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Investigator 24plex GO! Kit Handbook

For multiplex amplification of the CODIS core loci, the European standard set of loci, plus SE33, DYS391, and Amelogenin

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Kit Contents

Investigator 24plex GO! Kit	(200)	(1000)
Catalog number	382426	382428
No. of 25 μL reactions	200	1000
Fast Reaction Mix 2.0*	2 x 750 μ L	10 x 750 μ L
Primer Mix 24plex GO!	2 x 1250 μ L	10 x 1250 μ L
Control DNA 9948 (5 ng/ μ L)	50 μ L	50 μ L
DNA size standard 24plex (BTO)	110 μ L	5 x 110 μ L
Allelic ladder 24plex	2 x 25 μ L	6 x 25 μ L
Quick-Start Protocol	1	1

* Contains DNA Polymerase, dNTPs, MgCl₂, and bovine serum albumin (BSA).

Shipping and Storage

The Investigator 24plex GO! Kit is shipped on dry ice. It should be stored immediately upon receipt at -30°C to -15°C in a constant-temperature freezer. Avoid repeated thawing and freezing. Primer mix and allelic ladder must be stored protected from the light. DNA samples and post-PCR reagents (allelic ladder and DNA size standard) should be stored separately from the PCR reagents. Under these conditions, the components are stable until the expiration date indicated on the kit.

Once opened, the Investigator 24plex GO! Kit should be stored at $2-8^{\circ}\text{C}$ for a maximum of 6 months.

Intended Use

The Investigator 24plex GO! Kit is intended for molecular biology applications in forensic, human identity, and paternity testing. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the Investigator 24plex GO! Kit is tested against predetermined specifications to ensure consistent product quality. Investigator 24plex GO! Kits meet ISO 18385 requirements.

Introduction

The Investigator 24plex GO! Kit is used for multiplex PCR in forensic, human identity, and paternity testing. The PCR simultaneously amplifies the 22 polymorphic STR markers listed below along with the gender-specific marker Amelogenin. These 22 markers are recommended by the CODIS (Combined DNA Index System) Core Loci Working Group, the European Network of Forensic Science Institutes (ENFSI), and the European DNA Profiling Group (EDNAP).

The Investigator 24plex GO! Kit Primer Mix contains 2 innovative internal PCR controls (Quality Sensor QS1 and QS2) to provide helpful information about the efficiency of the PCR and the presence of PCR inhibitors. The Quality Sensors are amplified simultaneously with the polymorphic STR markers.

The Investigator 24plex GO! Kit has been developed specifically for rapid direct amplification from blood or buccal cells on FTA[®] and other paper, and buccal swabs. The kit utilizes QIAGEN's fast-cycling PCR technology, allowing amplification in around 45 minutes. Punches from FTA and other filter papers can be used without the need for pretreatment. For buccal swabs, a fast and convenient lysis protocol generates a crude lysate for amplification in around 5 minutes. The primers are fluorescence-labeled with the following dyes:

- 6-FAM[™]: Amelogenin, TH01, D3S1358, vWA, D21S11
- BTG: TPOX, DYS391, D1S1656, D12S391, SE33
- BTY: D10S1248, D22S1045, D19S433, D8S1179, D2S1338
- BTR2: D2S441, D18S51, FGA
- BTP: QS1, D16S539, CSF1PO, D13S317, D5S818, D7S820, QS2

The recommended amount of sample is one punch of 1.2 mm diameter for FTA and other filter papers, or 2 µL of a buccal swab lysate.

The Investigator 24plex GO! Kit was validated using the GeneAmp® PCR System 9700 (with Gold-plated 96-Well Silver Block) and the Applied Biosystems® 3500™ Genetic Analyzer.

Table 1 shows the STR loci with their chromosomal mapping and repeat motifs, which are concordant with the International Society for Forensic Genetics (ISFG) guidelines for the use of microsatellite markers (1).

For information about known microvariants not contained in the Investigator 24plex allelic ladder, see the National Institute of Standards and Technology (NIST) website (www.cstl.nist.gov/biotech/strbase/).

Table 1. Locus-specific information of the Investigator 24plex GO! Kit

Locus	GenBank® accession number	Repeat motif of the reference allele	Chromosomal mapping
Amelogenin X	M55418	–	Xp22.1-22.3
Amelogenin Y	M55419	–	Yp11.2
DYS391	AC011302	[TCTA] ₁₁	Yq11.21
D1S1656	NC_000001.9	[TAGA] ₁₆ [TGA][TAGA][TAGG] ₁ [TG] ₅	1q42
D2S441	AL079112	[TCTA] ₁₂	2p14
D2S1338	G08202	[TGCC] ₆ [TTCC] ₁₁	2q35
D3S1358	11449919	TCTA [TCTG] ₂ [TCTA] ₁₅	3p25.3
D5S818	G08446	[AGAT] ₁₁	5q23.2
D7S820	G08616	[GATA] ₁₂	7q21.11
D8S1179	G08710	[TCTA] ₁₂	8q23.1-23.2
D10S1248	AL391869	[GGAA] ₁₃	10q26.3

Table 1. Locus-specific information of the Investigator 24plex GO! Kit (continued)

Locus	GenBank® accession number	Repeat motif of the reference allele	Chromosomal mapping
D12S391	G08921	[AGAT] ₅ GAT [AGAT] ₇ [AGAC] ₆ AGAT	12p13.2
D13S317	G09017	[TATC] ₁₃	13q31.1
D16S539	G07925	[GATA] ₁₁	16q24.1
D18S51	L18333	[AGAA] ₁₃	18q21.3
D19S433	G08036	AAGG [AAAG] AAGG TAGG [AAGG] ₁₁	19q12
D21S11	AP000433	[TCTA] ₄ [TCTG] ₆ [TCTA] ₃ TA [TCTA] ₃ TCA [TCTA] ₂ TCCATA [TCTA] ₁₁	21q21.1
D22S1045	AL022314	[ATT] ₁₄ ACT [ATT] ₂	22q12.3
CSF1PO	X14720	[AGAT] ₁₂	5q33.1
FGA (FIBRA)	M64982	[TTTC] ₃ TTTTTTCT [CTTT] ₁₃ CTCC [TTCC] ₂	4q28.2
SE33 (ACTBP2)	NG000840	[AAAG] ₉ AA [AAAG] ₁₆	6q14.2
TH01 (TC11)	D00269	[TCAT] ₉	11p15.5
TPOX	M68651	[AATG] ₁₁	2p25.3
vWA	M25858	TCTA [TCTG] ₄ [TCTA] ₁₃	12p13.31

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

All protocols

- Hi-Di™ Formamide, 25 mL (Applied Biosystems, cat. no. 4311320)
- Matrix Standards BT6 for multi-capillary instruments (e.g., 3500 Genetic Analyzers)
- Pipettes and pipette tips
- One of the following DNA analyzers¹ :
 - Applied Biosystems 3500 Genetic Analyzer
 - Applied Biosystems 3500xL Genetic Analyzer
- One of the following PCR thermal cyclers¹ :
 - GeneAmp PCR System 9700
 - Veriti™ 96-Well Thermal Cycler
 - ProFlex™ 96-well PCR System
 - QIAamplifier® 96
 - Bio-Rad® PTC-200
 - Biometra UNO-Thermoblock
 - Eppendorf® Mastercycler® ep

¹This is not a complete list of suppliers and does not include many important vendors of biological supplies.

- PCR tubes or plates
- Microcentrifuge for PCR tubes or plates

For protocols based on blood or buccal cells on paper

- UniCore Punch Kit 1.2 mm (cat. no. WB100028)

For protocols based on buccal cells on paper

- Investigator STR GO! Punch Buffer (1000) or (200) (QIAGEN, cat. no. 386528 or 386526)

For the protocol based on buccal cells on Bode Buccal DNA Collectors™

- Investigator STR GO! Lysis Buffer (QIAGEN, cat. no. 386516)

For protocols based on buccal swab lysates

- Investigator STR GO! Lysis Buffer (QIAGEN, cat. no. 386516)
- 2 mL microcentrifuge tubes
- Shaker for 2 mL microcentrifuge tubes

Validity analysis software for human identification products

Investigator Human Identification PCR Kits require calibration with an allelic ladder. Therefore, the software used must be compatible with human identification products for forensic applications. We recommend GeneMapper® ID-X. The Investigator Template Files facilitate data analysis and are valid with this software.

Important Notes

The experimental conditions outlined in the protocols have been found to give the best results. However, depending on the sample material, PCR cycle numbers may be adapted to ensure the highest possible first round success rates. We recommend running a representative batch of samples in order to confirm that the cycle numbers given in this protocol are optimal. Increase the cycle number by one if the signals in the resulting electropherograms are too low. Decrease the cycle number by one if the signals in the resulting electropherograms are too high.

