TECHNICAL MANUAL

PowerPlex® Y23 System for Use on the Applied Biosystems® Genetic Analyzers

Instructions for Use of Products
DC2305 and DC2320

Revised 10/19
TMD035

Promega

PowerPlex® Y23 System
for Use on the Applied Biosystems® Genetic Analyzers

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 2
2. Product Components and Storage Conditions 4
3. Before You Begin 5
   - 3.A. Precautions 5
   - 3.B. Spectral Calibration 6
4. Protocols for DNA Amplification Using the PowerPlex® Y23 System 6
   - 4.A. **Amplification of Extracted DNA in a 25µl Reaction Volume** 7
   - 4.B. Direct Amplification of DNA from Storage Card Punches in a 25µl Reaction Volume 10
   - 4.C. **Direct Amplification of DNA from Swabs** in a 25µl Reaction Volume 13
5. Instrument Setup and Sample Preparation 16
   - 5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer with 3500 Data Collection Software, Version 1.0 16
   - 5.B. Detection of Amplified Fragments Using POP-4® Polymer and the ABI PRISM® 3100 or 3100-***Avant*** Genetic Analyzer with Data Collection Software, Version 2.0, or the Applied Biosystems® 3130 or 3130***xl*** Genetic Analyzer with Data Collection Software, Version 3.0 or 4.0 27
6. Data Analysis 30
   - 6.A. Importing PowerPlex® Y23 Panels, Bins and Stutter Text Files into GeneMapper® ***ID***-X Software, Version 1.2 30
   - 6.B. Importing the WEN ILS 500 IDX Size Standard into GeneMapper® ***ID***-X Software, Version 1.2 31
   - 6.C. Creating a Size Standard with GeneMapper® ***ID***-X Software, Version 1.2 31
   - 6.D. Creating a Casework Analysis Method with GeneMapper® ***ID***-X Software, Version 1.2 33
   - 6.E. Creating a Databasing or Paternity Analysis Method Using a Global Filter with GeneMapper® ***ID***-X Software, Version 1.2 37
   - 6.F. Importing PowerPlex® Y23 Panels and Bins Text Files with GeneMapper® ***ID*** Software, Version 3.2 40
   - 6.G. Importing the WEN ILS 500 Size Standard into GeneMapper® ***ID*** Software, Version 3.2 41
   - 6.H. Creating a Size Standard with GeneMapper® ***ID*** Software, Version 3.2 41
   - 6.I. Creating a Casework Analysis Method with GeneMapper® ***ID*** Software, Version 3.2 42
   - 6.J. Creating a Databasing or Paternity Analysis Method Using a Global Filter with GeneMapper® ***ID*** Software, Version 3.2 45
   - 6.K. Controls 47
   - 6.L. Results 48

7. Troubleshooting 51
7.A. Amplification and Fragment Detection 51
7.B. Amplification of Extracted DNA 55
7.C. Direct Amplification of DNA From Storage Card Punches 56
7.D. Direct Amplification of DNA From Swabs 59
7.E. GeneMapper® *ID*-X Software 62
7.F. GeneMapper® *ID* Software 64

8. References 67

9. Appendix 69
9.A. Advantages of Using the Loci in the PowerPlex® Y23 System 69
9.B. Detection of Amplified Fragments Using the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with POP-7® Polymer and Data Collection Software, Version 3.0 or 4.0 73
9.C DNA Extraction and Quantification Methods and Automation Support 76
9.D. The WEN Internal Lane Standard 500 Y23 76
9.E. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume 77
9.F. Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume 81
9.G. Composition of Buffers and Solutions 84
9.H. Related Products 84
9.I. Summary of Changes 84

1. Description

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

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–c] 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 5X Master Mix. This manual contains a protocol for use of the PowerPlex® Y23 System with the GeneAmp® PCR System 9700 and Veriti® 96-Well Thermal Cycler in addition to protocols to separate amplified products and detect separated material on the ABI PRISM® 3100 and 3100-*Avant* Genetic Analyzers and Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers (Figure 1). Protocols to operate the fluorescence-detection instruments should be obtained from the instrument manufacturer. Amplification and detection instrumentation may vary. You may need to optimize protocols including amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. In-house validation should be performed.

Information about other Promega fluorescent STR systems is available upon request from Promega or online at: **www.promega.com/products/genetic-identity/**

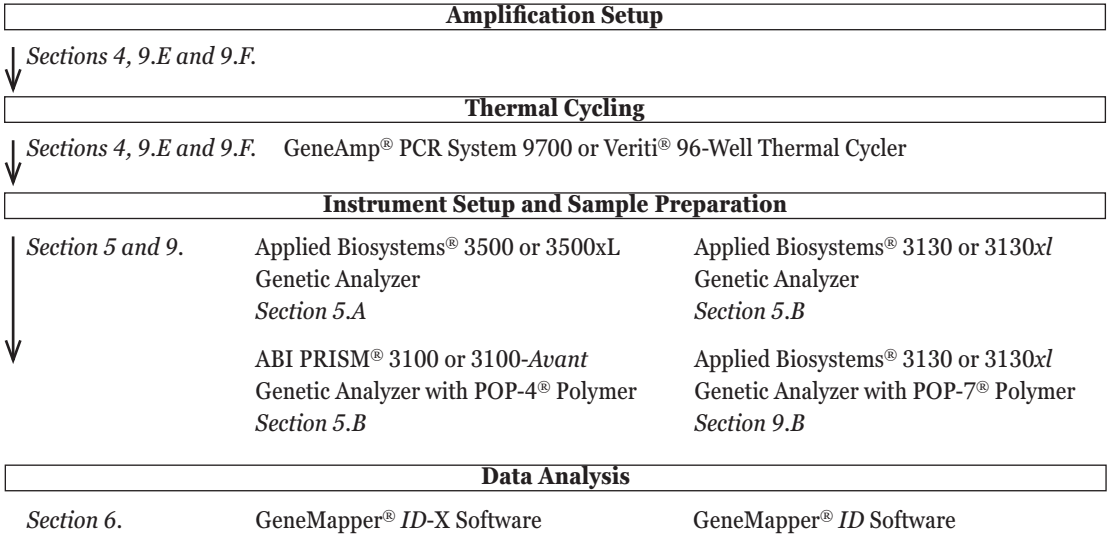

Figure 1. An overview of the PowerPlex® Y23 System protocol.

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

Storage Conditions: Upon receipt, store all components except the 2800M Control DNA at –30°C to –10°C in a nonfrost-free freezer. Store the 2800M Control DNA at 2–10°C. Make sure that the 2800M Control DNA is stored at 2–10°C for at least 24 hours before use. After the first use, store the WEN Internal Lane Standard 500 Y23 (WEN ILS 500 Y23) at 2–10°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 may be stored for up to 1 year at 2–10°C without loss of activity.

Available Separately

PRODUCT	SIZE	CAT.#
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
5X AmpSolution™ Reagent	100 preps	DM1231
Stabilizer Reagent	500 preps	DM6571

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

If you anticipate storing samples in loading cocktail in the injection plate for up to 48 hours prior to injection, we strongly recommend including Stabilizer Reagent in the loading cocktail. Omission of 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 the amplified samples.

The proper panels, bins and stutter text files and size standard .xml file for use with GeneMapper® *ID* and *ID*-X software can be downloaded at: **www.promega.com/resources/software-firmware/genemapper-id-software-panels-and-bin-sets/**

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

3. Before You Begin

3.A. Precautions

The application of PCR-based typing for forensic or paternity casework requires validation studies and quality control measures that are not contained in this manual (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, quality of plasticware, small changes in buffers, ionic strength, primer concentrations, reaction volume, choice of thermal cycler and thermal cycling conditions can affect PCR success. We suggest strict adherence to recommended procedures for amplification and fluorescence detection. Additional research and validation are required if any modifications are made to the recommended protocols.

PCR-based STR analysis is subject to contamination by very small amounts of human DNA. Extreme care should be taken to avoid cross-contamination when preparing template DNA, handling primer pairs, assembling amplification reactions and analyzing amplification products. Reagents and materials used prior to amplification (PowerPlex® Y23 5X Master Mix, PowerPlex® Y23 10X Primer Pair Mix, 2800M Control DNA and Water, Amplification Grade) are provided in a separate box and should be stored separately from those used following amplification (PowerPlex® Y23 Allelic Ladder Mix and WEN Internal Lane Standard 500 Y23). 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.

3.A. Precautions (continued)

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

3.B. Spectral Calibration

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

For protocols and additional information about spectral calibration on these instruments, see the *PowerPlex® 5C Matrix Standard Technical Manual* #TMD049. This manual is available online 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.A), FTA® and nonFTA storage card punches (Section 4.B) and swabs (Section 4.C) are included in the following amplification sections. Protocols for amplification in a 12.5µl reaction volume using FTA® and nonFTA storage card punches and swabs are included in Sections 9.E and 9.F, respectively.

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

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is NOT recommended with the PowerPlex® 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 7.

4.A. 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 or Veriti® 96-Well Thermal Cycler (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips

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 Amplification-Grade Water completely.

   Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 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 appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.

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

4.A. Amplification of Extracted DNA in a 25µl Reaction Volume (continued)

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	**25µl**				

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

[2]Store 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.

[3]Apparent DNA concentrations can differ, depending on the DNA quantification method used (22). The amount of DNA template recommended here is based on DNA concentrations determined by measuring absorbance at 260nm. We strongly recommend that you perform experiments to determine the optimal DNA amount based on your DNA quantification method.

[4]The PowerPlex® Y23 System was 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.

5. Vortex the PCR amplification mix for 5–10 seconds, and then pipet 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 DNA as soon as possible to each well and follow immediately by thermal cycling.

6. Add template DNA for each sample to the respective well or tube 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.

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

8. For the negative amplification control, pipet Amplification-Grade Water or TE^{-4} buffer instead of template DNA into a reaction well containing PCR amplification mix.
9. Seal or cap the plate, or close the tubes. **Optional:** Briefly centrifuge the plate or tubes 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 30 cycles works well for 0.5ng of purified DNA template.

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

   Notes:

   1. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
   2. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a silver or gold-plated 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 with fragment analysis or store amplified samples at –20°C in a light-protected box.

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

Promega

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

Materials to Be Supplied by the User

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

This section contains a protocol for direct amplification of DNA from storage card punches in a 25µl reaction volume using the PowerPlex® Y23 System and GeneAmp® PCR System 9700 or Veriti® 96-Well Thermal Cycler. A protocol for direct amplification of DNA from storage card punches in a 12.5µl reaction volume is provided in Section 9.E.

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

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

FTA®-based sample types include:

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

NonFTA sample types include:

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

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

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

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

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

Amplification Setup

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

   Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 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 appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.

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

Table 2. PCR Amplification Mix for Direct Amplification of DNA from 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	**25µl**				

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

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

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

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

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

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

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

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:

   1. Optimization of the amount of 2800M Control DNA may be required, depending on thermal cycling conditions and laboratory preferences.
   2. 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 plate 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 each laboratory instrument. Testing at Promega shows that 26 cycles works well for a variety of sample types. Buccal samples may require more amplification cycles than blood samples. Cycle number will need to be optimized in each laboratory for each sample type that is amplified (see below).

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

   Notes:

   1. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
   2. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a silver or gold-plated 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 in a light-protected box.

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

PCR Optimization

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

1. Choose several samples that represent 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 in each well of a reaction plate. Be sure to pretreat nonFTA samples with the PunchSolution™ Kit (Cat.# DC9271).
3. Prepare three identical reaction plates with punches from the same samples.
4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number.

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

   Blood sample on one 1.2mm FTA® or pretreated nonFTA punch: 25, 26 and 27 cycles

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

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

   Buccal cells on one 1.2mm pretreated nonFTA 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.C. Direct Amplification of DNA from Swabs in a 25µl Reaction Volume

Materials to Be Supplied by the User

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

This section contains a protocol for amplifying DNA from swab extracts in a 25µl reaction volume using the PowerPlex® Y23 System and GeneAmp® PCR System 9700 or Veriti® 96-Well Thermal Cycler. A protocol for direct amplification of DNA from swab extracts in a 12.5µl reaction volume is provided in Section 9.F.

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

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

Amplification Setup

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

   Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 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 appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.

4. Add the final volume of each reagent listed in Table 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	**25µl**				

[1]Add 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 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.

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

7. For the positive amplification control, vortex the tube of 2800M 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.

8. For the negative amplification control, pipet Amplification-grade Water or TE^{-4} buffer instead of swab extract into a reaction well containing PCR amplification mix.

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

9. Seal or cap the plate, or close the tubes. **Optional:** Briefly centrifuge the plate or tubes to bring contents to the bottom of the wells and remove any air bubbles.

Thermal Cycling

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

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

   Notes:

   1. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
   2. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a silver or gold-plated 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 in a light-protected box.

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

PCR Optimization

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

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

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

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. Instrument Setup and Sample Preparation

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer with 3500 Data Collection Software, Version 1.0

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice, ice-water bath or a freezer plate block
- centrifuge compatible with a 96-well plate
- aerosol-resistant pipette tips
- 3500/3500xL capillary array, 36cm
- plate retainer and base set (standard)
- POP-4® polymer for the Applied Biosystems® 3500 or 3500xL Genetic Analyzer
- anode buffer container
- cathode buffer container
- 96-well plate and septa (e.g., Plate, Barcoded, Semi-Skirted, 96-Well [Cat.# V7845] and Septa Mat, 96-Well [Cat.# CE2696] or MicroAmp® optical 96-well plate [or equivalent] and septa [Applied Biosystems])
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)
- 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 you anticipate storing samples in loading cocktail in the injection plate for up to 48 hours prior to injection, we strongly recommend including Stabilizer Reagent in the loading cocktail. Omission of 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 the amplified samples.

Sample Preparation

1. Prepare a loading cocktail by combining and mixing WEN ILS 500 Y23 and formamide as follows:

   [(0.5µl WEN ILS 500 Y23) × (# samples)] + [(9.5µl formamide) × (# samples)]

2. If samples in injection plate are not to be injected immediately, or if there is the possibility that the samples may be reinjected at any time within 48 hours of setting up the injection plate, prepare a loading cocktail by combining and mixing WEN ILS 500 Y23, Stabilizer Reagent and formamide as follows:

   [(0.5µl WEN ILS 500 Y23) × (# samples)] + [(0.5µl Stabilizer Reagent) × (# samples)] + [(9.5µl formamide) × (# 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 you anticipate storing samples in loading cocktail in the injection plate for up to 48 hours prior to injection, we strongly recommend including Stabilizer Reagent in the loading cocktail. Omission of 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 the amplified samples.

