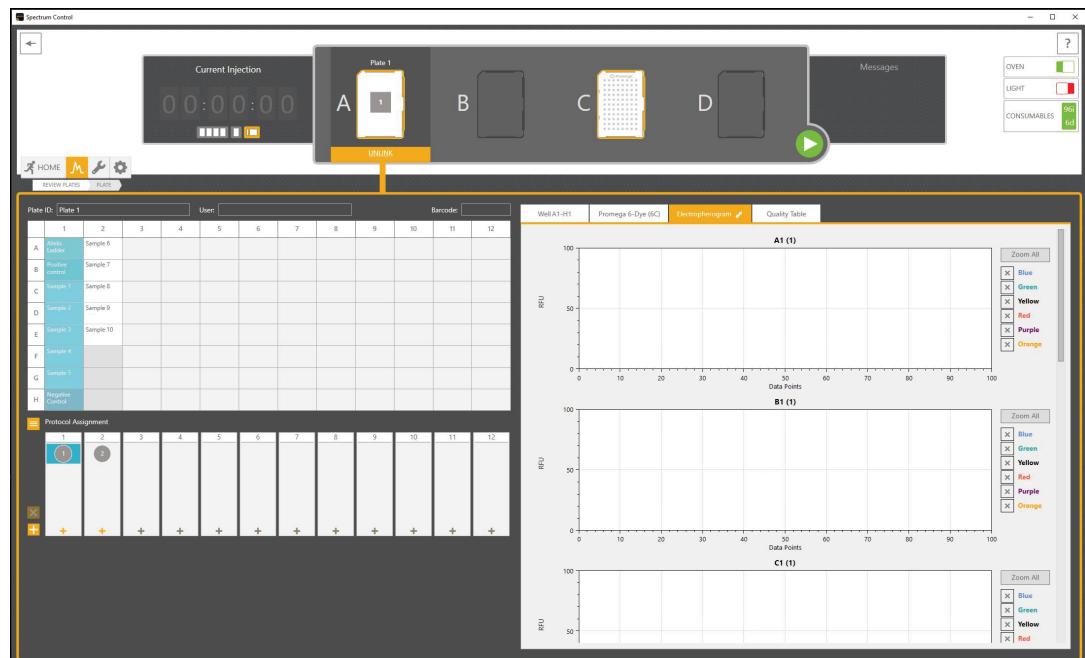


Figure 12. Linked plate in the Spectrum Control Software.

5.6 Monitoring a Run

1. The Plate Status is indicated by the icon in the Plate 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 and .fsa 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. Select **Activate FSA Export** to export .fsa data files in addition to the .promega data files.

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

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

6 Data Analysis Using GeneMapper® ID-X Software

Due to potential differences between software versions, some of the instructions may not apply to all versions of GeneMapper® ID-X software.

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

6.1 Importing PowerPlex® Y23 Panels, Bins and Stutter Text Files into GeneMapper® ID-X Software

To facilitate analysis of data with the PowerPlex® Y23 System, we have created panels, bins and stutter text files to allow automatic assignment of genotypes using GeneMapper® ID-X Software. We recommend that users receive training from Applied Biosystems on the GeneMapper® ID-X Software to familiarize themselves with proper operation of the software.

Note: The GeneMapper® ID-X stutter files include filters for the plus stutter associated with the two trinucleotide repeat loci (DYS481 and DYS392) as well as filters for plus-2- and minus-2-base artifacts associated with the DYS19 locus.

Getting Started

1. To obtain the proper panels, bins and stutter text files and WEN_ILS_500_IDX.xml file for the PowerPlex® Y23 System, go to: **www.promega.com/PowerPlexPanels**
2. Select the PowerPlex® System that you are using, and then select **GeneMapper ID-X**. Enter your contact information, and then select **Submit**.
3. Save the PowerPlex_Y23_Panels_IDX_vX.x.txt, PowerPlex_Y23_Bins_IDX_vX.x.txt and PowerPlex_Y23_Stutter_IDX_vX.x.txt files, where "X.x" refers to the most recent version of the panels, bins and stutter text files, to a known location on your computer.
4. Save the WEN_ILS_500_IDX.xml file to a known location on your computer.

Importing Panels, Bins and Stutter Files

1. Open the GeneMapper® ID-X software.
2. Select **Tools**, and then **Panel Manager**.
3. Highlight the Panel Manager icon in the upper left navigation pane.
4. Select **File**, and then **Import Panels**.
5. Navigate to the panels text file downloaded in the Getting Started section above. Select the file, and then **Import**.
6. Select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.
7. In the navigation pane, highlight the PowerPlex Y23 panels folder that you imported in Step 5.
8. Select **File**, and then **Import Bin Set**.
9. Navigate to the bins text file downloaded in the Getting Started section above. Select the file, and then **Import**.
10. In the navigation pane, highlight the PowerPlex Y23 panels folder that you imported in Step 5.
11. Select **File**, and then **Import Marker Stutter**. A warning box will appear asking if you want to overwrite current values. Select **Yes**.
12. Navigate to the stutter text file downloaded in the Getting Started section above. Select the file, and then **Import**.
13. At the bottom of the Panel Manager window, select **OK**. This will save the panels, bins and stutter text files, and then close the window.

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

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

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

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

1. Select **Tools**, and then **GeneMapper ID-X Manager**.
2. Select the 'Size Standard' tab.
3. Select **New**.
4. In the 'Size Standard Editor' window (Figure 13), select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.

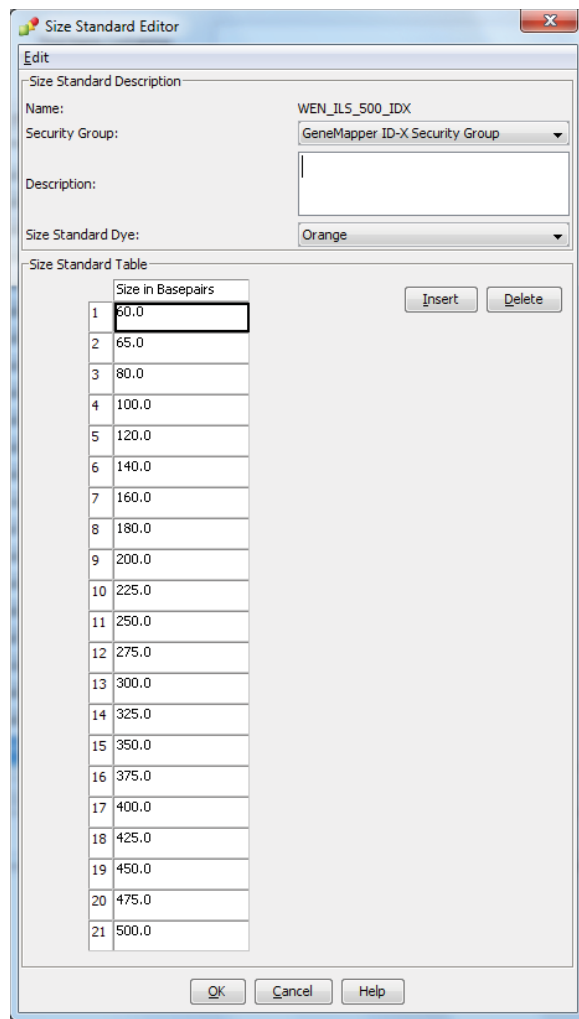


Figure 13. The GeneMapper® ID-X Size Standard Editor.