Protocol: PCR Amplification from Blood on FTA and Other Paper

This protocol is for direct PCR amplification of STR loci from punches of blood on FTA and other paper using the Investigator 24plex GO! Kit.

Important points before starting

- Set up all reaction mixtures in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination risks.

Thing to do before starting

- Before opening the tubes containing PCR components, vortex and then centrifuge briefly to collect the contents at the bottom of the tubes.

Procedure

1. Take a 1.2 mm punch from the center of the blood spot with a suitable tool (e.g., UniCore Punch Kit 1.2 mm, cat. no. WB100028).

Important: Do not use more than one punch at a time.

2. Prepare a master mix according to Table 2 on page 16.

As some loss of reagents can occur during transfers, prepare the mix with additional reactions included. Also include positive and negative control reactions. The master mix contains all of the components needed for PCR except the template (sample) DNA.

Table 2. Master mix setup

Component	Volume per reaction (µL)
Fast Reaction Mix 2.0	7.5
Primer Mix	12.5
Total volume	20.0

Note: In case blood on FTA samples has been stored for longer periods, we recommend the Investigator STR GO! Punch Buffer be used to overcome potential inhibition. Add 3 µL Investigator STR GO! Punch Buffer per reaction.

3. Vortex the reaction mix thoroughly, and dispense 20 µL (or 23 µL, if optional Investigator STR GO! Punch Buffer, cat. no. 386526 or 386528, is used) into PCR tubes or the wells of a PCR plate.
4. Transfer one 1.2 mm disk to each reaction.

Note: Do not mix the reaction after disc transfer.

5. Prepare the positive and negative controls.
 - Positive control: Use 2 µL Control DNA (i.e., 10 ng).

Note: The amount of Control DNA may need to be adapted after setting the optimal PCR cycle number in your laboratory if signals are too low or too high. Do not add a blank disc to the positive control well.
 - Negative control: Do not add any template DNA. Do not add a blank disc or water to the negative control PCR tube or well.
6. Briefly centrifuge reactions to ensure discs are fully submerged.
7. Program the thermal cycler according to the manufacturer’s instructions, using the conditions given in Table 3 on page 18.

Note: If using the GeneAmp 9700 thermal cycler with an Aluminum block, use **Std Mode**; or with a Silver block or Gold-plated Silver block, use **Max Mode**. Do not use **9600 Emulation Mode**.

8. After the cycling protocol is completed, store samples at -30°C to -15°C protected from light, or proceed directly with electrophoresis.

Table 3a. Standard cycling protocol for blood on FTA and other paper

Temperature (°C)	Time	Number of cycles
98*	30 s	
64	40 s	3 cycles
72	5 s	
96	10 s	
61	40 s	22 cycles
72	5 s	
68	5 min	–
60	5 min	–
10	∞	–

* Hot start to activate DNA polymerase.

Table 3b. Optional cycling protocol for blood on FTA and other paper

Temperature (°C)	Time	Number of cycles
98*	30 s	
64	40 s	3 cycles
72	5 s	
96	10 s	
61	40 s	22 cycles
72	5 s	
68	2 min	–
60	2 min	–
10	∞	–

* Hot start to activate DNA polymerase.

Table 3b details previously published cycling conditions which may continue to be used if incomplete adenylation is not visible within the electropherograms.

Protocol: PCR Amplification from Buccal Cells on FTA and Other Paper

This protocol is for direct PCR amplification of STR loci from punches of buccal cells on FTA and other paper using the Investigator 24plex GO! Kit. (For Bode Buccal DNA Collectors, see “Protocol: PCR Amplification from Buccal Cells from Bode Buccal DNA Collectors” on page 23).

Important points before starting

- Set up all reaction mixtures in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination risks.

Thing to do before starting

- Before opening the tubes containing PCR components, vortex and then centrifuge briefly to collect the contents at the bottom of the tubes.

Procedure

1. Take a 1.2 mm punch with a suitable tool (e.g., UniCore Punch Kit 1.2 mm, cat. no. WB100028).

Note: For buccal cells collected using a Indicating FTA Cards, take the punch from a white area. This color indicates successful sample transfer.

Important: Do not use more than one punch at a time.

2. Prepare a master mix according to Table 4 on the next page.

As some loss of reagents can occur during transfers, prepare the mix with additional reactions included. Also include positive and negative control reactions. The master mix contains all of the components needed for PCR except the template (sample) DNA.

Table 4. Master mix setup

Component	Volume per reaction (µL)
Fast Reaction Mix 2.0	7.5
Primer Mix	12.5
Investigator STR GO! Punch Buffer	2.0
Total volume	22.0

- Vortex the reaction mix thoroughly, and dispense 22 µL into PCR tubes or the wells of a PCR plate.
- Transfer one 1.2 mm disk to each reaction.
Note: Do not mix the reaction after disc transfer.
- Prepare positive and negative controls.
 - Positive control: Use 1 µL Control DNA (i.e., 5 ng).
Note: The amount of Control DNA may need to be adapted after setting the optimal PCR cycle number in your laboratory if signals are too low or too high. Do not add a blank disc to the positive control well.
 - Negative control: Do not add any template DNA. Do not add a blank disc or water to the negative control PCR tube or well.
- Briefly centrifuge reactions to ensure discs are fully submerged.
- Program the thermal cycler according to the manufacturer's instructions, using the conditions given in Table 5 on page 22.

Note: If using the GeneAmp 9700 thermal cycler with an Aluminum block, use “Std Mode”; with a Silver block or Gold-plated Silver block, use “Max Mode”. Do not use “9600 Emulation Mode”.

8. After the cycling protocol is completed, store samples at -30°C to -15°C protected from light, or proceed directly with electrophoresis.

Table 5a. Standard cycling protocol for buccal cells on FTA and other paper

Temperature (°C)	Time	Number of cycles
98*	30 s	
64	40 s	3
72	5 s	
96	10 s	
61	40 s	23
72	5 s	
68	5 min	–
60	5 min	–
10	∞	–

* Hot start to activate DNA polymerase.

Table 5b. Optional cycling protocol for buccal cells on FTA and other paper

Temperature (°C)	Time	Number of cycles
98*	30 s	
64	40 s	3
72	5 s	
96	10 s	
61	40 s	23
72	5 s	
68	2 min	–
60	2 min	–
10	∞	–

* Hot start to activate DNA polymerase.

Table 5b details previously published cycling conditions which may continue to be used if incomplete adenylation is not visible within the electropherograms.

Protocol: PCR Amplification from Buccal Cells from Bode Buccal DNA Collectors

This protocol is for direct PCR amplification of STR loci from punches of buccal cells on Bode Buccal DNA Collectors using the Investigator 24plex GO! Kit.

Important points before starting

- Set up all reaction mixtures in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination risks.

Thing to do before starting

- Before opening the tubes containing PCR components, vortex and then centrifuge briefly to collect the contents at the bottom of the tubes.

Procedure

1. Collect a 1.2 mm punch from the tip (rounded end) of the Bode Buccal DNA Collector with a suitable tool (e.g., UniCore Punch Kit 1.2 mm, cat. no. WB100028) into a 0.2 mL PCR-grade plate or 0.2 mL PCR-grade tube.

Important: Do not use more than one punch at a time, per well or per tube.

2. Add 2 μ L of Investigator STR GO! Kit Lysis Buffer directly onto the 1.2 mm punch. Centrifuge briefly if necessary to collect the punch and buffer at the bottom of the plate or tube.

3. Incubate the sample at 95°C for 5 min. Do not seal the plate.

Note: The Lysis Buffer will evaporate.

4. Prepare a master mix according to Table 6. Vortex the reaction mix thoroughly.

As some loss of reagents can occur during transfers, prepare the mix with additional reactions included. Also include positive and negative control reactions. The master mix contains all of the components needed for PCR except the template (sample) DNA.

Table 6. Master mix setup

Component	Volume per reaction (µL)
Fast Reaction Mix 2.0	7.5
Primer Mix	12.5
Total volume	20.0

5. After incubation, dispense 20 µL of the master mix into each well of the PCR plate or the PCR tubes, containing the 1.2 mm punch.

Note: Do not mix the reaction after distributing the master mix.

6. Prepare positive and negative controls.

- Positive control: Use 2 µL Control DNA (i.e., 10 ng).

Note: The amount of Control DNA may need to be adapted after setting the optimal PCR cycle number in your laboratory, if signals are too low or too high. Do not add a blank disc to the positive control well.

- Negative control: Do not add any template DNA. Do not add a blank disc or water to the negative control PCR tube or well.

