

Paradyme™ 27GY STR System for Use on the Spectrum CE System Technical Manual

Instructions for Use of Products RS2705 and RS2720



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

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

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

1 Description

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

The Paradyme™ 27GY STR System^(a,b) is a 27-locus multiplex for human identification applications including forensic analysis, relationship testing and research use. This eight-color system provides co-amplification and fluorescent detection of the 20 autosomal loci in the expanded CODIS loci (CSF1PO, FGA, TH01, TPOX, vWA, D1S1656, D2S1338, D2S441, D3S1358, D5S818, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11 and D22S1045). The Penta D, Penta E, and SE33 loci are also included to increase discrimination and database searches. In addition, two rapidly mutating Y-STR loci (DYS570 and DYS576), Amelogenin and DYS391 are included for gender determination.

The Paradyme™ 27GY STR System uses Reduced Stutter Polymerase (RSP) technology to reduce the formation of stutter artifacts, sometimes called repeat slippage, during PCR amplification of STR loci.

The Paradyme™ 27GY STR 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 Paradyme™ 27GY 5X Master Mix.

This manual contains protocols for using the Paradyme™ 27GY STR System with the ProFlex® and Mastercycler® X50s PCR Systems in addition to protocols to separate amplified products and detect separated material on the Spectrum CE System. Amplification and detection instrumentation may vary. Protocols may need to be optimized for template DNA amount, cycle number, loading volume and injection conditions for your laboratory instrumentation. Perform in-house validation. A protocol to operate the Spectrum CE System is available separately. See the *Spectrum CE System Operating Manual #TMD052*.

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

2 Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Paradyme™ 27GY STR System	200 (or 400 direct-amp) reactions	RS2720

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

Refrigerated components stored at +2°C to +10°C (Box 1)

Pre-Amplification Components

- 4 × 125µl Paradyme™ 27GY 10X Primer Pair Mix
- 5 × 1,250µl Water, Amplification Grade
- 2 × 25µl 2800M Control DNA, 10ng/µl

Post-Amplification Components

- 2 × 250µl CCO Internal Lane Standard 500
- 4 × 25µl Paradyme™ 27GY Allelic Ladder Mix

Frozen components stored at –30°C to –10°C (Box 2)

Pre-Amplification Components

- 4 × 250µl Paradyme™ 27GY 5X Master Mix
- 800µl TCEP, Concentrated
- 1,000µl TCEP Dilution Buffer

PRODUCT	SIZE	CAT.#
Paradyme™ 27GY STR System	50 (or 100 direct-amp) reactions	RS2705

Not For Medical Diagnostic Use. Contains sufficient reagents for 50 reactions of 25µl each or 100 direct-amplification reactions of 12.5µl each. Includes:

Refrigerated components stored at +2°C to +10°C (Box 1)

Pre-Amplification Components

- 125µl Paradyme™ 27GY 10X Primer Pair Mix
- 5 × 1,250µl Water, Amplification Grade
- 25µl 2800M Control DNA, 10ng/µl

Post-Amplification Components

- 250µl CCO Internal Lane Standard 500
- 25µl Paradyme™ 27GY Allelic Ladder Mix

Frozen components stored at –30°C to –10°C (Box 2)

Pre-Amplification Components

- 250µl Paradyme™ 27GY 5X Master Mix
- 800µl TCEP, Concentrated
- 1,000µl TCEP Dilution Buffer



The Paradyme™ 27GY Allelic Ladder Mix is provided in a separate, sealed bag for shipping. This component should be moved to the post-amplification box after opening. The Water, Amplification Grade, is provided in a separate, sealed bag for shipping. Store this component with the pre-amplification components after opening.

Storage Conditions

Upon receipt, store the Box 1 components of the Paradyme™ 27GY System at +2°C to +10°C. Store the 10X Primer Pair Mix, Water, Amplification Grade, and 2800M Control DNA at +2°C to +10°C in a pre-amplification location. Store the Paradyme™ 27GY Allelic Ladder Mix, and CCO Internal Lane Standard 500 in a post-amplification location.

Upon receipt, store the Box 2 components of the Paradyme™ 27GY System at –30°C to –10°C. Store the 5X Master Mix, TCEP, Concentrated, and TCEP Dilution Buffer in a pre-amplification location. TCEP, Concentrated, and TCEP Dilution Buffer may be stored at –30°C to –10°C, or at +2°C to +10°C after the first thaw.

The Paradyme™ 27GY 10X Primer Pair Mix, TCEP, Concentrated, Paradyme™ 27GY Allelic Ladder Mix and CCO Internal Lane Standard 500 (CCO ILS 500) are light-sensitive and must be stored in the dark.

We strongly recommend that pre-amplification and post-amplification reagents be stored and used separately with different pipettes, tube racks, etc.

Available Separately

PRODUCT	SIZE	CAT. #
8C Matrix Standard	5 preps	DG5050
Paradyme™ 27GY QI Reagent	800µl	DM1701
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
GeneMarker®HID Software for Spectrum CE Systems, Local	1 seat	CE3001
GeneMarker®HID Software for Spectrum CE Systems, Network	1 seat	CE3010
GeneMarker®HID Software for Spectrum CE Systems, Client	1 seat	CE3011

The PunchSolution™ Kit is required to process nonlytic storage card punches prior to direct amplification. The SwabSolution™ Kit is required to process swabs prior to direct amplification.

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

www.promega.com/resources/software-firmware/

The 8C Matrix Standard (Cat.# DG5050), available separately, is required for initial setup of the color separation matrix (see Section 3.2).

3 Before You Begin

3.1 Precautions

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

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

PCR-based STR analysis is subject to contamination by very small amounts of human DNA. Extreme care should be taken to avoid cross contamination when preparing template DNA, handling primer pairs, assembling amplification reactions and analyzing amplification products. The reagents and materials used prior to amplification are provided in two separate boxes based on storage temperature.

Box 1	Pre-Amplification	Special Instructions
(Store at +2°C to +10°C)	Paradyme™ 10X Primer Pair Mix	Store in the dark.
	Water, Amplification Grade	None
	2800M Control DNA	None
	Post-Amplification	Special Instructions
	Paradyme™ 27GY Allelic Ladder Mix	Store in the dark.
	CCO Internal Lane Standard 500	Store in the dark.

Box 2	Pre-Amplification	Special Instructions
(Store at –30°C to –10°C)	Paradyne™ 5X Master Mix	None
	TCEP, Concentrated	Store in the dark; can store at +2°C to +10°C after first thaw.
	TCEP Dilution Buffer	Store in the dark; can store at +2°C to +10°C after first thaw.

Always include a negative control reaction (e.g., no template) to detect reagent contamination. We highly recommend the use of gloves and aerosol-resistant pipette tips.

Some reagents used in the analysis of STR products are potentially hazardous and should be handled accordingly.

- The Paradyne™ 27GY 5X Master Mix contains a hazardous component; avoid oral exposure and contact with skin. Always wear appropriate PPE and use caution. Refer to Safety Data Sheet (SDS).
- TCEP is a hazardous material that can cause skin corrosion and eye damage. When handling TCEP, Concentrated and 25X TCEP Working Dilution, always wear appropriate PPE and use caution.
- Formamide is an irritant and a teratogen; avoid inhalation and contact with skin. Read the warning label, and take appropriate precautions when handling this substance. Always wear gloves and safety glasses when working with formamide.

3.2 Spectral Calibration

Proper spectral calibration is critical to evaluate multicolor systems with the Spectrum CE System. Generate a matrix for each individual instrument.

For protocol and additional information about matrix generation and spectral calibration, see the *8C Matrix Standards for Use on the Spectrum CE System Technical Manual #TMD068*. This manual is available online at: www.promega.com/protocols/

4 DNA Amplification Protocol Using the Paradyme™ 27GY STR System

The Paradyme™ 27GY STR System is optimized for use with purified DNA and direct-amplification samples. Suboptimal performance may be observed with crude lysates or unpurified extracts. Slight protocol variations are recommended for optimal performance with each template source. Adding the Paradyme™ 27GY QI Reagent to direct-amplification reactions may be necessary to ensure appropriate peak heights for the two Quality Indicators, QIS and QIL. Protocols for amplification in a 25µl reaction volume using extracted DNA (Section 4.1) and a 12.5µl reaction volume using lytic and nonlytic storage card punches (Section 4.2) and swabs (Section 4.3) are included in the following sections.

Protocols for amplification in a 25µl reaction volume using lytic and nonlytic storage card punches and swabs are included in Sections 10.3 and 10.4, respectively.

The Paradyme™ 27GY STR System is compatible with the ProFlex® and Mastercycler® X50s PCR Systems.

Note: Thermal cyclers other than the ones listed in this technical manual can be used. Using thermal cyclers not listed here requires optimization of cycling conditions and in-house validation.

We recommend using gloves and aerosol-resistant pipette tips to prevent cross contamination. Keep all pre- and post-amplification reagents in separate rooms. Prepare amplification reactions in a room dedicated for reaction setup. Use equipment and supplies dedicated for amplification setup.



Take meticulous care to ensure successful amplification. See Section 8 for amplification troubleshooting.

4.1 Amplifying Extracted DNA in a 25µl Reaction Volume

4.1.1 Materials to be Supplied by the User

- ProFlex® PCR System (Applied Biosystems)
- Mastercycler® X50s PCR System (Eppendorf)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plates or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips

We routinely amplify 0.5ng of template DNA in a 25µl reaction volume at 30 cycles using the following protocol.

4.1.2 Amplification Setup

1. Upon receipt, store the Paradyme™ 27GY reagents as follows:
 - Store the Paradyme™ 27GY 5X Master Mix, TCEP, Concentrated, and TCEP Dilution Buffer at –30°C to –10°C. TCEP, Concentrated and TCEP Dilution Buffer may be stored at +2°C to +10°C after the first thaw.
 - Store the Paradyme™ 27GY 10X Primer Pair Mix, Water, Amplification Grade and 2800M Control DNA at +2°C to +10°C.

Note: Thaw the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated (if frozen) completely before use. Ensure the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated are thoroughly mixed after thawing. Allow all pre-amplification components to equilibrate to room temperature before preparing the PCR amplification mix. Centrifuge tubes briefly to move contents to the tube 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 can concentrate the reagents at the bottom of the tube.

2. Prepare a 25X TCEP Working Dilution by combining the following components listed in Table 1.

Table 1. Preparing the 25X TCEP Working Dilution.

Component	Volume per Reaction	×	Number of Reactions	=	Final Volume
TCEP Dilution Buffer	0.975µl	×		=	
TCEP, Concentrated	0.025µl	×		=	

Notes:

- a. Use only the TCEP Dilution Buffer provided with the kit.
 - b. We do not recommend preparing the 25X TCEP Working Dilution for fewer than 80 reactions due to pipetting limitations and variability associated with small volumes. For example, an 80-reaction preparation requires 78µl of TCEP Dilution Buffer and 2µl of TCEP, Concentrated.
 - c. The 25X TCEP Working Dilution may be stored protected from light for up to 1 week at 4°C, or up to 1 month at –20°C. Vortex for 15 seconds before each use.
3. Determine the number of reactions to be set up, including positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach consumes a small amount of each reagent, it ensures that there is enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
 4. Use a new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new 0.2ml reaction tubes required, and label them appropriately.

5. Add the final volume of each reagent listed in Table 2 to a new tube.

Table 2. PCR Amplification Mix for 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	×		=	
25X TCEP Working Dilution	1.0µl				
Paradyme™ 27GY 5X Master Mix	5.0µl	×		=	
Paradyme™ 27GY 10X Primer Pair Mix	2.5µl	×		=	
template DNA (0.5ng) ^{2,3}	up to 16.5µl				
total reaction volume	25.0µl				

¹Add Water, Amplification Grade, to the tube first, and then add 25X TCEP Working Dilution, Paradyme™ 27GY 5X Master Mix and Paradyme™ 27GY 10X Primer Pair Mix. The template DNA will be added at Step 7.

²Store DNA templates in TE⁻⁴ buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE⁻⁴ buffer with 20µg/ml glycogen. PCR amplification efficiency and quality can be greatly altered by changes in pH (due to added Tris-HCl), available magnesium concentration (due to chelation by EDTA) or other PCR inhibitors, which may be present at low concentrations depending on the source of the template DNA and the extraction procedure used.

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

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



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

Notes:

- Protect samples from light before and after amplification. Light exposure increases likelihood for FAM dye channel artifacts, with a more pronounced effect prior to amplification.
- 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 immediately start thermal cycling.

7. Add the template DNA (0.5ng) for each sample to the respective well containing PCR amplification mix.

Note: The Paradyme™ 27GY STR System is optimized and balanced using 0.5ng of DNA template. The DNA template amount used should be based on internal validation results and may differ.

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

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

4.1.3 Thermal Cycling on the ProFlex® PCR System

Amplification and detection instrumentation can vary. You may need to optimize protocols for the template DNA amount, cycle number, injection conditions and loading volume for your laboratory instrumentation. Our tests have shown that 30 cycles work well for 0.5ng of purified DNA templates. Perform in-house validation.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the protocol provided below and in Figure 1. The total cycling time is approximately 70 minutes.

Notes:

- a. Set the ramp speed to 6°C/second and the reaction volume to 25µl.
- b. If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 2 minutes and
30 seconds, then:

97°C for 5 seconds
60°C for 1 minute
66°C for 15 seconds for
30 cycles, then:

60°C for 10 minutes

4°C hold

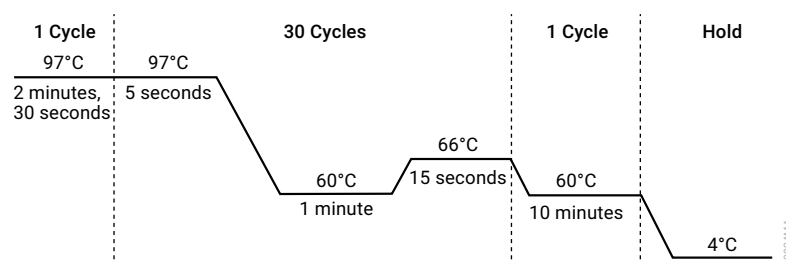


Figure 1. The thermal cycling protocol for the ProFlex® PCR System.

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

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

4.1.4 Thermal Cycling on the Mastercycler® X50s PCR System

Amplification and detection instrumentation can vary. You may need to optimize protocols for the template DNA amount, cycle number, injection conditions and loading volume for your laboratory instrumentation. Our tests have shown that 30 cycles work well for 0.5ng of purified DNA templates. Perform in-house validation.

- Place the reaction plate or tubes in the thermal cycler.
- Select and run the protocol provided below and in Figure 2. The total cycling time is approximately 75 minutes.

Notes:

- Set the ramp speed to $5^{\circ}\text{C}/\text{second}$, the temperature mode to “Safe” and the block setting to “Silver 96”.
- If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 3 minutes, then:

97°C for 10 seconds

60°C for 1 minute

66°C for 15 seconds for

30 cycles, then:

60°C for 10 minutes

4°C hold

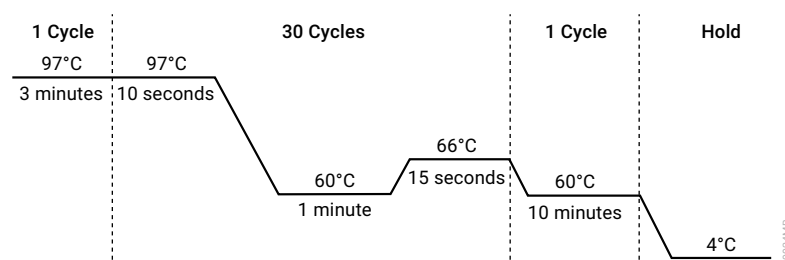


Figure 2. The thermal cycling protocol for the Mastercycler® X50s PCR System.

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

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

4.2 Directly Amplifying DNA from Storage Card Punches in a 12.5µl Reaction Volume

4.2.1 Materials to be Supplied by the User

- ProFlex® PCR System (Applied Biosystems)
- Mastercycler® X50s PCR System (Eppendorf)
- centrifuge compatible with 96-well plates or reaction tubes
- ClickFit Microtube, 1.5ml (Cat.# V4745)
- MicroAmp® optical 96-well reaction plates or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- SwabSolution™ Kit (Cat.# DC8271)
- PunchSolution™ Kit (Cat.# DC9271) for nonlytic card punches
- Paradyme™ 27GY QI Reagent (Cat.# DM1701)
- 1.2mm Harris Micro-Punch or equivalent manual punch and cutting mat or automated punch system

This section contains two protocols for amplifying DNA from storage card punches in a 12.5µl reaction volume using the Paradyme™ 27GY STR System on the ProFlex® and Mastercycler® X50s PCR Systems:

- SwabSolution™ processing of DNA from storage card punches followed by amplification. We recommend this protocol for blood and buccal samples on lytic and nonlytic storage cards.
- Direct amplification of DNA from storage card punches. We recommend this protocol for buccal samples only.

A protocol for directly amplifying DNA from storage card punches in a 25µl reaction volume is provided in Section 10.3.

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

Lytic storage card sample types include:

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

Nonlytic storage card sample types include:

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

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.

4.2.2 SwabSolution™ DNA Processing from Storage Card Punches

1. Using a 1.2mm Harris punch tool, create one punch (for blood samples) or two punches (for buccal samples) from the center of the stained area. This applies to both lytic and nonlytic cards. Place the punch(es) into a 1.5ml ClickFit microtube, a 0.2ml reaction tube, or a 96-well plate.
2. Add 150µl of SwabSolution™ Reagent to the tube or well, ensuring the punch is completely submerged, then cap it.

3. Place the reaction tube(s) or 96-well plate on a heat block and incubate at 70°C for 30 minutes.

Note: Using plates and tubes other than those recommended may affect heat transfer and lysis efficiency.

4. Following incubation, use the extract immediately or store at 4°C.

4.2.3 Amplification Setup for SwabSolution™ Extracts from Storage Card Punches

1. Upon receipt, store the Paradyme™ 27GY reagents as follows:
 - Store the Paradyme™ 27GY 5X Master Mix, TCEP, Concentrated, and TCEP Dilution Buffer at –30°C to –10°C. TCEP, Concentrated and TCEP Dilution Buffer may be stored at +2°C to +10°C after the first thaw.
 - Store the Paradyme™ 27GY 10X Primer Pair Mix, Water, Amplification Grade and 2800M Control DNA at +2°C to +10°C.