3. Vortex for 10–15 seconds to mix.
4. Pipet 10µl of formamide/internal lane standard mix (or 10.5µl for formamide/stabilizer reagent/internal lane standard mix if Stabilizer Reagent is used) into each well.
5. Add 1µl of amplified sample (or 1µl of PowerPlex® Y23 Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

   Notes:

   1. Instrument detection limits vary; therefore, injection time, injection voltage or the amount of product mixed with loading cocktail may need to be adjusted. To modify the injection time in the run module, select "Instrument Protocol" from the Library menu in the data collection software. If peak heights are higher than desired, use less DNA template in the amplification reactions or reduce the number of cycles in the amplification program to achieve the desired signal intensity.
   2. Use a volume of allelic ladder that results in peak heights that are all consistently above the peak amplitude threshold determined as part of your internal validation.

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.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer with 3500 Data Collection Software, Version 1.0 (continued)

Instrument Preparation

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

1. Open the 3500 Data Collection Software. The Dashboard screen will launch (Figure 2). To ensure that you are viewing the most up-to-date information, press the Refresh button. Ensure that the Consumables Information and Maintenance Notifications are acceptable.

   Set the oven temperature to 60°C, and then select "Start Pre-Heat". When the Oven Temperature and Detection Cell Temperature turn green, you may proceed with the first injection.

Figure 2. The Dashboard.

2. Prior to the first analysis using the PowerPlex® Y23 System, you must create an Instrument Protocol, Size Standard, QC Protocol, Assay, File Name Convention and Results Group.

   2.a. To create a new Instrument Protocol, navigate to the Library, select "Instrument Protocols", and then select "Create". Alternatively, a previously created Instrument Protocol may be used.

   Figure 3 shows the settings used at Promega for the Applied Biosystems® 3500xL Genetic Analyzer for the application type, dye set, capillary length, polymer, run module and appropriate protocol information. The only setting that was changed from the default settings is dye set.

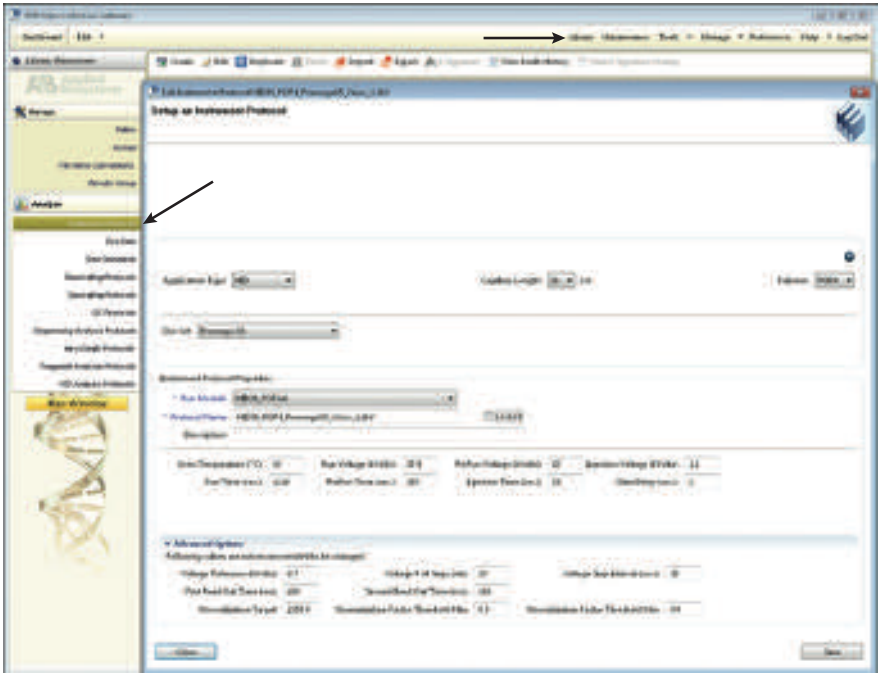

Figure 3. The Create New Instrument Protocol window.

The recommended settings are:

Application Type	HID
Capillary Length	36cm
Polymer	POP-4®
Dye Set	G5 (Promega G5 spectral)
Run Module	HID36_POP4(xl)
Injection Time[1]	15 seconds for the Applied Biosystems® 3500 Genetic Analyzer 24 seconds for the Applied Biosystems® 3500xL Genetic Analyzer
Injection Voltage	1.2kV
Run Time	1,210–1,500 seconds

[1]Injection time may be modified to increase or decrease peak heights.

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

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

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer with 3500 Data Collection Software, Version 1.0 (continued)

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

Assign a descriptive protocol name.

Note: For more detailed information refer to the *Applied Biosystems 3500/3500xL Genetic Analyzers User Guide*.

2.b. To create a new Size Standard for the QC protocol, navigate to the Library. Select "Size Standards", and then select "Create". Alternatively, a previously created Size Standard may be used.

Assign the size standard the name "ILS500" or another appropriate name. Choose "Orange" as the Dye Color. The fragments in the size standard are 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases. See Figure 4.

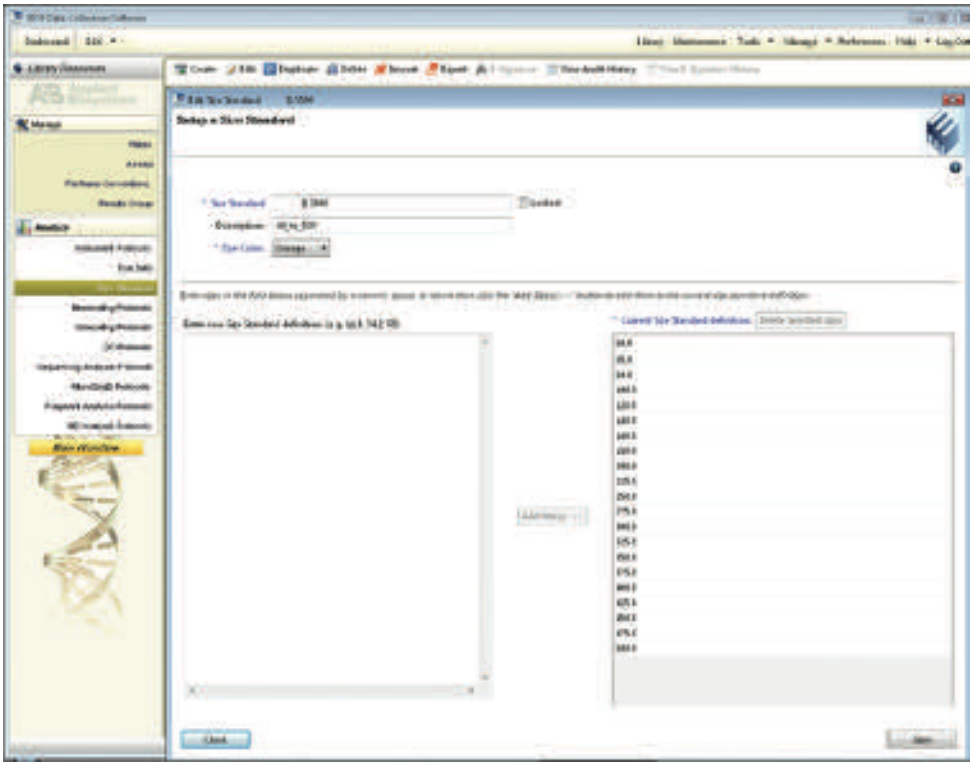

Figure 4. The Create New Size Standard window.

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

Assign a descriptive protocol name such as WEN ILS 500 Y23. Select the size standard created in Step 2.b. The settings for the QC protocol should be based on the internally validated conditions for the PowerPlex® Y23 System on the Applied Biosystems® 3500 or 3500xL Genetic Analyzer. Figure 5 shows one option for these settings.

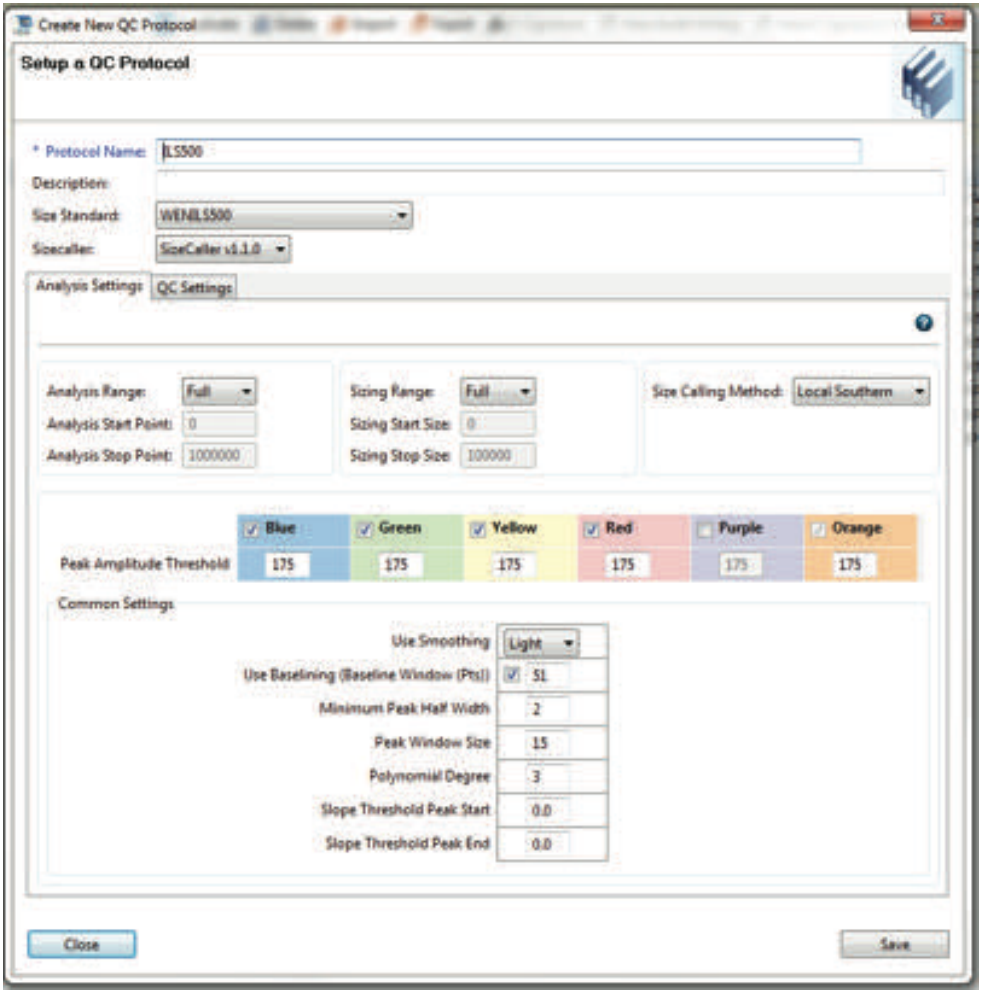

Figure 5. The Create New QC Protocol window.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer with 3500 Data Collection Software, Version 1.0 (continued)

2.d. To create a new Assay, navigate to the Library. Select "Assays", and then select "Create". Alternatively, a previously created Assay may be used.

In the Create New Assay window (Figure 6), select the Instrument Protocol created in Step 2.a and the QC Protocol created in Step 2.c. Assign a descriptive assay name. Select the application type "HID". An Assay is required for all named samples on a plate.

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

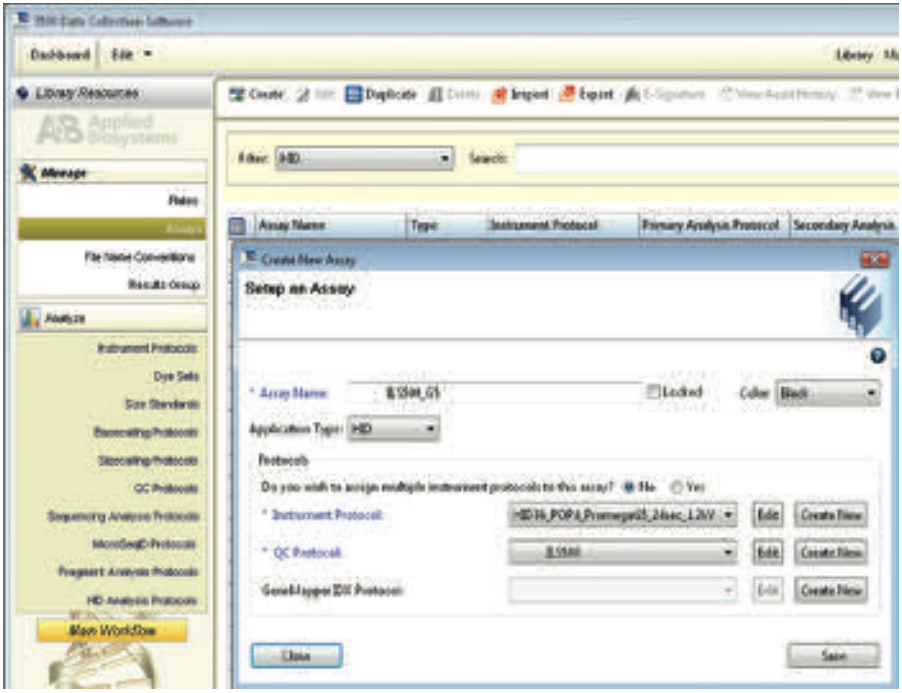

Figure 6. The Create New Assay window.

2.e. To create a new File Name Convention (Figure 7), navigate to the Library. Select “File Name Conventions”, and then select “Create”. Alternatively, a previously created File Name Convention may be used.

Select the File Name Attributes according to your laboratory practices, and save with a descriptive name.