7. Briefly centrifuge reactions to ensure discs are fully submerged.

8. Program the thermal cycler according to the manufacturer's instructions, using the conditions given in Table 7a on the next page.

Note: If using the GeneAmp PCR system 9700 with an Aluminum Sample Block Module, use "Std Mode". If using a Silver Block or Gold-Plated Silver Block Module, use "Max Mode". Do not use "9600 Emulation Mode".

9. After the cycling protocol is completed, store samples at -30°C to -15°C protected from light, or proceed directly with electrophoresis.

Table 7a. Standard cycling protocol for blood on FTA and other paper

Temperature (°C)	Time	Number of cycles
98*	30 s	
64	40 s	3
72	5 s	
96	10 s	
61	40 s	24
72	5 s	
68	5 min	–
60	5 min	–
10	∞	–

* Hot start to activate DNA polymerase.

Table 7b. Optimal cycling protocol for blood on FTA and other paper

Temperature (°C)	Time	Number of cycles
98*	30 s	
64	40 s	3
72	5 s	
96	10 s	
61	40 s	24
72	5 s	
68	2 min	–
60	2 min	–
10	∞	–

* Hot start to activate DNA polymerase.

Table 7b details previously published cycling conditions which may continue to be used if incomplete adenylation is not visible within the electropherograms.

Protocol: PCR Amplification from Buccal Swab Lysates

This protocol is for direct PCR amplification of STR loci from crude lysates of buccal swabs using the Investigator 24plex GO! Kit.

Important points before starting

- Set up all reaction mixtures in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination risks.

Thing to do before starting

- Before opening the tubes containing PCR components, vortex and then centrifuge briefly to collect the contents at the bottom of the tubes.

Procedure

1. Place the swab in a 2 mL microcentrifuge tube.

Carefully cut, break off, or eject the end part of the swab.

Note: Prepare a blank swab as negative control.

2. Add 500 μ L STR GO! Lysis Buffer to the sample.
3. Incubate at 95°C for 5 min shaking at 1200 rpm in a thermomixer.

Optional: Incubate at room temperature for 5 min shaking at 1200 rpm in a thermomixer.

4. Prepare a master mix according to Table 8.

As some loss of reagents can occur during transfers, prepare the mix with additional reactions included. Also include positive and negative control reactions. The master mix contains all of the components needed for PCR except the template (sample) DNA.

Table 8. Master mix setup

Component	Volume per reaction (µL)
Fast Reaction Mix 2.0	7.5
Primer Mix	12.5
Total volume	20.0

5. Vortex the reaction mix thoroughly, and dispense 20 µL into PCR tubes or the wells of a PCR plate.
6. Mix the swab lysate thoroughly and transfer 2 µL of swab lysate directly to each reaction.
7. Prepare positive and negative controls.

- Positive control: Use 1 µL Control DNA (i.e., 5 ng).

Note: The amount of Control DNA may need to be adapted after setting the optimal PCR cycle number in your laboratory if signals are too low or too high. Do not add a blank disc to the positive control well.

- Negative control: Use a blank swab lysate.

8. Program the thermal cycler according to the manufacturer's instructions, using the conditions given in Table 9 on page 27.

Note: If using the GeneAmp 9700 thermal cycler with an Aluminum block, use "Std Mode"; with a Silver block or Gold-plated Silver block, use "Max Mode". Do not use "9600 Emulation Mode".

9. After the cycling protocol is completed, store samples at -30°C to -15°C protected from light, or proceed directly with electrophoresis.

Table 9a. Standard cycling protocol for buccal swab lysates

Temperature (°C)	Time	Number of cycles
98*	30 s	
64	40 s	3
72	5 s	
96	10 s	
61	40 s	24
72	5 s	
68	5 min	–
60	5 min	–
10	∞	–

* Hot start to activate DNA polymerase.

Table 9b. Optional cycling protocol for buccal swab lysates

Temperature (°C)	Time	Number of cycles
98*	30 s	
64	40 s	3
72	5 s	
96	10 s	
61	40 s	24
72	5 s	
68	2 min	–
60	2 min	–
10	∞	–

* Hot start to activate DNA polymerase.

Table 9b details previously published cycling conditions which may continue to be used if incomplete adenylation is not visible within the electropherograms.

Protocol: Electrophoresis Using the Applied Biosystems 3500/3500xL Genetic Analyzer

The Investigator 24plex GO! Kit is validated for use on the 3500/3500xL Genetic Analyzer, which requires the 3500 Data Collection Software v1 or v2 or HID Updater 3500 Data Collection v2.0 software.

Note: The user must be logged on to the PC as local administrator or with equivalent access rights to allow data to be written to the appropriate files.

For detailed instructions on instrument setup, spectral calibration, or application of the Applied Biosystems 3500 Series Data Collection Software v1 or v2 and the GeneMapper ID-X Software v1.2, refer to the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide*. The system with 8 capillaries is the Applied Biosystems 3500 Genetic Analyzer; the system with 24 capillaries is the Applied Biosystems 3500xL Genetic Analyzer.

The virtual filter set AnyDye is used for combined application of the 6 fluorescent labels 6-FAM, BTG, BTY, BTR2, BTP, and BTO. This matrix standard is BT6.

The materials required for electrophoresis are given in Table 10.

Table 10. Materials required for electrophoresis

Component	Volume per reaction
Capillary	36 cm Array for Applied Biosystems 3500/3500xL Genetic Analyzer
Polymer	POP-4™ for Applied Biosystems 3500/3500xL Genetic Analyzer
Buffer	Anode Buffer Container (ABC) 3500 Series Cathode Buffer Container (CBC) 3500 Series

Spectral calibration/matrix generation

Before conducting DNA fragment size analysis, perform a spectral calibration with the 6 fluorescent labels 6-FAM, BTG, BTY, BTR2, BTP, and BTO for each analyzer (Table 11).

The calibration procedure creates a matrix that is used to correct the overlapping of the fluorescence emission spectra of the dyes.

Important: Spectral calibration must be performed for each new capillary array. It comprises the following steps:

- Preparation of the instrument
- Preparation of the standard calibration plate
- Plate assembly and loading into the instrument
- Software setup of dye set BT6
- Performing a spectral calibration run
- Checking the matrix

Preparation of the instrument

Before the spectral calibration process, ensure that the spatial calibration has been performed. This process is described in detail in the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide*.

Table 11. The 6 fluorescent labels of BT6

Color	Matrix standard
Blue (B)	6-FAM
Green (G)	BTG
Yellow (Y)	BTY
Red (R)	BTR2
Purple (P)	BTP
Orange (O)	BTO

Preparation of the standard calibration plate for 8 capillaries (Applied Biosystems 3500 Genetic Analyzer)

1. Before opening the tubes, vortex and then centrifuge briefly to collect the contents at the bottom of the tubes.
2. Set up a mixture of formamide and Matrix Standard BT6 according to the information in Table 12.

Table 12. Setup of formamide and Matrix Standard BT6 mixture for 8 capillaries

Component	Volume (µL)
Hi-Di Formamide	90
Matrix Standard BT6 multi cap.	10

3. Vortex and then centrifuge the mixture briefly.
4. Load 10 µL of the mixture into each of the 8 wells in a 96-well plate at positions A1–H1.
5. Denature for 3 min at 95°C.
6. Snap freeze by placing the plate on ice for 3 min.

A thermal cycler set to 4°C may be used to cool the plate instead.

Preparation of the standard calibration plate for 24 capillaries (Applied Biosystems 3500xL Genetic Analyzer)

1. Before opening the tubes, vortex and then centrifuge briefly to collect the contents at the bottom of the tubes.
2. Set up a mixture of formamide and Matrix Standard BT6 according to the information in Table 13.

Table 13. Setup of formamide and Matrix Standard BT6 mixture for 24 capillaries

Component	Volume (µL)
Hi-Di Formamide	225
Matrix Standard BT6 multi cap.	25

3. Vortex and then centrifuge the mixture briefly.
4. Load 10 µL of the mixture into each of the 24 wells in a 96-well plate at positions A1–H1, A2–H2, and A3–H3.
5. Denature for 3 min at 95°C.
6. Snap freeze by placing the plate on ice for 3 min.

A thermal cycler set to 4°C may be used to cool the plate instead.

Plate assembly and loading the plate in the instrument

The necessary steps are described in detail in the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide*.

Software setup of dye set BT6

Prior to the spectral calibration, a dye set for the Matrix Standard BT6 must be set up.

1. To create a new dye set, select **Library**. Under **Analyze**, go to **Dye Sets** and click **Create**.
2. Enter a Dye Set Name (for example, "BT6").
3. Under **Chemistry** select **Matrix Standard**, and as a **Dye Set Template**, select **AnyDye Template**.
4. Under **Calibration Peak Order** arrange the colors as follows: 6 – blue, 5 – orange, 4 – green, 3 – yellow, 2 – red, and 1 – purple.