5. Enter a detailed name, such as "WEN_ILS_500_IDX."
6. Choose **Orange** for the Size Standard Dye.
7. Enter the sizes of the internal lane standard fragments (60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases). See Section 11.2, Figure 25.
8. Select **OK**.

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

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

1. Select **Tools**, and then **GeneMapper ID-X Manager**.
2. Select the 'Analysis Methods' tab.
3. Select **New**, and a new analysis method dialog box will open.
4. In the 'Analysis Method Editor' window, select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.
5. Enter a descriptive name for the analysis method, such as "PowerPlex Y23."
6. Select the 'Allele' tab (Figure 14).

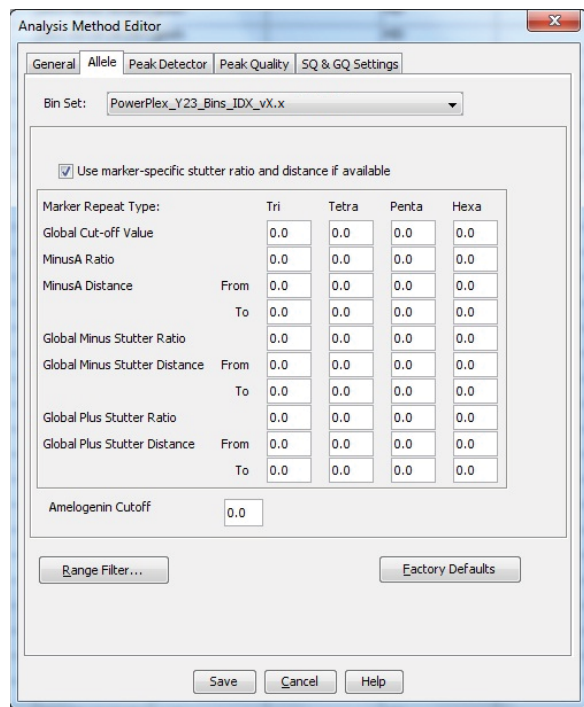


Figure 14. The GeneMapper® ID-X 'Allele' tab.

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

Note: The GeneMapper® ID-X stutter files include filters for the plus stutter associated with the two trinucleotide repeat loci (DYS481 and DYS392) as well as filters for the plus-2- and minus-2-base artifacts associated with the DYS19 locus.

- Select the 'Peak Detector' tab (Figure 15). You will need to optimize these settings. In-house validation should be performed.

Notes:

- Select full range or partial range for the analysis range. When using a partial range, choose an appropriate analysis range based on your data. Choose a start point after the primer peak and just before the first defined internal lane standard peak to help ensure proper sizing of the internal lane standard.
- The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Individual laboratories should determine their peak amplitude thresholds from internal validation studies.
- The normalization box can be checked or unchecked.

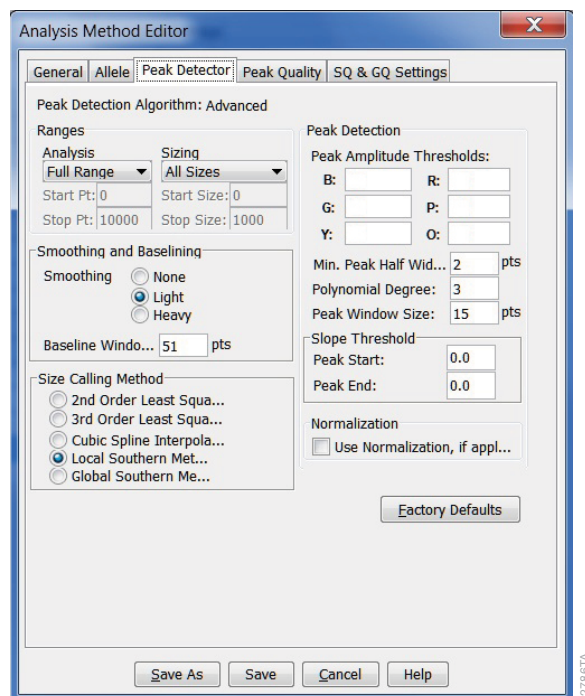


Figure 15. The GeneMapper® ID-X 'Peak Detector' tab.

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

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

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

Processing Data for Casework Samples

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

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

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

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

9. If all analysis requirements are met, the 'Save Project' window will open (Figure 16).

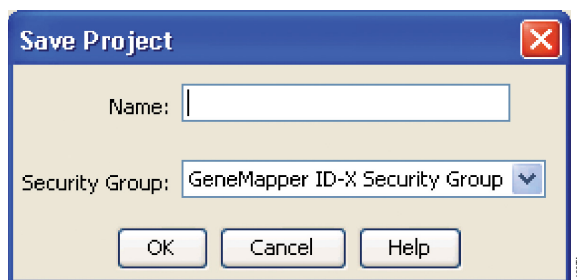


Figure 16. 'Save Project' Window.

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

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

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

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

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

1. Select **Tools**, and then **GeneMapper ID-X Manager**.
2. Select the 'Analysis Methods' tab.
3. Select **New**, and a new analysis method dialog box will open.
4. In the 'Analysis Method Editor' window, select **GeneMapper ID-X Security Group** as the Security Group. This allows access for all users of the software. Other security groups may be used.
5. Enter a descriptive name for the analysis method, such as "PowerPlexY23 20% Filter."
6. Select the 'Allele' tab (Figure 17).
7. Select the bins text file that was imported in Section 6.1.
8. Ensure that the "Use marker-specific stutter ratio and distance if available" box is checked. Doing this will assign locus-specific stutter files and distances from the imported stutter file. Ensure that the appropriate global filter is applied to this analysis method. For example, for a 20% filter, enter "0.20" for the Global Cut-off Value for Tri, Tetra and Penta repeats (Figure 17).

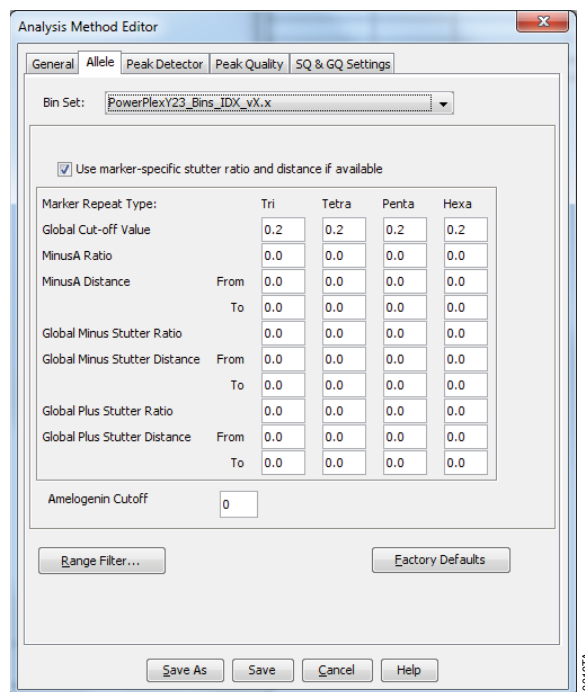


Figure 17. The GeneMapper® ID-X 'Allele' tab with settings for using a 20% peak filter.