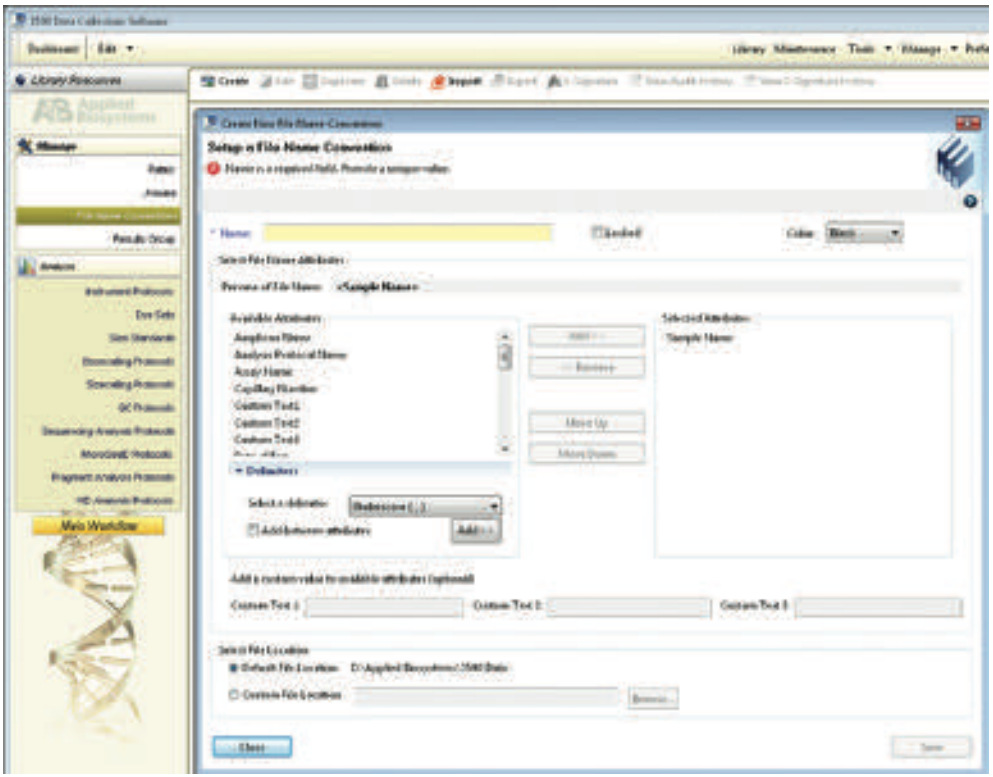

Figure 7. The Create New File Name Convention window.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer with 3500 Data Collection Software, Version 1.0 (continued)

2.f. To create a new Results Group (Figure 8), navigate to the Library. Select "Results Group", and then select "Create". Alternatively, a previously created Results Group may be used.

Select the Results Group Attributes according to your laboratory practices. Save with a descriptive name.

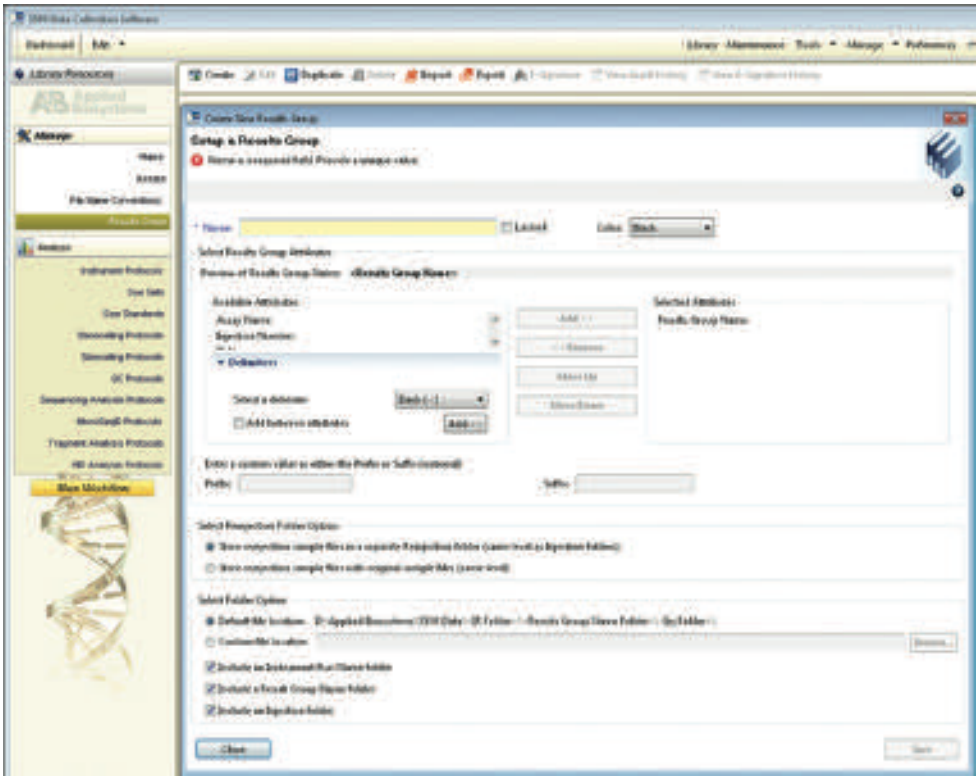

Figure 8. The Create New Results Group window.

3. To create a New Plate, navigate to the Library, and from the Manage menu, select “Plates”, and then “Create”.
4. Assign a descriptive plate name. Select the plate type “HID” from the drop-down menu (Figure 9).

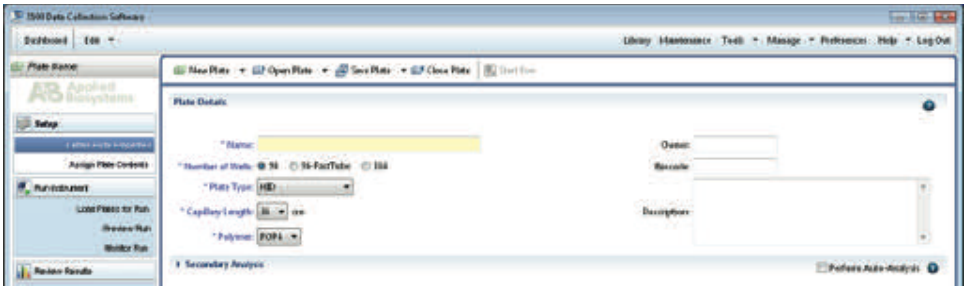

Figure 9. Defining plate properties.

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

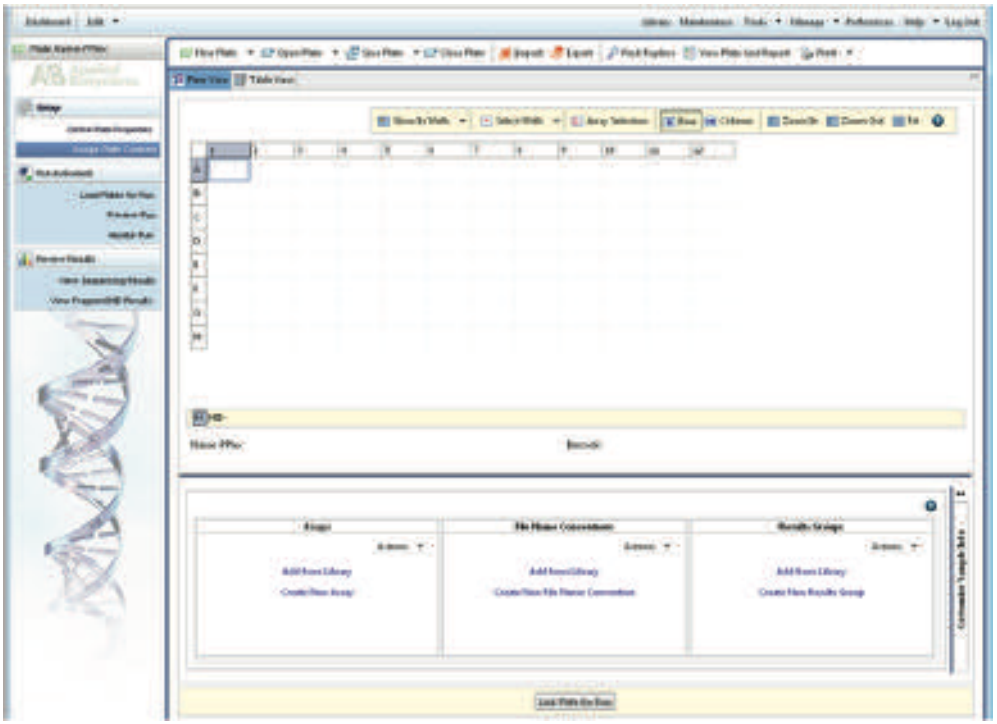

Figure 10. Assigning plate contents.

5.A. Detection of Amplified Fragments Using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer with 3500 Data Collection Software, Version 1.0 (continued)

6. Assign sample names to wells.
7. In the lower left portion of the screen, under "Assays", use the Add from Library option to select the Assay created in Step 2.d or one previously created. Click on the Add to Plate button, and close the window.
8. Under "File Name Conventions", use the Add from Library option to select the File Name Convention created in Step 2.e or one previously created. Click on the Add to Plate button, and close the window.
9. Under "Results Groups", use the Add from Library option to select the Results Group created in Step 2.f or one previously created. Click on the Add to Plate button, and close the window.
10. Highlight the sample wells, and then select the boxes in the Assays, File Name Conventions and Results Groups that pertain to those samples.
11. Select "Link Plate for Run".
12. The Load Plate window will appear. Select "Yes".
13. In the Run Information window (Figure 11), assign a Run Name. Select "Start Run" (not shown).

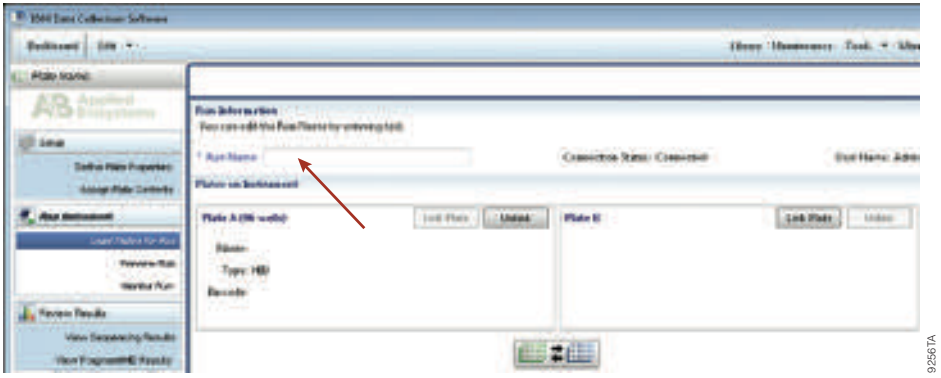

Figure 11. Assigning a run name.

5.B. Detection of Amplified Fragments Using POP-4® Polymer and the ABI PRISM® 3100 or 3100-*Avant* Genetic Analyzer with Data Collection Software, Version 2.0, or the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with Data Collection Software, Version 3.0 or 4.0

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice, ice-water bath or freezer plate block
- centrifuge compatible with 96-well plates
- aerosol-resistant pipette tips
- 3100 or 3130 capillary array, 36cm
- plate retainer and base set (standard)
- POP-4® polymer for the 3130/3130*xl* Genetic Analyzers
- 10X genetic analyzer buffer with EDTA
- 96-well plate and septa (e.g., Plate, Barcoded, Semi-Skirted, 96-Well [Cat.# V7845] and Septa Mat, 96-Well [Cat.# CE2696] or MicroAmp® optical 96-well plate [or equivalent] and septa [Applied Biosystems])
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)
- 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 you anticipate storing samples in loading cocktail in the injection plate for up to 48 hours prior to injection, we strongly recommend including Stabilizer Reagent in the loading cocktail. Omission of 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 the amplified samples.

5.B. Detection of Amplified Fragments Using POP-4® Polymer and the ABI PRISM® 3100 or 3100-*Avant* Genetic Analyzer with Data Collection Software, Version 2.0, or the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with Data Collection Software, Version 3.0 or 4.0 (continued)

Sample Preparation

1. Prepare a loading cocktail by combining and mixing WEN ILS 500 Y23 and formamide as follows:

   [(0.5µl WEN ILS 500 Y23) × (# samples)] + [(9.5µl formamide) × (# samples)]

2. If samples in injection plate are not to be injected immediately, or if there is the possibility that the samples may be reinjected at any time within 48 hours of setting up the injection plate, prepare a loading cocktail by combining and mixing WEN ILS 500 Y23, Stabilizer Reagent and formamide as follows:

   [(0.5µl WEN ILS 500 Y23) × (# samples)] + [(0.5µl Stabilizer Reagent) × (# samples)] + [(9.5µl formamide) × (# 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 you anticipate storing samples in loading cocktail in the injection plate for up to 48 hours prior to injection, we strongly recommend including Stabilizer Reagent in the loading cocktail. Omission of 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 the amplified samples.

3. Vortex for 10–15 seconds to mix.
4. Pipet 10µl of formamide/internal lane standard mix (or 10.5µl for formamide/stabilizer reagent/internal lane standard if Stabilizer Reagent is used) into each well.
5. Add 1µl of amplified sample (or 1µl of PowerPlex® Y23 Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

   Note: Instrument detection limits vary; therefore, injection time, injection voltage or the amount of sample mixed with loading cocktail may need to be adjusted. Use the Module Manager in the data collection software to modify the injection time or voltage in the run module (see Instrument Preparation below).

6. Centrifuge 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.

Instrument Preparation

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

Analyze samples as described in the user's manual for the ABI PRISM® 3100 or 3100-*Avant* Genetic Analyzer and the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with the following exceptions.

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

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

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

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

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

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

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

5. Place samples in the instrument, and close the instrument doors.

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

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

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

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

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

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

10. Click on the green Run Instrument arrow on the toolbar to start the sample run.

11. Monitor electrophoresis by observing the run, view, array or capillaries viewer window in the data collection software. Each injection will take approximately 40 minutes.

6. Data Analysis

The instructions in this section are for use with GeneMapper® *ID*-X software, version 1.2, or GeneMapper® *ID* software, version 3.2. Due to potential differences between software versions, some of the instructions may not apply to all software versions.

6.A. Importing PowerPlex® Y23 Panels, Bins and Stutter Text Files into GeneMapper® *ID*-X Software, Version 1.2

To facilitate analysis of data generated 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.

Notes:

1. The panels, bins and stutter text files mentioned here are compatible with earlier versions of the GeneMapper® *ID*-X software.
2. 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/resources/software-firmware/genemapper-id-software-panels-and-bin-sets/**
2. Select the PowerPlex® System that you are using, and select "GeneMapper ID-X". Enter your contact information, and select "Submit".
3. Save the PowerPlexY23_Panels_IDX_vX.x.txt, PowerPlexY23_Bins_IDX_vX.x.txt and PowerPlexY23_Stutter_IDX_vX.x.txt files, where "X.x" refers to the most recent version of the panels, bins and stutter text files, to a known location on your computer.
4. Save the WEN_ILS_500_IDX.xml file to a known location on your computer.