Note: This is the correct instrument setting of the peak order, although the peak order of the Matrix Standard BT6 is different.

5. Alter the **Parameters** settings as follows:
 - Matrix Condition Number Upper Limit: 13.5
 - Locate Start Point After Scan: 1000
 - Locate Start Point Before Scan: 5000
 - Limit Scans To: 2750
 - Sensitivity: 0.4
 - Minimum Quality Score: 0.95
6. Click **Save** to confirm the changes.

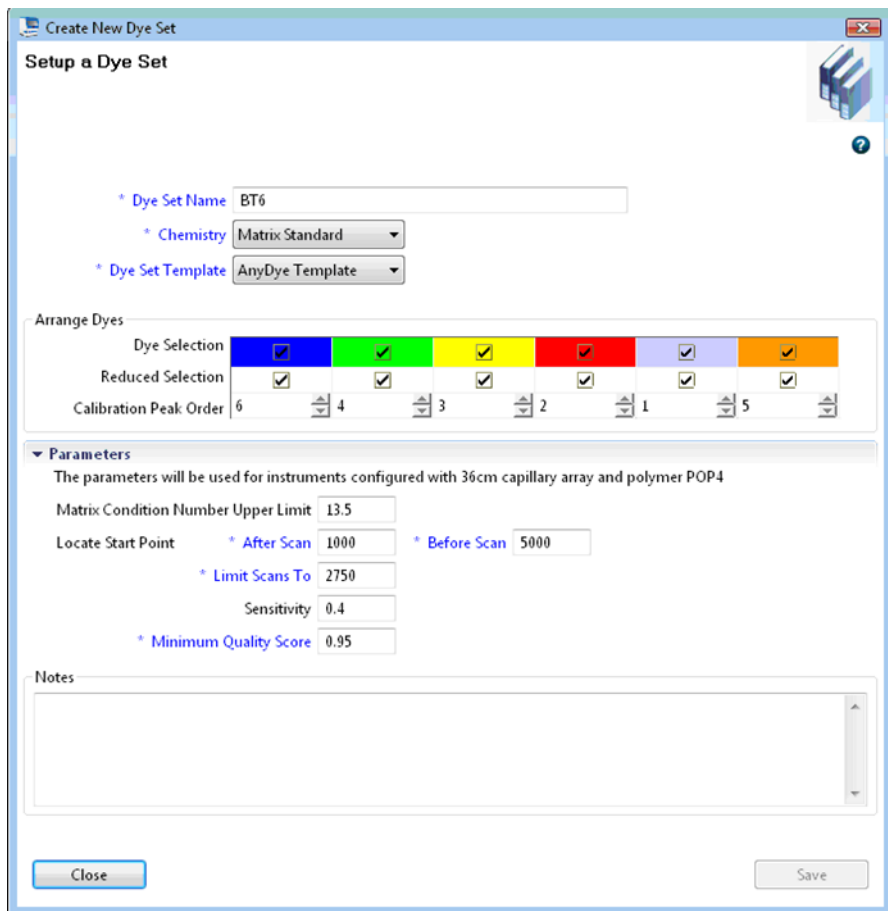


Figure 1. Setup of dye set BT6.

Performing a spectral calibration run

Once the multiwell plates containing the spectral calibration mixture are placed in the autosampler tray, the spectral calibration process can be started.

1. To access the Spectral Calibration screen, select **Maintenance** on the Dashboard of the 3500 Series Data Collection software.
2. To set up a calibration run go to **Calibrate**, followed by **Spectral** and select **Calibration Run**.
3. The number of wells in the spectral calibration plate and the position in the instrument must be specified.
4. Under **Chemistry Standard** select **Matrix Standard**, and as a Dye Set Name select, for example, the previously created BT6 (refer to “Software setup of dye set BT6” on page 33).
5. Optional: Enable **Allow Borrowing**.
6. Click **Start Run**.

Checking the matrix

Click a capillary in the table to display the results for each capillary below the run results table (Capillary, Quality value, and Condition Number).

- The quality value (Q value) of each capillary must be greater than 0.95 and the condition number range (C value) must be between 1 and 13.5.
- Check the matrix samples for a flat baseline. As shown in the figure, there should be 6 peaks with peak heights of about 1000–6000 RFU for each matrix sample (Note: The optimal range is 3000–5000 RFU).

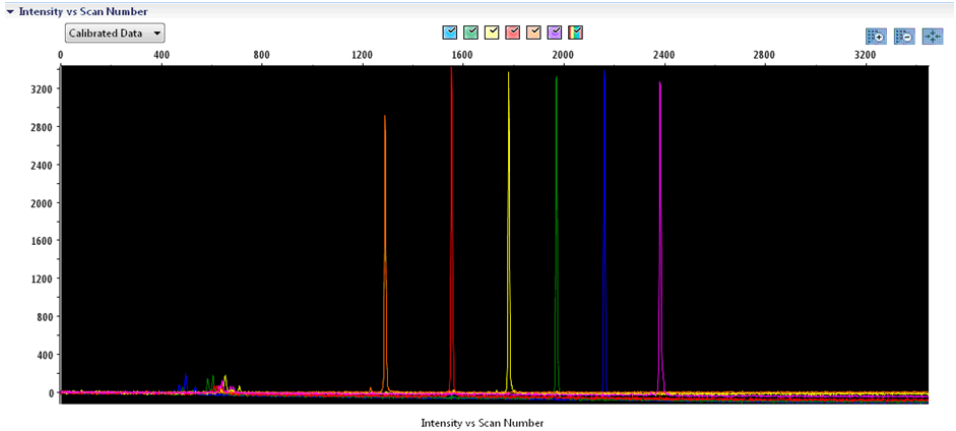


Figure 2. Electropherogram of spectral calibration of the matrix standard BT6 on an Applied Biosystems 3500 Genetic Analyzer.

When a spectral calibration is successfully completed, the “Overall” row displays green results. If the “Overall” row displays red results, refer to the “Spectral calibration troubleshooting” section of the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide*.

▼ Capillary Run Data

Capillary	1	2	3	4	5	6	7	8
Run 1	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Run 2	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Run 3	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Overall	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed

■ Passed
 ■ Failed
 ■ Borrowed
 Not Calibrated

Figure 3. Example of successful spectral calibration of the matrix standard BT6 for all capillaries on an Applied Biosystems 3500 Genetic Analyzer.

For each capillary, select and display the spectral and raw data. Check that the data meet the following criteria:

- The order of the peaks in the spectral profile from left to right should read orange–red–yellow–green–blue–purple
- No extraneous peaks should appear in the raw data profile
- Peak morphology in the spectral profile should show no gross overlaps, dips, or other irregularities. Separate and distinct peaks should be visible

If the data for all capillaries meet the criteria above, click **Accept**. If any capillary data do not meet the criteria above, click **Reject**, and refer to the “Spectral calibration troubleshooting” section of the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide*.

Sample preparation

1. Before opening the tubes, vortex and then centrifuge briefly to collect the contents at the bottom of the tubes.
2. Set up a mixture of formamide and DNA size standard according to the information in Table 14 on the facing page.
3. Vortex and then centrifuge the mixture briefly.
4. Aliquot 12 μL of the mixture to a tube for each sample to be analyzed.
5. Add 1 μL PCR product or allelic ladder (diluted, if necessary).
6. Denature for 3 min at 95°C.
7. Snap freeze by placing the plate on ice for 3 min.
A thermal cycler set to 4°C may be used to cool the plate instead.
8. Load the samples on the tray.

Table 14. Setup of formamide and DNA size standard mixture

Component	Volume per sample (µL)
Hi-Di Formamide	12.0
DNA Size Standard 24plex (BTO)	0.5

Note: Since injections take place simultaneously on all capillaries, a minimum of 1 entire column (8-sample protocol) or 3 entire columns (24-sample protocol) must be pipetted onto the plate of multi-capillary analyzers. If fewer samples are analyzed, the empty positions must be filled with 12 µL Hi-Di Formamide.

To ensure a reliable allelic assignment on multi-capillary analyzers, inject one allelic ladder for each set of 24 samples:

- 8-capillary instruments: One allelic ladder per 3 injections
- 24-capillary instruments: One allelic ladder per injection

Important: The actual room temperature may influence the performance of PCR products on multi-capillary instruments, so shoulder peaks or split peaks can occur, especially at lower temperatures. **Ensure that the ambient conditions are maintained as recommended by the instrument manufacturer.** Also, ensure buffers are equilibrated to ambient conditions.