Note: Thaw the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated (if frozen) completely before use. Ensure the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated are thoroughly mixed after thawing. Allow all pre-amplification components to equilibrate to room temperature before preparing the PCR amplification mix. Centrifuge tubes briefly to move contents to the tube 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 can concentrate the reagents at the bottom of the tube.

2. Prepare a 25X TCEP Working Dilution by combining the following components listed in Table 3.

Table 3. Preparing the 25X TCEP Working Dilution.

Component	Volume per Reaction	×	Number of Reactions	=	Final Volume
TCEP Dilution Buffer	0.975µl	×		=	
TCEP, Concentrated	0.025µl	×		=	

Notes:

- a. Use only the TCEP Dilution Buffer provided with the kit.
- b. We do not recommend preparing the 25X TCEP Working Dilution for fewer than 80 reactions due to pipetting limitations and variability associated with small volumes. For example, an 80-reaction preparation requires 78µl of TCEP Dilution Buffer and 2µl of TCEP, Concentrated.
- c. The 25X TCEP Working Dilution may be stored protected from light for up to 1 week at 4°C, or up to 1 month at –20°C. Vortex for 15 seconds before each use.

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

Table 4. PCR Amplification Mix for Amplifying DNA from Storage Card Punches in a 12.5µl Reaction Volume.

PCR Amplification Mix Component ¹	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 12.5µl	×		=	
25X TCEP Working Dilution	0.5µl				
Paradyme™ 27GY 5X Master Mix	2.5µl	×		=	
Paradyme™ 27GY 10X Primer Pair Mix	1.25µl	×		=	
SwabSolution™ extract	up to 2.0µl				
total reaction volume	12.5µl				

¹Add Water, Amplification Grade, to the tube first, and then add 25X TCEP Working Dilution, Paradyme™ 27GY 5X Master Mix and Paradyme™ 27GY 10X Primer Pair Mix.

6. Vortex the PCR amplification mix for 5–10 seconds. If using the maximum volume of 2µl of SwabSolution™ extract, then pipet 10.5µl of PCR amplification mix into each reaction well.



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

Notes:

- a. Protect samples from light before and after amplification. Light exposure increases likelihood for FAM dye channel artifacts, with a more pronounced effect prior to amplification.
 - b. 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 immediately start thermal cycling.
7. Pipet up to 2µl of swab extract for each sample into the appropriate well of the reaction plate.

Note: Sample input volume can be adjusted to achieve the desired peak heights.

8. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 0.125ng/μl in the desired template DNA volume. Add 2μl (0.25ng total) of the 2800M Control DNA to a reaction well containing 10.5μl of PCR amplification mix.

Note: The control DNA input in Step 8 is optimized for 30 cycles. Optimization may be required, depending on cycling conditions and laboratory preferences.

9. For the negative amplification control, pipet 2μl of Water, Amplification Grade, or TE⁻⁴ buffer instead of SwabSolution™ extract into a reaction well containing 10.5μl of PCR amplification mix.

Note: Include additional negative extraction controls, if necessary. Assemble a reaction containing the SwabSolution™ extract prepared from a blank punch, or assemble a reaction where the SwabSolution™ Reagent is processed as a blank without a punch.

10. Seal or cap the plate, or close the tubes. Briefly centrifuge the reactions to remove any air bubbles.

4.2.4 Thermal Cycling on the ProFlex® PCR System

Amplification and detection instrumentation can vary. You will need to optimize protocols for cycle number (29–30 cycles), injection conditions and loading volume for your laboratory instrumentation. Tests at Promega show that 30 cycles work well for a variety of sample types. Perform in-house validation.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the protocol provided below and in Figure 3. The total cycling time is approximately 70 minutes.

Notes:

- a. Set the ramp speed to 6°C/second and the reaction volume to 13μl.
- b. If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 2 minutes and
30 seconds, then:

97°C for 5 seconds
60°C for 1 minute
66°C for 15 seconds
for 30 cycles, then:

60°C for 10 minutes

4°C hold

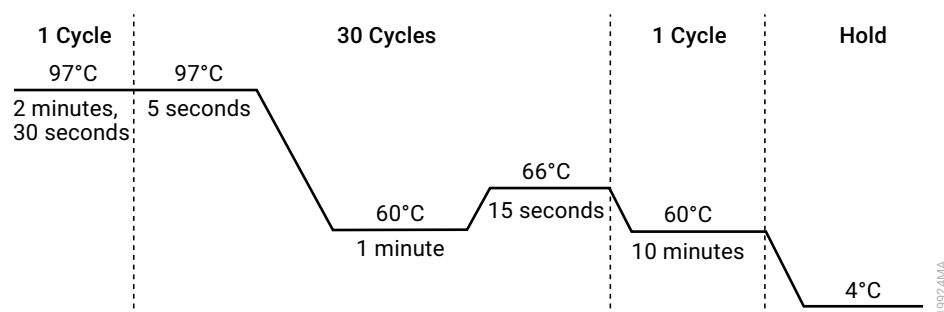


Figure 3. Thermal cycling protocol for the ProFlex® PCR System.

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

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

4.2.5 Thermal Cycling on the Mastercycler® X50s PCR System

Amplification and detection instrumentation can vary. You will need to optimize protocols for cycle number (29–30 cycles), injection conditions and loading volume for your laboratory instrumentation. Tests at Promega show that 30 cycles work well for a variety of sample types. Perform in-house validation.

- Place the reaction plate or tubes in the thermal cycler.
- Select and run the protocol provided below and in Figure 4. The total cycling time is approximately 75 minutes.

Notes:

- Set the ramp speed to $5^{\circ}\text{C}/\text{second}$, the temperature mode to “Safe” and the block setting to “Silver 96”.
- If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 3 minutes, then:

97°C for 10 seconds

60°C for 1 minute

66°C for 15 seconds

for 30 cycles, then:

60°C for 10 minutes

4°C hold

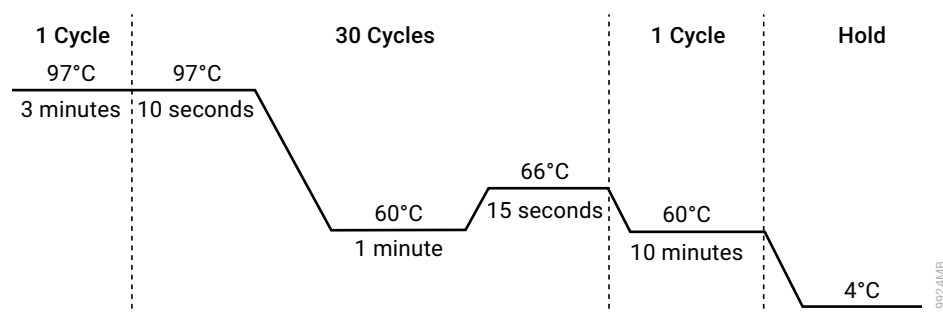


Figure 4. Thermal cycling protocol for the Mastercycler® X50s System.

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

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

4.2.6 Amplification Setup for Direct Amplification of Buccal DNA from Storage Card Punches

Note: This protocol is specific for buccal samples on lytic and nonlytic cards.

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

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

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

1. Upon receipt, store the Paradyme™ 27GY reagents as follows:
 - Store the Paradyme™ 27GY 5X Master Mix, TCEP, Concentrated, and TCEP Dilution Buffer at -30°C to -10°C . TCEP, Concentrated and TCEP Dilution Buffer may be stored at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$ after the first thaw.
 - Store the Paradyme™ 27GY 10X Primer Pair Mix, Water, Amplification Grade and 2800M Control DNA at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$.

Note: Thaw the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated (if frozen) completely before use. Ensure the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated are thoroughly mixed after thawing. Allow all pre-amplification components to equilibrate to room temperature before preparing the PCR amplification mix. Centrifuge tubes briefly to move contents to the tube 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 can concentrate the reagents at the bottom of the tube.

2. Prepare a 25X TCEP Working Dilution by combining the following components listed in Table 5.

Table 5. Preparing the 25X TCEP Working Dilution.

Component	Volume per Reaction	×	Number of Reactions	=	Final Volume
TCEP Dilution Buffer	0.975µl	×		=	
TCEP, Concentrated	0.025µl	×		=	

Notes:

- a. Use only the TCEP Dilution Buffer provided with the kit.
- b. We do not recommend preparing the 25X TCEP Working Dilution for fewer than 80 reactions due to pipetting limitations and variability associated with small volumes. For example, an 80-reaction preparation requires 78µl of TCEP Dilution Buffer and 2µl of TCEP, Concentrated.
- c. The 25X TCEP Working Dilution may be stored protected from light for up to 1 week at 4°C, or up to 1 month at –20°C. Vortex for 15 seconds before each use.
3. Determine the number of reactions to be set up, including positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach consumes a small amount of each reagent, it ensures that there is enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
4. Use a new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new 0.2ml reaction tubes required, and label them appropriately.

5. Add the final volume of each reagent listed in Table 6 to a new tube. The Paradyme™ 27GY QI Reagent volume will depend on the cycle number:
 - For 25 cycles, use 1µl of Paradyme™ 27GY QI Reagent
 - For 26 cycles, use 0.5µl of Paradyme™ 27GY QI Reagent

Table 6. PCR Amplification Mix for Directly Amplifying DNA from Storage Card Punches in a 12.5µl Reaction Volume.

PCR Amplification Mix Component ¹	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 12.5µl	×		=	
25X TCEP Working Dilution	0.5µl				
Paradyme™ 27GY 5X Master Mix	2.5µl	×		=	
Paradyme™ 27GY 10X Primer Pair Mix	1.25µl	×		=	
Paradyme™ 27GY QI Reagent	up to 1.0µl				
total reaction volume	12.5µl				

¹Add Water, Amplification Grade, to the tube first, and then add 25X TCEP Working Dilution, Paradyme™ 27GY 5X Master Mix, Paradyme™ 27GY 10X Primer Pair Mix and Paradyme™ 27GY QI Reagent. For lytic card punches, the template DNA will be added at Step 7.

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



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

Notes:

- a. Protect samples from light before and after amplification. Light exposure increases likelihood for FAM dye channel artifacts, with a more pronounced effect prior to amplification.
 - b. 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 immediately start thermal cycling.
7. For lytic storage cards, add one 1.2mm punch from a card containing buccal cells to the appropriate wells of the reaction plate. For nonlytic card punches, add the PCR amplification mix to the PunchSolution™ Reagent-treated punch.

Note: You can also add the lytic card punch first, and then add the PCR amplification mix.

8. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 2ng/μl in the desired template DNA volume. Add 2μl (4ng total) of the 2800M Control DNA to a reaction well containing 10.5μl of PCR amplification mix.

Notes:

- a. The Control DNA input in Step 8 is optimized for 26 cycles. Optimization may be required depending on cycling conditions and laboratory preferences.
 - b. Do not include blank storage card punches in the positive control reactions.
9. Reserve a well containing PCR amplification mix as a negative amplification control.

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

10. Seal or cap the plate, or close the tubes. Briefly centrifuge the reactions to move storage card punches to the well bottoms and remove any air bubbles.

4.2.7 Thermal Cycling on the ProFlex® PCR System

Amplification and detection instrumentation can vary. You will need to optimize protocols for the number of storage card punches, cycle number (25–26 cycles), injection conditions and loading volume for your laboratory instrumentation. Tests at Promega show that 26 cycles work well for a variety of sample types. Perform in-house validation.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the protocol provided below and in Figure 5. The total cycling time is approximately 65 minutes.

Notes:

- a. Set the ramp speed to 6°C/second and the reaction volume to 13μl.
- b. If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 2 minutes and
30 seconds, then:

97°C for 5 seconds
60°C for 1 minute
66°C for 15 seconds
for 26 cycles, then:

60°C for 10 minutes

4°C hold

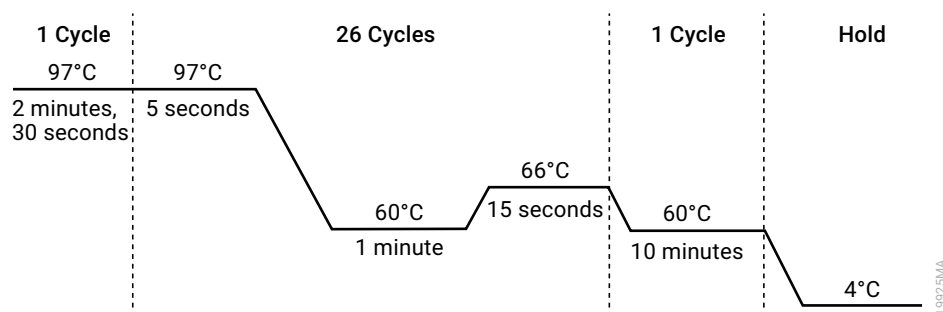


Figure 5. Thermal cycling protocol for the ProFlex® PCR System.

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

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

4.2.8 Thermal Cycling on the Mastercycler® X50s PCR System

Amplification and detection instrumentation can vary. You will need to optimize protocols for the number of storage card punches, cycle number (25–26 cycles), injection conditions and loading volume for your laboratory instrumentation. Tests at Promega show that 26 cycles work well for a variety of sample types. Perform in-house validation.

- Place the reaction plate or tubes in the thermal cycler.
- Select and run the protocol provided below and in Figure 6. The total cycling time is approximately 70 minutes.

Notes:

- Set the ramp speed to $5^{\circ}\text{C}/\text{second}$, the temperature mode to “Safe” and the block setting to “Silver 96”.
- If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 3 minutes, then:

97°C for 10 seconds

60°C for 1 minute

66°C for 15 seconds

for 26 cycles, then:

60°C for 10 minutes

4°C hold

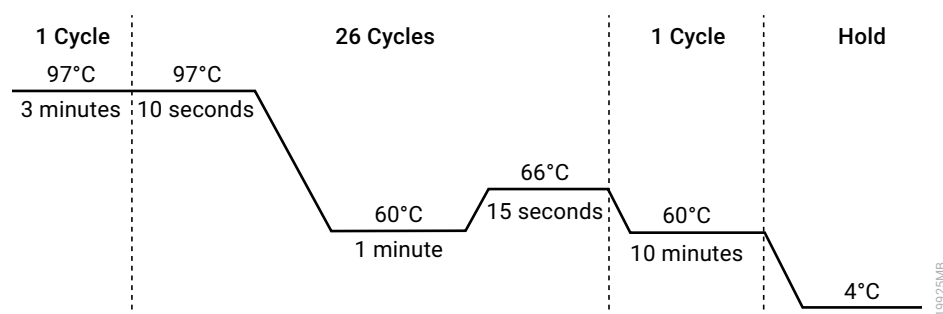


Figure 6. Thermal cycling protocol for the Mastercycler® X50s System.

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

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

4.2.9 PCR Optimization

Optimize the cycle number 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 using your normal workflow.
2. Place one 1.2mm storage card punch containing buccal cells in each well of a reaction plate. Be sure to pretreat nonlytic samples with the PunchSolution™ Kit (Cat.# DC9271).
3. Prepare two reaction plates with punches from the same samples. Use the appropriate amount of Paradyme™ 27GY QI reagent (Cat.# DM1701) for the desired cycle number.
4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (25–26 cycles).
5. Following amplification, use the laboratory validated separation and detection protocols to determine the optimal cycle number for the sample type.

4.3 Directly Amplifying DNA from Swabs in a 12.5µl Reaction Volume

4.3.1 Materials to be Supplied by the User

- ProFlex® PCR System (Applied Biosystems)
- Mastercycler® X50s PCR System (Eppendorf)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plates or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- Paradyme™ 27GY QI Reagent (Cat.# DM1701)
- SwabSolution™ Kit (Cat.# DC8271)

This section contains a protocol for amplifying DNA from swab extracts in a 12.5µl reaction volume using the ProFlex® and Mastercycler® X50s PCR Systems. Protocols for directly amplifying DNA from swab extracts in a 25µl reaction volume are provided in Section 10.4.

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

4.3.2 Amplification Setup

1. Upon receipt, store the Paradyme™ 27GY reagents as follows:
 - Store the Paradyme™ 27GY 5X Master Mix, TCEP, Concentrated, and TCEP Dilution Buffer at –30°C to –10°C. TCEP, Concentrated and TCEP Dilution Buffer may be stored at +2°C to +10°C after the first thaw.
 - Store the Paradyme™ 27GY 10X Primer Pair Mix, Water, Amplification Grade and 2800M Control DNA at +2°C to +10°C.

Note: Thaw the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated (if frozen) completely before use. Ensure the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated are thoroughly mixed after thawing. Allow all pre-amplification components to equilibrate to room temperature before preparing the PCR amplification mix. Centrifuge tubes briefly to move contents to the tube 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 can concentrate the reagents at the bottom of the tube.

2. Prepare a 25X TCEP Working Dilution by combining the following components listed in Table 7.

Table 7. Preparing the 25X TCEP Working Dilution.

Component	Volume per Reaction	×	Number of Reactions	=	Final Volume
TCEP Dilution Buffer	0.975µl	×		=	
TCEP, Concentrated	0.025µl	×		=	

Notes:

- a. Use only the TCEP Dilution Buffer provided with the kit.
 - b. We do not recommend preparing the 25X TCEP Working Dilution for fewer than 80 reactions due to pipetting limitations and variability associated with small volumes. For example, an 80-reaction preparation requires 78µl of TCEP Dilution Buffer and 2µl of TCEP, Concentrated.
 - c. The 25X TCEP Working Dilution may be stored protected from light for up to 1 week at 4°C, or up to 1 month at –20°C. Vortex for 15 seconds before each use.
3. Determine the number of reactions to be set up, including positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach consumes a small amount of each reagent, it ensures that there is enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
 4. Use a new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new, 0.2ml reaction tubes required, and label them appropriately.

5. Add the final volume of each reagent listed in Table 8 to a new tube. The Paradyme™ 27GY QI Reagent volume will depend on the cycle number:
 - For 24 cycles, use 2µl of Paradyme™ 27GY QI Reagent
 - For 25 cycles, use 1µl of Paradyme™ 27GY QI Reagent
 - For 26 cycles, use 0.5µl of Paradyme™ 27GY QI Reagent

Table 8. PCR Amplification Mix for Directly Amplifying DNA from Swabs in a 12.5µl Reaction Volume.