9. Select the 'Peak Detector' tab (Figure 15). You will need to optimize these settings. In-house validation should be performed.

Notes:

- a. Select full range or partial range for the analysis range. When using a partial range, choose an appropriate analysis range based on your data. Choose a start point after the primer peak and just before the first defined internal lane standard peak to help ensure proper sizing of the internal lane standard.
 - b. The peak amplitude thresholds are the minimum peak heights at which the software will call a peak. Individual laboratories should determine their peak amplitude thresholds from internal validation studies.
 - c. The normalization box can be checked or unchecked.
10. Select the 'Peak Quality' tab. You can change the settings for peak quality.
 11. Select the 'SQ & GQ Settings' tab. You can change these settings.
Note: For Steps 10 and 11, see the GeneMapper® ID-X user's manual for more information. The settings in Steps 10 and 11 should be based on the results of your internal validation.
 12. Select **Save** to save the new analysis method.
 13. Select **Done** to exit the GeneMapper® ID-X Manager.

Processing Data for Databasing or Paternity Samples

1. Select **File**, and then **New Project**.
2. Select **Edit**, and then **Add Samples to Project**.
3. Browse to the location of the run files. Highlight the desired files, and then select **Add to List**, followed by **Add**.
Note: The Spectrum CE System generates .fsa and .promega files for each sample run. The .promega files are not compatible with GeneMapper® ID-X Software.
4. The sample types assigned in Section 5.3 will be included in the 'Sample Type' column. If needed, the drop-down menu in the 'Sample Type' column can be used to select **Allelic Ladder**, **Sample**, **Positive Control** or **Negative Control** as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as **Allelic Ladder** in the 'Sample Type' column for proper genotyping.
5. In the 'Analysis Method' column, select the analysis method created previously in this section.
6. In the 'Panel' column, select the panels text file that was imported in Section 6.1.
7. In the 'Size Standard' column, select the size standard that was imported in Section 6.2 or created in Section 6.3.
8. Select **Analyze** (green arrow button) to start data analysis.
Note: By default, the software is set to display the 'Analysis Requirements Summary' window if an analysis requirement is not met.

9. If all analysis requirements are met, the 'Save Project' window will open (Figure 16).
10. Enter the project name.
11. Choose the applicable security group from the drop-down menu, and then select **OK**.

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

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

6.6 Controls in GeneMapper® ID-X Software

1. Observe the results for the negative control. Using the protocols defined in this manual, the negative control should be devoid of amplification products.
2. Observe the results for the 2800M Control DNA. The expected 2800M DNA allele designations for each locus are listed in Table 6 (Section 11.1).

7 Data Analysis Using GeneMarker®HID Software for Spectrum CE Systems

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

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

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

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

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

4. Select **Open**, and the selected files will appear in the Data File List (Figure 18).

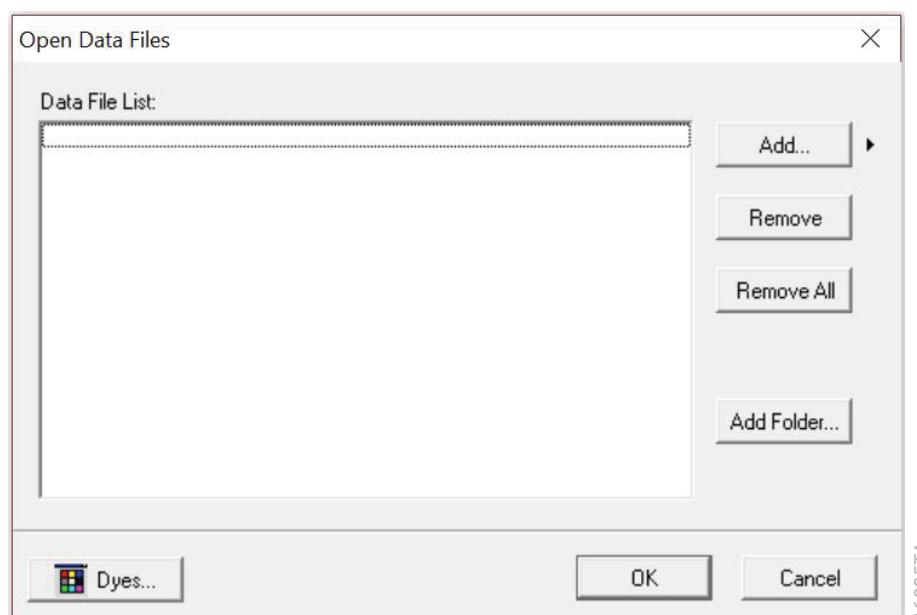


Figure 18. The GMHID-Spectrum Data File List.

5. Select **OK** in the 'Open Data Files' window, and the data will be uploaded into GMHID-Spectrum. In the 'Raw Data' folder in the File Navigator, verify that the sample types (allelic ladder, positive control and negative control) are designated correctly in Section 5. 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_Y23 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 19). This will set values for peaks within the marker range. Refer to *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555* for more information.

Notes:

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

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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 %

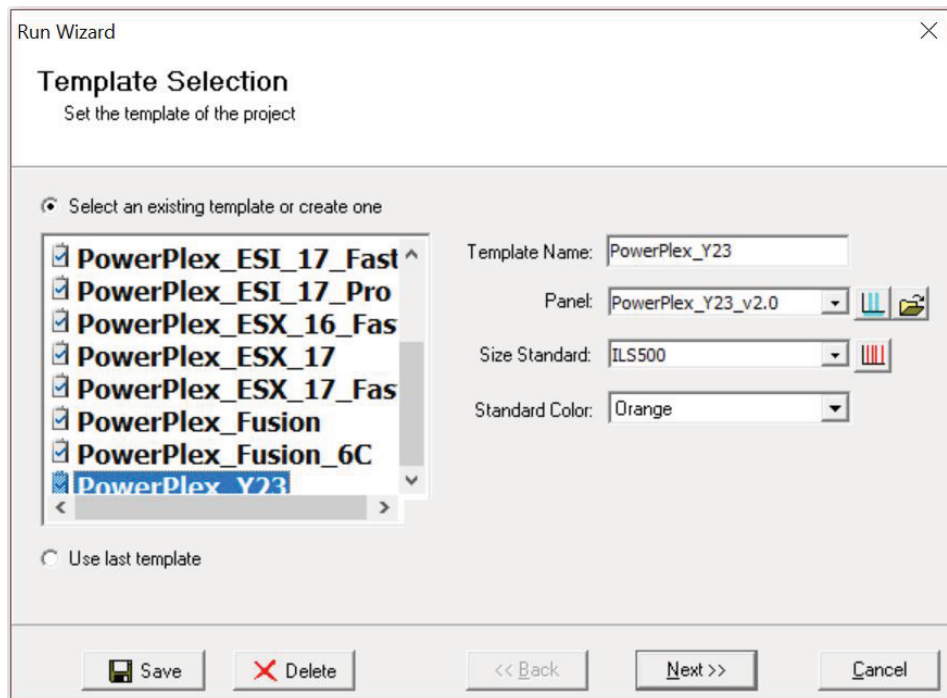
Use Allele-Specific Values(From Panel)

Apply Stutter Settings to All Markers

Figure 19. The 'Edit Marker' window for the DYS576 marker.