Importing Panels, Bins and Stutter Text Files

1. Open the GeneMapper® *ID*-X software.
2. Select "Tools", 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. Select the file, and then "Import".
6. In the navigation pane, highlight the PowerPlex Y23 panels folder that you just imported in Step 5.
7. Select "File", and then "Import Bin Set".
8. Navigate to the bins text file downloaded in the Getting Started section. Select the file, and then "Import".

9. In the navigation pane, highlight the PowerPlex Y23 panels folder that you just imported in Step 5.
10. Select "File", and then "Import Marker Stutter". A warning box will appear asking if you want to overwrite current values. Select "Yes".
11. Navigate to the stutter text file imported in the Getting Started section. Select the file, and then "Import".
12. At the bottom of the Panel Manager window, select "OK". This will save the panels, bins and stutter text files and close the window.

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

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

The WEN_ILS_500_IDX.xml file can be used to assign fragment sizes for the WEN ILS 500 Y23 and can be downloaded at: **www.promega.com/resources/software-firmware/genemapper-id-software-panels-and-bin-sets/**

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 on your computer.
5. Highlight the file, and then select "Import".
6. Select "Done" to save changes and close the GeneMapper® *ID*-X Manager.

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

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 12), select "GeneMapper ID-X Security Group" as the Security Group. This allows access for all users of the software. Other security groups may be used.
5. Enter a detailed name, such as "WEN_ILS_500_IDX".

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

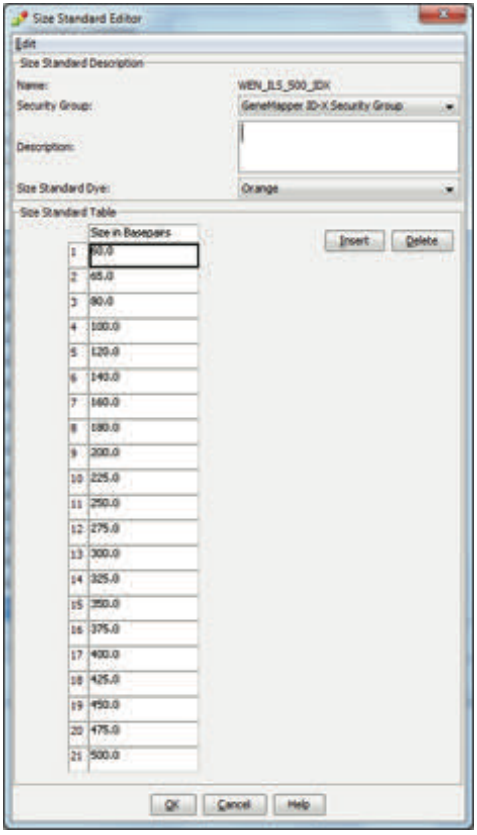

Figure 12. The GeneMapper® *ID*-X Software Size Standard Editor.

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 9.D, Figure 24.
8. Select “OK”.

6.D. Creating a Casework Analysis Method with GeneMapper® *ID*-X Software, Version 1.2

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

1. Select “Tools”, 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”.
6. Select the Allele tab (Figure 13).

6.D. Creating a Casework Analysis Method with GeneMapper® *ID*-X Software, Version 1.2 (continued)

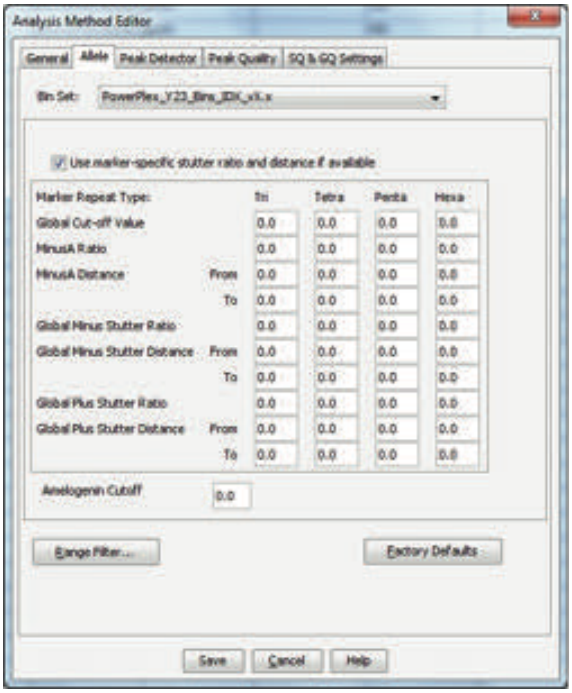

Figure 13. The GeneMapper® *ID*-X Software Allele tab.

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

8. Ensure that the "Use marker-specific stutter ratio and distance if available" box is checked. Doing this will assign locus-specific stutter filters and distances from the imported stutter file. We recommend the settings shown in Figure 13 for proper filtering of stutter peaks when using the PowerPlex® Y23 System.

   Notes:

   1. 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.
   2. If you do not check the "Use marker-specific stutter ratio and distance if available" box, you will need to optimize these settings. In-house validation should be performed.

9. Select the Peak Detector tab (Figure 14). You will need to optimize these settings. In-house validation should be performed.

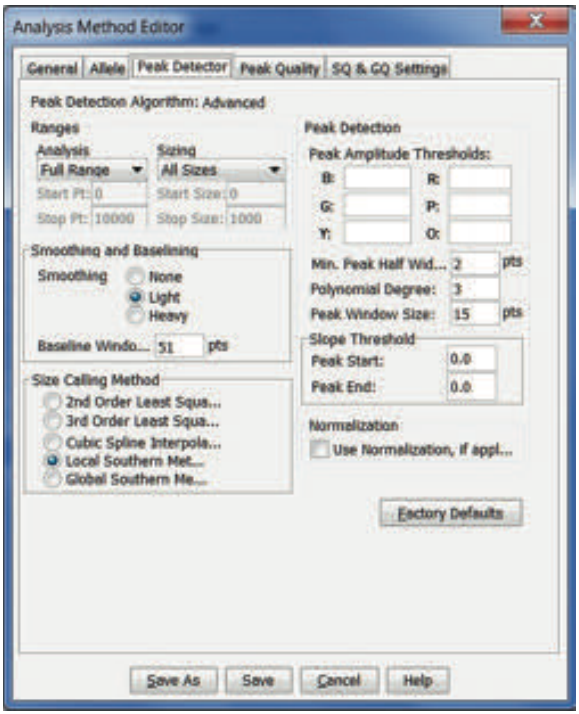

Figure 14. The GeneMapper® *ID*-X Software Peak Detector tab.

Notes:

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

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

    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.

11. Select the SQ & GQ Settings tab. You may change these settings.
12. Select "Save" to save the new analysis method.
13. Select "Done" to exit the GeneMapper® *ID*-X Manager.

6.D. Creating a Casework Analysis Method with GeneMapper® *ID*-X Software, Version 1.2 (continued)

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".
4. In the Sample Type column, use the drop-down menu to select "Allelic Ladder", "Sample", "Positive Control" or "Negative Control" as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as "Allelic Ladder" in the Sample Type column for proper genotyping.

   Note: The positive control DNA defined in the GeneMapper® *ID*-X panel file is the 2800M Control DNA. Redefine the genotype in the panel file if using a different positive control DNA.
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.A.
7. In the Size Standard column, select the size standard that was imported in Section 6.B or created in Section 6.C.
8. Select "Analyze" (green arrow button) to start data analysis.

   Note: By default, the software is set to display the Analysis Requirements Summary window and Allelic Ladder Analysis Summary window if an issue is detected. After analysis is complete, the default setting is to show the Analysis Summary tab. If these default settings are changed, manual troubleshooting may be necessary.
9. If all analysis requirements are met, the Save Project window will open (Figure 15).

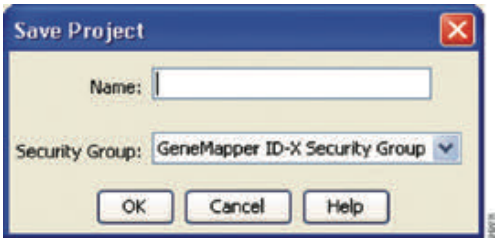

Figure 15. The Save Project window.

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.E. Creating a Databasing or Paternity Analysis Method Using a Global Filter with GeneMapper® *ID*-X Software, Version 1.2

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

1. Select "Tools", 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 16).
7. Select the bins text file that was imported in Section 6.A.

6.E. Creating a Databasing or Paternity Analysis Method Using a Global Filter with GeneMapper® *ID*-X Software, Version 1.2 (continued)

8. Ensure that the "Use marker-specific stutter ratio and distance if available" box is checked. Doing this will assign locus-specific stutter filters 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, Penta and Hexa repeats (Figure 16).

   Note: If you do not check the "Use marker-specific stutter ratio and distance if available" box, you will need to optimize these settings. In-house validation should be performed.

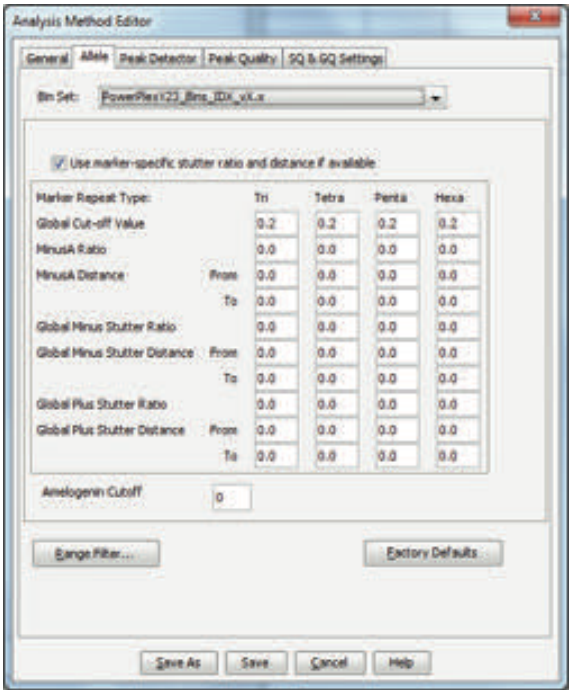

Figure 16. The GeneMapper® *ID*-X Software Allele tab with settings for using a 20% peak filter.

9. Select the Peak Detector tab (Figure 14). You will need to optimize these settings. In-house validation should be performed.

   Notes:

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

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

    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.

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

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 run files. Highlight desired files, and then select "Add to list" followed by "Add".

4. In the Sample Type column, use the drop-down menu to select "Allelic Ladder", "Sample", "Positive Control" or "Negative Control" as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as "Allelic Ladder" in the Sample Type column for proper genotyping.

   Note: The positive control DNA defined in the GeneMapper® *ID*-X panel file is the 2800M Control DNA. Redefine the genotype in the panel file if using a different positive control DNA.

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.A.

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

8. Select "Analyze" (green arrow button) to start data analysis.

   Note: By default, the software is set to display the Analysis Requirements Summary window and Allelic Ladder Analysis Summary window if an issue is detected. After analysis is complete, the default setting is to show the Analysis Summary tab. If these default settings are changed, manual troubleshooting may be necessary.

9. If all analysis requirements are met, the Save Project window will open (Figure 15).

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.F. Importing PowerPlex® Y23 Panels and Bins Text Files with GeneMapper® *ID* Software, Version 3.2

To facilitate analysis of data generated with the PowerPlex® Y23 System, we have created panels and bins text files to allow automatic assignment of genotypes using GeneMapper® *ID* software, version 3.2. We recommend that users of GeneMapper® *ID* software, version 3.2, complete the Applied Biosystems *GeneMapper®* ID *Software Human Identification Analysis Tutorial* to familiarize themselves with proper operation of the software. For GeneMapper® *ID* software, version 3.1, users we recommend upgrading to version 3.2.

For analysis using GeneMapper® *ID* software, version 3.2, you will need the proper panels and bins text files: PowerPlexY23_Panels_vX.x.txt and PowerPlexY23_Bins_vX.x.txt files, where "X.x" refers to the most recent version of the panels and bins text files.

Note: Run files generated using the Applied Biosystems® 3500 or 3500xL Genetic Analyzer cannot be analyzed using GeneMapper® *ID* Software, version 3.2. You must analyze these files with GeneMapper® *ID*-X software, version 1.2 or higher.

Getting Started

1. To obtain the proper panels and bins text files and WEN_ILS_500.xml file for the PowerPlex® Y23 System go to: **www.promega.com/resources/software-firmware/genemapper-id-software-panels-and-bin-sets/**
2. Select the PowerPlex® System that you are using, and select "GeneMapper ID". Enter your contact information, and select "Submit".
3. Save the PowerPlexY23_Panels_vX.x.txt and PowerPlexY23_Bins_vX.x.txt files, where "X.x" refers to the most recent version of the panels and bins text files, to a known location on your computer.
4. Save the WEN_ILS_500.xml file to a known location on your computer.

Importing Panels and Bins Text Files

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

1. Open the GeneMapper® *ID* software, version 3.2.
2. Select "Tools", 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. Select the file, and then "Import".
6. In the navigation pane, highlight the PowerPlex Y23 panels folder that you just imported in Step 5.
7. Select "File", and then "Import Bin Set".
8. Navigate to the bins text file downloaded in the Getting Started section. Select the file, and then "Import".
9. At the bottom of the Panel Manager window, select "OK". This will save the panels and bins text files and close the window.

6.G. Importing the WEN ILS 500 Size Standard into GeneMapper® *ID* Software, Version 3.2

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

The WEN_ILS_500.xml file can be used to assign fragment sizes for the WEN ILS 500 Y23 and can be downloaded at: **www.promega.com/resources/software-firmware/genemapper-id-software-panels-and-bin-sets/**

1. Select "Tools", and then "GeneMapper Manager".
2. Select the Size Standard tab.
3. Select "Import".
4. Browse to the location of the WEN_ILS_500.xml file on your computer.
5. Highlight the file, and then select "Import".
6. Select "Done" to save changes and close the GeneMapper® Manager.

6.H. Creating a Size Standard with GeneMapper® *ID* Software, Version 3.2

1. Select "Tools", and then "GeneMapper Manager".
2. Select the Size Standard tab.
3. Select "New".
4. Select "Basic or Advanced" (Figure 17). The type of analysis method selected must match the type of analysis method created earlier. Select "OK".

