June 2021

Investigator® 26plex QS Handbook

For multiplex amplification of the CODIS core loci, the European standard set of loci, plus Penta D, Penta E, D6S1043, DYS391, and Amelogenin

Sample to Insight

QIAGEN

Contents

Kit Contents

Investigator 26plex QS Kit	**(100)**	**(400)**
Catalog no.	**382615**	**382617**
Number of 25 µl reactions	**100**	**400**
Fast reaction mix 3.0*	750 µl	4 x 750 µl
Nuclease-free water	1.9 ml	4 x 1.9 ml
Primer mix 26plex QS	250 µl	4 x 250 µl
Control DNA 9948 (0.5 ng/µl)	40 µl	40 µl
Allelic ladder 26plex	25 µl	3 x 25 µl

* Contains DNA Polymerase, dNTPs, $MgCl_2$, and bovine serum albumin (BSA).

Storage

The Investigator 26plex QS Kit is shipped on dry ice. It should be stored immediately upon receipt at –30 to –15°C in a constant-temperature freezer. Avoid repeated thawing and freezing. The primer mix and allelic ladder must be stored protected from light. DNA samples and post-PCR reagents (allelic ladder and DNA size standard) should be stored separately from PCR reagents. Under these conditions, the components are stable until the expiration date indicated on the kit.

Once opened, the Investigator 26plex QS Kit should be stored at 2–8°C for a maximum of 6 months.

Intended Use

The Investigator 26plex QS 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 26plex QS Kit is tested against predetermined specifications to ensure consistent product quality. Investigator 26plex QS Kits meet ISO 18385 requirements.

Introduction

The Investigator 26plex QS Kit is used for multiplex PCR in forensic, human identity, and paternity testing. The PCR simultaneously amplifies the 24 polymorphic STR markers listed below, along with the gender-specific marker Amelogenin.

The Investigator 26plex QS 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 26plex QS Kit is specifically designed for rapid and reliable generation of DNA profiles from blood, buccal swabs, and forensic stains. The kit utilizes QIAGEN's fast-cycling PCR technology, allowing amplification in around 65 min. It provides highly robust results with inhibitor-resistant chemistry. The primers are fluorescence-labeled with these dyes:

- 6-FAM™: Amelogenin, TH01, D3S1358, Penta D, D6S1043, D21S11
- BTG: TPOX, DYS391, D1S1656, D12S391, Penta E
- BTY: D10S1248, D22S1045, D19S433, D8S1179, D2S1338
- BTR2: D2S441, D18S51, vWA, FGA
- BTP: QS1, D16S539, CSF1PO, D13S317, D5S818, D7S820, QS2

The recommended amount of DNA under standard conditions is 0.5 ng.

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 26plex allelic ladder, see the National Institute of Standards and Technology (NIST) website (**strbase.nist.gov/**).

Table 1. Locus-specific information of the Investigator 26plex QS 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
D6S1043	G08539	[AGAT]$_{11}$	6q15
D7S820	G08616	[GATA]$_{12}$	7q21.11
D8S1179	G08710	[TCTA]$_{12}$	8q23.1-23.2
D10S1248	AL391869	[GGAA]$_{13}$	10q26.3
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
Penta D	AP001752	[AAAGA]$_{13}$	21q22.3
Penta E	AC027004	[AAAGA]$_5$	15q26.2
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 Standard BT6 for multicapillary instruments, e.g., 3500 Genetic Analyzers (see "Ordering Information")
- Pipettes and pipette tips
- One of the following DNA analyzers:*
  - Applied Biosystems® 3500 Genetic Analyzer
  - Applied Biosystems 3500xL Genetic Analyzer
- One of the following PCR thermal cyclers:*
  - QIAamplifier® 96
  - GeneAmp® PCR System 9700
  - Veriti™ 96-Well Thermal Cycler
  - ProFlex™ 96-well PCR System
  - Bio-Rad® PTC-200
  - Biometra® UNO-Thermoblock
  - Eppendorf® Mastercycler® ep
- PCR tubes or plates
- Microcentrifuge for PCR tubes or plates
- DNA size standard (BTO), see Ordering Information and "Protocol: Analysis" on page 27

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

Validity of 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* Software. The Investigator template files facilitate data analysis and are valid with this software.