8. Select **Run** in the Magic Wizard or choose the **Run Project** icon (green arrow) in the toolbar. The 'Template Selection' screen will appear (Figure 20). Select the PowerPlex_Y23 template and the settings shown in Figure 20. Verify the panel that you created in Steps 6 and 7 is selected in the panel drop-down field. The 'Size Standard' must be ILS500 and the 'Standard Color' must be Orange. Select **Next**.

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



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Figure 20. The 'Template Selection' window.

9. The 'Data Process – HID Analysis' window will appear (Figure 21). 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

Data Process - HID Analysis

Set data process options

Raw Data Analysis

Auto Range (frame)
Start: 0 End: 10000

Smooth Enhanced Smooth

Baseline Subtraction:
 Superior Classic Enhanced

Pull-up Correction Spike Removal

Saturation Detection Saturation Repair

Allele Call

Auto Range (bps)
Start: 50 End: 550

Max Intensity: 32500

Peak Detection Threshold: ? Dye Specific

Min Intensity: 50

Percentage > 20 Global Max

Min Intensity: for Standard Color

Apply Nearest Marker Settings

Note: Use Panel Editor to set Min Intensity and % Global Max for peaks within Markers

Size Call
 Local Southern Cubic Spline

Save Delete << Back Next >> Cancel

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Figure 21. The 'Data Process' window for an analysis method.

10. The 'Additional Settings – HID Analysis' window will appear (Figure 22). Select **Y23_2800M** as the P.C. Template 1 and verify that the boxes are checked for "Auto Select Best Ladder" and "Auto Panel Adjustment". The values displayed in the 'Allele Evaluation' dialogue box are defaults and will affect the quality values displayed in the plot settings. For more information on quality values, use of a second positive control and mixture evaluation, refer to the *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555*. Select **OK** to analyze the raw data.

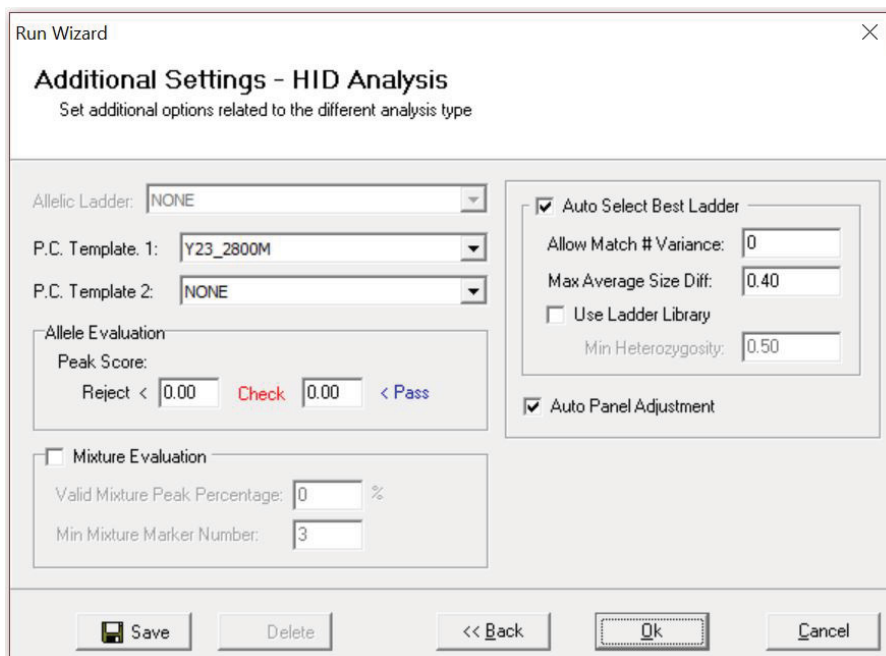


Figure 22. The 'Additional Settings' window.

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

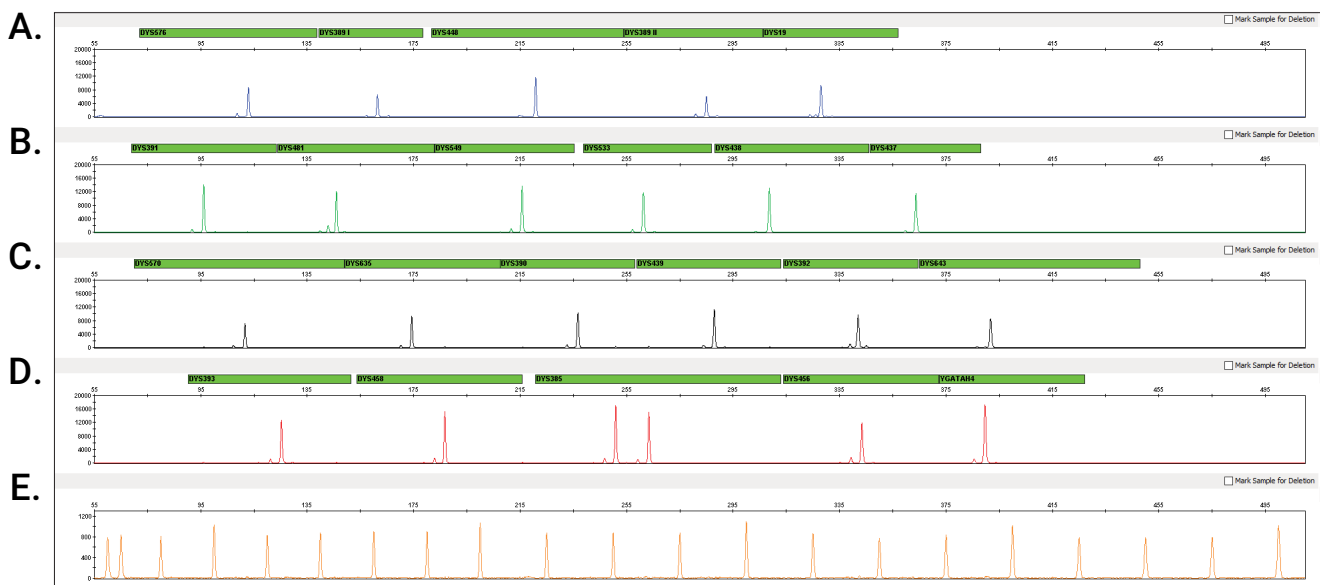
Note: Quality and display settings are set using Preferences under the View menu. Refer to *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555* for more information.