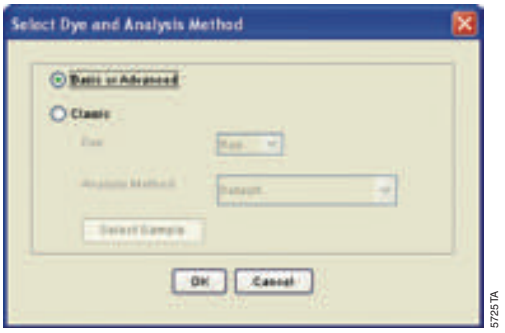

Figure 17. The Select Dye and Analysis Method window.

6.H. Creating a Size Standard with GeneMapper® *ID* Software, Version 3.2 (continued)

5. Enter a detailed name, such as "WEN ILS 500", in the Size Standard Editor (Figure 18).

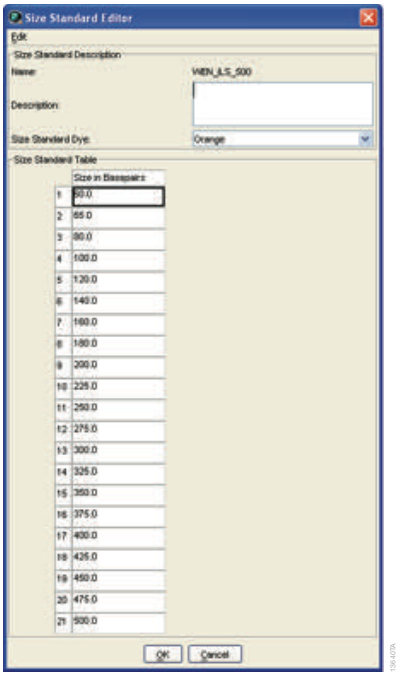

Figure 18. The GeneMapper® *ID* Software Size Standard Editor.

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 9.D, Figure 24.
8. Select "OK".

6.I. Creating a Casework Analysis Method with GeneMapper® *ID* Software, Version 3.2

These instructions are intended as a guide to start analyzing data in GeneMapper® *ID* software. They are not intended as a comprehensive guide for using GeneMapper® *ID* software. We recommend that users contact Applied Biosystems for training on the software. These instructions loosely follow the Applied Biosystems GeneMapper® *ID* software tutorial, pages 5–11.

1. Select "Tools", and then "GeneMapper Manager".
2. Select the Analysis Methods tab.
3. Select "New", and a new analysis method dialog box will open.
4. Select "HID", and select "OK".

   Note: If you do not see the HID option, you do not have the GeneMapper® *ID* software. Contact Applied Biosystems.

5. Enter a descriptive name for the analysis method, such as "PowerPlexY23".
6. Select the Allele tab (Figure 19).
7. Select the bins text file that was imported in Section 6.F.
8. Ensure that the "Use marker-specific stutter ratio if available" box is checked.
9. Enter the values shown in Figure 19 for proper filtering of stutter peaks when using the PowerPlex® Y23 System. For an explanation of the proper usage and effects of these settings, refer to the Applied Biosystems user bulletin titled "*Installation Procedures and New Features for GeneMapper* ID *Software 3.2*".

Notes:

1. The PowerPlex® Y23 System includes two trinucleotide repeat loci (DYS481 and DYS392). Both of these loci exhibit plus stutter. The plus-stutter filter of 0.06 will filter the majority of the plus stutter for DYS481 but not for DYS392. A filter value of 0.1 is needed to filter most of the plus stutter for DYS392.
2. Some of these settings have been optimized and are different from the recommended settings in the user bulletin. You will need to optimize these settings. In-house validation is required.

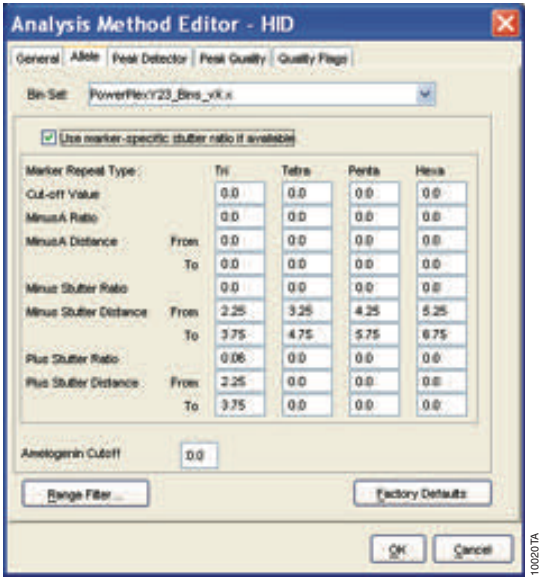

Figure 19. The GeneMapper® *ID* Software Allele tab.

6.I. Creating a Casework Analysis Method with GeneMapper® *ID* Software, Version 3.2 (continued)

10. Select the Peak Detector tab (Figure 20). You will need to optimize these settings. In-house validation should be performed.

Notes:

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

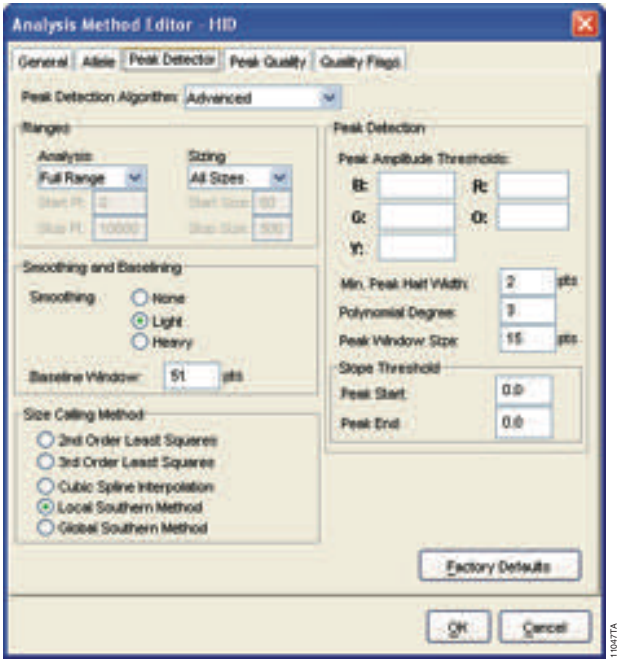

Figure 20. The GeneMapper® *ID* Software Peak Detector tab

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

    Note: See the GeneMapper® *ID* user's manual for more information. The settings in Steps 11 and 12 should be based on the results of your internal validation.

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

13. Select "OK" to save your settings.

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".
4. In the Sample Type column, use the drop-down menu to select "Ladder", "Sample", "Positive Control" or "Negative Control" as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as "Ladder" in the Sample Type column for proper genotyping.

   Note: The positive control DNA defined in the GeneMapper® *ID* panel file is the 2800M Control DNA. Redefine the genotype in the panel file if using a different positive control DNA.
5. In the Analysis Method column, select the analysis method created previously in this section.
6. In the Panel column, select the panels text file that was imported in Section 6.F.
7. In the Size Standard column, select the size standard that was imported in Section 6.G or created in Section 6.H.
8. Select "Analyze" (green arrow button) to start data analysis.

6.J. Creating a Databasing or Paternity Analysis Method Using a Global Filter with GeneMapper® *ID* Software, Version 3.2

1. Select "Tools", and then "GeneMapper Manager".
2. Select the Analysis Methods tab.
3. Select "New", and a new analysis method dialog box will open.
4. Select "HID", and select "OK".

   Note: If you do not see the HID option, you do not have the GeneMapper® *ID* software. Contact Applied Biosystems.
5. Enter a descriptive name for the analysis method, such as "PowerPlexY23_20%filter".
6. Select the Allele tab (Figure 21).
7. Select the bins text file that was imported in Section 6.F.
8. Ensure that the "Use marker-specific stutter ratio if available" box is checked. 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, Penta and Hexa repeats (Figure 21).

   Note: If you do not check the "Use marker-specific stutter ratio if available" box, you will need to optimize these settings. In-house validation should be performed.

6.J. Creating a Databasing or Paternity Analysis Method Using a Global Filter with GeneMapper® *ID* Software, Version 3.2 (continued)

9. Enter the values shown in Figure 21 for proper filtering of peaks when using the PowerPlex® Y23 System. For an explanation of the proper usage and effect of these settings, refer to the Applied Biosystems user bulletin titled "*Installation Procedures and New Features for GeneMapper* ID *Software 3.2*".

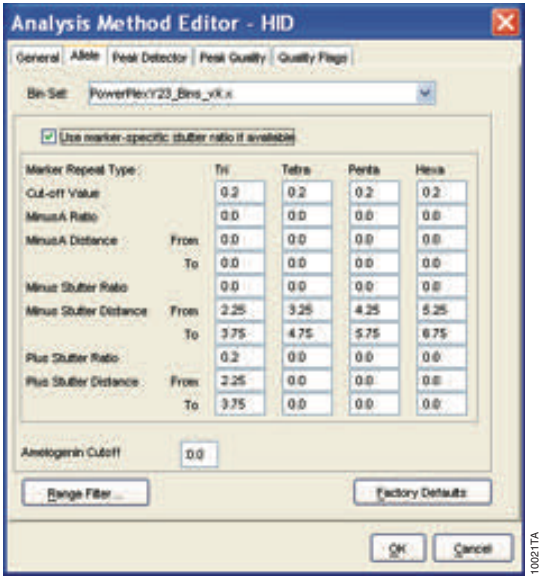

Figure 21. The GeneMapper® *ID* Software Allele tab with settings for using a 20% peak filter.

10. Select the Peak Detector tab (Figure 20). You will need to optimize these settings. In-house validation should be performed.

    Notes:

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

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

    Note: See the GeneMapper® *ID* user's manual for more information. The settings in Steps 11 and 12 should be based on the results of your internal validation.

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

13. Select "OK" to save your settings.

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 desired files, and then select “Add to list” followed by “Add”.
4. In the Sample Type column, use the drop-down menu to select “Ladder”, “Sample”, “Positive Control” or “Negative Control” as appropriate for the sample. Every folder in the project must contain at least one allelic ladder injection that is designated as “Ladder” in the Sample Type column for proper genotyping.

   Note: The positive control DNA defined in the GeneMapper® *ID* panel file is the 2800M Control DNA. Redefine the genotype in the panel file if using a different positive control DNA.
5. In the Analysis Method column, select the analysis method created previously in this section.
6. In the Panel column, select the panels text file that was imported in Section 6.F.
7. In the Size Standard column, select the size standard that was imported in Section 6.G or created in Section 6.H.
8. Select “Analyze” (green arrow button) to start the data analysis.

6.K. Controls

1. Observe the results for the negative control. Using the protocols defined in this manual, the negative control should be devoid of amplification products.
2. Observe the results for the 2800M Control DNA. The expected 2800M DNA allele designations for each locus are listed in Table 8 (Section 9.A).

6.L. Results

Figure 23. The PowerPlex® Y23 Allelic Ladder Mix. The PowerPlex® Y23 Allelic Ladder Mix was analyzed using an Applied Biosystems® 3500xL Genetic Analyzer and a 1.2kV, 24-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.

Representative results of the PowerPlex® Y23 System are shown in Figure 22. The PowerPlex® Y23 Allelic Ladder Mix is shown in Figure 23.

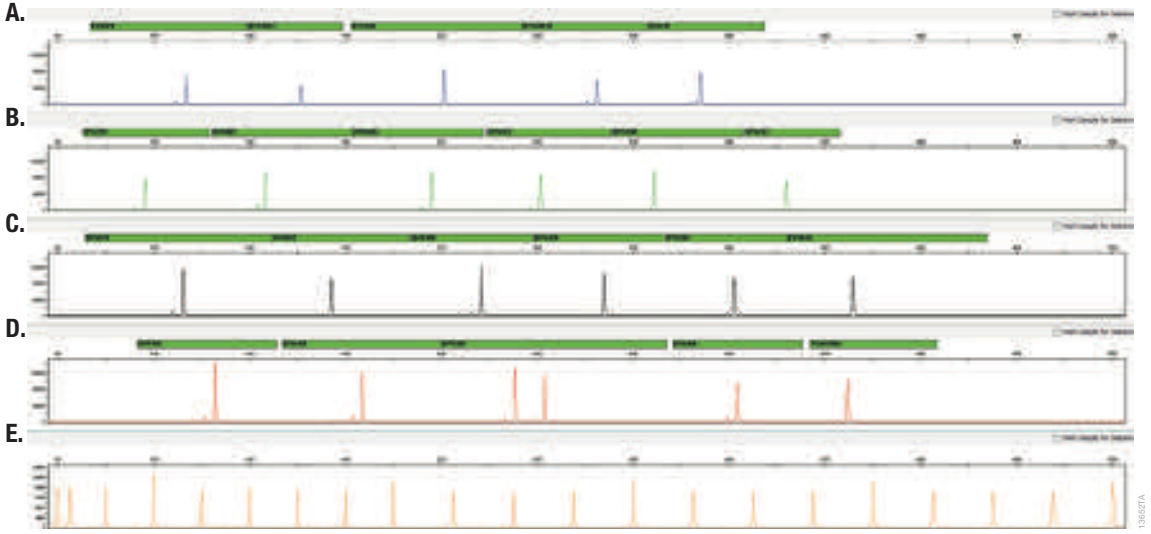

Figure 22. The PowerPlex® Y23 System. The 2800M Control DNA (0.5ng) was amplified using the PowerPlex® Y23 System. Amplification products were mixed with WEN Internal Lane Standard 500 Y23 and analyzed with an Applied Biosystems® 3500xL Genetic Analyzer using a 1.2kV, 24-second injection. Results were analyzed using GeneMapper® *ID*-X software, version 1.4. **Panel A.** An electropherogram showing the peaks of the fluorescein-labeled loci: 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.

6.L. Results (continued)

Artifacts and Stutter

Stutter products are a common amplification artifact associated with STR analysis (23,24). Stutter products often are 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 plus three standard deviations at each locus is used in the PowerPlex® Y23 panels text files for locus-specific filtering in the GeneMapper® *ID* software, version 3.2, and in the PowerPlex® Y23 stutter text files for locus-specific filtering in GeneMapper® *ID*-X software. The GeneMapper® *ID*-X stutter 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, DNA-dependent artifact peaks (Table 4) and DNA-independent artifact peaks (Table 5) may be observed with the PowerPlex® Y23 System.