Protocol: PCR Amplification

This protocol is for PCR amplification of STR loci from forensic samples using the Investigator 26plex QS 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 analysis).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination risks.
- The recommended amount of DNA under standard conditions is 0.5 ng.

Things 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. Thaw PCR components and template nucleic acid.

   Mix thoroughly. Centrifuge briefly before use.

2. Prepare a Master Mix according to Table 2.

   The Master Mix contains all of the components needed for PCR except the template (sample) DNA and nuclease-free water.

   As some loss of reagents can occur during transfers, prepare the mix with additional reactions included. Also include positive and negative control reactions.

3. Vortex the Master Mix thoroughly, centrifuge briefly, and then dispense appropriate volumes into PCR tubes or the wells of a PCR plate.

4. Add template DNA and nuclease-free water to the Master Mix to give a final sample volume of 25 µl.

5. Prepare positive and negative controls.

   Positive control: Use 1 µl of the Control DNA (i.e., 0.5 ng).

   Negative control: Use nuclease-free water instead of template DNA in the reaction.

Table 2. Reaction setup

Component	Volume per reaction
Fast reaction mix 3.0	7.5 µl
Primer mix	2.5 µl
Nuclease-free water (added in step 4)	Variable
Template DNA (added in step 4)	Variable
Total volume	25 µl

6. If template DNA was pipetted onto the rim or lid of the PCR tube, then centrifuge briefly to collect the contents at the bottom of the tubes.
7. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 3.

   Note: If using the GeneAmp PCR System 9700 with an aluminum block, use "Std Mode". With a silver 96-well block or a gold-plated silver 96-well block, use "Max Mode". Do not use "9600 Emulation Mode".
8. After the cycling protocol is completed, store samples at –30 to –15°C protected from the light, or proceed directly to electrophoresis.

Table 3. Standard cycling protocol recommended for all DNA samples

Temperature	Time	Number of cycles
96°C*	8 Min	–
96°C 64°C 72°C	10 s 55 s 5 s	3 cycles
96°C 61°C 72°C	10 s 55 s 5s	27 cycles
68°C	2 min	–
60°C	2 min	–
10°C	∞	–

* Hot-start to activate DNA polymerase.

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

The Investigator 26plex QS Kit is intended for use on the 3500/3500xL Genetic Analyzer, which requires the following software:

- 3500 Data Collection 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 and the GeneMapper *ID-X* Software, refer to the *Applied Biosystems 3500/3500xL Genetic Analyzers User Guide* (**tools.thermofisher.com/content/sfs/manuals/4401661.pdf**).

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 4.

Table 4. Materials required for electrophoresis

Material	Specifications
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 5). 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 Analyzers User Guide*.

Table 5. 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 Table 6.

Table 6. Setup of formamide and Matrix Standard BT6 mixture for 8 capillaries

Component	Volume
Hi-Di Formamide	90 µl
Matrix Standard BT6 multi cap.	10 µl

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)

7. Before opening the tubes, vortex and then centrifuge briefly to collect the contents at the bottom of the tubes.
8. Set up a mixture of formamide and Matrix Standard BT6 according to Table 7.

Table 7. Setup of formamide and Matrix Standard BT6 mixture for 24 capillaries

Component	Volume
Hi-Di Formamide	225 µl
Matrix Standard BT6 multi cap.	25 µl

9. Vortex and then centrifuge the mixture briefly.

10. 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.
11. Denature for 3 min at 95°C.
12. 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 Analyzers 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. Input the "Dye Set Name", for example, "BT6".
3. Under "Chemistry" select "Matrix Standard", and in "Dye Set Template" select "AnyDye Template".
4. In "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.