PCR Amplification Mix Component ¹	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 12.50µl	×		=	
25X TCEP Working Dilution	0.50µl	×		=	
Paradyme™ 27GY 5X Master Mix	2.50µl	×		=	
Paradyme™ 27GY 10X Primer Pair Mix	1.25µl				
Paradyme™ 27GY QI Reagent	up to 2.00µl				
swab extract	up to 2.00µl				
total reaction volume	12.50µl				

¹Add Water, Amplification Grade, to the tube first, and then add 25X TCEP Working Dilution, Paradyme™ 27GY 5X Master Mix and Paradyme™ 27GY 10X Primer Pair Mix and Paradyme™ 27GY QI Reagent. The swab extract will be added at Step 7.

6. Vortex the PCR amplification mix for 5–10 seconds. If using the maximum volume of 2µl of swab extract, pipet 10.5µl of PCR amplification mix into each reaction well.



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

Note:

- a. Protect samples from light before and after amplification. Light exposure increases likelihood for FAM dye channel artifacts, with a more pronounced effect prior to amplification.
 - b. 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 immediately start thermal cycling.
7. Pipet up to 2µl of swab extract for each sample into the appropriate well of the reaction plate.

8. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 4ng/μl in the desired template DNA volume. Add 2μl (8ng total) of the 2800M Control DNA to a reaction well containing 10.5μl of PCR amplification mix.

Note: The Control DNA input in Step 8 is optimized for 25 cycles. Optimization may be required, depending on thermal cycling conditions and laboratory preferences.

9. For the negative amplification control, pipet 2μl of Water, Amplification Grade, or TE⁻⁴ buffer instead of swab extract into a reaction well containing 10.5μl of PCR amplification mix.

Note: Include additional negative controls if necessary. 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.

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

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

4.3.3 Thermal Cycling on the ProFlex® PCR System

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

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the protocol provided below and in Figure 7. The total cycling time is approximately 65 minutes.

Notes:

- a. Set the ramp speed to 6°C/second and the reaction volume to 13μl.
- b. If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 2 minutes and
30 seconds, then:

97°C for 5 seconds
60°C for 1 minute
66°C for 15 seconds
for 25 cycles, then:

60°C for 10 minutes

4°C hold

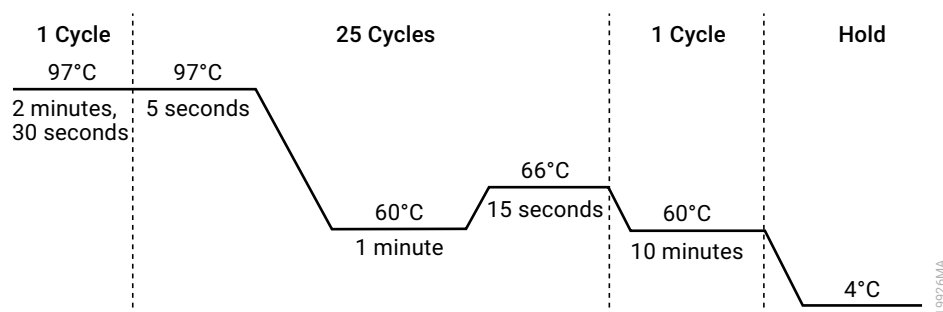


Figure 7. Thermal cycling protocol for the ProFlex® PCR System.

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

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

4.3.4 Thermal Cycling on the Mastercycler® X50s PCR System

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

- Place the reaction plate or tubes in the thermal cycler.
- Select and run the protocol provided below and in Figure 8. The total cycling time is approximately 70 minutes.

Notes:

- Set the ramp speed to $5^{\circ}\text{C}/\text{second}$, the temperature mode to “Safe” and the block setting to “Silver 96”.
- If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 3 minutes, then:

97°C for 10 seconds

60°C for 1 minute

66°C for 15 seconds

for 25 cycles, then:

60°C for 10 minutes

4°C hold

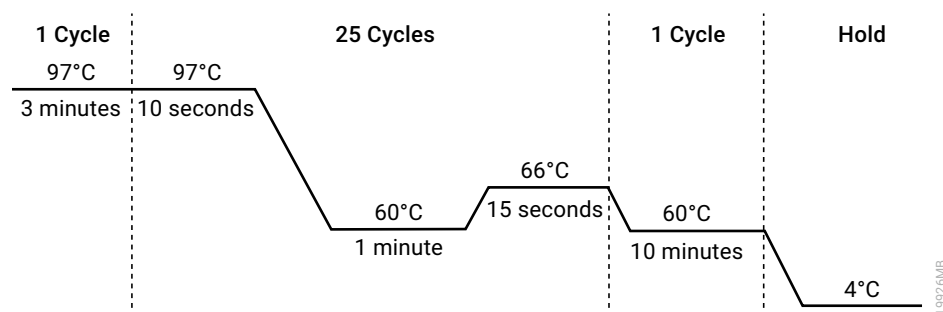


Figure 8. Thermal cycling protocol for the Mastercycler® X50s PCR System.

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

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

4.3.5 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 using your normal workflow.
2. Prepare three identical reaction plates with aliquots of the same swab extracts. Use the appropriate amount of Paradyme™ 27GY QI Reagent (Cat.# DM1701) for the desired cycle number.
3. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (24–26 cycles).

Note: This recommendation is for 2µl of swab extract. If a different volume of swab extract is used, additional cycle number testing may be required.

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

5 Detecting Amplified Fragments Using the Spectrum CE System

Materials to be Supplied by the User

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



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



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

Note: Wear gloves when handling consumables.

5.1 Preparing Samples

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

Note: Adjust the internal lane standard volume in the loading cocktail to change the size standard peak intensity based on laboratory preferences.

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

Notes:

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

5.2 Preparing the Instrument

Refer to the *Spectrum CE System Operating Manual #TMD052* for the instrument maintenance schedule and instructions to install the capillary array, anode buffer, cathode buffer and polymer bottle. Perform and activate the appropriate spectral calibration on the instrument before using it for samples. For instructions on performing a spectral calibration, see the *8C Matrix Standard for Use on the Spectrum CE System Technical Manual #TMD068*.

Open the Spectrum Control Software (SCS). The 'Home' screen appears upon launch (Figure 9), providing access to four workflow menus (Home, Review Plates, Maintenance and Settings; see Figure 10) and status indicators. The screen is divided into three sections: Header, Task Pane and Status Bar.

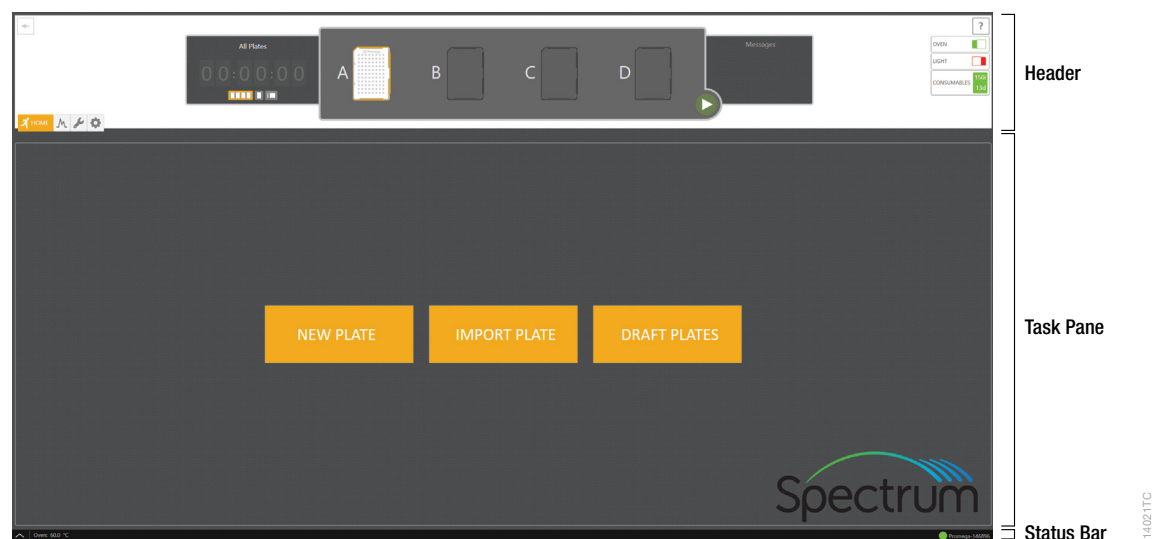


Figure 9. Spectrum Control Software 'Home' screen.

The Header (Figure 10) is fixed and remains available to the user throughout all navigation processes. This area contains Status Indicators, Message Center, Start Button, Run Time Indicator and Workflow Menu. The Header also shows the status of the plate positions within the Plate Drawer.

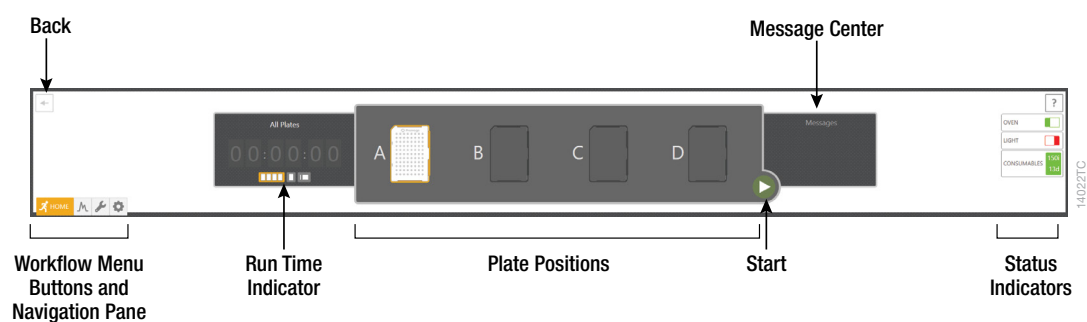


Figure 10. Header.

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

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

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

Before starting a run, ensure all consumables are installed and in sufficient supply. For best results, use unexpired reagents that are within the recommended use range. Refer to the 'Consumables' screen (Figure 11) to determine if any consumables need to be replaced. To access the 'Consumables' screen, select the Consumables status indicator in the Header on the SCS screen (Figure 10). The 'Consumables' screen (Figure 11) displays information for the four RFID-tagged consumables on the instrument: Polymer, Capillary Array and Anode and Cathode Buffers.

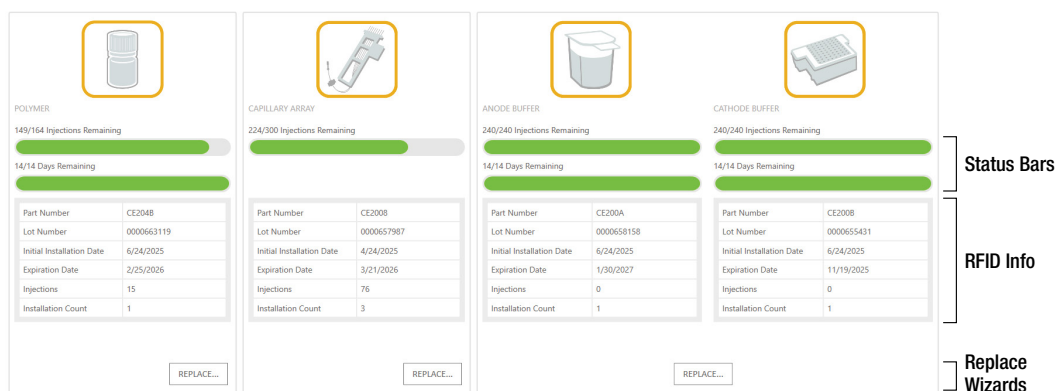


Figure 11. 'Consumables' screen.

The following information is tracked through these tags:

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

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

Select **Oven Heater Indicator** in the Header (Figure 10) to start preheating the oven to 60°C. The oven temperature will be displayed in the status bar at the bottom of the screen and the indicator will change from red to green when the oven preheating is complete.

Notes:

- a. Preheat the oven for a minimum of 2 hours prior to starting a run.
- b. The oven will automatically turn off after 2 hours if a run is not started. Inspect the fluid block, tubing, polymer syringe and polymer bottle for bubbles, and perform a bubble purge if necessary.

5.3 Creating a Plate Record

On the 'Home' screen, there are three options displayed for plate setup: New Plate, Import Plate and Draft Plates (Figure 9).

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

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

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

1. Select **New Plate** from the 'Home' screen. This will open a blank 'Plate' screen that is divided into three sections: Plate Information, Sample Information and Injection Information (Figure 12).

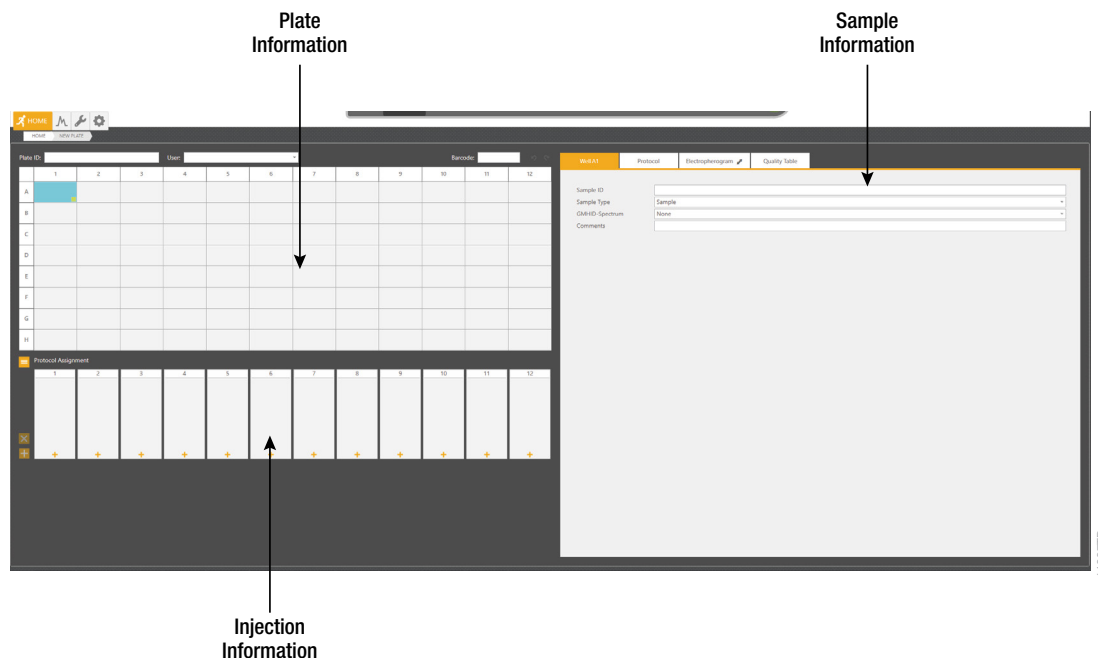


Figure 12. 'Plate' screen.

2. Use the default Plate ID that includes date and time or enter information into the 'Plate ID' field (Figure 13).
3. If desired, enter user information into the 'User' field (Figure 13).

Note: The 'User' field is a user-defined section that can be used to search or filter previously created or completed plates (refer to the *Spectrum CE System Operating Manual #TMD052* for information on reviewing plate records). Previously entered user information is available via the drop-down option on the right side of the field (Figure 13).

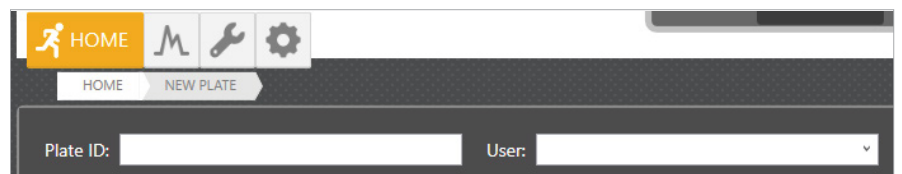


Figure 13. 'Plate ID' and 'User' fields of the plate screen.

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

Alternatively, enter Sample IDs in the 'Sample ID' field of the Sample Information section (Figure 12).

The green box in the lower right corner of a sample well (Figure 14) can be used to fill other wells with the same information by left clicking and dragging the green box to the other wells.

Select and drag to fill other wells with the same information.

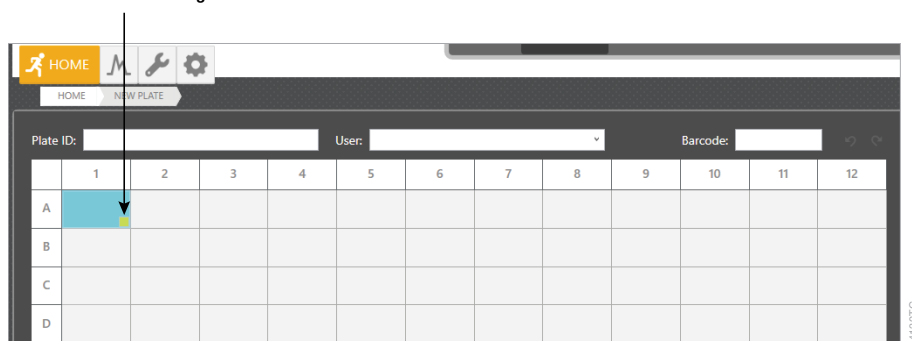


Figure 14. Filling information across multiple sample wells.

5. Select sample types for each sample or group of samples on the plate. Select the sample(s) then use the drop-down menu (Figure 15) to designate the well(s) as Sample, Ladder, Positive Control or Negative Control. These designations will be included in the output file for downstream analysis.

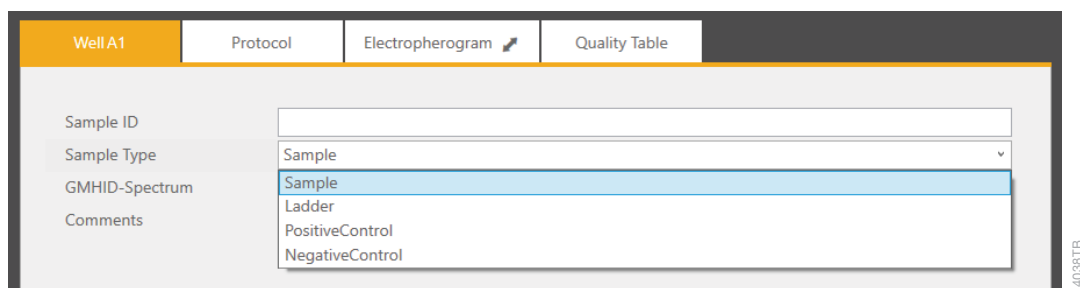


Figure 15. Drop-down options for sample type.