7.2 Controls in GeneMarker®HID Software for Spectrum CE Systems

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 6 (Section 11.1).

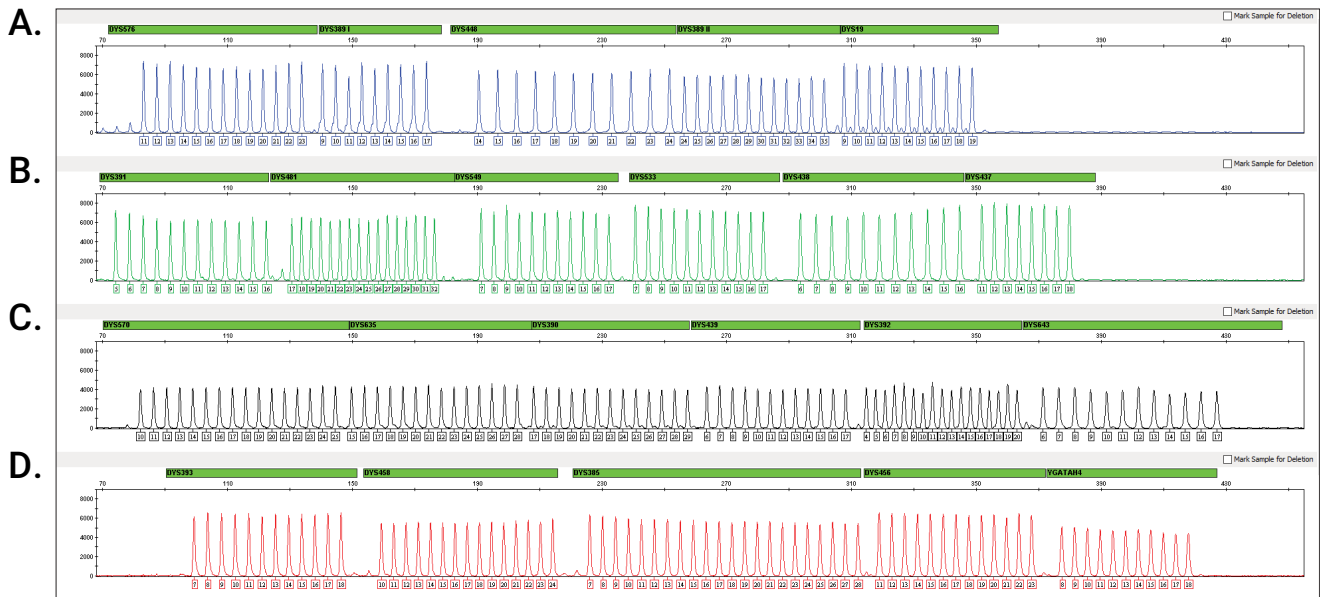
8 Results

Representative results of the PowerPlex® Y23 System are shown in Figure 23. The PowerPlex® Y23 Allelic Ladder Mix is shown in Figure 24.



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Figure 23. The PowerPlex® Y23 System. The 2800M Control DNA (0.5ng) was amplified using the PowerPlex® Y23 System. Amplification products were mixed with WEN Internal Lane Standard 500 Y23 and analyzed with a Spectrum CE System using a 2kV, 15-second injection. Results were analyzed using GeneMapper® ID-X software, version 1.4, and PowerPlex® Y23 panels and bins text files. **Panel A.** An electropherogram showing the peaks of the fluorescein-labeled loci: DYS576, DYS389I, DYS448, DYS389II and DYS19. **Panel B.** An electropherogram showing the peaks of the JOE-labeled loci: DYS391, DYS481, DYS549, DYS533, DYS438 and DYS437. **Panel C.** An electropherogram showing the peaks of the TMR-ET-labeled loci: DYS570, DYS635 DYS390, DYS439, DYS392 and DYS643. **Panel D.** An electropherogram showing the peaks of the CXR-ET-labeled loci: DYS393, DYS458, DYS385a/b, DYS456 and Y-GATA-H4. **Panel E.** An electropherogram showing the 60bp to 500bp fragments of the WEN Internal Lane Standard 500 Y23.



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Figure 24. The PowerPlex® Y23 Allelic Ladder Mix. The PowerPlex® Y23 Allelic Ladder Mix was analyzed with a Spectrum CE System using a 2kV, 15-second injection. The sample file was analyzed with the GeneMapper® ID-X software, version 1.4, and PowerPlex® Y23 panels and bins text files. **Panel A.** The fluorescein-labeled allelic ladder components and their allele designations. **Panel B.** The JOE-labeled allelic ladder components and their allele designations. **Panel C.** The TMR-ET-labeled allelic ladder components and their allele designations. **Panel D.** The CXR-ET-labeled allelic ladder components and their allele designations.

Artifacts and Stutter

Stutter products are a common amplification artifact associated with STR analysis (23,24). 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 will generally exhibit higher stutter than loci with longer repeat lengths. *DYS481* is a trinucleotide repeat and exhibits exceptionally high stutter. The pattern and intensity of stutter may differ slightly between primer sets for the same locus. Increased forward stutter (i.e., $n+4$ and $n+8$) can be observed at the *DYS389I* and *DYS389II* loci when using the cell-line derived 2800M Control DNA. This forward stutter is inherent to this cell line DNA and is not seen in amplification of DNA from human blood, body fluid or other human-derived samples.

The mean stutter plus three standard deviations observed at each locus is used in the PowerPlex® Y23 stutter text file for locus-specific filtering in GeneMapper® ID-X software and the panel file included in GMHID-Spectrum. These files also include filters for the plus stutter associated with the two trinucleotide repeat loci (*DYS481* and *DYS392*) as well as filters for the plus-2- and minus-2-base artifacts associated with the *DYS19* locus.

In addition to stutter peaks, you may observe the following low-level DNA-dependent artifact peaks (Table 4) and DNA-independent (with or without human genomic DNA) artifact peaks (Table 5) with the PowerPlex® Y23 System.

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

Dye Label or Locus	Artifact Size ¹
JOE	~163 bases ² ~187 bases ² ~253 bases ² ~272 bases ²
TMR-ET	~159 bases ² ~428 bases ² ~441 bases ²
CXR-ET	201 bases ²
DYS448	n-9 to n-15 ³
DYS19	n-2, n+2

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

²Artifact is observed more often with samples that contain relatively higher levels of female DNA.

³These variably sized peaks may represent double-stranded DNA derived from the *DYS448* amplicon. (Double-stranded DNA is known to migrate faster than single-stranded DNA on capillary electrophoresis instruments.) This low-level DNA-dependent artifact is noticeable with high input template amounts and peak heights.