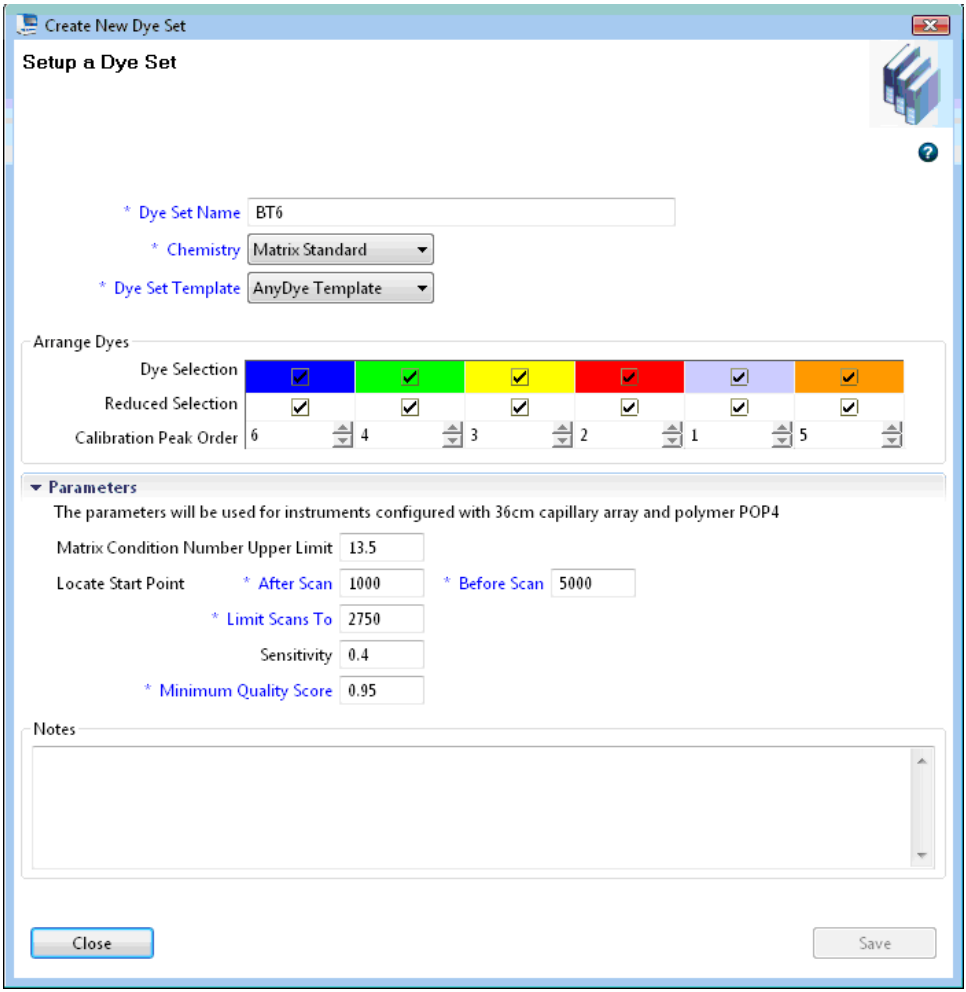

Figure 1. Setup of dye set BT6.

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" select the previously created BT6 (see "Software setup of dye set BT6", page 16).
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 Figure 2, 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.

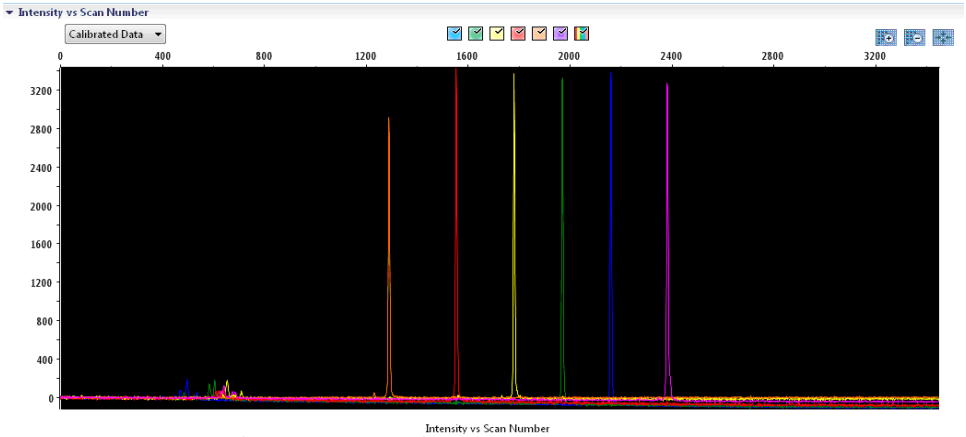

Figure 2. Electropherogram of spectral calibration of the Matrix Standard BT6 on an Applied Biosystems 3500 Genetic Analyzer.