6. **Optional:** Select **Paradyne_27GY** in the GMHID-Spectrum drop-down menu (Figure 16).

Notes:

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

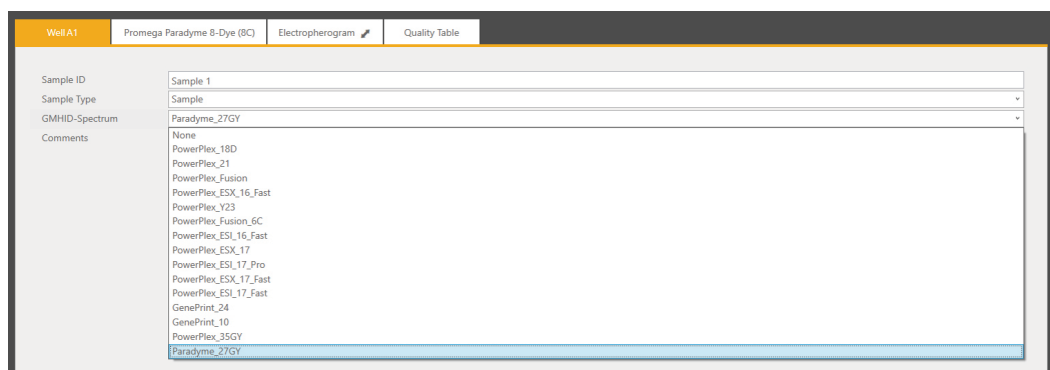


Figure 16. Drop-down options for GeneMarker®HID Software for Spectrum CE Systems run wizard templates.

7. When a run protocol has been assigned to an injection set, an injection number will be assigned and displayed in a circle within the injection set box. There are three methods to assign run protocol(s) to samples:
 - To add a run protocol to selected samples, highlight the desired wells (Ctrl + right-click or drag with the mouse). Then right-click, mouse over **Add Injection To Selected Wells** then select the **Promega Paradyne 8-Dye (8C)** protocol from the pop-out menu.
 - To add a run protocol to individual injections, select the **+** button under the box for that injection set then select the **Promega Paradyne 8-Dye (8C)** protocol from the pop-out menu.
 - To add a run protocol to multiple injections, highlight the desired injections in the plate map (click-drag, Ctrl + left-click or click the upper left cell). Then select the **+** button on the left side of the injection information section and select **Promega Paradyne 8-Dye (8C)** (Figure 17) from the pop-out menu.

Notes:

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

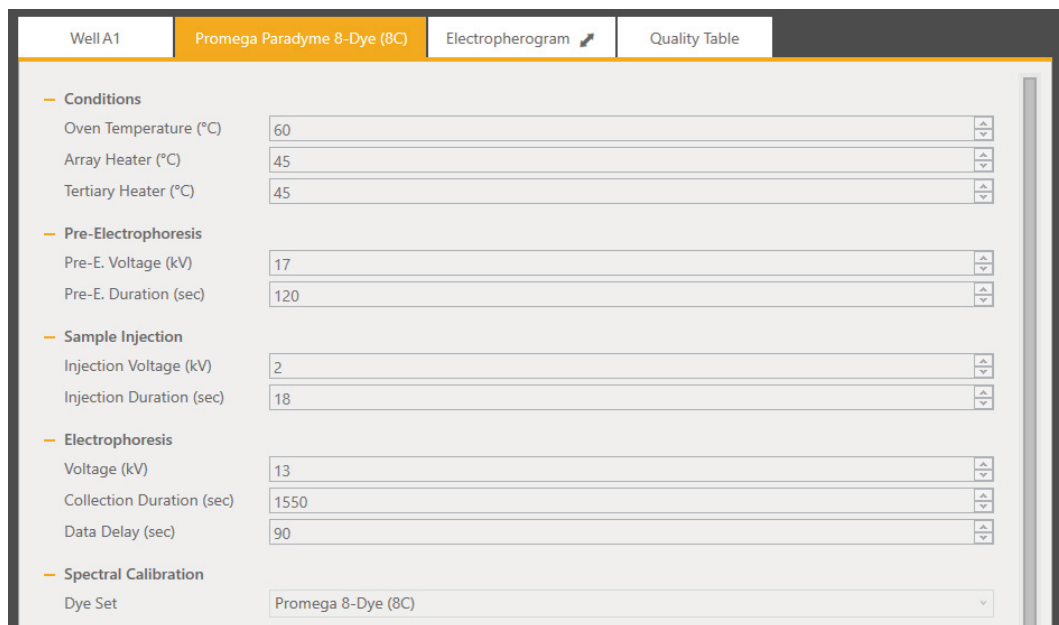
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8. Verify the assigned run protocol(s).

To verify the parameters of the assigned run protocol(s), select the injection number or a sample well in the plate map then select the 'Protocol' tab of the Sample Information section (Figure 18), which now displays the name of the assigned protocol. The information in this tab is read-only.

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



Well A1	Promega Paradyne 8-Dye (8C)	Electropherogram	Quality Table
Conditions			
Oven Temperature (°C)	60		
Array Heater (°C)	45		
Tertiary Heater (°C)	45		
Pre-Electrophoresis			
Pre-E. Voltage (kV)	17		
Pre-E. Duration (sec)	120		
Sample Injection			
Injection Voltage (kV)	2		
Injection Duration (sec)	18		
Electrophoresis			
Voltage (kV)	13		
Collection Duration (sec)	1550		
Data Delay (sec)	90		
Spectral Calibration			
Dye Set	Promega 8-Dye (8C)		

Figure 18. The 'Protocol' tab of the Sample Information section.

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

5.4 Preparing and Loading the Plate Assembly

1. Place the 96-well plate created in Section 5.1 into the Spectrum plate base, lining up the notch above well A12 with the notch on the base.
2. To complete the plate assembly, place the Spectrum plate retainer over the plate/base assembly, lining up the notch on the retainer with the notch on the plate and base. Verify that the retainer is locked in place on both sides of the plate, sitting evenly on top of the base (Figure 19).



Figure 19. The Spectrum plate assembly.

3. Confirm the drawer handle light is illuminated, indicating that the drawer is unlocked and ready for plate loading. Alternatively, check the Message Center to verify that the drawer is unlocked. The Drawer Status indicator is in the Message Center located in the Header (Figure 10).
4. Check the Plate Position Status indicator to note which of the four plate positions (A, B, C or D) is open and does not already contain a plate.
5. Open the plate drawer and place the plate assembly in an open plate position, verify that the notch on the plate base retainer aligns with the notch in the plate drawer then close the drawer.

Notes:

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

5.5 Linking a Plate for a Run

1. A plate record must be linked to the plate before adding it to the run queue.
2. Open the draft plate record, then select **Link** in the Plate Position indicator under the position where the plate was placed. Once the plate is successfully linked, the text under the position will change status to **Unlink** and the Plate ID will appear above the position (Figure 20).
3. Select the **Start** button to begin the run.

Notes:

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

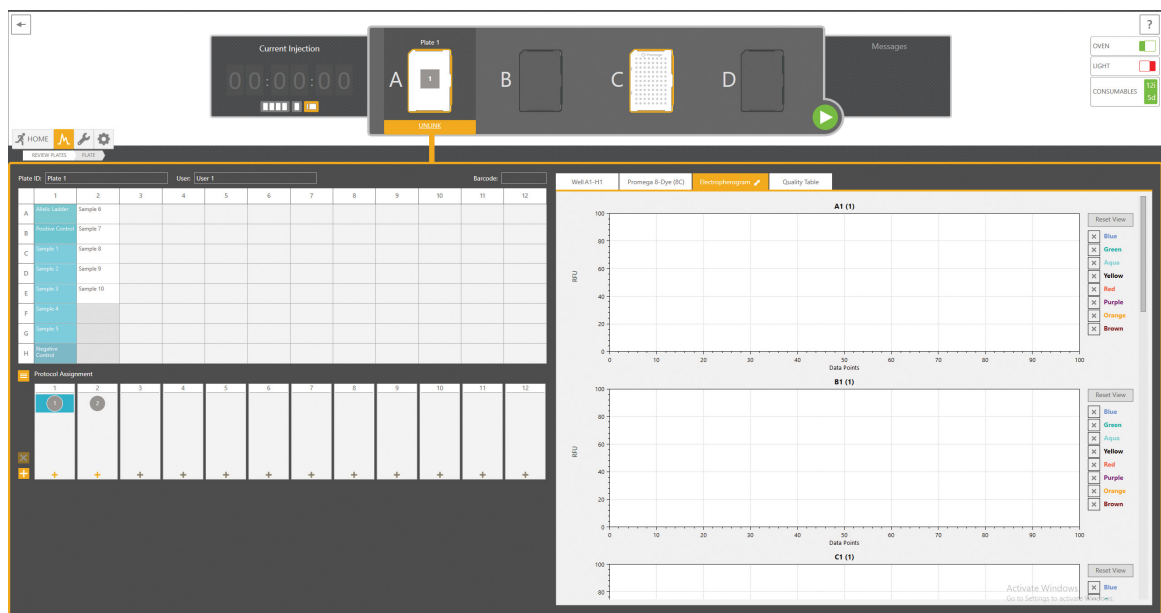


Figure 20. Linked plate in the Spectrum Control Software.

5.6 Monitoring a Run

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

5.7 Exporting Data

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

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

6 Data Analysis Using GeneMarker®HID Software

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

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

The current Paradyme27GY.xml panel files are available at:

www.promega.com/resources/software-firmware/str-analysis/panels-and-bin-sets/

1. Open GMHID-Spectrum.
2. To access your data files, select **Open Data** in the Magic Wizard or under the File menu.
3. Select **Add**, then navigate to the directory containing your raw data files and select the .promega files.
4. Select **Open**, and the selected files will appear in the Data File List (Figure 21).

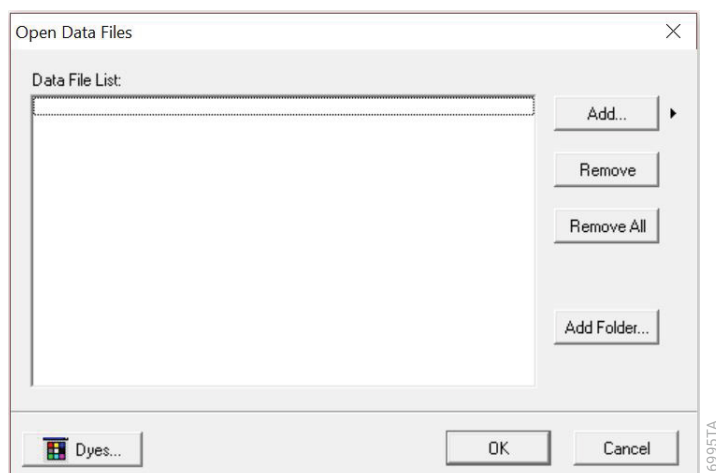


Figure 21. The GMHID-Spectrum Data File List.

5. Select **OK** in the 'Open Data Files' window, and the data will be imported into GMHID-Spectrum. In the 'Raw Data' folder in the File Navigator, verify that the sample types (ladder sample, positive control and negative control) assigned in Section 5 are designated correctly. If sample types are not correct, designate sample types by right-clicking on the file name and selecting **Set Sample Type**.
6. Use the Panel Editor in the Tools menu to import the Paradyme_27GY panel by selecting **File** and then **Import Panels**. Refer to *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555* for more information (Section 8.D.3).
7. Select the Paradyme_27GY panel from the Panel Template list. Click on the plus symbol to expand the list, select a marker name, then right-click and select **Edit**. Enter laboratory-specified values for Min Homozygote Intensity, Homozygote Inconclusive Range, Min Heterozygote Intensity, Heterozygote Inconclusive Range, Min Heterozygote Imbalance and Max Heterozygote Imbalance (Figure 22). This will set values for peaks within the marker range. These settings can be applied to all markers by checking the "Apply Homo/Hetero Settings to All Markers" check box, or by dye channel by checking the "Apply Homo/Hetero Settings to Markers in this dye" check box.

There are two options for setting stutter filters: marker-specific and allele-specific. To use marker-specific stutter filters, select **Use Marker-Specific Values**. Marker-specific stutter filters are preloaded in the Paradyme_27GY panel file. Stutter filters for one marker can be applied to all markers in a kit by checking the box "Apply Stutter Settings to All Markers". To use allele-specific values, select the radio button next to "Use Allele-Specific Values (From Panel)" in the 'Edit Marker' window, then enter the appropriate stutter filters in the Panel Table of the 'Panel Editor' window. For more information, refer to *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555*.

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

8. Select **OK** and close the 'Panel Editor' window. Select **Save Changes** or **Save as New Panel** under the File menu then close the window.

Edit Marker [X]

Marker Parameters:

Marker Name:

Nucleotide Repeats (x):

Boundary: To

Min Homozygote Intensity:

< = Inconclusive <=

Min Heterozygote Intensity:

< = Inconclusive <=

Max Heterozygote Imbalance(%):

Min Heterozygote Imbalance(%):

☐ Apply Homo/Hetero Settings to All Markers

☐ Apply Homo/Hetero Settings to Markers in this dye

Stutter Filter:

☒ Use Marker-Specific Values

N - 2x %

N + x % N - x %

N+0.5x % N-0.5x %

☐ Use Allele-Specific Values(From Panel)

☐ Apply Stutter Settings to All Markers

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Figure 22. The 'Edit Marker' window for the D3S1358 marker.

9. Select **Run** in the Magic Wizard or click the **Run Project** icon (green arrow) in the toolbar. The 'Template Selection' screen will appear (Figure 23). Select the Paradyme_27GY template and the settings shown in Figure 23. If the Paradyme_27GY template is not available, check the "Select an existing template or create one" box and provide a new name. Select the Paradyme_27GY panel that you created in Steps 6–8 from the panel drop-down field. The Size Standard must be ILS500 and the Standard Color must be Brown. Select **Save** to ensure this template will be stored among the other analysis run templates. Select **Next**.

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

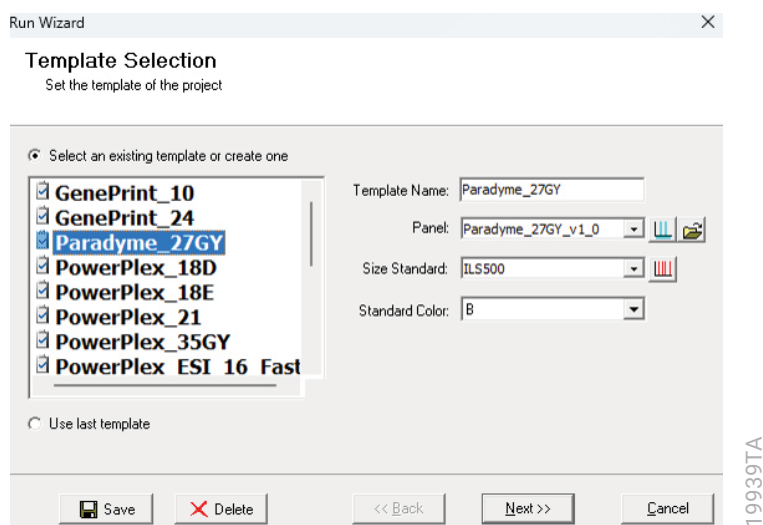


Figure 23. The 'Template Selection' window.

10. The 'Data Process – HID Analysis' window will appear (Figure 24). For the Raw Data Analysis, we recommend using Auto Range, Smooth, Superior Baseline Subtraction and Local Southern for the Size Call.

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

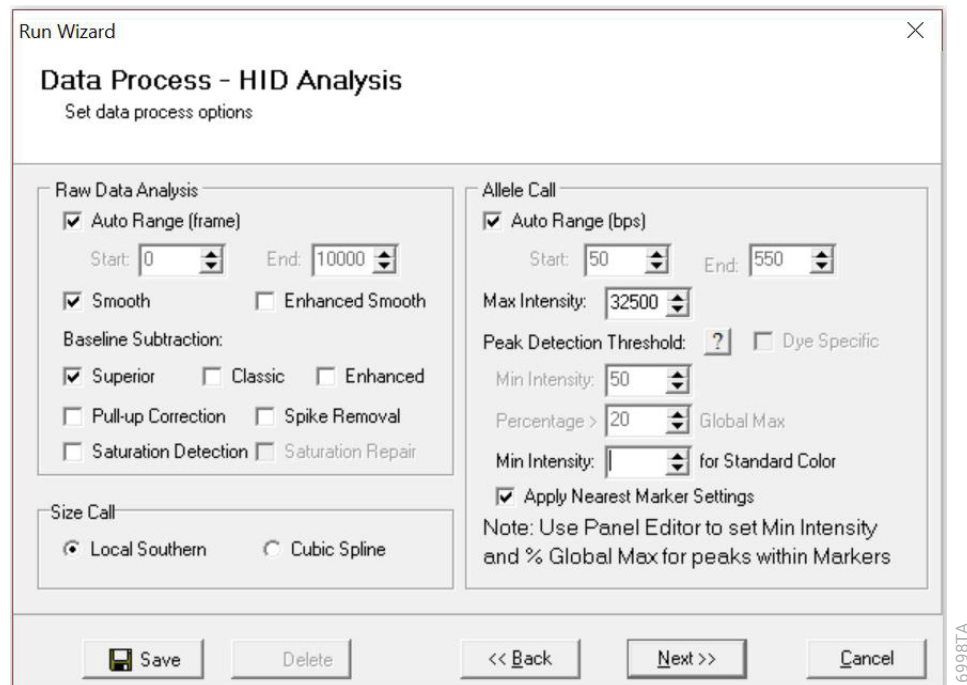


Figure 24. The 'Data Process' window for an analysis method.