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

Dye Label	Artifact Size ¹
Fluorescein	~55–60 bases ² ~64–68 bases ² ~142–150 bases
JOE	~142–150 bases

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

²The signal strength of these artifacts increases with storage of the amplification plate at 4°C, sometimes in as short a time period as overnight but more commonly when plates are left at 4°C for a few days. We recommend storing amplification products at -20°C.

9 Troubleshooting

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

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

9.1 Amplification and Fragment Detection

Symptoms	Causes and Comments
Faint or absent allele peaks	The Master Mix was not vortexed well before use. Vortex the Master Mix for 15 seconds before dispensing into the PCR amplification mix.
	Primer concentration was too low. Use the recommended primer concentration. Vortex the Primer Pair Mix for 15 seconds before use.
	An air bubble formed at the bottom of the reaction tube. Use a pipette to remove the air bubble or centrifuge the reactions briefly before thermal cycling.
	Thermal cycler, plate or tube problems. Review the thermal cycling protocol. We have not tested reaction tubes, plates or thermal cyclers that are not listed. Calibrate the thermal cycler heating block if necessary.
	Samples stored in a plate for prolonged period (e.g., 48 hours) in loading cocktails without Stabilizer Reagent. If you anticipate that samples will be stored for a prolonged time in the plate, we strongly recommend including Stabilizer Reagent in the loading cocktail.
	Repeat sample preparation using fresh formamide. Long-term storage of amplified sample in formamide can result in loss of signal.
	Poor capillary electrophoresis injection (ILS peaks also affected). Re-inject the sample.
	Laser is starting to fail. Check laser power.
	Poor-quality formamide was used. Use only Hi-Di™ formamide.

Symptoms	Causes and Comments
Faint or absent allele peaks for the positive control reaction	Improper storage of the 2800M Control DNA. Store the 2800M Control DNA at +2°C to +10°C. Make sure that the 2800M Control DNA is stored at +2°C to +10°C for at least 24 hours before use. Vortex before use.
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.
	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. <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.
	Dye blob artifacts. The signal strength of certain dye blob artifacts increases with storage of the amplification plate at 4°C, sometimes in as short a time period as overnight but more commonly when left at 4°C for a few days. We recommend storing amplification products at -20°C.
An incorrect internal lane standard was used. Use the size standard provided in the kit.	

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

9.2 Amplification of Extracted DNA

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

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

Symptoms	Causes and Comments
Faint or absent allele peaks (continued)	The reaction volume was too low. This system is optimized for a final reaction volume of 25µl for extracted DNA. Decreasing the reaction volume may result in suboptimal performance.
Extra peaks visible in one or all color channels	Amplification of more than the recommended amount of purified DNA can result in a higher number of artifact peaks due to overamplification, resulting in saturating signal. Use the recommended amount of template DNA. See 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>Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3' A residue.</p> <ul style="list-style-type: none"> • Be sure to perform the recommended extension step at 60°C after thermal cycling. • Decrease the amount of template DNA. Using more than the recommended amount of template DNA can result in incomplete adenylation. • Decrease cycle number. • Increase the final extension time.
Peak height imbalance	Amplification of greater than the recommended amount of template can result in an imbalance, with smaller loci showing more product than larger loci. Use less template or fewer cycles. The amount of template will need to be optimized if you are using reduced reaction volumes.
	Degraded DNA sample. DNA template was degraded, and larger loci showed diminished yield.
	Insufficient template DNA. Use the recommended amount of template DNA if available. Stochastic effects can occur when amplifying low amounts of template.
	Impure template DNA. Inhibitors that may be present in forensic samples can lead to allele dropout or imbalance.
	Imbalance may be seen more often when using the maximum template volume or a reduced amplification reaction volume.
	The reaction volume was too low. This system is optimized for a final reaction volume of 25µl. Decreasing the reaction volume may result in suboptimal performance.

9.3 Direct Amplification of DNA from Storage Card Punches

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

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

Symptoms	Causes and Comments
Faint or absent allele peaks in the positive control reaction	<p>If the positive control reaction failed to amplify, check to make sure that the correct amount of 2800M Control DNA was added to the reaction. Due to the reduced cycle number, it is necessary to increase the mass of 2800M Control DNA to obtain a profile. Use the recommended amount of 2800M Control DNA per amplification reaction. This mass of DNA should be reduced if cycle number is increased and increased if the cycle number is decreased. Increase or decrease by twofold the mass of 2800M Control DNA for every one-cycle decrease or increase, respectively. We do not recommend including blank punches in the 2800M Control DNA reaction.</p>
Extra peaks visible in one or all color channels	<p>Punch contaminated with DNA from another sample. Perform punches on a blank card between samples to minimize potential for carryover between samples.</p>
	<p>Amplification of processed punches with high amounts of DNA can result in artifact peaks due to overamplification, resulting in saturating signal on the CE instrument. Be sure to use the recommended number of punches. Use of a larger punch size or a smaller reaction volume may result in overamplification and signal saturation. If the signal is saturated, repeat the amplification with a smaller punch, a larger reaction volume or reduced cycle number.</p>
	<p>Amplification of excess template for a given cycle number can result in overloading of the capillary upon electrokinetic injection. The presence of excess DNA in the capillary makes it difficult to maintain the DNA in a denatured single-stranded state. Some single-stranded DNA renatures and becomes double-stranded. Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis and appears as 'shadow' peaks migrating in front of the main peaks. If this occurs at a heterozygous locus, it is sometimes possible to see two 'shadow' peaks that differ in size from one another by approximately the same distance as the single-stranded alleles.</p>
	<p>Artifacts of STR amplification. Direct amplification of high amounts of template can result in a higher number of artifact peaks. Use the recommended punch size and number of punches. Optimize the cycle number. See Results section for additional information on stutter and artifacts.</p>
	<p>Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3' A residue.</p> <ul style="list-style-type: none"> • Be sure to perform the recommended extension step at 60°C after thermal cycling. • Decrease cycle number. • Increase the final extension time.

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

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

9.4 Direct Amplification of DNA from Swabs

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

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

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

Symptoms	Causes and Comments
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 reagent 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>

9.5 GeneMapper® ID-X Software

Symptoms	Causes and Comments
Stutter peaks not filtered	<p>Stutter text file was not imported into the Panel Manager when the panels and bins text files were imported.</p> <p>Be sure that the “Use marker-specific stutter ratio and distance if available” box is checked. If the “Use marker-specific stutter ratio and distance if available” box is not checked, stutter distance must be defined in the Analysis Method ‘Allele’ tab.</p>
Samples in the project not analyzed	<p>The ‘Analysis Requirement Summary’ window was not active, and there was an analysis requirement that was not met. Turn on ‘Analysis Requirement Summary’ in the ‘Project Options’ menu under File, and correct the necessary analysis requirements to continue analysis.</p>
Edits in label edit viewer cannot be viewed	<p>To view edits made to a project, the project must first be saved. Close the plot view window, return to the main GeneMapper® ID-X page and save the project. Display the plot window again, and then view the label edit table.</p>
Marker header bar for some loci are gray	<p>When an edit is made to a locus, the quality flags and marker header bar automatically changes to gray. To change the GQ and marker header bar for a locus to green, override the GQ in the plot window.</p>
Alleles not called	<p>To analyze samples with GeneMapper® ID-X software, at least one allelic ladder must be defined per folder of sample files being analyzed in the project.</p> <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. <p>A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.</p>

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

Symptoms	Causes and Comments
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.