Table 4. DNA-Dependent Artifacts Observed with the PowerPlex® Y23 System.

Dye	Artifact
Fluorescein	DYS448 n−9 to n−15[1,2] DYS19 n−2; n+2[3]
JOE	163 bases[4] 187 bases[4] 253 bases[4] 272 bases[4]
TMR	159 bases[4] 428 bases[4] 441 bases[4]
CXR	201 bases[4]

[1]These variably sized peaks on the Applied Biosystems® 3130 and 3500 Genetic Analyzers 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 [CE] instruments.)

[2]The low-level, DNA-dependent artifact is noticeable only with high input template amounts and allele peak heights.

[3]Two bases below and above the true allele peak, respectively.

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

6.L. Results (continued)

Table 5. DNA-Independent Artifacts Observed with the PowerPlex® Y23 System.

Dye	Artifact
Fluorescein	61–65 bases[1] 58–63 bases[1] 136–144 bases[2]
JOE	136–144 bases[2]

[1]The 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.

[2]Artifact may appear as a dye blob or a peak in sample reaction and negative control reaction.

7. 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**

7.A. Amplification and Fragment Detection

This section provides information about general amplification and detection. For questions about amplification of extracted DNA, see Section 7.B. For questions about direct amplification, see Sections 7.C and 7.D.

Symptoms	Causes and Comments
Faint or absent allele peaks	The PowerPlex® Y23 5X Master Mix was not vortexed well before use. Vortex the 5X Master Mix for 15 seconds before dispensing into the PCR amplification mix.
	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 in Section 4 or 9. We have not tested other reaction tubes, plates or thermal cyclers. Calibrate the thermal cycler heating block if necessary.
	Samples stored in injection plate for prolonged period (e.g., 48 hours) in loading cocktails devoid of Stabilizer Reagent. If you anticipate that samples will be stored for a prolonged time in the injection plate, we strongly recommend including Stabilizer Reagent in the loading cocktail.
	Primer concentration was too low. Use the recommended primer concentration. Vortex the PowerPlex® Y23 10X Primer Pair Mix for 15 seconds before use.

7.A. Amplification and Fragment Detection (continued)

Symptoms	Causes and Comments
Faint or absent allele peaks (continued)	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.
	Poor capillary electrophoresis injection (WEN ILS 500 Y23 peaks also affected). Re-inject the sample. Check the laser power.
	Poor-quality formamide was used. Use only the recommended formamide when analyzing samples.
Faint or absent allele peaks for the positive control reaction	Improper storage of the 2800M Control DNA.
Extra peaks visible in one or all color channels	Contamination with another template DNA or previously amplified DNA. Cross-contamination can be a problem. Use aerosol-resistant pipette tips, and change gloves regularly.
	Samples were not denatured completely. Heat denature samples for the recommended time, and then cool on crushed ice or 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.
	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´ A residue. • Be sure to perform a 20-minute extension step at 60°C after thermal cycling (Section 4 or 9). • 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.

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels	Artifacts. The signal strength of certain artifacts increases with storage of the amplification plate at 4°C (see Table 5), 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.
	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.
	CE-related artifacts (contaminants). Contaminants in the water used with the instrument or to dilute the 10X genetic analyzer buffer may generate peaks in the fluorescein and JOE channels. Use autoclaved deionized water; change vials and wash buffer reservoir.
	An incorrect internal lane standard was used. 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 or WEN_ILS_500_IDX.xml file can be used to assign fragment sizes for the WEN ILS 500 Y23.
	Incorrect G5 spectral was active when analyzing samples with the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer. Re-run samples, and confirm that the PowerPlex® 5-dye spectral is set for G5. See instructions for instrument preparation in Section 5.
	The wrong spectral calibration was used. Make sure that the spectral calibration was performed using the same polymer type as that for sample analysis. (e.g., do not use a POP-4®-generated spectral calibration for a POP-7® run).
	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. • Perform a new spectral calibration, and re-run the samples. • Instrument sensitivities can vary. Optimize the injection conditions. See Section 5. • Reboot the Applied Biosystems® 3500 or 3500xL Genetic Analyzer and the instrument's computer. Repeat the spectral calibration. Do not allow borrowing when running the spectral calibration on the Applied Biosystems® 3500 or 3500xL Genetic Analyzer.

7.A. Amplification and Fragment Detection (continued)

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels (continued)	Repeat sample preparation using fresh formamide. Long-term storage of amplified sample in formamide can result in artifacts.
	The CE polymer was beyond its expiration date, or polymer was stored at room temperature for more than one week.
	Maintain instrumentation on a daily or weekly basis, as recommended by the manufacturer.
	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.
Allelic ladder not running the same as samples	Allelic ladder and primer pair mix were not compatible. Ensure that the allelic ladder is from the same kit as the primer pair mix.
	Poor-quality formamide. Use only the recommended formamide when analyzing samples.
	Be sure the allelic ladder and samples are from the same instrument run.
	Migration of samples changed slightly over the course of a CE run with many samples. This may be due to changes in temperature or the CE column over time. Use a different injection of allelic ladder to determine sizes.
	Poor injection of allelic ladder. Include more than one ladder per instrument run.
	Internal size standard was not assigned correctly. Evaluate the sizing labels on the WEN ILS 500 Y23, and correct if necessary.
Peak height imbalance	Miscellaneous balance problems. At the first use, thaw the 10X Primer Pair Mix and 5X Master Mix completely. Vortex the 10X Primer Pair Mix and 5X Master Mix for 15 seconds before use; do not centrifuge the 10X Primer Pair Mix or 5X Master Mix after mixing. Calibrate thermal cyclers and pipettes routinely.
	PCR amplification mix prepared in Section 4 or 9 was not mixed well. Vortex the PCR amplification mix for 5–10 seconds before dispensing into the reaction tubes or plate.
	Tubes of 5X Master Mix and 10X Primer Pair Mix from different lots were used. The PowerPlex® Y23 5X Master Mix and PowerPlex® Y23 10X 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.

7.B. Amplification of Extracted DNA

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

Symptoms	Causes and Comments
Faint or absent allele peaks	Impure template DNA. Because a small amount of template is used, this is rarely a problem. Depending on the DNA extraction procedure used and sample source, inhibitors might be present in the DNA sample. Faint or absent peaks may be seen more often when using the maximum template volume or reduced amplification reaction volume.
	Insufficient template. Use the recommended amount of template DNA if available.
	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), TE^{-4} buffer with 20µg/ml glycogen or nuclease-free water. Faint or absent peaks may be seen more often when using the maximum template volume or 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.
Extra peaks visible in one or all color channels	Artifacts of STR amplification. Amplification of excess amounts of purified DNA can result in a higher number of artifact peaks . Use the recommended amount of template DNA. See Section 6.L for additional information about stutter and artifacts. Amplification of excess amounts also may result in overamplification and signal saturation. If signal is saturated, repeat amplification with less swab extract or reduced cycle number.
	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.

7.B. Amplification of Extracted DNA (continued)

Symptoms	Causes and Comments
Peak height imbalance	Excessive amount of DNA. Amplification of >0.5ng of template can result in an imbalance, with smaller loci showing more product than larger loci. Use less template or fewer cycles.
	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.
	The reaction volume was too low. This system is optimized for a final reaction volume of 25µl to overcome inhibitors present in DNA samples. Decreasing the reaction volume can result in suboptimal performance.
	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.

7.C. 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 7.A.

Symptoms	Causes and Comments
Faint or absent allele peaks	DNA was not accessible on nonlytic material. Pretreat nonFTA materials with PunchSolution™ Reagent to ensure that DNA is liberated 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 improve low peak heights.
	Too much sample in the reaction. Use one or two 1.2mm storage card punches (see Section 4.B). 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.

Symptoms	Causes and Comments
Faint or absent allele peaks (continued)	Active PunchSolution™ Reagent carried over into amplification reactions with nonFTA 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 nonFTA punches. Thaw the PunchSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
Faint or absent allele peaks for the positive control reaction	Positive control did not amplify. Check to make sure that the correct amount of 2800M Control DNA was added to the reaction. We recommend 5ng of 2800M Control DNA per 25µl amplification reaction and 2.5ng of 2800M Control DNA per 12.5µl reaction. • Do not include a blank punch in the positive control reaction. Presence of a blank punch may inhibit amplification of 2800M Control DNA. • Optimize the amount of 2800M Control DNA for your thermal cycling conditions and laboratory preferences.
	Improper storage of the 2800M Control DNA.
Extra peaks visible in one or all color channels	Punch was contaminated. Take punches from blank paper between samples.
	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.
	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 (i.e., smaller in size).

7.C. Direct Amplification of DNA From Storage Card Punches (continued)

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels (continued)	Artifacts of STR amplification. Direct amplification of >20ng of template can result in a higher number of artifact peaks. Use the recommended punch size and number of punches. Do not reduce the reaction volume below 25µl. Optimize the cycle number. See Section 6.L for additional information about stutter and artifacts.
	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´ A residue. • Be sure to perform a 20-minute extension step at 60°C after thermal cycling (Section 4). • Decrease cycle number. • Increase the final extension time.
	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 punch as soon as possible to each well, and follow immediately by thermal cycling.
Peak height imbalance	Excessive amount of DNA. Amplification of >20ng of template can result in an imbalance, with smaller loci showing more product than larger loci. • Be sure to use the recommended number of punches. Follow the manufacturer's recommendations when depositing sample onto the card. • Decrease cycle number.
	The cycle number was too high. Decrease the cycle number by one cycle, and repeat the amplification.
	Amplification was inhibited when using more than one storage card punch with blood. Use only one 1.2mm storage card punch with blood.
	DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat nonFTA materials with PunchSolution™ Reagent to ensure that DNA is liberated from cellular proteins.

Symptoms	Causes and Comments
Peak height imbalance (continued)	Active PunchSolution™ Reagent carried over into amplification reactions with nonFTA card punches. Larger loci are most susceptible to carryover and will drop out before the smaller loci. • Ensure that the heat block reached 70°C and samples were incubated for 30 minutes or until wells are dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. • We recommend treating one 1.2mm nonFTA card punch with 10µl of PunchSolution™ Reagent and using one punch per 25µl or 12.5µl amplification reaction. Reducing the PunchSolution™ Reagent volume may improve results for reactions with reduced amplification volumes. Optimization and validation are required.
	Inactive PunchSolution™ Reagent was used to pretreat nonFTA punches. Thaw the PunchSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
Extreme variability in sample-to-sample peak heights	There can be significant individual-to-individual variability in the number of cells on a punch, resulting in peak height variability between samples. The PunchSolution™ Kit maximizes the recovery of amplifiable DNA from nonFTA punches but does not normalize the amount of DNA present.

7.D. 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 7.A.

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 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.

7.D. Direct Amplification of DNA From Swabs (continued)

Symptoms	Causes and Comments
Faint or absent allele peaks (continued)	Active SwabSolution™ Reagent carried over into the amplification reaction. Ensure that the heat block reached 70°C (90°C if using a 2.2ml, Square-Well Deep Well Plate) and samples were incubated for the full 30 minutes. Incubation for shorter time periods may result in incomplete reagent inactivation. Do not use an incubator to incubate tubes or plates; heat transfer is inefficient and will result in poor performance. 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 liberated 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. We recommend 5ng of 2800M Control DNA per 25µl amplification reaction and 2.5ng of 2800M Control DNA per 12.5µl 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.
	Improper storage of the 2800M Control DNA.
Extra peaks visible in one or all color channels	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.
	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. If signal is saturated, repeat amplification with less swab extract or reduced cycle number.
	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 (i.e., smaller in size).

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels (continued)	Artifacts of STR amplification. Amplification of STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete addition of the 3´ A residue. • Be sure to perform a 20-minute extension step at 60°C after thermal cycling (Section 4) • Use 2µl of swab extract in a PowerPlex® Y23 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.
	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.
Peak height imbalance	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.
	The cycle number was too high. Decrease cycle number by one cycle, and repeat the amplification.
	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. Incubation for shorter time periods may result in incomplete reagent inactivation. Do not use an incubator to incubate tubes or plates; heat transfer is inefficient and will result in poor performance. Use only a heat block to maintain efficient heat transfer.
	Inactive SwabSolution™ Reagent. Thaw the SwabSolution™ Reagent completely in a 37°C water bath, and mix by gentle inversion. Store the SwabSolution™ Reagent at 2–10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not re-freeze, as this may reduce activity.
	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 liberated from cellular proteins.

7.D. Direct Amplification of DNA From Swabs (continued)

Symptoms	Causes and Comments
Extreme variability in sample-to-sample peak heights	There can be significant individual-to-individual variability in cell deposition onto buccal swabs. This will appear as variability in peak heights between swab extracts. The extraction process maximizes recovery of amplifiable DNA from buccal swabs but does not normalize the amount of DNA present. If variability is extreme, quantify the DNA using a fluorescence-based double-stranded DNA quantification method or qPCR-based quantification method. The quantification values can be used to normalize input template amounts to minimize variation in signal intensity.

7.E. GeneMapper® *ID*-X Software

Symptoms	Causes and Comments
Stutter peaks not filtered	Stutter text file was not imported into the Panel Manager when the panels and bins text files were imported.
	Be sure that the "Use marker-specific stutter ratio and distance if available" box is checked. If the "Use marker-specific stutter ratio and distance if available" box is not checked, stutter distance must be defined in the Analysis Method Allele tab.
Samples in the project not analyzed	The Analysis Requirement Summary window was not active, and there was an analysis requirement that was not met. Turn on Analysis Requirement Summary in the Options menu, and correct the necessary analysis requirements to continue analysis.
Edits in label edit viewer cannot be viewed	To view edits made to a project, the project first must be saved. Close the plot view window, return to the main GeneMapper® *ID*-X page and save the project. Display the plot window again, and then view the label edit table.
Marker header bar for some loci are gray	When an edit is made to a locus, the quality flags and marker header bar automatically change to gray. To change the GQ and marker header bar for a locus to green, override the GQ in the plot window.
Alleles not called	To analyze samples with GeneMapper® *ID*-X software, at least one allelic ladder must be defined.
	Peaks in ILS were not captured. Not all WEN ILS 500 Y23 peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Re-run samples using a longer run time.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.