11. The 'Additional Settings – HID Analysis' window will appear (Figure 25). Select **PD27GY_2800M** as the P.C. Template 1 and verify that the boxes are checked for "Auto Select Best Ladder", "Auto Panel Adjustment" and "Sample Quality Peak(s)". If the Paradyme 27GY positive control template is not available, consult the *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555* and use the Positive Control Template Editor to set up a new template. Ensure that "QIS:QIS" is selected for the Small Allele and "QIL:QIL" for the Large Allele in the Sample Quality Peak(s) Section. The values displayed in the Allele Evaluation dialogue box are defaults and will affect the quality values displayed in the plot settings. For more information on quality values, use of a second positive control and mixture evaluation, refer to the *GeneMarker®HID Software for Spectrum CE Systems User Manual #TM555*. Select **OK** to analyze the raw data.

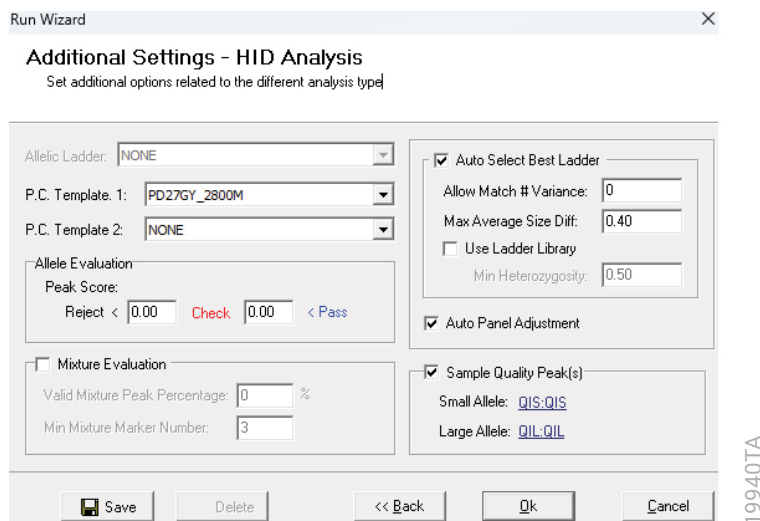


Figure 25. The 'Additional Settings' window for an analysis method.

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

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

6.2 Controls in GeneMarker®HID Software for Spectrum CE Systems

1. Observe the results for the negative control. Using the protocols defined in this manual, the negative controls should be devoid of amplification products except for QIS and QIL.
2. Observe the results for the 2800M Control DNA. The expected 2800M allele designations for each locus are listed in Table 14 (Section 10.1).

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

The Paradyme™ 27GY STR System includes PCR primers and synthetic DNA templates used to amplify two Quality Indicators, assisting in STR data analysis and interpretation. The Quality Indicator Small (QIS) migrates at approximately 75bp while the Quality Indicator Large (QIL) migrates at approximately 435bp. QIS and QIL cover the lower and higher base pair range of the amplification product. The presence or absence of QIS and QIL can be used to assess the overall success of the PCR amplification while their relative peak heights are also useful in determining sample inhibition or degradation. Table 9 provides additional information to assist with the interpretation of QIS and QIL data.

Table 9. Interpretation of QIS and QIL Peaks.

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

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

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

7 Results

Representative results of the Paradyme™ 27GY STR System are shown in Figure 26. The Paradyme™ 27GY Allelic Ladder Mix is shown in Figure 27.

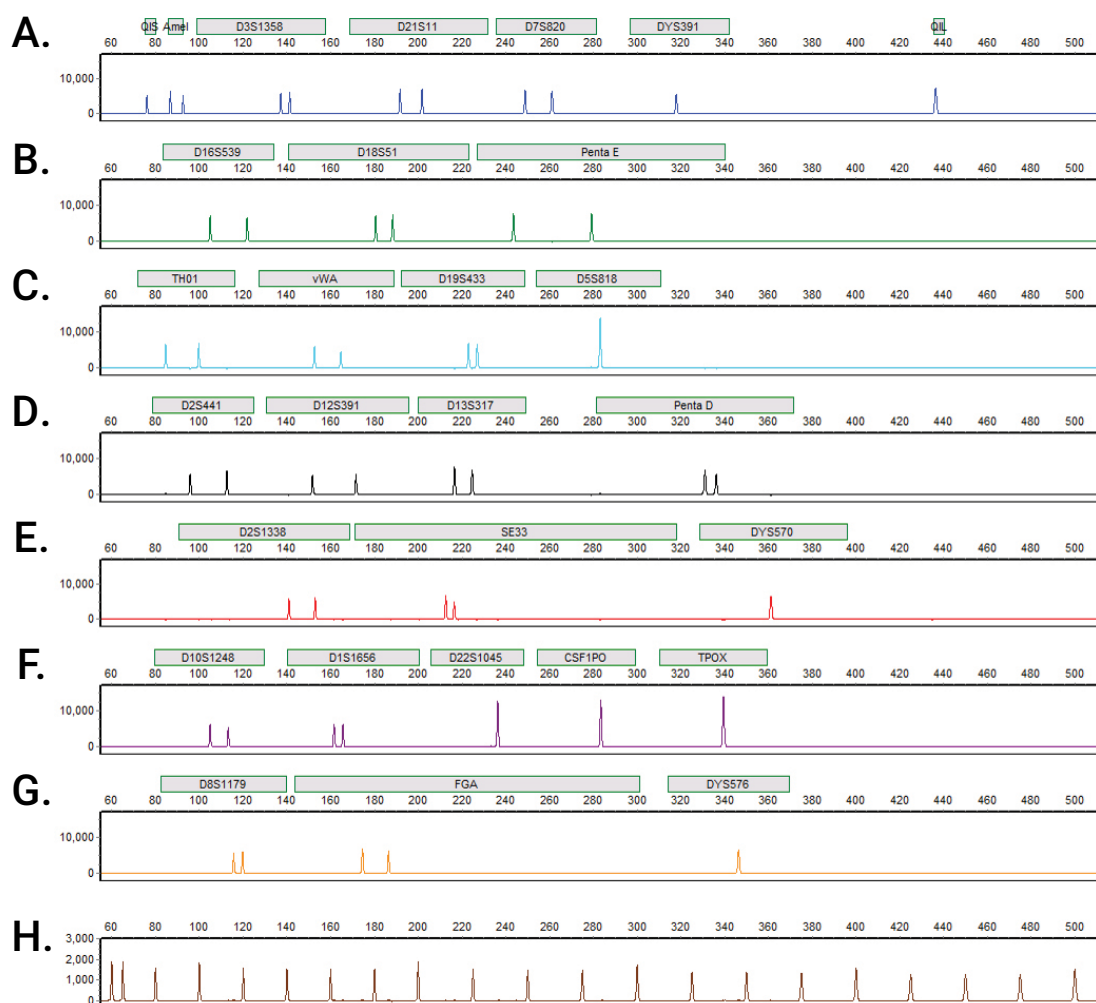


Figure 26. An electropherogram of 2800M Control DNA amplified by the Paradyme™ 27GY STR System. The 2800M Control DNA (0.5ng) was amplified using the Paradyme™ 27GY STR System. Amplification products were mixed with CCO Internal Lane Standard 500 and analyzed with a Spectrum CE System and a 2kV, 18-second injection. Results were analyzed using GeneMarker®HID Software for Spectrum CE Systems, version 3.2.0 and the Paradyme™ 27GY panel file. **Panel A.** The peaks of the FL-8C-labeled loci: QIS, Amelogenin, D3S1358, D21S11, D7S820, DYS391 and QIL. **Panel B.** The peaks of the JOE-8C-labeled loci: D16S539, D18S51 and Penta E. **Panel C.** The peaks of the AQA-8C-labeled loci: TH01, vWA, D19S433 and D5S818. **Panel D.** The peaks of the TMR-8C-labeled loci: D2S441, D12S391, D13S317 and Penta D. **Panel E.** The peaks of the CXR-8C-labeled loci: D2S1338, SE33 and DYS570. **Panel F.** The peaks of the TOM-8C-labeled loci: D10S1248, D1S1656, D22S1045, CSF1PO and TPOX. **Panel G.** The peaks of the WEN-8C-labeled loci: D8S1179, FGA and DYS576. **Panel H.** The 60bp to 500bp fragments of the CCO Internal Lane Standard 500.

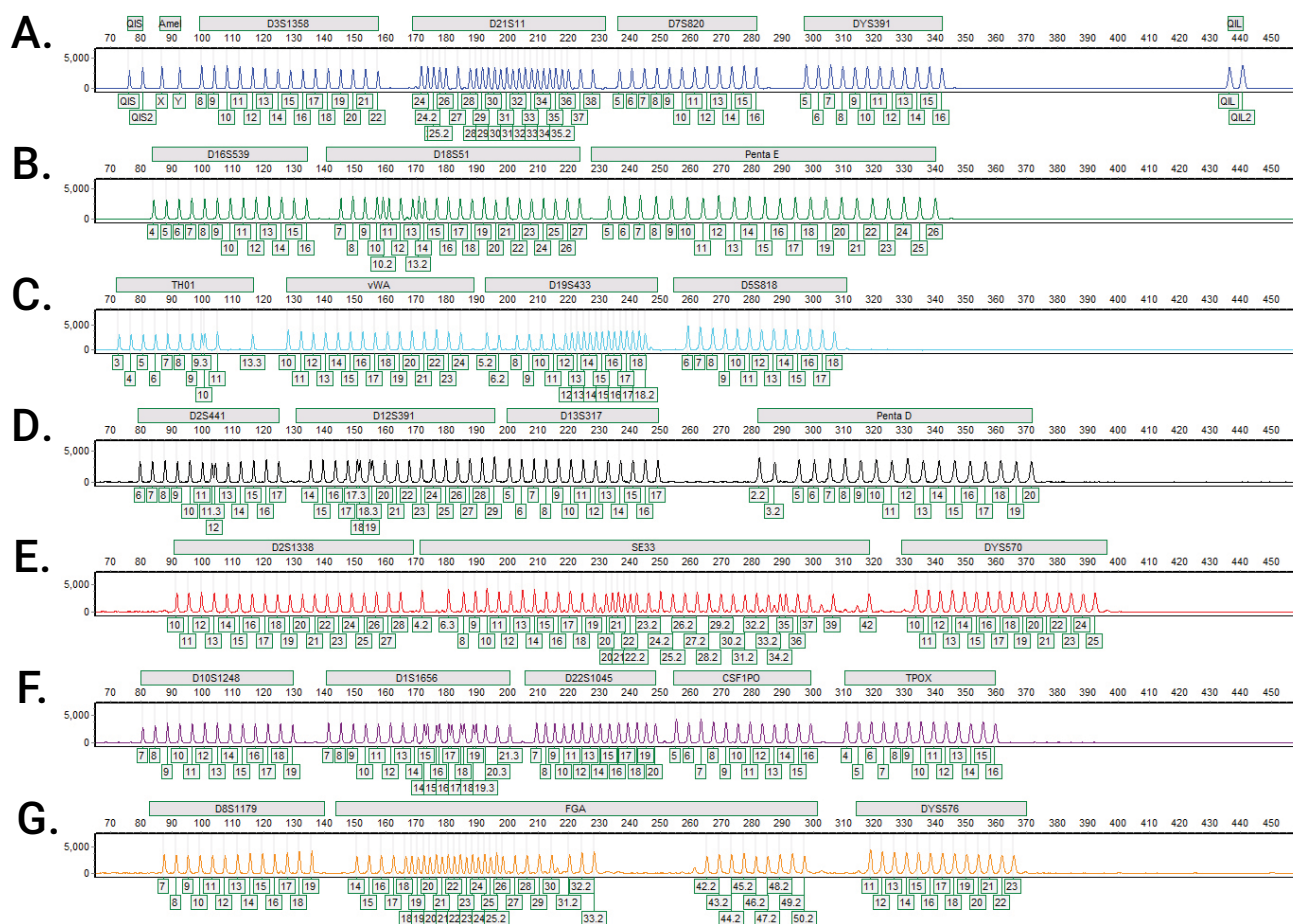


Figure 27. The Paradyme™ 27GY Allelic Ladder Mix. The Paradyme™ 27GY Allelic Ladder Mix was analyzed with a Spectrum CE System and a 2kV, 18-second injection. The sample file was analyzed with the GeneMarker®HID Software for Spectrum CE Systems, version 3.2.0, and the Paradyme™ 27GY panel file. **Panel A.** The FL-8C-labeled allelic ladder components and their allele designations. **Panel B.** The JOE-8C-labeled allelic ladder components and their allele designations. **Panel C.** The AQA-8C-labeled allelic ladder components and their allele designations. **Panel D.** The TMR-8C-labeled allelic ladder components and their allele designations. **Panel E.** The CXR-8C-labeled allelic ladder components and their allele designations. **Panel F.** The TOM-8C-labeled allelic ladder components and their allele designations. **Panel G.** The WEN-8C-labeled allelic ladder components and their allele designations.

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Artifacts and Stutter

Stutter products are a common amplification artifact associated with STR analysis. While the Paradyme™ 27GY STR System uses Reduced Stutter Polymerase (RSP) technology to substantially decrease the formation of stutter, some residual stutter artifacts may still be observed and can vary in intensity depending on locus and the DNA sequence being amplified. Stutter products are often observed one repeat unit below and above the true allele peak. Alleles with a greater number of repeat units will frequently exhibit a higher percent stutter. Trinucleotide repeat loci and rapidly mutating Y-STR loci can have more pronounced stutter than a typical tetranucleotide repeat locus. The pattern and intensity of stutter may differ slightly between primer sets for the same loci.

The 99.7th percentile stutter value observed at each locus is used in the Paradyme_27GY panel file for marker-specific filtering and is preloaded in GMHID-Spectrum.

In addition to stutter peaks, you may observe the following low-level DNA-dependent artifact peaks (Table 10) and DNA-independent (with or without human genomic DNA) artifact peaks (Table 11) with the Paradyme™ 27GY STR System.

Table 10. DNA-Dependent Artifacts Observed in Amplification Reactions with Human Genomic DNA.

Locus	Artifact Size
QIS ¹	n-3 to n-4
AMEL ¹	n-3 to n-4
D3S1358 ¹	n-2 to n-3
D21S11 ¹	n-2 to n-3
D7S820 ¹	n-1 to n-2
DYS391 ¹	n-1 to n-2
QIL ¹	n-1
D19S433	n-2, n+2
Penta D	n+1, n+2
SE33	n-2, n+2
TPOX	n-15 to n-23

¹FAM dye channel artifacts at these loci are more likely to form as a result of elevated TCEP concentration and/or prolonged exposure to light. To minimize their appearance, use the recommended volume of TCEP working dilution and minimize prolonged exposure of the amplification reaction mix to light (before and after amplification).

Table 11. DNA-Independent Artifacts Observed in Amplification Reactions with and without Human Genomic DNA.

Dye Label	Artifact Size¹
FL-8C	~58–63bp ~71–75bp ~118–125bp ~143–148bp ~413–422bp
JOE-8C	~58–80bp
AQA-8C	~58–60bp
TMR-8C	~58–60bp
CXR-8C	~58–60bp
TOM-8C	~58–65bp
WEN-8C	~58–83bp

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

A variety of nonhuman DNA templates from bacteria, yeast, plants, insects, fish, amphibians, avians and mammals were tested to characterize potential artifacts with the Paradyme™ 27GY STR System. No artifacts were noted above a 150RFU threshold with 5ng of template DNA using the Spectrum CE System.

A variety of primate DNA templates were tested using 0.5ng of template DNA. Partial profiles were obtained with all primate species tested, but these profiles can be distinguished from a human profile because many of the alleles were called as off-ladder or were outside of the locus panels.

8 Troubleshooting

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

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

8.1 Amplification and Fragment Detection

Symptoms	Causes and Comments
Faint or absent allele peaks	The Master Mix was not vortexed well before use. Vortex the Master Mix for 15 seconds before dispensing into the PCR amplification mix.
	Primer concentration was too low. Use the recommended primer concentration. Vortex the Primer Pair Mix for 15 seconds before use.
	TCEP concentration was too low. Ensure that TCEP, Concentrated, was diluted appropriately and use the recommended volume of the 25X TCEP Working Dilution in 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. We have not tested reaction tubes, plates or thermal cyclers that are not listed. Calibrate the thermal cycler heating block if necessary.
	Repeat sample preparation using fresh formamide. Long-term storage of amplified sample in formamide can result in loss of signal.
	Poor capillary electrophoresis injection (ILS peaks also affected). Re-inject the sample.
	Poor-quality formamide was used. Use only Hi-Di™ formamide when analyzing samples.

Symptoms	Causes and Comments
Faint or absent allele peaks for the positive control reaction	Improper storage of the 2800M Control DNA. Store the 2800M Control DNA at +2°C to +10°C. Vortex before use.
	Repeat sample preparation using fresh formamide. Long-term storage of amplified sample in formamide can result in loss of signal.
	Poor capillary electrophoresis injection (ILS peaks also affected). Reinject the sample.
	Poor-quality formamide was used. Use only Hi-Di™ formamide when analyzing samples.
Extra peaks visible in one or all color channels	Contamination with another template DNA or previously amplified DNA. Cross-contamination can be a problem. Use aerosol-resistant pipette tips and change gloves regularly.
	Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the DNA source as soon as possible to each well and follow immediately by thermal cycling.
	Samples were not denatured completely. Heat-denature samples for the recommended time, and cool on crushed ice or a freezer plate block or in an ice-water bath immediately prior to capillary electrophoresis. Do not cool samples in a thermal cycler set at 4°C, as this may lead to artifacts due to DNA reannealing. Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis. Appearance of 'shadow' peaks migrating in front of the main peaks, especially if the shadow peaks are separated by the same distance as the main peaks in a heterozygote, can indicate the presence of double-stranded DNA due to incomplete denaturation or post-injection re-annealing.
	CE-related artifacts ('spikes'). Minor voltage changes or urea crystals passing by the laser can cause 'spikes' or unexpected peaks. Spikes sometimes appear in one color but often are easily identified by their presence in more than one color. Reinject samples to confirm. Ensure no urea crystals are present in the polymer bottle before installing on the CE.