Setting up a run

If you are using the Investigator 24plex GO! Kit for the first time on an Applied Biosystems 3500 Genetic Analyzer, you will first need to set up a number of protocols:

- Instrument Protocol
- Size Standard
- QC Protocol
- Assay

All protocols can be set up via the Dashboard of the 3500 Series Data Collection software.

Instrument Protocol

1. To set up the Instrument Protocol, select **Library**, under **Analyze**. Then go to **Instrument Protocols** and click **Create**.

Note: Modify the Run Module Default settings from "HID36_POP4" as shown in Table 15 on the facing page.

2. The parameters from Table 15 must be entered or selected.
3. Click **Save** to confirm the changes.

Table 15. Instrument Protocol parameters for Applied Biosystems 3500/3500xL Genetic Analyzer

Parameter	3500 Setting	3500xL Setting
Application Type	HID	HID
Capillary Length	36 cm	36 cm
Polymer	POP4	POP4
Dye Set	e.g., BT6	e.g., BT6
Run Module	HID36_POP4	HID36_POP4
Protocol Name	e.g., Investigator 24plex	e.g., Investigator 24plex
Oven Temperature (°C)	Default (60)	Default (60)
Run Voltage (kV)	13.0	13.0
PreRun Voltage (kV)	Default (15)	Default (15)
Injection Voltage (kV)	1.2	1.6
Run Time (s)	1550	1550
PreRun Time (s)	Default (180)	Default (180)
Injection Time (s)	30.0*	33.0*
Data Delay (s)	Default (1)	Default (1)
Advanced Options	Default	Default

* Deviating from the standard settings, the injection time may range between 1 and 35 s depending on the type of sample. If samples with very high signal intensities are recorded, a shorter injection time may be selected. For samples with low DNA content, an injection time of up to 35 s may be necessary.

Size Standard

1. To set up the Size Standard, select **Library**, under **Analyze**. Then go to **Size Standards**, and click **Create**.
2. The parameters in Table 16 on the next page must be entered or selected.

The DNA Size Standard 24plex (BTO) should be used with the following lengths of fragments: 60, 80, 90, 100, 120, 140, 160, 180, 200, 220, 240, 250, 260, 280, 300, 320, 340, 360, 380, 400, 425, 450, 475, 500, 525, and 550 bp.

- Alternatively, import the DNA Size Standard 24plex (BTO) parameters using the recommended Investigator template file “SST-BTO_60-500bp” (Table 21 on page 47).
- Click **Save** to confirm the changes.

Table 16. Size standard parameters

Parameter	Setting
Size Standard	e.g., SST-BTO_60-500bp
Dye Color	Orange

QC Protocol

- To set up the QC Protocol, select **Library**, under **Analyze**. Then go to **QC Protocols**, and click **Create**.
- The parameters in Table 17 must be entered or selected.

Table 17. QC Protocol parameters

Parameter	Setting
Protocol Name	e.g., BTO_550
Size Standard	SST-BTO_60-500bp
Sizecaller	SizeCaller v1.1.0

- Go to **Analysis Settings**, followed by **Peak Amplitude Threshold** and ensure that all colors are enabled.

Check the recommended analysis settings in Table 20 on page 45. All other settings

should remain as **Default**.

4. Click **Save** to confirm the changes.

Assay

1. To set up an Assay, go to **Library**, under **Manage**. Then go to **Assays**, and click **Create**.
2. To analyze Investigator 24plex fragments, the parameters in Table 18 below must be selected.
3. Click **Save** to confirm the changes.

Table 18. Assay parameters

Parameter	Setting
Assay Name	e.g., Investigator 24plex
Color	Default
Application Type	HID
Instrument Protocol	e.g., Investigator 24plex
QC Protocols	e.g., BTO_550

Starting the run

1. In the Dashboard, click **Create New Plate**.
2. Go to **Setup**, followed by **Define Plate Properties** and select **Plate Details**. Select or enter the parameters in Table 19.

Table 19. Plate properties

Property	Setting
Name	e.g., Investigator 24plex
Number of Wells	96
Plate Type	HID
Capillary Length	36 cm
Polymer	POP4

3. Click **Assign Plate Contents** to confirm the changes.
4. Enter the designated sample name in each well containing a sample or allelic ladder. This will identify the well positions of each sample for the data collection and processing.
5. Under **Assay**, choose the correct Assay for the analysis. If you followed the steps under "Setting up a run", click **Add from Library** and select **Investigator 24plex** as Instrument Protocol. All named wells on the plate must have an assigned assay.
6. Repeat for **File name conventions** and **Results group**.
7. Select the wells that an assay should be specified for. Check the boxes next to the names of **Assay**, **File name conventions**, and **Results group** to assign those to the selected wells.
8. If not already done, load the assembled plate into the instrument and close the instrument door to re-initialize the instrument. Then click **Link Plate for Run**. In the next screen, enter the desired Run Name and click **Start Run**.

Analysis parameters/analysis method

Table 20 on the facing page lists the recommended analysis parameters in the worksheet Peak Detector.

Table 20. Recommended settings for the Applied Biosystems 3500/3500xL Genetic Analyzer

Parameter	Settings
Peak Detection Algorithm	Advanced
Ranges	Analysis: Partial Range Start Point: 1000; Stop Point: 20,000 Sizing: All Sizes
Smoothing and Baselineing	Smoothing: Light Baseline Window: 51 pts
Size Calling Method	Local Southern Method
Peak Detection	Peak Amplitude Thresholds B:* Y:* G:* R:* P:* O:* Min. Peak Half Width: 2 pts Polynomial Degree: 3 Peak Window Size: 11 pts† Slope Thresholds: 0.0

* The peak amplitude threshold (cutoff value) corresponds to the minimum peak height that will be filtered by the GeneMapper ID-X Software. The thresholds are usually 50–200 RFU and should be determined individually by the laboratory.

Recommendation: The minimal peak height should be 3 times higher than the background noise of the baseline.

† Only the setting for Peak Window Size is different to the Applied Biosystems defaults for HID analysis.

Protocol: Analysis

For general instructions on automatic sample analysis, refer to the appropriate user guide for GeneMapper ID-X Software.

Finding the exact lengths of the amplified products depends on the device type, the conditions of electrophoresis, as well as the DNA size standard used. Due to the complexity of some loci, determining the size should be based on evenly distributed references. The DNA Size Standard 24plex (BTO) should be used with the following lengths of fragments: 60, 80, 90, 100, 120, 140, 160, 180, 200, 220, 240, 250, 260, 280, 300, 320, 340, 360, 380, 400, 425, 450, 475, 500, 525, and 550 bp.

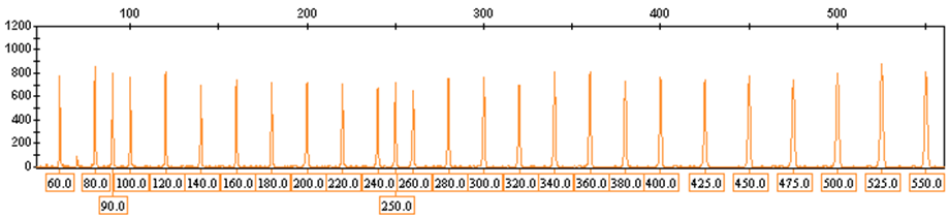


Figure 4. Electropherogram of the DNA Size Standard 24plex (BTO) fragments, with lengths in bp.

Analysis software

Allele allocation should be carried out using suitable analysis software (e.g., GeneMapper ID-X Software in combination with the Investigator Template Files, which are available for download from www.qiagen.com; see Table 21 on the facing page).

Table 21. Recommended Investigator Template Files for GeneMapper ID-X

File type	File name
Panels	24plex_Panels_x
BinSets	24plex_Bins_x
Stutter	24plex_Stutter_x
Size standard	SST-BTO_60–500bp
Analysis Method	Analysis_HID_3500_200rfu
Plot Settings	Plots_6dyes

Panels and BinSets must always be used; other template files are optional.

Controls

The alleles listed in Table 22 represent the Control DNA 9948 (included in the Investigator 24plex GO! Kit) and DNA from other commercially available standard cell lines.