Symptoms	Causes and Comments
Off-ladder alleles	An allelic ladder from a different run than the samples was used. Re-analyze samples with an allelic ladder from the same run.
	The GeneMapper® *ID*-X software requires that the allelic ladder be imported from the same folder as the sample. Be sure that the allelic ladder is in the same folder as the sample. Create a new project and re-analyze, as described in Section 6.D or 6.E.
	Panels text file selected for analysis was incorrect for the STR system used. Assign correct panels text file that corresponds to the STR system used for amplification.
	The allelic ladder was not identified as an allelic ladder in the Sample Type column.
	The internal lane standard was not properly identified in the sample. Manually redefine the sizes of the size standard fragments in the sample.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.
Size standard not called correctly	Starting data point was incorrect for the partial range chosen in Section 6.D or 6.E. Adjust the starting data point in the analysis method. Alternatively, 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".
	Peaks in ILS were not captured. Not all WEN ILS 500 Y23 peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Re-run samples using a longer run time.
Peaks in size standard missing	If peaks are low-quality, redefine the size standard for the sample to skip these peaks.
	An incorrect size standard was used.

7.E. GeneMapper® *ID*-X Software (continued)

Symptoms	Causes and Comments
Significantly raised baseline	Poor spectral calibration. Perform a new spectral calibration, and re-run the samples.
	Incorrect G5 spectral was active. Re-run samples, and confirm that the PowerPlex® 5-dye G5 spectral is set for G5. See instructions for instrument preparation in Section 5.
	The wrong spectral calibration was used. Make sure that the spectral calibration was performed using the same polymer type as that for sample analysis. (e.g., do not use a POP-4®-generated spectral calibration for a POP-7® run).

7.F. GeneMapper® *ID* Software

Symptoms	Causes and Comments
Alleles not called	To analyze samples with GeneMapper® *ID* software, the analysis parameters and size standard must both have "Basic or Advanced" as the analysis type. If they are different, an error is obtained.
	To analyze samples with GeneMapper® *ID* software, at least one allelic ladder must be defined.
	Peaks in ILS were not captured. Not all WEN ILS 500 Y23 peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Re-run samples using a longer run time.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.
Off-ladder alleles	An allelic ladder from a different run than the samples was used. Re-analyze samples with an allelic ladder from the same run.
	The GeneMapper® *ID* software requires that the allelic ladder be imported from the same folder as the sample. Be sure that the allelic ladder is in the same folder as the sample. Create a new project and re-analyze as described in Section 6.I or 6.J.
	Panels text file selected for analysis was incorrect for the STR system used. Assign correct panels 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.

Symptoms	Causes and Comments
Off-ladder alleles (continued)	The wrong analysis type was chosen for the analysis method. Be sure to use the HID analysis type.
	The internal lane standard was not properly identified in the sample. Manually redefine the sizes of the size standard fragments in the sample.
	A low-quality allelic ladder was used during analysis. Ensure that only high-quality allelic ladders are used for analysis.
Size standard not called correctly	Starting data point was incorrect for the partial range chosen in Section 6.I or 6.J. Adjust the starting data point in the analysis method. Alternatively, use a full range for the analysis.
	Extra peaks in advanced mode size standard. Open the Size Match Editor. Highlight the extra peak, select "Edit" and select "delete size label". Select "auto adjust sizes".
	Peaks in ILS were not captured. Not all WEN ILS 500 Y23 peaks defined in the size standard were detected during the run. • Create a new size standard using the internal lane standard fragments present in the sample. • Re-run samples using a longer run time.
Peaks in size standard missing	If peaks are low-quality, redefine the size standard for the sample to skip these peaks.
	An incorrect size standard was used.
Error message: "Either panel, size standard, or analysis method is invalid"	The size standard and analysis method were not in the same mode ("Classic" vs. "Basic or Advanced"). Be sure both files are set to the same mode, either Classic or Basic or Advanced mode.
No alleles called, but no error message appears	Panels text file was not selected for sample. In the Panel column, select the appropriate panels text file for the STR system that was used.
	No size standard was selected. In the Size Standard column, be sure to select the appropriate size standard.
	Size standard was not correctly defined, or size peaks were missing. Redefine size standard to include only peaks present in your sample. Terminating analysis early or using short run times will cause larger ladder peaks to be missing. This will cause your sizing quality to be flagged as "red", and no allele sizes will be called.

7.F. GeneMapper® *ID* Software (continued)

Symptoms	Causes and Comments
Error message: "Both the Bin Set used in the Analysis Method and the Panel must belong to the same Chemistry Kit"	The bins text file assigned to the analysis method was deleted. In the GeneMapper® Manager, select the Analysis Methods tab, and open the analysis method of interest. Select the Allele tab, and select an appropriate bins text file.
	The wrong bins text file was chosen in the analysis method Allele tab. Be sure to choose the appropriate bins text file, as shown in Figure 19.
Significantly raised baseline	Poor spectral calibration. Perform a new spectral calibration, and re-run the samples.
	Use of Classic mode analysis method. Use of Classic mode analysis on samples can result in baselines with more noise than those analyzed using the Basic or Advanced mode analysis method. Advanced mode analysis methods and size standards are recommended.
	Incorrect G5 spectral was active. Re-run samples, and confirm that the PowerPlex® 5-dye G5 spectral is set for G5. See instructions for instrument preparation in Section 5.
	The wrong spectral calibration was used. Make sure that the spectral calibration was performed using the same polymer type as that for sample analysis. (i.e., do not use a POP-4®-generated spectral calibration for a POP-7® run).
Error message after attempting to import panels and bins text files: "Unable to save panel data: java.SQLEException:ORA-00001: unique constraint (IFA.CKP_NNN) violated".	There was a conflict between different sets of panels and bins text files. Check to be sure that the bins are installed properly. If not, delete all panels and bins text files, and re-import files in a different order.
Allelic ladder peaks labeled off-ladder	GeneMapper® *ID* software was not used, or microsatellite analysis settings were used instead of HID analysis settings. GeneMapper® software does not use the same algorithms as GeneMapper® *ID* software and cannot correct for sizing differences using the allelic ladder. We recommend using GeneMapper® *ID* software to analyze PowerPlex® reactions. If using GeneMapper® *ID* software, version 3.2, be sure that the analysis method selected is an HID method. This can be verified by opening the analysis method using the GeneMapper® Manager, and then selecting the General tab. The analysis type cannot be changed. If the method is not HID, delete it and create a new analysis method. Contact Promega Technical Services at: **genetic@promega.com** with questions.

8. References

1. Edwards, A. *et al.* (1991) DNA typing with trimeric and tetrameric tandem repeats: Polymorphic loci, detection systems, and population genetics. In: *The Second International Symposium on Human Identification 1991*, Promega Corporation, 31–52.
2. Edwards, A. *et al.* (1991) DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *Am. J. Hum. Genet.* **49**, 746–56.
3. Edwards, A. *et al.* (1992) Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. *Genomics* **12**, 241–53.
4. Warne, D. *et al.* (1991) Tetranucleotide repeat polymorphism at the human β-actin related pseudogene 2 (actbp2) detected using the polymerase chain reaction. *Nucleic Acids Res.* **19**, 6980.
5. Ausubel, F.M. *et al.* (1996) Unit 15: The polymerase chain reaction. In: *Current Protocols in Molecular Biology*, Vol. 2, John Wiley and Sons, NY.
6. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Chapter 14: In vitro amplification of DNA by the polymerase chain reaction. In: *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
7. *PCR Technology: Principles and Applications for DNA Amplification* (1989) Erlich, H.A., ed., Stockton Press, New York, NY.
8. *PCR Protocols: A Guide to Methods and Applications* (1990) Innis, M.A. *et al.* eds., Academic Press, San Diego, CA.
9. Butler, J.M. (2005) *Forensic DNA Typing*, 2nd ed., Elsevier Academic Press, London.
10. Gusmão, L. and Carracedo, A. (2003) Y chromosome-specific STRs. *Profiles in DNA* **6**, 3–6.
11. Jobling, M.A., Pandya, A. and Tyler-Smith, C. (1997) The Y chromosome in forensic analysis and paternity testing. *Int. J. Legal Med.* **110**, 118–24.
12. Gill, P. *et al.* (2001) DNA Commission of the International Society of Forensic Genetics: Recommendations on forensic analysis using Y-chromosome STRs. *Int. J. Legal Med.* **114**, 305–9.
13. Roewer, L. *et al.* (2001) Online reference database of European Y-chromosomal short tandem repeat (STR) haplotypes. *Forensic Sci. Int.* **118**, 106–13.
14. Butler, J.M. *et al.* (2002) A novel multiplex for simultaneous amplification of 20 Y chromosome STR markers. *Forensic Sci. Int.* **129**, 10–24.
15. Kayser, M. *et al.* (1997) Evaluation of Y-chromosomal STRs: A multicenter study. *Int. J. Legal Med.* **110**, 125–33.
16. Ruitberg, C.M., Reeder, D.J. and Butler, J.M. (2001) STRBase: A short tandem repeat DNA database for the human identity testing community. *Nucleic Acids Res.* **29**, 320–2.
17. Prinz, M. *et al.* (1997) Multiplexing of Y chromosome specific STRs and performance for mixed samples. *Forensic Sci. Int.* **85**, 209–18.

8. References (continued)

18. Prinz, M. *et al.* (2001) Validation and casework application of a Y chromosome specific STR multiplex. *Forensic Sci. Int.* **120**, 177–88.

19. Presley, L.A. *et al.* (1992) The implementation of the polymerase chain reaction (PCR) HLA DQ alpha typing by the FBI laboratory. In: *The Third International Symposium on Human Identification 1992*, Promega Corporation, 245–69.

20. Hartmann, J.M. *et al.* (1991) Guidelines for a quality assurance program for DNA analysis. *Crime Laboratory Digest* **18**, 44–75.

21. *Internal Validation Guide of Y-STR Systems for Forensic Laboratories* #GE713, Promega Corporation.

22. Kline, M.C. *et al.* (2005) Results from the NIST 2004 DNA quantitation study. *J. Forensic Sci.* **50**, 570–8.

23. Levinson, G. and Gutman, G.A. (1987) Slipped-strand mispairing: A major mechanism for DNA sequence evolution. *Mol. Biol. Evol.* **4**, 203–21.

24. Schlötterer, C. and Tautz, D. (1992) Slippage synthesis of simple sequence DNA. *Nucleic Acids Res.* **20**, 211–5.

25. Lim, S.K. *et al.* (2007) Variation of 52 new Y-STR loci in the Y chromosome consortium worldwide panel of 76 diverse individuals. *Int. J. Legal Med.* **121**, 124–7.

26. Kayser, M. *et al.* (2004) A comprehensive survey of human Y-chromosomal microsatellites. *Am. J. Hum. Genet.* **74**, 1183–97.

27. Vermeulen, M. *et al.* (2009) Improving global and regional resolution of male lineage differentiation by simple single-copy Y-chromosomal short tandem repeat polymorphisms. *Forensic Sci. Int. Genet.* **3**, 205–13.

28. Rodig, H. *et al.* (2008) Evaluation of haplotype discrimination capacity of 35 Y-chromosomal short tandem repeat loci. *Forensic Sci. Int.* **174**, 182–8.

29. Geppert, M., Edelmann, J. and Lessig, R. (2009) The Y-chromosomal STRs DYS481, DYS570, DYS576 and DYS643. *Leg. Med. (Tokyo)* **11**, S109–10.

30. Smith, J.R. *et al.* (1995) Approach to genotyping errors caused by nontemplated nucleotide addition by *Taq* DNA polymerase. *Genome Res.* **5**, 312–7.

31. Magnuson, V.L. *et al.* (1996) Substrate nucleotide-determined non-templated addition of adenine by *Taq* DNA polymerase: Implications for PCR-based genotyping. *BioTechniques* **21**, 700–9.

32. Walsh, P.S., Fildes, N.J. and Reynolds, R. (1996) Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. *Nucleic Acids Res.* **24**, 2807–12.

33. Bär, W. *et al.* (1997) DNA recommendations. Further report of the DNA Commission of the ISFH regarding the use of short tandem repeat systems. *Int. J. Legal Med.* **110**, 175–6.

34. Gill, P. *et al.* (1997) Considerations from the European DNA Profiling Group (EDNAP) concerning STR nomenclature. *Forensic Sci. Int.* **87**, 185–92.

35. Budowle, B. *et al.* (2008) Null allele sequence structure at the DYS448 locus and implications for profile interpretation. *Int. J. Legal Med.* **122**, 421–7.

36. Mandrekar, P.V., Krenke, B.E. and Tereba, A. (2001) DNA IQ™: The intelligent way to purify DNA. *Profiles in DNA* **4(3)**, 16.

37. Krenke, B.E. *et al*. (2005) Development of a novel, fluorescent, two-primer approach to quantitative PCR. *Profiles in DNA* **8(1)**, 3–5.

38. Ewing, M.M. *et al*. (2016) Human DNA quantification and sample quality assessment: Developmental validation of the PowerQuant® System. *Forensic Sci. Int. Genet.* **23**, 166–77.

9. Appendix

9.A. Advantages of Using the Loci in the PowerPlex® Y23 System

The loci included in the PowerPlex® Y23 System (Tables 6 and 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 (SWGDAM)-recommended Y-STR panel (European minimal haplotype plus DYS438 and DYS439) and the loci included in the US Y-STR database (SWGDAM-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 band one base shorter than expected (i.e., missing the terminal addition) is sometimes seen. We have modified primer sequences and added a final extension step at 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	TTTTC
DYS437	JOE	Y	TCTA
DYS570	TMR-ET	Y	TTTC
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

[1]Information about most of these loci can be found at: **www.cstl.nist.gov/biotech/strbase/chrom.htm**

[2]The 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".

9.A. Advantages of Using the Loci in the PowerPlex® Y23 System (continued)

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 Components[3]
DYS576	Fluorescein	97–145	11–23
DYS389I	Fluorescein	147–179	9–17
DYS448	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

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

[2]When 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.

[3]For a current list of microvariants, see the Variant Allele Report published at the U.S. National Institute of Standards and Technology (NIST) web site at: **www.cstl.nist.gov/div831/strbase/** and the Y Chromosome Haplotype Reference Database at: **www.yhrd.org**

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

STR Locus	2800M
DYS576[1]	18
DYS389I	14
DYS448[1]	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

[1]A 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.

9.B. Detection of Amplified Fragments Using the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with POP-7® Polymer and Data Collection Software, Version 3.0 or 4.0

The PowerPlex® Y23 System is optimized for POP-4® polymer. We recognize that some laboratories use POP-7® polymer and therefore have included a protocol in this manual.