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels (continued)	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 rerun the samples. • Confirm that the correct spectral was used for the sample run. • Instrument sensitivities can vary. Optimize the injection conditions. Refer to the instrument user manual.
	Dye blob artifacts. The signal strength of certain dye blob artifacts increases with storage of the amplification plate at 4°C, sometimes in as short a time period as overnight but more commonly when left at 4°C for a few days. We recommend storing amplification products at –20°C.
	FAM Artifacts from light exposure and excess TCEP. Protect samples from light both before and after amplification. Prolonged light exposure increases FAM dye channel artifacts, with a more pronounced effect prior to amplification. Too much TCEP in the PCR (e.g., using undiluted TCEP) can generate artifacts in the FAM dye channel. These artifacts may increase in amplitude when the amplified material is stored. Artifacts are associated with amplicons in the FAM dye channel and migrate smaller than allelic and QI peaks: • Artifacts associated with QIS and AMEL migrate approximately 3-4 bases smaller than the main amplicons. • Artifacts associated with D3S1358 and D21S11 migrate approximately 2-3 bases smaller than the main amplicons. • Artifacts associated with D7S820 and DYS391 migrate approximately 1-2 bases smaller than the main amplicons. • Artifacts associated with QIL migrate <1 base smaller than the main amplicon. Please ensure that TCEP is appropriately diluted, and that the correct volume of TCEP Working Dilution is used.
	N-1 artifacts. Amplification with excess template DNA can cause N-1 artifacts. Use the recommended amount of template DNA.
	An incorrect internal lane standard was used. Use the size standard provided in the kit.

Symptoms	Causes and Comments
Allelic ladder not running the same as samples	Be sure the allelic ladder and samples are from the same instrument run.
	Allelic ladder and primer pair mix were not compatible. Ensure that the allelic ladder is from the same kit as the primer pair mix.
	Migration of samples changed slightly over the course of a CE run with many samples. This may be due to changes in temperature over time. Use a different injection of allelic ladder to determine sizes.
	Poor injection of allelic ladder. Include more than one ladder per instrument run. Include one allelic ladder for every 16 samples.
	Internal size standard was not assigned correctly. Evaluate the sizing labels on the ILS and correct if necessary.
	Ensure the oven is preheated for a minimum of 2 hours prior to starting a CE run for complete temperature equilibration.
Peak height imbalance	Miscellaneous balance problems. Vortex the Primer Pair Mix and Master Mix for 15 seconds before use; do not centrifuge the Primer Pair Mix or Master Mix after mixing. Calibrate thermal cyclers and pipettes routinely.
	PCR amplification mix was not mixed well. Vortex the PCR amplification mix for 5–10 seconds before dispensing into the reaction tubes or plate.

8.2 Amplifying Extracted DNA

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

Symptoms	Causes and Comments
Faint or absent allele peaks	Impure template DNA. Depending on the DNA purification procedure used and sample source, inhibitors might be present in the DNA sample. This may be more of an issue as DNA sample volume increases as a percentage of the total amplification reaction volume.
	Insufficient template. Use the recommended amount of template DNA if available. Quantify template DNA before use if possible.
	High salt concentration or altered pH. - Carryover of K⁺, Na⁺, Mg²⁺ or EDTA from the DNA sample can negatively affect PCR. - A change in pH also may affect PCR.
	Store DNA in TE ⁻⁴ buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE ⁻⁴ buffer with 20µg/ml glycogen.
	The reaction volume was too low. This system is optimized for a final reaction volume of 25µl for extracted DNA. Decreasing the reaction volume may result in suboptimal performance.
Extra peaks visible in one or all color channels	Amplifying more than the recommended amount of purified DNA can result in a higher number of artifact peaks due to overamplification, resulting in saturating signal. Use the recommended amount of template DNA. See Results section for additional information about stutter and artifacts. The amount of template will need to be optimized if you are using reduced reaction volumes.
	STR amplification artifacts. Amplifying STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete adenylation. - Be sure to perform the recommended extension step at 60°C after thermal cycling. - Decrease the amount of template DNA. Using more than the recommended amount of template DNA can result in incomplete adenylation. - Decrease cycle number. - Increase the final extension time

Symptoms	Causes and Comments
Peak height imbalance	Amplifying greater than the recommended amount of template can result in an imbalance, with smaller loci showing more product than larger loci. Use less template or fewer cycles. The amount of template will need to be optimized if you are using reduced reaction volumes.
	Degraded DNA sample. DNA template was degraded, and larger loci showed diminished yield.
	Insufficient template DNA. Use the recommended amount of template DNA if available. Stochastic effects can occur when amplifying low amounts of template.
	Impure template DNA. Inhibitors that may be present in forensic samples can lead to allele dropout or imbalance.
	Imbalance may be seen more often when using the maximum template volume or a reduced amplification reaction volume.
	The reaction volume was too low. This system is optimized for a final reaction volume of 25µl. Decreasing the reaction volume may result in suboptimal performance.

8.3 Directly Amplifying DNA from Storage Card Punches

The following information is specific to amplifying DNA from SwabSolution™ extracts of storage card punches, or punches used directly in PCR. For additional information about general amplification and detection, see Section 8.1.

Symptoms	Causes and Comments
Faint or absent allele peaks	Poor sample deposition. Donor cell shedding and collection was variable. Increasing the cycle number can increase peak heights.
	Poor sample transfer to storage card or variable sampling from storage card. Take punches from a different portion of the card. Increasing cycle number can increase peak heights.
	Too much sample in the reaction can result in inhibition, decreasing intensity of peaks (especially larger amplicons).
	The reaction volume was too low. Decreasing the reaction volume from that recommended in the protocol may result in suboptimal performance.
Faint or absent allele peaks when amplifying DNA from SwabSolution™ extracts of storage card punches	Active SwabSolution™ Reagent carried over into amplification reactions. Ensure that the heat block reached 70°C and samples were incubated for 30 minutes. Incubation for shorter time periods may result in incomplete inactivation of the SwabSolution™ Reagent. We have not tested longer incubation times.
	Inactive SwabSolution™ Reagent was used to extract punches. Thaw the SwabSolution™ Reagent completely in a 37°C water bath and mix by gentle inversion. Store the SwabSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
	Heat transfer during incubation can be influenced by the vessel used for incubation (e.g., microtubes vs. 96-well plates).
	Adjusting the cycle number or the volume of SwabSolution™ extract per reaction may increase peak heights.

Symptoms	Causes and Comments
Elevated allele peaks when amplifying DNA from SwabSolution™ extracts of storage card punches	Heat transfer during incubation can be influenced by the vessel used for incubation (e.g., microtubes vs. 96-well plates). Adjusting the cycle number or the volume of SwabSolution™ extract per reaction may decrease peak heights. We do not recommend shortening the incubation period below 30 minutes.
Faint or absent allele peaks when amplifying DNA directly from buccal punches	DNA was not accessible on nonlytic material. Pretreat nonlytic materials with PunchSolution™ Reagent to ensure that DNA is released from cellular proteins. Increasing the cycle number can increase peak heights.
	Active PunchSolution™ Reagent carried over into amplification reactions with nonlytic card punches. Ensure that the heat block reached 70°C and samples were incubated for 30 minutes or until wells are dry. Incubation for shorter time periods may result in incomplete inactivation of the PunchSolution™ Reagent. We have not tested longer incubation times.
	Inactive PunchSolution™ Reagent was used to pretreat nonlytic punches. Thaw the PunchSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.

Symptoms	Causes and Comments
Faint or absent allele peaks in the positive control reaction	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. If cycle number was reduced, it is necessary to increase the mass of 2800M Control DNA to obtain a profile. Use the recommended amount of 2800M Control DNA per amplification reaction. This mass of DNA should be reduced if cycle number is increased and increased if the cycle number is decreased. Increase or decrease by twofold the mass of 2800M Control DNA for every one-cycle decrease or increase, respectively. We do not recommend including blank punches in the 2800M Control DNA reaction.
Extra peaks visible in one or all color channels	Punch contaminated with DNA from another sample. Perform punches on a blank card between samples to minimize potential for carryover between samples.
	Amplification of processed punches with high amounts of DNA can result in artifact peaks due to overamplification, resulting in excess signal on the CE instrument. Using a larger punch size or a smaller reaction volume may result in overamplification and signal saturation. • When using SwabSolution™ extracts of storage card punches, use the recommended volume of extract per reaction. • When amplifying directly from buccal punches, use the recommended number of punches. • If the signal is saturated, repeat the amplification with less SwabSolution™ extract, 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. If this occurs at a heterozygous locus, it is sometimes possible to see two 'shadow' peaks that differ in size from one another by approximately the same distance as the single-stranded alleles.

Symptoms	Causes and Comments
Extra peaks visible in one or all color channels (continued)	STR amplification artifacts. Directly amplifying high amounts of template can result in a higher number of artifact peaks. Use the recommended SwabSolution™ extract volume, punch size and number of punches. Optimize the cycle number. See Results (Section 7) for additional information on stutter and artifacts.
	STR amplification artifacts. Amplifying STRs can result in artifacts that appear as peaks one base smaller than the allele due to incomplete adenylation. • Be sure to perform the recommended extension step at 60°C after thermal cycling. • Decrease cycle number. • Increase the final extension time.
	N–1 artifacts. Amplification with excess template DNA can cause additional N–1 artifacts. Repeat the amplification with less SwabSolution™ extract, a smaller punch, a larger reaction volume or reduced cycle number.
Peak height imbalance	Excessive DNA amount. Amplifying high amounts of template can result in an imbalance, with smaller loci showing more product than larger loci. • Be sure to use the recommended SwabSolution™ extract volume or 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 storage card punch with blood. Storage cards with blood must be processed using the SwabSolution™ extraction method. Use the recommended volume of SwabSolution™ extract in the reaction.
	The reaction volume was too low. Decreasing the reaction volume can result in suboptimal performance due to inhibitors present in lytic cards and PunchSolution™ Reagent.
	DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat nonlytic materials with PunchSolution™ Reagent to ensure that DNA is released from cellular proteins.

Symptoms	Causes and Comments
Peak height imbalance (continued)	Active SwabSolution™ or PunchSolution™ Reagent (for nonlytic punch cards) carried over into amplification reactions. Larger loci are most susceptible to carryover and will drop out before the smaller loci. - When processing DNA from storage cards using the SwabSolution™ method, use the recommended number of punches, SwabSolution™ volume, and plates or tubes. Ensure that the heat block reaches 70°C and samples are incubated for 30 minutes. Incubation for shorter time periods may result in incomplete inactivation of SwabSolution™ Reagent. - When processing DNA from nonlytic card punches using PunchSolution™ Reagent, treat one 1.2 mm punch with 10µl of PunchSolution™ Reagent and use one punch per 12.5µl or 25µl amplification reaction. Ensure the heat block reaches 70°C and samples are incubated for 30 minutes or until wells are dry. Shorter incubation times may result in incomplete inactivation of PunchSolution™ Reagent. Reducing the PunchSolution™ Reagent volume may improve results for reduced amplification volumes; optimization and validation are required.
	Inactive SwabSolution™ PunchSolution™ Reagent was used. Thaw the SwabSolution™ Reagent completely in a 37°C water bath and mix by gentle inversion. Store the SwabSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity. Thaw the PunchSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.

Symptoms	Causes and Comments
Extreme variability in sample-to- sample peak heights	There can be significant individual-to-individual variability in the number of cells on a card, resulting in peak height variability between samples. The PunchSolution™ Kit maximizes the recovery of amplifiable DNA from nonlytic punches but does not normalize the amount of DNA present.
Low peak heights for QIS and QIL from buccal samples.	Peak heights for QIS and QIL are simultaneously reduced while STR allele peak heights are unaffected. Take punches from a different portion of the card.

8.4 Directly Amplifying DNA from Swabs

The following information is specific to directly amplifying DNA from swabs after pretreating with the SwabSolution™ Kit. For additional information about general amplification and detection, see Section 8.1.

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

Symptoms	Causes and Comments
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.
	STR amplification artifacts. Amplifying swab extracts with high DNA concentrations can result in artifact peaks due to overamplification, resulting in saturated signal on the CE instrument. We recommend 2µl of swab extract per reaction. Using more than 2µl may result in overamplification and signal saturation. If signal is saturated, repeat amplification with less swab extract or reduced cycle number.
	Amplifying excess template for a given cycle number resulted in overloading of the capillary upon electrokinetic injection. Excess DNA in the capillary is difficult to maintain in a denatured single-stranded state. Some single-stranded DNA renatures and becomes double-stranded. Double-stranded DNA migrates faster than single-stranded DNA during capillary electrophoresis and appears as 'shadow' peaks migrating in front of the main peaks. If this occurs at a heterozygous locus it is possible to observe the presence of two 'shadow' peaks that differ in size by approximately the same distance as the single-stranded alleles.
	STR amplification artifacts. Amplifying 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 the recommended extension step at 60°C after thermal cycling. • Use 2µl of swab extract in an amplification reaction. A larger volume of swab extract may contain more than the recommended amount of DNA template, resulting in incomplete adenylation. • Decrease cycle number. • Increase the final extension time.

Symptoms	Causes and Comments
Peak height imbalance	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.
	Active SwabSolution™ Reagent carried over from swab extracts into the amplification reaction. Larger loci are most susceptible to reagent carryover and will drop out before the smaller loci. Ensure that the heat block reached 70°C (90°C if using 2.2ml, Square-Well Deep Well Plates) and samples were incubated for the full 30 minutes. Shorter incubations may result in incomplete reagent inactivation. Do not use an incubator for tubes or plates; heat transfer is inefficient and will result in poor performance. Use only a heat block to maintain efficient heat transfer.
	Inactive SwabSolution™ Reagent. Thaw the SwabSolution™ Reagent completely in a 37°C water bath and mix by gentle inversion. Store the SwabSolution™ Reagent at +2°C to +10°C. Do not store reagents in the refrigerator door, where the temperature can fluctuate. Do not refreeze, as this may reduce activity.
	DNA was not accessible on nonlytic material. Small loci may amplify preferentially, with large loci dropping out. Pretreat swabs with SwabSolution™ Reagent to ensure that DNA is released from cellular proteins.
Extreme variability in sample-to- sample peak heights	There can be significant individual-to-individual variability in cell deposition onto buccal swabs. This can appear as variability in peak heights between swab extracts. The extraction process maximizes recovery of amplifiable DNA from buccal swabs but does not normalize the amount of DNA present. If variability is extreme, quantitate the DNA using a fluorescence-based double- stranded DNA quantitation method or qPCR-based quantitation method. The quantitation values can be used to normalize input template amounts to minimize variation in signal intensity.

8.5 GeneMarker®HID Software for Spectrum CE Systems

Symptoms	Causes and Comments
Stutter peaks not filtered	Be sure the "Use Marker-Specific Values" box in the Panel Editor is checked. Alternatively, select the "Use Allele-Specific Values (From Panel)" box in the 'Edit Marker' window and enter the appropriate stutter filters in the Panel Table of the 'Panel Editor' window.
Alleles not called	To analyze samples with GMHID-Spectrum, at least one allelic ladder must be present in the data set. Ensure that the allelic ladder is designated as such in Section 5.3 or 6.1.
	Run was too short and larger peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run. - Create a new size standard using the internal lane standard fragments present in the sample. - Rerun samples using a longer run time.
	A low-quality allelic ladder was used during analysis. Ensure that only high- quality allelic ladders are used for analysis. See the 'Off-ladder or off-bin alleles' column below for more information.
Off-ladder or off-bin alleles	Ensure the oven is preheated for a minimum of two hours prior to starting a run to allow complete temperature equilibration.
	An allelic ladder from a different run than the samples was used. Re-analyze samples with an allelic ladder from the same run.
	The panel file selected for analysis was incorrect for the STR system used. Assign a correct panel file that corresponds to the STR system used for amplification.
	The allelic ladder was not designated as an allelic ladder in Section 5.3 or 6.1.
	The internal lane standard was not properly identified in the sample. Manually redefine the sizes of the size standard fragments in the sample.

Symptoms	Causes and Comments
Off-ladder or off-bin alleles (continued)	A low-quality allelic ladder was used during analysis. Ensure that only high- quality allelic ladders are used for analysis. Allelic ladders are flagged for Analysis Quality when: • There is an error in bin shifting • Peaks in virtual bins have greater than expected peak heights • The expected peaks cannot be identified in a marker. When expected peaks cannot be identified in a marker, the marker bar will be shaded dark green, indicating the marker failed analysis. When errors in bin shifting occur or when peaks in virtual bins have higher than expected peak heights, the marker bars will be outlined in red. Any peaks that cause an 'Analysis Quality' flag are indicated in the electropherogram and Peak Table. When Flag Variant Alleles in Ladder is selected in the 'Display' tab of the 'Preferences' window, peaks in virtual bins with higher than expected peak heights are indicated by yellow vertical bars and green allele labels. You can set your preferences to automatically delete these peaks by selecting Auto-Delete Alleles in Virtual Bins in Allelic Ladder in the 'Forensics' tab of the 'Preferences' window. Peaks that are not present in the expected bins are marked with red vertical bars and red allele labels
Size standard not called or poor quality	If a partial range was chosen for the Allele Call analysis in the 'Data Process - HID Analysis' window of the Run Wizard, the chosen starting or end point was incorrect. Adjust the starting point or end point or use the Auto Range setting for the analysis. The incorrect Standard Color was selected in the 'Template Selection' window of the Run Wizard. The Standard Color should be Brown.

Symptoms	Causes and Comments
Size standard not called or poor quality (continued)	If the quality of the size standard peaks is not sufficient (and the 'Low ILS Quality Flag 'SQ"' section in the 'Sample Quality' tab of the 'Preferences' window is selected), the size standard is marked with a red SQ flag in the Sample File Tree of the 'Main Analysis' window and in the upper left corner of the sample electropherogram. There are three criteria that determine whether a size standard fails the size quality requirements: - The sample size standard does not contain all peaks that are marked as "Enabled" in the chosen size standard template. - Not all of the expected size standard peaks are called in the 'Main Analysis' window. If the expected size standard peaks are present but are not labeled, open the Calibration Charts and select the affected sample from the Sample List. Edit the size calls of the sample size standard to remove the extra peak(s) and define the correct peak(s). Right-click anywhere in the Sample Size Standard Chart and select Update Calibration. - Not all of the expected size standard peaks are of high quality such that the absolute difference in size between the actual size standard peaks and the expected, Enabled size standard peaks is less than 0.2bp.
	An incorrect size standard was used.
	Run was too short, and larger peaks in ILS were not captured. Not all ILS peaks defined in the size standard were detected during the run. - Create a new size standard using the internal lane standard fragments present in the sample. - Rerun samples using a longer run time.
Peaks in size standard missing	If peaks are low-quality, redefine the size standard for the sample to skip these peaks.
	An incorrect size standard was used.
Significantly raised baseline	Poor spectral calibration. Perform a new spectral calibration and rerun the samples.
	Confirm that the correct spectral was used to run the samples.