Table 22. Allele assignment of the Investigator 24plex GO! Kit

Locus	CCR 9948	CCR 9947A	CCR 3657
Amelogenin	X/Y	X/X	X/Y
DYS391	10/10	–	10/10
D1S1656	14/17	18.3/18.3	13/18.3
D2S441	11/12	10/14	14/14
D2S1338	23/23	19/23	18/22
D3S1358	15/17	14/15	16/18
D5S818	11/13	11/11	11/11
D7S820	11/11	10/11	10/11
D8S1179	12/13	13/13	15/16
D10S1248	12/15	13/15	14/16
D12S391	18/24	18/20	18/19
D13S317	11/11	11/11	11/13
D16S539	11/11	11/12	13/13
D18S51	15/18	15/19	12/20
D19S433	13/14	14/15	13/14
D21S11	29/30	30/30	28/29
D22S1045	16/18	11/14	11/17
CSF1PO	10/11	10/12	11/11
FGA	24/26	23/24	18/23
SE33	23.2/26.2	19/29.2	22.2/27.2
TH01	6/9.3	8/9.3	7/9.3
TPOX	8/9	8/8	8/11
vWA	17/17	17/18	14/19

For further confirmation, Table 22 displays the alleles of the reference DNA purchased from Coriell Cell Repositories (CCR), as well as 2 reference DNAs purchased from CCR to the standard of Szibor et al. (2).

Quality Sensor

The Investigator 24plex GO! Kit contains 2 internal PCR controls (Quality Sensor QS1 and QS2), which provide helpful information about the PCR amplification efficiency in general, and about the presence of PCR inhibitors. The internal Quality Sensors are included in the Primer Mix and are amplified simultaneously with the polymorphic STR markers. The Quality Sensors are labeled with BTP and appear at fragment sizes of 74 bp (QS1) and 435 bp (QS2).

To address the issue of sequence similarity and the possibility of non-specific binding, a synthetic internal control DNA template was designed using a random algorithm. The template sequence differs from all known DNA sequences, and in particular bears no similarity to human DNA. The chance of non-specific binding in the context of a multiplex PCR amplification reaction is therefore very low.

In general, the successful amplification of the small Quality Sensor (QS1) indicates that the PCR was set up and conducted correctly, regardless of whether DNA was present or absent in the sample. If no Quality Sensor is detected in the analysis of the amplification products, this means that pipetting of the PCR setup or the PCR itself was performed incorrectly. This indicates that the experiment should be repeated, following the instructions in the protocol carefully.

Sensitivity experiments revealed that the internal controls have no effect on the performance of the PCR. The amplification of low DNA template amounts showed similar results for primer mixes with or without the Quality Sensors.

In addition, analysis of the 2 internal control fragments, QS1 and QS2, and of the STR target amplification products enables differential identification of the presence of inhibitors or the presence of DNA degradation in an amplification reaction.

In the case of sample degradation, the amplification of smaller target fragments is more efficient than the amplification of larger target fragments. However, degradation of the target template does not hamper amplification of the internal control fragments from the internal control template (Electropherogram of STR analysis in the presence of degraded DNA (fragments of 150 bp). Genomic DNA was sheared to fragments of 150 bp using ultrasound. The large STR fragments were amplified with a very low PCR yield, but QS1 and QS2 were amplified normally with equal peak heights. The markers are shown at the top of the electropherogram. The Quality Sensors are labeled with BTP (panel 5) and appear at fragment sizes of 74 bp (QS1) and 435 bp (QS2).). Thus, an equal ratio of QS1 and QS2, together with a ratio in favor of small STR target products suggests sample degradation.

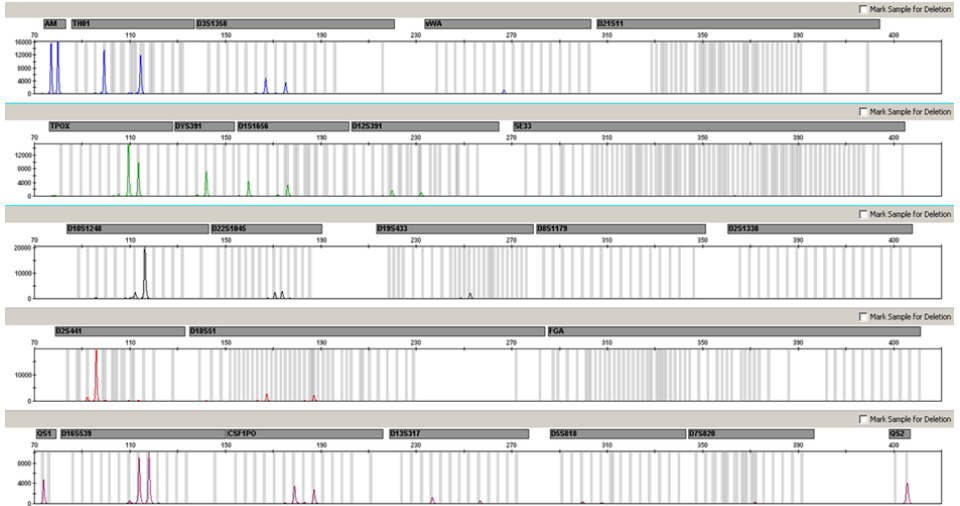


Figure 5. Electropherogram of STR analysis in the presence of degraded DNA (fragments of 150 bp). Genomic DNA was sheared to fragments of 150 bp using ultrasound. The large STR fragments were amplified with a very low PCR yield, but QS1 and QS2 were amplified normally with equal peak heights. The markers are shown at the top of the electropherogram. The Quality Sensors are labeled with BTP (panel 5) and appear at fragment sizes of 74 bp (QS1) and 435 bp (QS2).

If inhibitors such as hematin are present in the sample in high concentrations, amplification is less efficient and larger DNA fragments are amplified less well than smaller ones. If the analysis of the amplification products indicates inefficient amplification of the larger STR target sequences and the larger Quality Sensor (QS2) fragment, but the smaller Quality Sensor (QS1) is amplified successfully, the sample is likely to have been contaminated with inhibitors. This means that a shift of the ratio in favor of the small Quality Sensor (QS1) suggests the presence of inhibitors (Figure 6).

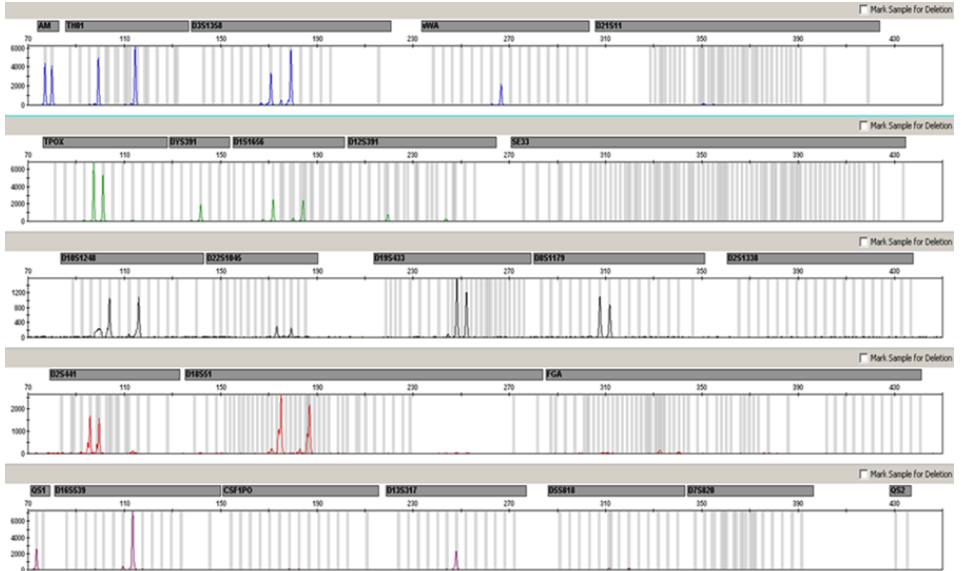


Figure 6. Electropherogram of STR analysis in the presence of hematin. Twenty-two STR markers, Amelogenin, and the 2 Quality Sensors were amplified in the presence of 1000 μM hematin and analyzed using capillary electrophoresis. The amplification of high-molecular-weight fragments, including STR makers of more than 250 bp and QS2, was inhibited by the high hematin content. The markers are shown at the top of the electropherogram. The Quality Sensors are labeled with BTP (panel 5) and appear at fragment sizes of 74 bp (QS1) and 435 bp (QS2 not visible).

Analysis of the presence of the 2 Quality Sensors allows the user to differentially identify the presence of PCR inhibitors or the occurrence of degradation in the forensic sample. This provides the user with helpful information for data interpretation and for planning of the next steps.

Table 23 on the facing page summarizes the possible profile appearances and their meanings.

Table 23. Profile appearances and their meanings

Allele peaks	QS1	QS2	Interpretation
Present	Present	Present	Successful profile
Absent	Present	Present	No DNA
Absent	Absent	Absent	Failed PCR
Ski-slope profile	Present	Dropdown	Inhibitors present
Ski-slope profile	Present	Present	Degraded DNA

Note: The amplification reactants for both Quality Sensors are limited in the Investigator 24plex GO! amplification reaction, which means that the maximum peak height is already achieved with 25 cycles. Therefore, increasing the number of cycles to, for example, 26 or 27 cycles will not increase the peak height of the Quality Sensors.