Some DNA-independent artifacts migrate in the panel range with the POP-7® polymer (see Table 9). Global filters used for database analysis will generally filter these artifact peaks. However, these peaks may be labeled with casework samples. Internal validation should be performed and interpretation guidelines created that describe the artifacts and their impact on data analysis. For information about DNA-dependent stutter products and artifacts, see Table 4 in Section 6.L.

Table 9. DNA-Independent Artifacts.

Dye	Artifact
Fluorescein	61–64 bases 70–72 bases 82–84 bases 99–103 bases[1]
JOE	60–63 bases 86–89 bases[1]

[1]The 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.

Note: For data analysis, follow the instructions in Section 6 except use POP-7®-specific panels and bins text files (e.g., use PowerPlexY23_POP7_Panels_IDX_vX.x.txt instead of PowerPlexY23_Panels_IDX_vX.x.txt).

Contact Promega Technical Services at: **genetic@promega.com** for the POP-7®-specific panels and bins text files for GeneMapper® and GeneMapper® *ID*-X software.

Materials to Be Supplied by the User

- 95°C dry heating block, water bath or thermal cycler
- crushed ice, ice-water bath or a freezer plate block
- centrifuge compatible with 96-well plates or reaction tubes
- aerosol-resistant pipette tips
- 3130 or 3130*xl* capillary array, 36cm
- plate retainer and base set (standard)
- POP-7® polymer for the Applied Biosystems® 3130 Genetic Analyzer
- 10X genetic analyzer buffer with EDTA
- 96-well plate and septa (e.g., Plate, Barcoded, Semi-Skirted, 96-Well [Cat.# V7845] and Septa Mat, 96-Well [Cat.# CE2696] or MicroAmp® optical 96-well plate [or equivalent] and septa [Applied Biosystems])
- Hi-Di™ formamide (Applied Biosystems Cat.# 4311320)
- Stabilizer Reagent (Cat.# DM6571)

9.B. Detection of Amplified Fragments Using the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with POP-7® Polymer and Data Collection Software, Version 3.0 or 4.0 (continued)

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 you anticipate storing samples in loading cocktail in the injection plate for up to 48 hours prior to injection, we strongly recommend including Stabilizer Reagent in the loading cocktail. Omission of 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 the amplified samples.

Sample Preparation

1. Prepare a loading cocktail by combining and mixing WEN ILS 500 Y23 and formamide as follows:

   [(0.5µl WEN ILS 500 Y23) × (# samples)] + [(9.5µl formamide) × (# samples)]

2. If samples in injection plate are not to be injected immediately, or if there is the possibility that the samples may be reinjected at any time within 48 hours of setting up the injection plate, prepare a loading cocktail by combining and mixing WEN ILS 500 Y23, Stabilizer Reagent and formamide as follows:

   [(0.5µl WEN ILS 500 Y23) × (# samples)] + [(0.5µl Stabilizer Reagent) × (# samples)] + [(9.5µl formamide) × (# 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 you anticipate storing samples in loading cocktail in the injection plate for up to 48 hours prior to injection, we strongly recommend including Stabilizer Reagent in the loading cocktail. Omission of 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 the amplified samples.

3. Vortex for 10–15 seconds to mix.

4. Pipet 10µl of formamide/internal lane standard mix (or 10.5µl for formamide/stabilizer reagent/internal lane standard mix if Stabilizer Reagent is used) into each well.

5. Add 1µl of amplified sample (or 1µl of PowerPlex® Y23 Allelic Ladder Mix) to each well. Cover wells with appropriate septa.

   Note: Instrument detection limits vary; therefore, injection time, injection voltage or the amount of product mixed with loading cocktail may need to be adjusted. Use the Module Manager in the data collection software to modify the injection time or voltage in the run module (see Instrument Preparation below).

6. Centrifuge 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.

Instrument Preparation

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

Analyze samples as described in the user's manual for the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with the following exceptions.

1. In the Module Manager, select "New". Select "Regular" in the Type drop-down list, and select "FragmentAnalysis36_POP7" in the Template drop-down list. Confirm that the injection time is 23 seconds and the injection voltage is 1.2kV. Change the run time to 1,500 seconds. Give a descriptive name to your run module, and select "OK".

   Note: Instrument sensitivities can vary. The injection time and voltage may be adjusted in the Module Manager.
2. In the Protocol Manager, select "New". Type a name for your protocol. Select "Regular" in the Type drop-down list, and select the run module you created in the previous step in the Run Module drop-down list. Lastly, select "G5" in the dye-set drop-down list. Select "OK".
3. In the Plate Manager, create a new plate record as described in the instrument user's manual. In the dialog box that appears, select "GeneMapper—Generic" in the Application drop-down list, and select the appropriate plate type (96-well). Add entries in the owner and operator windows, and select "OK".

   Note: If autoanalysis of sample data is desired, refer to the instrument user's manual for instructions.
4. In the GeneMapper® plate record, enter sample names in the appropriate cells. Scroll to the right. In the Results Group 1 column, select the desired results group. In the Instrument Protocol 1 column, select the protocol you created in Step 2. Be sure this information is present for each row that contains a sample name. Select "OK".

   Note: To create a new results group, select "New" in the drop-down menu in the Results Group column. Select the General tab, and enter a name. Select the Analysis tab, and select "GeneMapper—Generic" in the Analysis type drop-down list.
5. Place samples in the instrument, and close the instrument doors.
6. In the spectral viewer, select dye set G5, and confirm that the active dye set is the file generated for POP-7® polymer and PowerPlex® 5-dye chemistry.

   It is critical to select the correct G5 spectral for the PowerPlex® 5-dye chemistry and that the G5 spectral was generated using POP-7® polymer.

   If the PowerPlex® 5-dye chemistry is not the active dye set, locate the POP-7® PowerPlex® 5-dye spectral in the List of Calibrations for Dye Set G5, and select "Set".
7. In the run scheduler, locate the plate record that you just created in Steps 3 and 4, and click once on the name to highlight it.

9.B. Detection of Amplified Fragments Using the Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer with POP-7® Polymer and Data Collection Software, Version 3.0 or 4.0 (continued)

8. Once the plate record is highlighted, click the plate graphic that corresponds to the plate on the autosampler that contains your amplified samples.
9. When the plate record is linked to the plate, the plate graphic changes from yellow to green, and the green Run Instrument arrow becomes enabled.
10. Click on the green Run Instrument arrow on the toolbar to start the sample run.
11. Monitor electrophoresis by observing the run, view, array or capillaries viewer window in the data collection software. Each injection will take approximately 45 minutes.

9.C DNA Extraction and Quantification Methods and Automation Support

Promega offers a wide variety of reagents and automated methods for sample preparation, DNA purification and DNA quantification prior to STR amplification.

For analysis of database, reference and other single-source samples, we recommend direct amplification of DNA from FTA® card punches or direct amplification of DNA from swabs and nonFTA punches following a preprocessing step with the SwabSolution™ Kit or PunchSolution™ Kit, respectively. The SwabSolution™ Kit (Cat.# DC8271) contains reagents for rapid DNA preparation from buccal swabs prior to amplification. The procedure lyses cells contained on the swab head and releases into solution sufficient DNA for STR amplification. A small volume of the final swab extract is added to the PowerPlex® reaction. The PunchSolution™ Kit is used to process punches from nonFTA storage cards containing blood or buccal samples prior to direct amplification.

For casework or samples that require DNA purification, we recommend the DNA IQ™ System (Cat.# DC6700), which is a DNA isolation system designed specifically for forensic samples (36). This system uses paramagnetic particles to prepare clean samples for STR analysis easily and efficiently and can be used to extract DNA from stains or liquid samples, such as blood or solutions. The DNA IQ™ System eliminates PCR inhibitors and contaminants frequently encountered in casework samples. In additional, DNA has been isolated from casework samples such as tissue, differentially separated sexual assault samples and stains on support materials. The DNA IQ™ System has been tested with PowerPlex® Systems to ensure a streamlined process.

For applications requiring human-specific DNA quantification, the PowerQuant® System (Cat.# PQ5002, PQ5008) and the Plexor® HY System (Cat.# DC1000) were developed. These qPCR-based methods provide total human and male-specific DNA quantification in one reaction. (37,38).

For information about automation of Promega chemistries on automated workstations using Identity Automation™ solutions, contact your local Promega Branch Office or Distributor (contact information available at: **www.promega.com/support/worldwide-contacts/**), e-mail: **genetic@promega.com** or visit: **www.promega.com/idautomation/**

9.D. 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 24). 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.

Low-level artifact peaks at approximately 132 and 176 bases may be observed with the WEN ILS 500 Y23 in the orange channel. The peak height of these artifacts may vary from lot-to-lot and may be labeled by the software. These peaks are not used during sizing of the peaks present in the sample.

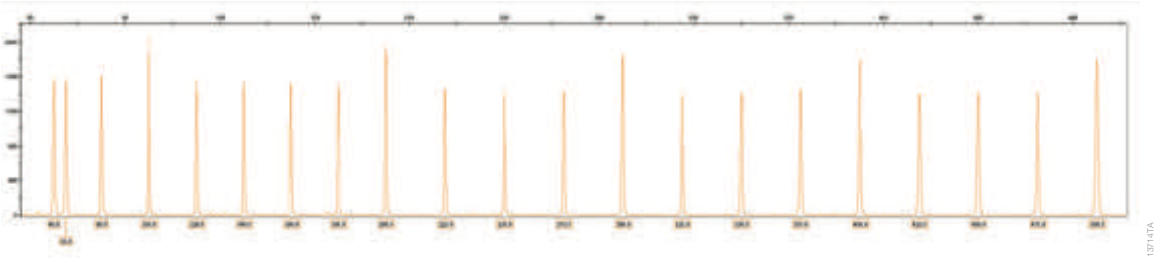

Figure 24. WEN Internal Lane Standard 500 Y23. An electropherogram showing the WEN Internal Lane Standard 500 Y23 fragments.

9.E. 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. When using the protocol detailed below, add only one 1.2mm storage card punch to each 12.5µl amplification reaction.

Materials to Be Supplied by the User

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

Notes:

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

9.E. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume (continued)

FTA®-based sample types include:

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

NonFTA sample types include:

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

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

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

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

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

Amplification Setup

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

   Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 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™ Reagent should be thawed completely, mixed by vortexing and stored at 2–10°C. The reagent may be turbid after thawing or storage at 4°C. If this occurs, warm the buffer briefly at 37°C, and then vortex until clear. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Storing reagents in the refrigerator door can compromise stability.

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

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

5. Add the final volume of each reagent listed in Table 10 to a clean tube.

Table 10. 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	2.5µl	×		=	
PowerPlex® Y23 10X Primer Pair Mix	1.25µl	×		=	
5X AmpSolution™ Reagent	2.5µl	×		=	
total reaction volume	12.5µl				

[1]Add 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 FTA® card punches, the template DNA will be added at Step 7.

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

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

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

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

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

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

   Notes:

   1. Do not include blank storage card punches in the positive control reactions.
   2. Optimization of the amount of 2800M Control DNA may be required depending on thermal cycling conditions and laboratory preferences. Typically, 5ng of 2800M Control DNA is sufficient to provide a robust profile using the cycle numbers recommended here. A one- cycle reduction in cycle number will require a twofold increase in mass of DNA template to generate similar signal intensity. Similarly, a one-cycle increase in cycle number will require a twofold reduction in the amount of 2800M Control DNA to avoid signal saturation.

9.E. Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume (continued)

9. Reserve a well containing PCR amplification mix as a negative amplification control.

   Note: An additional negative control with a blank punch may be performed to detect contamination from the storage card or punch device.

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

Thermal Cycling

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

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

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

   Note: The GeneAmp® PCR System 9700 program must be run with Max Mode as the ramp speed. This requires a silver or gold-plated 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

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

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

PCR Optimization

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

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

2. Depending on your preferred protocol, place one 1.2mm storage card punch containing a buccal sample or one 1.2mm punch of a storage card containing whole blood into each well of a reaction plate. Be sure to pretreat nonFTA samples with the PunchSolution™ Kit (Cat.# DC9271).
3. Prepare three identical reaction plates with punches from the same samples.
4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (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.

9.F. 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.

Materials to Be Supplied by the User

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

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

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

Amplification Setup

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

   Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 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 appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label appropriately.
4. Add the final volume of each reagent listed in Table 11 to a clean tube.

9.F. Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume (continued)

Table 11. 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	2.5µl	×		=	
PowerPlex® Y23 10X Primer Pair Mix	1.25µl	×		=	
swab extract	2.0µl				
total reaction volume	12.5µl				

[1]Add 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 2.0µl of swab extract for each sample into the appropriate well of the reaction plate.

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

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

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

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

9. Seal or cap the plate, or close the tubes. **Optional:** Briefly centrifuge the plate to bring contents to the bottom of the wells and remove any air bubbles.

Thermal Cycling

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

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

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

   Note: The GeneAmp® PCR System 9700 program must be run with Max Mode as the ramp speed. This requires a silver or gold-plated 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

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

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

PCR Optimization

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

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

Promega

9.G. Composition of Buffers and Solutions

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

1.21g Tris base
0.037g EDTA ($Na_2EDTA \cdot 2H_2O$)

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 ($Na_2EDTA \cdot 2H_2O$)
20µg/ml glycogen

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

9.H. Related Products

STR Systems

Accessory Components

Product	Size	Cat.#
PowerPlex® 5C Matrix Standard	5 preps	DG4850
WEN Internal Lane Standard 500 Y23	200µl	DG5201
2800M Control DNA (10ng/µl)	25µl	DD7101
2800M Control DNA (0.25ng/µl)	500µl	DD7251
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
5X AmpSolution™ Reagent	100 preps	DM1231
Water, Amplification Grade	6,250µl (5 × 1,250µl)	DW0991
Stabilizer Reagent	500 preps	DM6571

Not for Medical Diagnostic Use.

9.I. Summary of Changes

The following changes were made to the 10/19 revision of this document:

1. Added instructions for using Stabilizer Reagent.

[a]U.S. Pat. No. 6,242,235, Australian Pat. No. 761757, Canadian Pat. No. 2,335,153, Chinese Pat. No. ZL99808861.7, Hong Kong Pat. No. HK 1040262, Japanese Pat. No. 3673175, European Pat. No. 1088060 and other patents pending.

[b]U.S. Pat. No. 9,139,868 and other patents pending.

[c]TMR-ET, CXR-ET and WEN dyes are proprietary.