When a spectral calibration is successfully completed, the "Overall" row displays green results (Figure 3). If the "Overall" row displays red results, refer to the "Spectral calibration troubleshooting" section of the *Applied Biosystems 3500/3500xL Genetic Analyzers User Guide*.

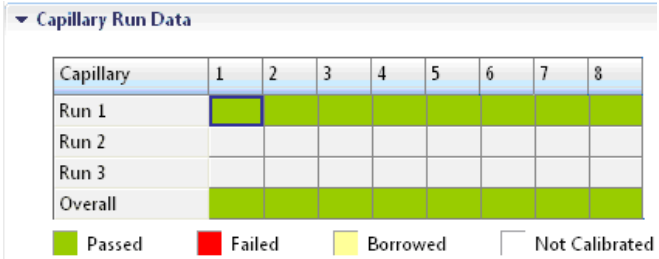

Figure 3. Example of successful spectral calibration of the Matrix Standard BT6 for all capillaries on an Applied Biosystems 3500 Genetic Analyzer.

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 Analyzers 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 Table 8.
3. Vortex and then centrifuge the mixture briefly.
4. Aliquot 12 µl of the mixture to a tube for each sample you plan to analyze.
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.
8. A thermal cycler set to 4°C may be used to cool the plate instead.
9. Load the samples on the tray.

Table 8. Setup of formamide and DNA size standard mixture

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

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 multicapillary 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 multicapillary 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 multicapillary 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 26plex QS 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", and then under "Analyze" go to "Instrument Protocols" and click "Create".

   Note: Modify the "Run Module" default settings from "HID36_POP4" as shown in Table 9.
2. The parameters from Table 9 must be entered or selected.
3. Click "Save" to confirm the changes.

Table 9. 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 26plex	e.g., Investigator 26plex
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*	27.0*
Data delay (s)	Default (1)	Default (1)
Advanced options	Default	Default

* Deviating from the settings above, the injection time can be varied depending on the type of samples and PCR cycle number used. The injection times designate maximum injection times at the given voltages. If samples with very high signal intensities are recorded, a shorter injection time may be selected to reduce risk of pull-up peaks.

Size standard

4. To set up the size standard, select “Library”, and then under “Analyze” go to “Size” ”Standards” and click “Create”.

5. The parameters in Table 10 must be entered or selected.

   DNA Size Standard 24plex (BTO) or DNA Size Standard (BTO) 450 should be used with the following lengths of fragments:

   - **DNA Size Standard 24plex (BTO)**: 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
   - **DNA Size Standard (BTO) 450**: 60, 80, 100, 140, 180, 200, 220, 260, 300, 340, 360, 400, and 450 bp

Table 10. Size standard parameters

Parameter	Setting
Size standard	e.g., SST-BTO_60-500bp or SST-BTO_60-450bp
Dye color	Orange

6. Alternatively, import the DNA size standard parameters using the recommended Investigator template files (Table 15).
7. Click "Save" to confirm the changes.

QC protocol

8. To set up the QC protocol, select "Library", and then under "Analyze" go to "QC Protocols" and click "Create".
9. The parameters in Table 11 must be entered or selected.

Table 11. QC protocol parameters

Parameter	Setting
Protocol name	e.g., BTO_550, or BTO_450
Size standard	SST-BTO_60-500bp, or SST-BTO_60-450bp
Sizecaller	SizeCaller v1.1.0

10. Go to “Analysis Settings”, followed by “Peak Amplitude Threshold”, and ensure that all colors are enabled.

    Check the recommended analysis settings in Table 14. All other settings should remain as “Default”.

11. Click “Save” to confirm the changes.

Assay

12. To set up an assay, go to “Library”, and then under “Manage” go to “Assays” and click “Create”.
13. To analyze Investigator 26plex fragments, select the parameters in Table 12.
14. Click “Save” to confirm the changes.