9 References

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10 Appendix

10.1 Advantages of Using the Loci in the Paradyme™ 27GY STR System

A single Paradyme™ 27GY STR System reaction amplifies all loci required for US expanded CODIS and European databases (Table 12 and Table 13). The male-specific DYS391 locus is included to identify null Y results for Amelogenin. Additionally, two rapidly mutating Y-STR loci (DYS570 and DYS576) are included in the system. Table 14 lists the Paradyme™ 27GY STR System alleles amplified from the 2800M standard DNA template.

The Paradyme™ 27GY STR System uses Reduced Stutter Polymerase (RSP) technology to reduce the formation of stutter artifacts, sometimes called repeat slippage, during PCR amplification of STR loci. These peaks are due to the loss of a repeat unit during DNA amplification, somatic variation within the DNA or both. While amplification-derived stutter artifacts are greatly reduced by RSP, some residual stutter artifacts may be observed and vary in intensity depending on locus and the DNA sequence being amplified. We have carefully selected primers to avoid or minimize artifacts, including those associated with DNA polymerases such as terminal nucleotide addition (14,15).

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

Table 12. The Paradyme™ 27GY STR System Locus-Specific Information.

STR Locus	Label	Chromosomal Location ¹	Repeat Sequence ² 5'→3'
Amelogenin ³	FL-8C	Xp22.1–22.3 and Y	NA
D3S1358	FL-8C	3p21.31 (45.557Mb)	TCTA Complex
D21S11	FL-8C	21q21.1 (19.476Mb)	TCTA Complex (20)
D7S820	FL-8C	7q21.11 (83.433Mb)	GATA
DYS391	FL-8C	Y	TCTA
D16S539	JOE-8C	16q24.1 (84.944Mb)	GATA
D18S51	JOE-8C	18q21.33 (59.1Mb)	AGAA (20)
Penta E	JOE-8C	15q26.2 (95.175Mb)	AAAGA
TH01	AQA-8C	11p15.5 (2.149Mb)	AATG (20)
vWA	AQA-8C	12p13.31 (5.963Mb)	TCTA Complex (20)
D19S433	AQA-8C	19q12 (35.109Mb)	AAGG Complex
D5S818	AQA-8C	5q23.2 (123.139Mb)	AGAT
D2S441	TMR-8C	2p14 (68.214Mb)	TCTA
D12S391	TMR-8C	12p12 (12.341Mb)	AGAT/AGAC Complex
D13S317	TMR-8C	13q31.1 (81.62Mb)	TATC
Penta D	TMR-8C	21q22.3 (43.88Mb)	AAAGA
D2S1338	CXR-8C	2q35 (218.705Mb)	TGCC/TTCC
SE33	CXR-8C	6q14 (89.043Mb)	AAAG Complex
DYS570	CXR-8C	Y	TTTC
D10S1248	TOM-8C	10q26.3 (130.567Mb)	GGAA
D1S1656	TOM-8C	1q42 (228.972Mb)	TAGA Complex
D22S1045	TOM-8C	22q12.3 (35.779Mb)	ATT
CSF1PO	TOM-8C	5q33.1 (149.436Mb)	AGAT
TPOX	TOM-8C	2p25.3 (1.472Mb)	AATG
D8S1179	WEN-8C	8q24.13 (125.976Mb)	TCTA Complex (20)
FGA	WEN-8C	4q28 (155.866Mb)	TTTC Complex (20)
DYS576	WEN-8C	Y	AAAG

¹Find information about chromosomal location of these loci in references 19, 20 and 21 and at:

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

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

³Amelogenin is not an STR.

NA = Not Applicable

Table 13. The Paradyme™ 27GY STR System Allelic Ladder Information.

STR Locus	Label	Size Range of Allelic Ladder Components^{1,2} (bases)	Repeat Numbers of Allelic Ladder Components³
QIS	FL-8C	82, 86	QIS, QIS2
Amelogenin	FL-8C	91, 97	X, Y
D3S1358	FL-8C	107–163	8–22
D21S11	FL-8C	173–229	24, 24.2, 25, 25.2, 26–28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36–38
D7S820	FL-8C	239–283	5–16
DYS391	FL-8C	298–342	5–16
QIL	FL-8C	439, 443	QIL, QIL2
D16S539	JOE-8C	91–139	4–16
D18S51	JOE-8C	152–232	7–10, 10.2, 11–13, 13.2, 14–27
Penta E	JOE-8C	237–342	5–26
TH01	AQA-8C	74–117	3–9, 9.3, 10–11, 13.3
vWA	AQA-8C	128–184	10–24
D19S433	AQA-8C	195–247	5.2, 6.2, 8–12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2, 18, 18.2
D5S818	AQA-8C	258–306	6–18
D2S441	TMR-8C	82–126	6–11, 11.3, 12–17
D12S391	TMR-8C	135–195	14–17, 17.3, 18, 18.3, 19–29
D13S317	TMR-8C	201–249	5–17
Penta D	TMR-8C	280–368	2.2, 3.2, 5–20
D2S1338	CXR-8C	93–165	10–28
SE33	CXR-8C	174–324	4.2, 6.3, 8–20, 20.2, 21, 21.2, 22, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 31.2, 32.2, 33.2, 34.2, 35–37, 39, 42
DYS570	CXR-8C	335–395	10–25
D10S1248	TOM-8C	81–129	7–19
D1S1656	TOM-8C	141–200	7–14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18, 18.3, 19, 19.3, 20.3, 21.3

STR Locus	Label	Size Range of Allelic Ladder Components ^{1,2} (bases)	Repeat Numbers of Allelic Ladder Components ³
D22S1045	TOM-8C	208–247	7–20
CSF1P0	TOM-8C	253–297	5–16
TPOX	TOM-8C	310–358	4–16
D8S1179	WEN-8C	77–125	7–19
FGA	WEN-8C	140–286	14–18, 18.2, 19, 19.2, 20, 20.2, 21, 21.2, 22, 22.2, 23, 23.2, 24, 24.2, 25, 25.2, 26–30, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 49.2, 50.2
DYS576	WEN-8C	306–362	10–24

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

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

³For a current list of microvariants, see the National Institute of Standards and Technology Variant Allele Report (24).

Table 14. The Paradyme™ 27GY STR System Allele Determinations for the 2800M Control DNA.

STR Locus	2800M
Amelogenin	X, Y
D3S1358	17, 18
D21S11	29, 31.2
D7S820	8, 11
DYS391	10
D16S539	9, 13
D18S51	16, 18
Penta E	7, 14
TH01	6, 9.3
vWA	16, 19
D19S433	13, 14
D5S818	12, 12
D2S441	10, 14
D12S391	18, 23
D13S317	9, 11
Penta D	12, 13
D2S1338	22, 25
SE33	15, 16
DYS570	17
D10S1248	13, 15
D1S1656	12, 13
D22S1045	16, 16
CSF1PO	12, 12
TPOX	11, 11
D8S1179	14, 15
FGA	20, 23
DYS576	18

10.2 The CCO Internal Lane Standard 500

The CCO Internal Lane Standard 500 contains 21 DNA fragments of 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 and 500 bases in length (Figure 28). Each fragment is labeled with CCO-8C dye and can be detected separately (as an eighth color) in the presence of Paradyme™ 27GY STR-amplified material. The CCO ILS 500 is designed for use in each CE injection to increase precision in analysis when using the Paradyme™ 27GY STR System. Section 5 provides protocols to prepare and use this internal lane standard.

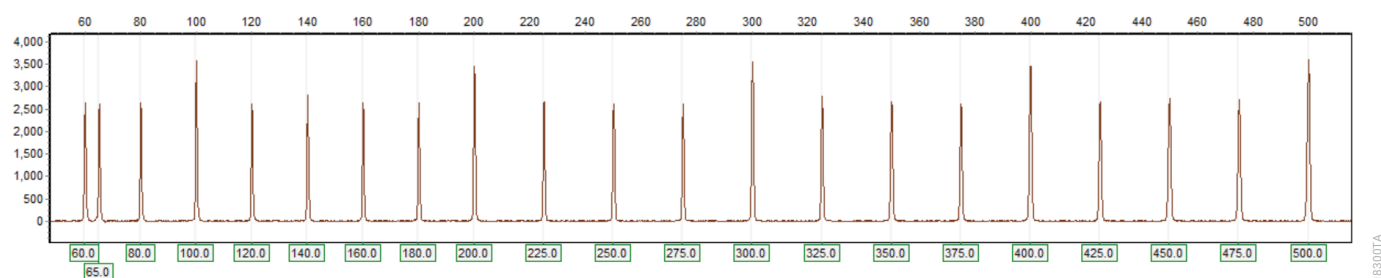


Figure 28. CCO Internal Lane Standard 500. An electropherogram showing the CCO Internal Lane Standard 500 fragments.

10.3 Directly Amplifying DNA from Storage Card Punches in a 25µl Reaction Volume

Depending on the procedure used to prepare storage cards, high cell numbers may be present in each punch. In these cases, directly amplifying in 25µl reaction volumes may improve results.

10.3.1 Materials to be Supplied by the User

- ProFlex® PCR System (Applied Biosystems)
- Mastercycler® X50s PCR System (Eppendorf)
- centrifuge compatible with 96-well plates or reaction tubes
- ClickFit Microtube, 1.5ml (Cat.# V4745)
- MicroAmp® optical 96-well reaction plates or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- SwabSolution™ Kit (Cat.# DC8271)
- PunchSolution™ Kit (Cat.# DC9271) for nonlytic card punches
- Paradyme™ 27GY QI Reagent (Cat.# DM1701)
- 1.2mm Harris Micro-Punch or equivalent manual punch and cutting mat or automated punch system

This section contains two protocols for amplifying DNA from storage card punches in a 25µl reaction volume using the Paradyme™ 27GY STR System on the ProFlex® and Mastercycler® X50s PCR Systems:

- SwabSolution™ processing of DNA from storage card punches followed by amplification. We recommend this protocol for blood and buccal samples on lytic and nonlytic storage cards.
- Direct amplification of DNA from storage card punches. We recommend this protocol for buccal samples only.

The Paradyme™ 27GY STR System (Cat.# RS2720) contains sufficient reagents for 400 direct-amplification reactions of 12.5µl each; when performing 25µl reactions, the system contains sufficient reagents for 200 reactions.

Note: You can use thermal cyclers other than the ones listed in this technical manual. Using thermal cyclers not listed here will require optimization of cycling conditions and in-house validation.

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

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

Lytic storage card sample types include:

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

Nonlytic storage card sample types include:

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

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.

10.3.2 SwabSolution™ Processing of DNA from Storage Card Punches

1. Using a 1.2mm Harris punch tool, create one punch (for blood samples) or two punches (for buccal samples) from the center of the stained area. This applies to both lytic and nonlytic cards. Place the punch(es) into a 1.5ml microcentrifuge tube, a 0.2ml reaction tube or a 96-well plate.
2. Add 150µl of SwabSolution™ Reagent to the tube or well, ensuring the punch is completely submerged, then cap it.
3. Place the reaction tube(s) or 96-well plate on a heat block and incubate at 70°C for 30 minutes.

Note: Using plates and tubes other than those recommended may affect heat transfer and lysis efficiency.

4. Following incubation, use the extract immediately or store at 4°C for short-term storage or –20°C for long-term storage.

10.3.3 Amplification Setup for SwabSolution™ Extracts from Storage Card Punches

- Upon receipt, store the Paradyme™ 27GY reagents as follows:
 - Store the Paradyme™ 27GY 5X Master Mix, TCEP, Concentrated, and TCEP Dilution Buffer at -30°C to -10°C . TCEP, Concentrated and TCEP Dilution Buffer may be stored at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$ after the first thaw.
 - Store the Paradyme™ 27GY 10X Primer Pair Mix, Water, Amplification Grade and 2800M Control DNA at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$.

Note: Thaw the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated (if frozen) completely before use. Ensure the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated are thoroughly mixed after thawing. Allow all pre-amplification components to equilibrate to room temperature before preparing the PCR amplification mix. Centrifuge tubes briefly to move contents to the tube 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 can concentrate the reagents at the bottom of the tube.

- Prepare a 25X TCEP Working Dilution by combining the following components listed in Table 15.

Table 15. Preparing the 25X TCEP Working Dilution.

Component	Volume per Reaction	×	Number of Reactions	=	Final Volume
TCEP Dilution Buffer	0.975µl	×		=	
TCEP, Concentrated	0.025µl	×		=	

Notes:

- Use only the TCEP Dilution Buffer provided with the kit.
 - We do not recommend preparing the 25X TCEP Working Dilution for fewer than 80 reactions due to pipetting limitations and variability associated with small volumes. For example, an 80-reaction preparation requires 78µl of TCEP Dilution Buffer and 2µl of TCEP, Concentrated.
 - The 25X TCEP Working Dilution may be stored protected from light for up to 1 week at 4°C , or up to 1 month at -20°C . Vortex for 15 seconds before each use.
- Determine the number of reactions to be set up, including positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach consumes a small amount of each reagent, it ensures that there is enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
 - Use a new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new 0.2ml reaction tubes required, and label them appropriately.

5. Add the final volume of each reagent listed in Table 16 to a new tube.

Table 16. PCR Amplification Mix for Amplifying SwabSolution™ Extracts in a 25µl Reaction Volume.

PCR Amplification Mix Component ¹	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25.0µl	×		=	
25X TCEP Working Dilution	1.0µl				
Paradyne™ 27GY 5X Master Mix	5.0µl	×		=	
Paradyne™ 27GY 10X Primer Pair Mix	2.5µl	×		=	
SwabSolution™ extract	up to 4.0µl				
total reaction volume	25.0µl				

¹Add Water, Amplification Grade, to the tube first, and then add 25X TCEP Working Dilution, Paradyne™ 27GY 5X Master Mix and Paradyne™ 27GY 10X Primer Pair Mix.

6. Vortex the PCR amplification mix for 5–10 seconds. If using the maximum volume of 4µl of SwabSolution™ extract, then pipet 21µl of PCR amplification mix into each reaction well.



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

Notes:

- Protect samples from light before and after amplification. Light exposure increases likelihood for FAM dye channel artifacts, with a more pronounced effect prior to amplification.
 - 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 immediately start thermal cycling.
7. Pipet up to 4µl of SwabSolution™ extract for each sample into the appropriate well of the reaction plate.

Note: Sample input volume can be adjusted to achieve the desired peak heights.

8. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 0.25ng/µl in the desired template DNA volume. Add 2µl (0.5ng total) of the 2800M Control DNA to a reaction well containing 23µl of PCR amplification mix.

Note: The control DNA input in Step 8 is optimized for 30 cycles. Optimization may be required, depending on cycling conditions and laboratory preferences.

9. For the negative amplification control, pipet 4µl of Water, Amplification Grade, or TE⁻⁴ buffer instead of SwabSolution™ extract into a reaction well containing 21µl of PCR amplification mix.

Note: Include additional negative extraction controls, if necessary. Assemble a reaction containing the SwabSolution™ extract prepared from a blank punch, or assemble a reaction where the SwabSolution™ Reagent is processed as a blank without a punch.

10. Seal or cap the plate, or close the tubes. Briefly centrifuge the reactions to remove any air bubbles.

10.3.4 Thermal Cycling on the ProFlex® PCR System

Amplification and detection instrumentation can vary. You will need to optimize protocols for cycle number (30–31 cycles), injection conditions and loading volume for your laboratory instrumentation. Tests at Promega show that 30 cycles work well for a variety of sample types. Perform in-house validation.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the protocol provided below and in Figure 29. The total cycling time is approximately 70 minutes.

Notes:

- a. Set the ramp speed to 6°C/second and the reaction volume to 25µl.
- b. If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 2 minutes and
30 seconds, then:

97°C for 5 seconds
60°C for 1 minute
66°C for 15 seconds
for 30 cycles, then:

60°C for 10 minutes

4°C hold

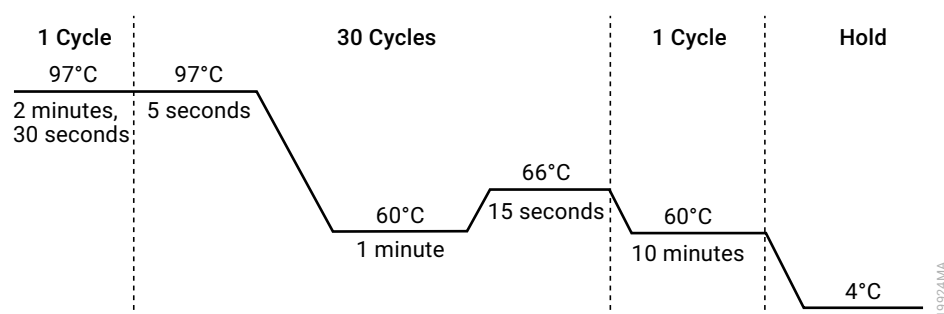


Figure 29. Thermal cycling protocol for the ProFlex® PCR System.

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

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

10.3.5 Thermal Cycling on the Mastercycler® X50s PCR System

Amplification and detection instrumentation can vary. You will need to optimize protocols for cycle number (30–31 cycles), injection conditions and loading volume for your laboratory instrumentation. Tests at Promega show that 30 cycles work well for a variety of sample types. Perform in-house validation.

- Place the reaction plate or tubes in the thermal cycler.
- Select and run the protocol provided below and in Figure 30. The total cycling time is approximately 75 minutes.

Notes:

- Set the ramp speed to $5^{\circ}\text{C}/\text{second}$, the temperature mode to “Safe” and the block setting to “Silver 96”.
- If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 3 minutes, then:

97°C for 10 seconds

60°C for 1 minute

66°C for 15 seconds

for 30 cycles, then:

60°C for 10 minutes

4°C hold



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

Note: This protocol is specific for buccal samples on lytic and nonlytic cards.

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

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

1. Upon receipt, store the Paradyme™ 27GY reagents as follows:
 - Store the Paradyme™ 27GY 5X Master Mix, TCEP, Concentrated, and TCEP Dilution Buffer at -30°C to -10°C . TCEP, Concentrated and TCEP Dilution Buffer may be stored at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$ after the first thaw.
 - Store the Paradyme™ 27GY 10X Primer Pair Mix, Water, Amplification Grade and 2800M Control DNA at $+2^{\circ}\text{C}$ to $+10^{\circ}\text{C}$.