The peak heights of QS1 and QS2 may vary slightly between different experiments. A slight peak height scattering is usual, and is not dependent on inhibitor influence. During the validation, the analyst should evaluate the usual variation spectrum in relation to their sample type, and should define a regular peak height range for both QS.

A drop down of the QS2 signal below 20% of the QS1 signal indicates inhibition of the PCR reaction.

Alleles

Table 24 shows the alleles of the allelic ladder. All analyses were performed using POP-4 polymer (Table 24 on page 56 and Figure 7 on the next page). Different analysis instruments, DNA size standards, or polymers may result in different fragment lengths. In addition, visual alignment with the allelic ladder is recommended.

Scaling

- Horizontal: 70–470 bp
- Vertical: Depending on signal intensity

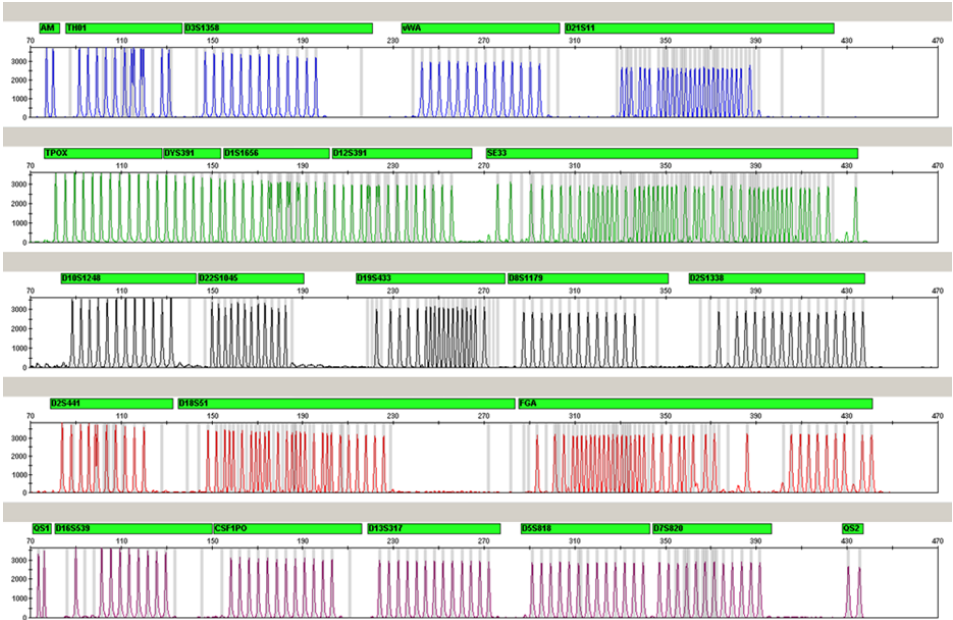


Figure 7. Electropherogram of the allelic ladder 24plex analyzed on an Applied Biosystems 3500 Genetic Analyzer. The allelic ladder includes 2 alleles for each Quality Sensor (QS1 and QS2). This allows automated calling of the QS peaks for sample analysis.

Table 24. Allelic ladder fragments included in the allelic ladder 24plex

Locus	Dye label	Repeat numbers of allelic ladder
Amelogenin	6-FAM	X, Y
TH01	6-FAM	4, 5, 6, 7, 8, 9, 9.3, 10, 10.3, 11, 13, 13.3
D3S1358	6-FAM	9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21
vWA	6-FAM	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24
D21S11	6-FAM	24, 24.2, 25, 26, 26.2, 27, 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, 36.2, 37, 38
TPOX	BTG	4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15
DYS391	BTG	7, 8, 9, 10, 11, 12, 13
D1S1656	BTG	10, 11, 12, 13, 14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18, 18.3, 19.3, 20.3
D12S391	BTG	14, 15, 16, 17, 17.3, 18, 18.3, 19, 20, 21, 22, 23, 24, 25, 26, 27
SE33	BTG	3, 4.2, 6.3, 8, 9, 10, 11, 12, 13, 13.2, 14, 14.2, 15, 15.2, 16, 17, 18, 18.2, 19, 19.2, 20, 20.2, 21, 21.2, 22, 22.2, 23.2, 24.2, 25, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 36, 36.2, 37, 38, 39, 42
D10S1248	BTY	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
D22S1045	BTY	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
D19S433	BTY	6.2, 8, 9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2, 18.2
D8S1179	BTY	7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
D2S1338	BTY	12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28
D2S441	BTR2	8, 9, 10, 11, 11.3, 12, 13, 14, 15, 16, 17
D18S51	BTR2	8, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 17.2, 18, 18.2, 19, 20, 21, 21.2, 22, 23, 24, 25, 26, 27, 28
FGA	BTR2	14, 16, 17, 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, 27, 28, 29, 30, 30.2, 31.2, 33, 34, 37.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2

Table 24. Allelic ladder fragments included in the allelic ladder 24plex (continued)

Locus	Dye label	Repeat numbers of allelic ladder
QS1	BTP	Q, S
D16S539	BTP	5, 8, 9, 10, 11, 12, 13, 14, 15
CSF1PO	BTP	5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16
D13S317	BTP	5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17
D5S818	BTP	6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18
D7S820	BTP	5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16
QS2	BTP	Q, S

For information about known microvariants not contained in the Investigator 24plex allelic ladder, see the National Institute of Standards and Technology (NIST) website (www.cstl.nist.gov/biotech/strbase/).

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page in our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook (for contact information, visit support.qiagen.com).

Comments and suggestions

Many samples show off-scale alleles

PCR cycle number too high	Determine optimal cycle number by running a representative batch of samples. Re-inject off-scale samples occurring under optimal cycle conditions using a reduced injection time.
---------------------------	---

Many samples show faint or no signal

PCR cycle number too low	Determine optimal cycle number by running a representative batch of samples.
--------------------------	--

Unbalanced profiles, low signals

- | | |
|--|---|
| a. Incorrect volume of Fast Reaction Mix 2.0 or Primer Mix | Check reaction setup and repeat amplification. |
| b. Master mix not vortexed before distribution | Vortex master mix thoroughly and centrifuge briefly. |
| c. Sample size too large | Do not use more than one 1.2 mm punch, or punches with a larger diameter. |
-

Dominance of Quality Sensor peaks

QS1 and QS2 peaks are too dominant	Using GeneMapper ID-X software choose under "Display Settings" a new setting for "all-dye range" to zoom in. The range should be between the QS1 and QS2. Important: In addition adjust in the "Analysis Method Editor" under "peak detector" the size-calling (Ranges; Sizing) to 75 -> 450.
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Comments and suggestions

Peak heights of QS1 and/or QS2 drop down in Standard experiments

Peak height drop-down of QS1 and QS2	<p>A slight peak height scattering is usual, and is not dependent on inhibitor influence. During the validation, the analyst should evaluate the usual variation spectrum in relation to their sample type, and should define a regular peak height range for both QS.</p> <p>A drop down of the QS2 signal below 20% of the QS1 signal indicates inhibition of the PCR reaction.</p>
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Swab samples show low signal for high-molecular-weight markers

Lysis incomplete	Perform optional lysis at 95°C.
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Size Standard peak heights too low

Instrument sensitivity and competition by the sample	If the size standard peak heights regularly fall below either 50 RFU or the laboratory analytical threshold due to increased competition from highly amplified samples or due to low CE instrument sensitivity, up to double the amount of DNA Size Standard 24plex (BTO) can be used (1 µL size standard to 11 µL HiDi).
--	---

Matrix/spectral calibration is not appropriate

There are pull-up peaks between the dye panels (B, G, Y, R, P, O) with the current matrix/ spectral calibration	This matrix cannot be used for the analysis. Repeat the matrix generation/spectral calibration. Be sure to carefully follow the correct protocol for the specific analysis instrument.
---	--

Many peaks are labeled as off-ladder (OL) alleles in the samples

a. DNA Size Standard 24plex (BTO) was not defined or identified correctly	<p>Click the orange "Size Match Editor" icon in the upper toolbar or the GeneMapper ID or GeneMapper ID-X Software. Mark the orange fragments of all samples.</p> <p>Always use the DNA Size Standard 24plex included in Investigator Human Identification PCR Kits.</p>
---	--