9.6 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 6 or 7.
	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.
	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.

Symptoms	Causes and Comments
<p>Off-ladder or off-bin alleles</p>	<p>An allelic ladder from a different run than the samples was used. Re-analyze samples with an allelic ladder from the same run.</p>
	<p>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.</p>
	<p>The allelic ladder was not designated as an allelic ladder in Section 6 or 7.</p>
	<p>The internal lane standard was not properly identified in the sample. Manually redefine the sizes of the size standard fragments in the sample.</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>

Symptoms	Causes and Comments
<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>
	<p>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:</p> <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.
	<p>An incorrect size standard was used.</p>
	<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.

Symptoms	Causes and Comments
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.

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11 Appendix

11.1 Advantages of Using the Loci in the PowerPlex® Y23 System

The loci included in the PowerPlex® Y23 System (Table 6 and Table 7) were selected because they represent well characterized loci generally accepted for forensic use. This multiplex includes all loci in the 'European minimal haplotype' (DYS19, DYS385a/b, DYS389I/II, DYS390, DYS391, DYS392 and DYS393; see www.yhrd.org), the Scientific Working Group—DNA Analysis Methods (SWGDM)-recommended Y-STR panel (European minimal haplotype plus DYS438 and DYS439) and the loci included in the US Y-STR database (SWGDM-recommended loci plus DYS437, DYS456, DYS458, DYS635, DYS448 and Y-GATA-H4). Six additional Y-STR loci are included in this multiplex; DYS481, DYS533, DYS549, DYS570, DYS576 and DYS643 were selected for their high genetic diversity (25–29). Table 8 lists the PowerPlex® Y23 System alleles amplified from 2800M Control DNA.

Terminal nucleotide addition (30,31) 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 of 60°C (32) to the amplification protocols to provide conditions for essentially complete terminal nucleotide addition when recommended amounts of template DNA are used.

Table 6. The PowerPlex® Y23 System Locus-Specific Information.

STR Locus	Label	Chromosomal Location ¹	Repeat Sequence ² 5'→3'
DYS576	Fluorescein	Y	AAAG
DYS389I/II	Fluorescein	Y	(TCTG) (TCTA)
DYS448	Fluorescein	Y	AGAGAT
DYS19	Fluorescein	Y	TAGA
DYS391	JOE	Y	TCTA
DYS481	JOE	Y	CTT
DYS549	JOE	Y	GATA
DYS533	JOE	Y	ATCT
DYS438	JOE	Y	TTTTTC
DYS437	JOE	Y	TCTA
DYS570	TMR-ET	Y	TTTTC
DYS635	TMR-ET	Y	TSTA compound
DYS390	TMR-ET	Y	(TCTA) (TCTG)
DYS439	TMR-ET	Y	AGAT
DYS392	TMR-ET	Y	TAT
DYS643	TMR-ET	Y	CTTTT
DYS393	CXR-ET	Y	AGAT
DYS458	CXR-ET	Y	GAAA
DYS385a/b	CXR-ET	Y	GAAA
DYS456	CXR-ET	Y	AGAT
Y-GATA-H4	CXR-ET	Y	TAGA

¹Information about most of these loci can be found at: <https://strbase.nist.gov/chrom.htm>

²The August 1997 report (33,34) 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.'

Table 7. The PowerPlex® Y23 System Allelic Ladder Information.

STR Locus	Label	Size Range of Allelic Ladder Components^{1,2} (bases)	Repeat Numbers of Allelic Ladder Components³
DYS576	Fluorescein	97–145	11–23
DYS389I	Fluorescein	147–179	9–17
DYS448 ⁴	Fluorescein	196–256	14–24
DYS389II	Fluorescein	259–303	24–35
DYS19	Fluorescein	312–352	9–19
DYS391	JOE	86–130	5–16
DYS481	JOE	139–184	17–32
DYS549	JOE	198–238	7–17
DYS533	JOE	245–285	7–17
DYS438	JOE	293–343	6–16
DYS437	JOE	344–380	11–18
DYS570	TMR-ET	90–150	10–25
DYS635	TMR-ET	150–202	15–28
DYS390	TMR-ET	207–255	17–29
DYS439	TMR-ET	263–307	6–17
DYS392	TMR-ET	314–362	4–20
DYS643	TMR-ET	368–423	6–17
DYS393	CXR-ET	101–145	7–18
DYS458	CXR-ET	159–215	10–24
DYS385a/b	CXR-ET	223–307	7–28
DYS456	CXR-ET	316–364	11–23
Y-GATA-H4	CXR-ET	374–414	8–18

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

²When using an internal lane standard, such as the WEN Internal Lane Standard 500 Y23, the calculated sizes of allelic ladder components may differ from those listed. This occurs because different sequences in allelic ladder and ILS components may cause differences in migration. The dye label also affects migration of alleles.

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: strbase.nist.gov/var_tab.htm and the Y Chromosome Haplotype Reference Database at: www.yhrd.org

⁴A deletion has been reported at the DYS448 locus (35). Samples with this deletion will show two peaks (i.e., duplication) in DYS576 and a null allele in DYS448.

Table 8. The PowerPlex® Y23 System Allele Determinations for the 2800M Control DNA.

STR Locus	2800M
DYS576	18
DYS389I	14
DYS448	19
DYS389II	31
DYS19	14
DYS391	10
DYS481	22
DYS549	13
DYS533	12
DYS438	9
DYS437	14
DYS570	17
DYS635	21
DYS390	24
DYS439	12
DYS392	13
DYS643	10
DYS393	13
DYS458	17
DYS385a/b	13, 16
DYS456	17
Y-GATA-H4	11

11.2 The WEN Internal Lane Standard 500 Y23

The WEN Internal Lane Standard 500 Y23 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 25). Each fragment is labeled with WEN dye and can be detected separately (as a fifth color) in the presence of PowerPlex® Y23-amplified material. The WEN ILS 500 Y23 is designed for use in each CE injection to increase precision in analyses when using the PowerPlex® Y23 System. Protocols to prepare and use this internal lane standard are provided in Section 5.

Be sure to use the WEN ILS 500 Y23 as the size standard for the PowerPlex® Y23 System. Do not use the WEN ILS 500 (Cat.# DG5001). The WEN_ILS_500.xml file can be used to assign fragment sizes for the WEN ILS 500 Y23.