Note: Thaw the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated (if frozen) completely before use. Ensure the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated are thoroughly mixed after thawing. Allow all pre-amplification components to equilibrate to room temperature before preparing the PCR amplification mix. Centrifuge tubes briefly to move contents to the tube 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 can concentrate the reagents at the bottom of the tube.

2. Prepare a 25X TCEP Working Dilution by combining the following components listed in Table 17.

Table 17. Preparing the 25X TCEP Working Dilution.

Component	Volume per Reaction	×	Number of Reactions	=	Final Volume
TCEP Dilution Buffer	0.975µl	×		=	
TCEP, Concentrated	0.025µl	×		=	

Notes:

- a. Use only the TCEP Dilution Buffer provided with the kit.
 - b. We do not recommend preparing the 25X TCEP Working Dilution for fewer than 80 reactions due to pipetting limitations and variability associated with small volumes. For example, an 80-reaction preparation requires 78µl of TCEP Dilution Buffer and 2µl of TCEP, Concentrated.
 - c. The 25X TCEP Working Dilution may be stored protected from light for up to 1 week at 4°C, or up to 1 month at –20°C. Vortex for 15 seconds before each use.
3. Determine the number of reactions to be set up, including positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach consumes a small amount of each reagent, it ensures that there is enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
 4. Use a new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new 0.2ml reaction tubes required, and label them appropriately.

5. Add the final volume of each reagent listed in Table 18 to a new tube. The Paradyme™ 27GY QI Reagent volume will depend on the cycle number:
 - For 26 cycles, use 1µl of Paradyme™ 27GY QI Reagent
 - For 27 cycles, use 0.5µl of Paradyme™ 27GY QI Reagent

Table 18. PCR Amplification Mix for Directly Amplifying DNA from Storage Card Punches in a 25µl Reaction Volume.

PCR Amplification Mix Component ¹	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25.0µl	×		=	
25X TCEP Working Dilution	1.0µl				
Paradyme™ 27GY 5X Master Mix	5.0µl	×		=	
Paradyme™ 27GY 10X Primer Pair Mix	2.5µl	×		=	
Paradyme™ 27GY QI Reagent	up to 1.0µl				
total reaction volume	25.0µl				

¹Add Water, Amplification Grade, to the tube first, and then add 25X TCEP Working Dilution, Paradyme™ 27GY 5X Master Mix, Paradyme™ 27GY 10X Primer Pair Mix and Paradyme™ 27GY QI Reagent. For lytic card punches, the template DNA will be added at Step 10.

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



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

Notes:

- a. Protect samples from light before and after amplification. Light exposure increases likelihood for FAM dye channel artifacts, with a more pronounced effect prior to amplification.
 - b. 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 immediately start thermal cycling.
7. For lytic storage cards, add one or two 1.2mm punch(es) from a card containing buccal cells to the appropriate wells of the reaction plate. For nonlytic card punches, add the PCR amplification mix to the PunchSolution™ Reagent-treated punch.

Note: You can also add the lytic card punch first, and then add the PCR amplification mix.

8. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 2ng/μl in the desired template DNA volume. Add 2μl (4ng total) of the 2800M Control DNA to a reaction well containing 23μl of PCR amplification mix.

Notes:

- a. The Control DNA input in Step 8 is optimized for 27 cycles. Optimization may be required depending on cycling conditions and laboratory preferences.
 - b. Do not include blank storage card punches in the positive control reactions.
9. Reserve a well containing PCR amplification mix as a negative amplification control.

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

10. Seal or cap the plate, or close the tubes. Briefly centrifuge the reactions to move storage card punches to the well bottoms and remove any air bubbles.

10.3.7 Thermal Cycling on the ProFlex® PCR System

Amplification and detection instrumentation can vary. You will need to optimize protocols for the number of storage card punches, cycle number (26–27 cycles), injection conditions and loading volume for your laboratory instrumentation. Tests at Promega show that 27 cycles work well for a variety of sample types. Perform in-house validation.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the protocol provided below and in Figure 31. The total cycling time is approximately 65 minutes.

Notes:

- a. Set the ramp speed to 6°C/second and the reaction volume to 25μl.
- b. If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 2 minutes and
30 seconds, then:

97°C for 5 seconds
60°C for 1 minute
66°C for 15 seconds
for 27 cycles, then:

60°C for 10 minutes

4°C hold

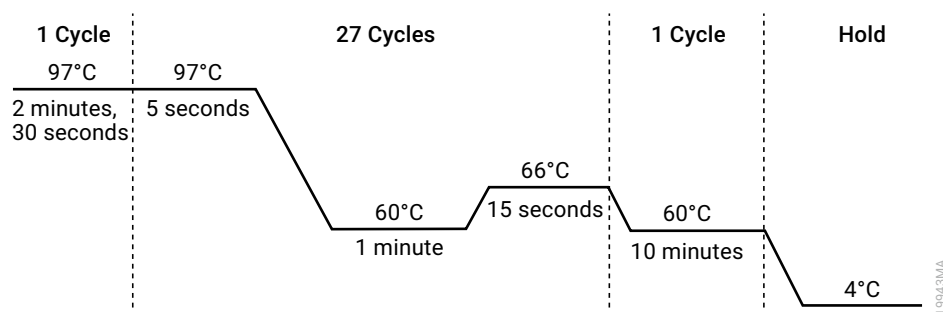


Figure 31. Thermal cycling protocol for the ProFlex® PCR System.

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

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

10.3.8 Thermal Cycling on the Mastercycler® X50s PCR System

Amplification and detection instrumentation can vary. You will need to optimize protocols for the number of storage card punches, cycle number (26–27 cycles), injection conditions and loading volume for your laboratory instrumentation. Tests at Promega show that 27 cycles work well for a variety of sample types. Perform in-house validation.

- Place the reaction plate or tubes in the thermal cycler.
- Select and run the protocol provided below and in Figure 32. The total cycling time is approximately 70 minutes.

Notes:

- Set the ramp speed to $5^{\circ}\text{C}/\text{second}$, the temperature mode to “Safe” and the block setting to “Silver 96”.
- If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 3 minutes, then:

97°C for 10 seconds

60°C for 1 minute

66°C for 15 seconds

for 27 cycles, then:

60°C for 10 minutes

4°C hold

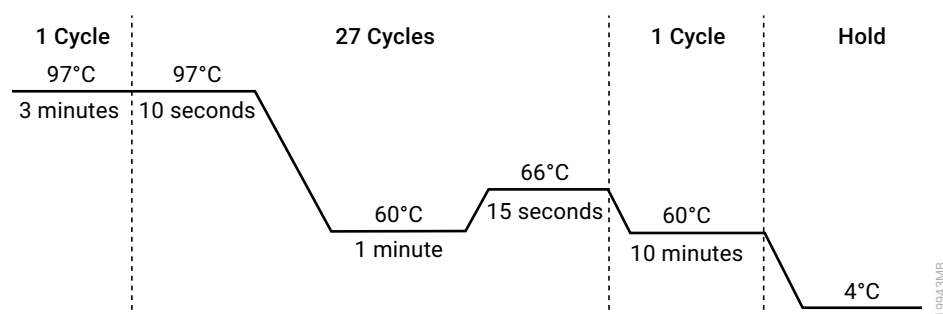


Figure 32. Thermal cycling protocol for the Mastercycler® X50s System.

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

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

10.3.9 PCR Optimization

Optimize the cycle number 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 using your normal workflow.
2. Depending on your preferred protocol, place one or two 1.2mm storage card punches containing buccal cells in each well of a reaction plate. Be sure to pretreat nonlytic samples with the PunchSolution™ Kit (Cat.# DC9271).
3. Prepare two identical reaction plates with punches from the same samples. Use the appropriate amount of Paradyme™ 27GY QI reagent (Cat.# DM1701) for the desired cycle number.
4. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (26–27 cycles).
5. Following amplification, use the laboratory validated separation and detection protocols to determine the optimal cycle number for the sample type.

10.4 Directly Amplifying DNA from Swabs in a 25µl Reaction Volume

Depending on the swab preparation procedure, high cell numbers may be present on each swab. In these cases, directly amplifying in 25µl reaction volumes may provide better results.

10.4.1 Materials to be Supplied by the User

- ProFlex® PCR System (Applied Biosystems)
- Mastercycler® X50s PCR System (Eppendorf)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plates or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- Paradyme™ 27GY QI Reagent (Cat.# DM1701)
- SwabSolution™ Kit (Cat.# DC8271)

This section contains a protocol for amplifying DNA from swab extracts in a 25µl reaction volume using the ProFlex® and Mastercycler® X50s PCR Systems. The Paradyme™ 27GY STR System (Cat.# RS2720) contains sufficient reagents for 200 direct-amplification reactions of 25µl each.

Note: Thermal cyclers other than the ones listed in this technical manual can be used. Using thermal cyclers not listed here requires optimization of cycling conditions and in-house validation.

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

10.4.2 Amplification Setup

1. Upon receipt, store the Paradyme™ 27GY reagents as follows:
 - Store the Paradyme™ 27GY 5X Master Mix, TCEP, Concentrated, and TCEP Dilution Buffer at –30°C to –10°C. TCEP, Concentrated and TCEP Dilution Buffer may be stored at +2°C to +10°C after the first thaw.
 - Store the Paradyme™ 27GY 10X Primer Pair Mix, Water, Amplification Grade and 2800M Control DNA at +2°C to +10°C.

Note: Thaw the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated (if frozen) completely before use. Ensure the Paradyme™ 27GY 5X Master Mix and TCEP, Concentrated are thoroughly mixed after thawing. Allow all pre-amplification components to equilibrate to room temperature before preparing the PCR amplification mix. Centrifuge tubes briefly to move contents to the tube 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 can concentrate the reagents at the bottom of the tube.

2. Prepare a 25X TCEP Working Dilution by combining the following components listed in Table 19.

Table 19. Preparing the 25X TCEP Working Dilution.

Component	Volume per Reaction	×	Number of Reactions	=	Final Volume
TCEP Dilution Buffer	0.975µl	×		=	
TCEP, Concentrated	0.025µl	×		=	

Notes:

- a. Use only the TCEP Dilution Buffer provided with the kit.
- b. We do not recommend preparing the 25X TCEP Working Dilution for fewer than 80 reactions due to pipetting limitations and variability associated with small volumes. For example, an 80-reaction preparation requires 78µl of TCEP Dilution Buffer and 2µl of TCEP, Concentrated.
- c. The 25X TCEP Working Dilution may be stored protected from light for up to 1 week at 4°C, or up to 1 month at –20°C. Vortex for 15 seconds before each use.
3. Determine the number of reactions to be set up, including positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach consumes a small amount of each reagent, it ensures that there is enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
4. Use a new plate for reaction assembly, and label it appropriately. Alternatively, determine the number of new, 0.2ml reaction tubes required, and label them appropriately.

5. Add the final volume of each reagent listed in Table 20 to a new tube. The Paradyme™ 27GY QI Reagent volume will depend on the cycle number:
 - For 25 cycles, use 2µl of Paradyme™ 27GY QI Reagent
 - For 26 cycles, use 1µl of Paradyme™ 27GY QI Reagent

Table 20. PCR Amplification Mix for Directly Amplifying DNA from Swabs in a 25µl Reaction Volume.

PCR Amplification Mix Component ¹	Volume per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25.0µl	×		=	
25X TCEP Working Dilution	1.0µl	×		=	
Paradyme™ 27GY 5X Master Mix	5.0µl	×		=	
Paradyme™ 27GY 10X Primer Pair Mix	2.5µl				
Paradyme™ 27GY QI Reagent	up to 2.00µl				
swab extract	up to 2.0µl				
total reaction volume	25.0µl				

¹Add Water, Amplification Grade, to the tube first, and then add 25X TCEP Working Dilution, Paradyme™ 27GY 5X Master Mix and Paradyme™ 27GY 10X Primer Pair Mix and Paradyme™ 27GY QI Reagent. The swab extract will be added at Step 7.

6. Vortex the PCR amplification mix for 5–10 seconds. If using the maximum volume of 2µl of swab extract, pipet 23µl of PCR amplification mix into each reaction well.



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

Notes:

- a. Protect samples from light before and after amplification. Light exposure increases likelihood for FAM dye channel artifacts, with a more pronounced effect prior to amplification.
 - b. 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 immediately start thermal cycling.
7. Pipet up to 2µl of swab extract for each sample into the appropriate well of the reaction plate.

8. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 4ng/μl in the desired template DNA volume. Add 2μl (8ng total) of the 2800M Control DNA to a reaction well containing 23μl of PCR amplification mix.

Note: The Control DNA input in Step 8 is optimized for 26 cycles. Optimization may be required, depending on thermal cycling conditions and laboratory preferences.

9. For the negative amplification control, pipet up to 2μl of Water, Amplification Grade, or TE⁻⁴ buffer instead of swab extract into a reaction well containing 23μl of PCR amplification mix.

Note: Include additional negative controls if necessary. 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.

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

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

10.4.3 Thermal Cycling on the ProFlex® PCR System

Amplification and detection instrumentation can vary. You will need to optimize protocols for the template DNA amount, cycle number (25–26 cycles), injection conditions and loading volume for your laboratory instrumentation. Tests at Promega show that 26 cycles works well for a variety of sample types. Optimize the cycle number in each laboratory for each sample type that is amplified.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the protocol provided below and in Figure 33. The total cycling time is approximately 65 minutes.

Notes:

- a. Set the ramp speed to 6°C/second and the reaction volume to 25μl.
- b. If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 2 minutes and
30 seconds, then:

97°C for 5 seconds
60°C for 1 minute
66°C for 15 seconds
for 26 cycles, then:

60°C for 10 minutes

4°C hold

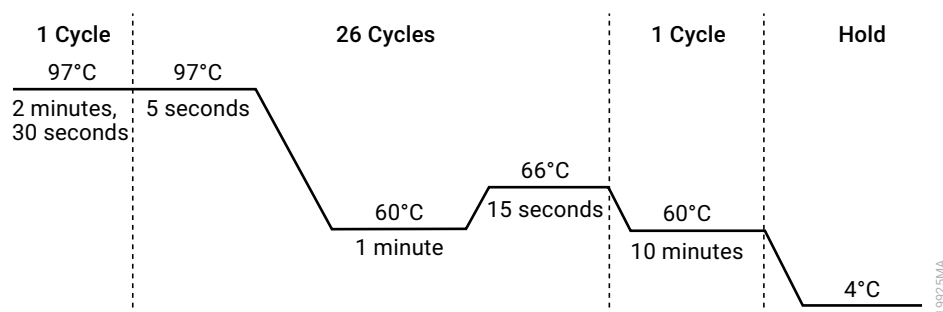


Figure 33. Thermal cycling protocol for the ProFlex® PCR System.

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

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

10.4.4 Thermal Cycling on the Mastercycler® X50s PCR System

Amplification and detection instrumentation can vary. You will need to optimize protocols for the template DNA amount, cycle number (25–26 cycles), injection conditions and loading volume for your laboratory instrumentation. Tests at Promega show that 26 cycles works well for a variety of sample types. Optimize the cycle number in each laboratory for each sample type that is amplified.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the protocol provided below and in Figure 34. The total cycling time is approximately 70 minutes.

Notes:

- a. Set the ramp speed to $5^{\circ}\text{C}/\text{second}$, the temperature mode to “Safe” and the block setting to “Silver 96”.
- b. If thermal cycling is interrupted at any stage after initiating the cycling protocol, prepare a new amplification plate.

Thermal Cycling Protocol

97°C for 3 minutes, then:

97°C for 10 seconds

60°C for 1 minute

66°C for 15 seconds

for 26 cycles, then:

60°C for 10 minutes

4°C hold

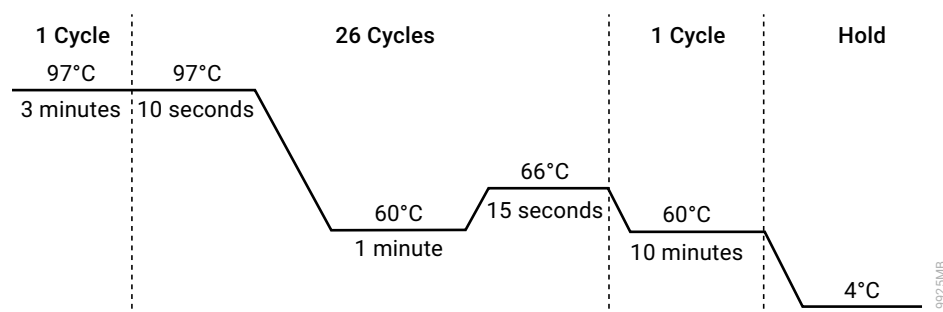


Figure 34. Thermal cycling protocol for the Mastercycler® X50s PCR System.

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

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

10.4.5 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 using your normal workflow.
2. Prepare three identical reaction plates with aliquots of the same swab extracts. Use the appropriate amount of Paradyme™ 27GY QI Reagent (Cat.# DM1701) for the desired cycle number.
3. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (25–26 cycles).

Note: This recommendation is for $2\mu\text{l}$ of swab extract. If a different volume of swab extract is used, additional cycle number testing may be required.

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

10.5 Composition of Buffers and Solutions

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

1.21g Tris base

0.037g EDTA (Na₂EDTA • 2H₂O)

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

TE⁻⁴ buffer with 20µg/ml glycogen

1.21g Tris base

0.037g EDTA (Na₂EDTA • 2H₂O)

20µg/ml glycogen

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

10.6 Related Products

PRODUCT	SIZE	CAT.#
8C Matrix Standard	5 preps	DG5050
CCO Internal Lane Standard 500	250µl	DG8571
Paradyne™ 27GY QI Reagent	800µl	DM1701
PunchSolution™ Kit	100 preps	DC9271
SwabSolution™ Kit	100 preps	DC8271
2800M Control DNA (10ng/µl)	25µl	DD7101
2800M Control DNA (0.25ng/µl)	500µl	DD7251
Water, Amplification Grade	6,250µl (5 × 1,250µl)	DW0991

Not for Medical Diagnostic Use.

Spectrum CE System Accessories and Consumables

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

Not for Medical Diagnostic Use.

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

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

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Class 1 Laser Product.