Comments and suggestions

- | | |
|---|--|
| b. Signal intensities are too high. If the peak heights of the samples are outside the linear detection range (>5000 RFU using Applied Biosystems 3500/3500xL Genetic Analyzers), stutters, split peaks, and artifacts may be increased | Reduce the injection time in increments to a minimum of 1 s, reduce the amount of the PCR amplification product for analysis, or reduce the quantity of DNA for PCR. |
| c. Bubbles in the capillary lead to pull-up peaks in all color panels ("spikes") that result in allele misnomer | Repeat electrophoresis to confirm results. Check the maximum number of injections recommended by the instrument manufacturer. Set up a new capillary array, if necessary. |
| d. Differences in the run performance among the capillaries of a multi-capillary analyzer may result in allelic assignment shift | For reliable allelic assignment on multi-capillary analyzers, a number of allelic ladders should be run. |
| e. Low temperature of the room air or of the CE buffers may result in fragment migration shifts or OL peaks | Ensure ambient conditions are kept, as recommended by the instrument manufacturer. Ensure buffers are equilibrated to ambient conditions as well. Preheating of the CE instrument (~30 min) is recommended by the instrument manufacturer. |

Injection/file of the allelic ladder is not appropriate

- | | |
|--|--|
| a. An additional signal can be identified as a peak of the allelic ladder because of dysfunctions during the electrophoresis. If peaks of the allelic ladder are miscalled, the ladder cannot be used for the analysis | Use a different injection/file of the allelic ladder and check the data of the analyzed sizes from the Size Standard (in bp) of the allelic ladder.
Always use the DNA Size Standard 24plex for Investigator Human Identification PCR Kits. |
| b. One peak of the allelic ladder is below the peak detection value (50–200 RFU) of the analysis method used, and thus, is not identified | The allelic ladder must be loaded onto the analysis instrument at a higher concentration than the samples to be analyzed.
Alternatively, allelic ladder data can be analyzed with a lower peak detection value in Analysis Software. |

Comments and suggestions

- | | |
|---|---|
| c. One peak of the allelic ladder is not identified because it is outside the expected size range of the software (in bp) | Compare the length of the fragments (in bp) of the first allele in one color of the allelic ladder with the corresponding value in the categories. Then, compare it with the other alleles. |
| d. Point alleles are not found | Point alleles are alleles with at least 1 bp difference to the next integer allele. Check the settings of the analysis method. Lower the Peak Window Size value to 11 points. |

References

1. Bär, W., et al. (1997) DNA recommendations. Further report of the DNA Commission of the ISFG regarding the use of short tandem repeat systems. *Int. J. Legal Med.* **110**, 175.
2. Szibor, R., et al. (2003) Cell line DNA typing in forensic genetics — the necessity of reliable standards. *Forensic Sci. Int.* **138**, 37.

Appendix A: Interpretation of Results

Post-PCR analysis and automatic allele assignment with suitable analysis software ensure precise and reliable discrimination of alleles.

General procedure for the analysis

1. Check the DNA size standard.
2. Check the allelic ladder.
3. Check the positive control.
4. Check the negative control.
5. Analyze and interpret the sample data.

Pull-up peaks

Pull-up peaks may occur if peak heights are outside the linear detection range (see “Troubleshooting Guide”, page 57), or if an incorrect matrix was applied. They appear at positions of specific peaks in other color channels, typically with lower signal intensities. Peak heights should not exceed thresholds in order to prevent pull-up peaks.

Stutter peaks

The occurrence of stutter peaks depends on the sequence of the repeat structure and the number of alleles. The $n - 4$ peaks are caused by a loss of a repeat unit during amplification of tetranucleotide STR motifs, caused by slippage effects of the Taq DNA Polymerase, whereas $n - 3$ peaks appear particularly during amplification of the trinucleotide STR motif D22S1045. These peaks should be interpreted using the Investigator Template Files for GeneMapper ID-X Software.

Template-independent addition of nucleotides

Because of its terminal transferase activity, the Taq DNA Polymerase may cause incomplete adenylation at the 3'-end of the amplified DNA fragments. The artifact peak is one base shorter than expected (-1 peaks). All primers included in the Investigator 24plex GO! Kit are designed to minimize these artifacts. Peak height of the artifact correlates with the amount of DNA. Laboratories should define their own limits for analysis of the peaks.

Artifacts

Room temperature may influence the performance of PCR products on multi-capillary instruments, so that shoulder peaks or split peaks occur. If shoulder or split peaks appear, we recommend injecting the sample again. Ensure ambient conditions are kept, as recommended by the instrument manufacturer. Ensure buffers are equilibrated to ambient conditions as well.

Appendix B: Varying PCR Volumes Using Investigator 24plex GO! Kit

The Investigator 24plex GO! Kit can be run with half reaction mix volumes (Fast Reaction Mix + Primer Mix). Note that while we have successfully tested the reduced reaction volume stated here, the highest overall success rates still have to be expected when using the full reaction volumes as recommended in the kit manual.

Blood or buccal cells on FTA or other paper

We recommend using the Investigator STR GO! Punch Buffer to overcome potential inhibition caused by the paper. Add 2 μL of STR GO! Punch Buffer regardless of the final reaction volume.

Buccal swab lysates

We recommend reducing the volume of lysate added according to the reaction volume reduction, e.g., 1 μL lysate for half reactions. Higher input volumes may result in inhibition of PCR amplifications.

Ordering Information

Product	Contents	Cat. no.
Investigator 24plex GO! Kit (100)	Primer Mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder 24plex, DNA size standard 24plex (BTO)	382426
Investigator 24plex GO! Kit (400)	Primer Mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder 24plex, DNA size standard 24plex (BTO)	382428
Related products		
Matrix Standard BT6 (50)	Matrix standard for 6-FAM, BTG, BTY, BTR2, BTP, and BTO, for Applied Biosystems 3500 Genetic Analyzers	386224
Investigator STR GO! Lysis Buffer (200)	Lysis buffer for 200 swab samples	386516
Investigator STR GO! Punch Buffer (1000)	Punch buffer for 1000 samples of epithelial cells on paper	386528
Investigator STR GO! Punch Buffer (200)	Punch buffer for 200 samples of epithelial cells on paper	386526
Sample Collection		
OmniSwab Sterile (100)	100 sterile collection swabs with ejectable brush-like head used for buccal and saliva samples	WB100035
EasiCollect Plus (50)	50 buccal sample collection devices: Includes a foam applicator for direct sample transfer to an integral QIAcard FTA Indicating.	WB120472
EasiCollect Buccal Collection Kit (50)	50 buccal sample collection kits: Includes EasiCollect (incl. barcode), return mailing envelope, multi-barrier pouch, nitrile gloves, tamper evident tape and desiccant	WB120237
Uni-Core Punch 1.2 mm	Manual punch for precise sample disc removal from FTA cards	WB100028
Investigator Human Identification PCR Kits		
Investigator Quantiplex Pro Kit (200)	For use on Applied Biosystems 7500 Real-Time Systems: Quantiplex Pro Reaction Mix, Quantiplex Pro Primer Mix, Quantiplex Pro Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387216

Product	Contents	Cat. no.
Investigator Quantiplex Pro FLX Kit (576)	6x single blistered 96-well optical plates with Master Mix, Control DNA M1, and QuantiTect Nucleic Acid Dilution Buffer	387516
Investigator Quantiplex Pro RGQ Kit (200)	For use on QIAGEN Rotor-Gene® Q Real-Time Systems: Quantiplex Pro RGQ Reaction Mix, Quantiplex Pro RGQ Primer Mix, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387316
Investigator 24plex QS Kit (100)*	Primer Mix, Fast Reaction Mix 2.0, Control DNA, allelic Ladder, DNA size standard, and Nuclease-free water	382415
Investigator 26plex QS (100)*	Primer Mix, Fast Reaction Mix 3.0, Control DNA, allelic ladder, and Nuclease-free water	382615
Investigator Argus X-12 QS (25)*	Primer Mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder, DNA size standard, Nuclease-free water	383223
Investigator Argus Y-28 QS (100)*	Primer Mix, Fast Reaction Mix 3.0, Control DNA, allelic ladder, DNA size standard, Nuclease-free water	383625

* Larger kit sizes available; please inquire.

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Document Revision History

Date	Changes
12/2017	Added optimized protocol and information about Bode Buccal DNA Collectors (pages 10, 14, 17–19)
11/2018	Added Appendix B
04/2019	Modified step 3 and added note to Table 2 in "Protocol: PCR Amplification from Blood on FTA and Other Paper"
12/2020	Added optional final extension. Updated "Ordering Information" section.
02/2021	Table 3 is split into Tables 3a and 3b, Table 5 is split into Tables 5a and 5b, Table 7 is split into Tables 7a and 7b, and Table 9 is split into Tables 9a and 9b. Added "Veriti™ 96-Well Thermal Cycler" and "ProFlex™ 96-well PCR System" in the "Equipment and Reagents to Be Supplied by User" section.
05/2024	Updated "Troubleshooting Guide" and "Ordering Information" sections. Layout and editing changes.

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