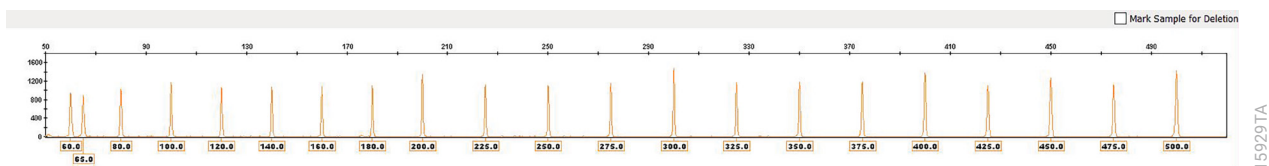


Figure 25. WEN Internal Lane Standard 500 Y23. An electropherogram showing the WEN Internal Lane Standard 500 Y23 fragments.

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

Testing at Promega has shown successful direct amplification of DNA from storage card punches in a 12.5µl reaction volume. This section contains a protocol for direct amplification of DNA from storage card punches in a 12.5µl reaction volume using the PowerPlex® Y23 System and GeneAmp® PCR System 9700 or ProFlex® PCR System. When using the protocol detailed below, add only one 1.2mm storage card punch to each 12.5µl amplification reaction.

Materials to Be Supplied by the User

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

Notes:

- a. It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is **not** recommended with the PowerPlex® Y23 System.
- b. You will need to optimize and validate the number of storage card punches per reaction in your laboratory. See the PCR Optimization recommendations at the end of this section.

Lytic card sample types include:

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

Nonlytic card sample types include:

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

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

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

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

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

Amplification Setup

1. Thaw the PowerPlex® Y23 5X Master Mix, PowerPlex® Y23 10X Primer Pair Mix and Water, Amplification Grade, completely.

Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 10X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

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

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

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

4. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean 0.2ml reaction tubes required and label them appropriately.
5. Add the final volume of each reagent listed in Table 9 to a clean tube.

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

PCR Amplification Mix Component ¹	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	6.25µl	×		=	
PowerPlex® Y23 5X Master Mix	2.50µl	×		=	
PowerPlex® Y23 10X Primer Pair Mix	1.25µl	×		=	
5X AmpSolution™ Reagent	2.00µl	×		=	
total reaction volume	12.50µl				

¹Add Water, Amplification Grade, to the tube first, and then add PowerPlex® Y23 5X Master Mix, PowerPlex® Y23 10X Primer Pair Mix and 5X AmpSolution™ Reagent. For lytic card punches, the template DNA will be added at Step 7.

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



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

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

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

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

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

Notes:

- a. Do not include blank storage card punches in the positive control reactions.
 - b. Optimization of the amount of 2800M Control DNA may be required, depending on thermal cycling conditions and laboratory preferences. Typically, 5ng of 2800M Control DNA is sufficient to provide a robust profile using the cycle numbers recommended here. A one-cycle reduction in cycle number will require a twofold increase in mass of DNA template to generate similar signal intensity. Similarly, a one-cycle increase in cycle number will require a twofold reduction in the amount of 2800M Control DNA to avoid signal saturation.
9. Reserve a well containing PCR amplification mix as a negative amplification control.
Note: An additional negative control with a blank punch may be performed to detect contamination from the storage card or punch device.
 10. Seal or cap the plate, or close the tubes. Briefly centrifuge the 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, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 25 cycles works well for a variety of storage card sample types. Cycle number will need to be optimized in each laboratory for each sample type.

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is **not** recommended with the PowerPlex® Y23 System.

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

Notes:

- a. When using the ProFlex® PCR System, use the 9700 Simulation Mode as the ramp speed.
- b. When using the GeneAmp® PCR System, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol

96°C for 2 minutes, then:

94°C for 10 seconds

61°C for 1 minute

72°C for 30 seconds

for 25 cycles, then:

60°C for 20 minutes

4°C soak

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

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

PCR Optimization

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

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Depending on your preferred protocol, place one 1.2mm storage card punch 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, 25 and 26 cycles).
5. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type and number of storage card punches.

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

Testing at Promega has shown successful direct amplification of DNA from swabs in a 12.5µl reaction volume. This section contains a protocol for amplifying DNA from swab extracts in a 12.5µl reaction volume using the PowerPlex® Y23 System and GeneAmp® PCR System 9700 or ProFlex® PCR System.

Materials to Be Supplied by the User

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

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

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is **not** recommended with the PowerPlex® Y23 System.

Amplification Setup

1. Thaw the PowerPlex® Y23 5X Master Mix, PowerPlex® Y23 10X Primer Pair Mix and Water, Amplification Grade, completely.

Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 10X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
3. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.
4. Add the final volume of each reagent listed in Table 10 to a clean tube.

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

PCR Amplification Mix Component ¹	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	6.75µl	×		=	
PowerPlex® Y23 5X Master Mix	2.50µl	×		=	
PowerPlex® Y23 10X Primer pair Mix	1.25µl	×		=	
swab extract	2.00µl				
total reaction volume	12.50µl				

¹Add Water, Amplification Grade, to the tube first, and then add PowerPlex® Y23 5X Master Mix and PowerPlex® Y23 10X Primer Pair Mix. The swab extract will be added at Step 6.

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



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

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

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

7. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 2.5ng/μl. Add 2μl (5ng) to a reaction well containing 10.5μl of PCR amplification mix.

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

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

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

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

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

Thermal Cycling

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

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is **not** recommended with the PowerPlex® Y23 System.

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

Notes:

- a. When using the ProFlex® PCR System, use the 9700 Simulation Mode as the ramp speed.
- b. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select **Max** for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol

96°C for 2 minutes, then:

94°C for 10 seconds

61°C for 1 minute

72°C for 30 seconds

for 25 cycles, then:

60°C for 20 minutes

4°C soak

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

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

PCR Optimization

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

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Prepare three identical reaction plates with aliquots of the same swab extracts.
3. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (24, 25 and 26 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.

11.5 Composition of Buffers and Solutions

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

1.21g Tris base

0.037g EDTA (Na₂EDTA • 2H₂O)

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

TE⁻⁴ buffer with 20µg/ml glycogen

1.21g Tris base

0.037g EDTA (Na₂EDTA • 2H₂O)

20µg/ml glycogen

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

11.6 Related Products

Accessory Components

PRODUCT	SIZE	CAT.#
PowerPlex® 5C Matrix Standard	5 preps	DG4850
Stabilizer Reagent	500 preps	DM6571
WEN Internal Lane Standard 500 Y23	200µl	DG5201
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
5X AmpSolution™ Reagent	500µl	DM1231
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 pairs	CE2001
Spectrum Cathode Septa Mat	10 each	CE2002
Spectrum Buffer and Cathode Septa Mat Bundle	1 each	CE2012
Septa Mat, 96-Well	2 each	CE2696
Spectrum Plate Base & Retainer, 96-Well	4 each	CE5004
Spectrum Wash Solution	1 each	CE2099

Not for Medical Diagnostic Use.

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

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

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