Table 12. Assay parameters

Parameter	Setting
Assay name	e.g., Investigator 26plex
Color	Default
Application type	HID
Instrument protocol	e.g., Investigator 26plex
QC protocols	e.g., BTO_550, or BTO_450

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 13.

Table 13. Plate properties

Property	Setting
Name	e.g., Investigator 26plex
Number of wells	96
Plate type	HID
Capillary length	36 cm
Polymer	POP4

3. Click “Assign Plate Contents” to implement 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 26plex” from “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 for which to specify an assay. Tick the boxes that are next to “the name of the Assay”, “File name conventions”, and “Results group” to assign those to the selected wells.
8. If not already done, load the assembled plate to the instrument and close the instrument door to reinitialize the instrument. Click “Link Plate for Run”. In the next screen, enter the desired run name and click “Start Run”.

Analysis parameters/analysis method

Table 14 lists the recommended analysis parameters in the worksheet Peak Detector.

Table 14. 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 baselining	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 detected 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 from the Applied Biosystems defaults for HID analysis.

Protocol: Analysis

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

Finding the exact lengths of the amplified products depends on the device type, the conditions of electrophoresis, and the DNA size standard used. Due to the complexity of some loci, size determination should be based on evenly distributed references. DNA Size Standard 24plex (BTO) or DNA Size Standard (BTO) 450 should be used with the following fragment lengths:

DNA Size Standard 24plex (BTO): 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

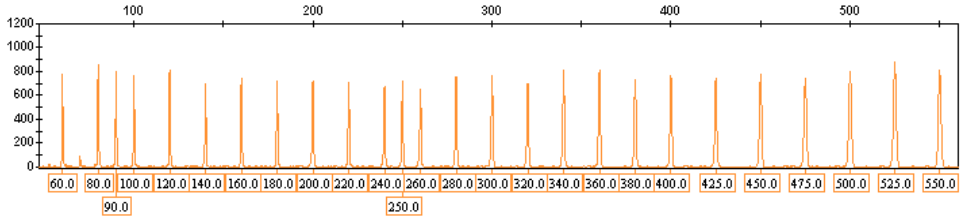

Figure 4a. Electropherogram of the DNA Size Standard 24plex (BTO). Fragments lengths in bp.

DNA Size Standard (BTO) 450: 60, 80, 100, 140, 180, 200, 220, 260, 300, 340, 360, 400, and 450 bp

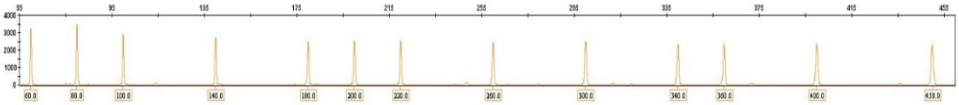

Figure 4b. Electropherogram of the DNA Size Standard (BTO) 450. Fragments lengths in bp.

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**.

Table 15. Recommended Investigator template files for GeneMapper *ID-X*

File type	File name
Panels*	26plex_Panels
BinSets*	26plex_Bins
Stutter	26plex_Stutter
Size standard	SST-BTO_60–500bp, or SST-BTO_60-450bp
Analysis method	Analysis_HID_3500_50rfu Analysis_HID_3500_200rfu
Plot settings	Plots_6dyes

* Panels and bin sets must always be used; other template files are optional.

Controls

The alleles listed in Table 16 represent the Control DNA 9948 (included in the Investigator 26plex QS Kit).

Table 16. Allele assignment of the Investigator 26plex QS Kit

Locus	CCR 9948
Amelogenin	X/Y
DYS391	10/10
D1S1656	14/17
D2S441	11/12
D2S1338	23/23
D3S1358	15/17
D5S818	11/13
D6S1043	12/12
D7S820	11/11
D8S1179	12/13
D10S1248	12/15
D12S391	18/24
D13S317	11/11
D16S539	11/11
D18S51	15/18
D19S433	13/14
D21S11	29/30
D22S1045	16/18
CSF1PO	10/11
FGA	24/26
Penta D	8/12
Penta E	11/11
TH01	6/9.3
TPOX	8/9
vWA	17/17

Quality Sensor

The Investigator 26plex QS Kit contains 2 internal PCR controls (quality sensors 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 enclosed 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 nonspecific 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 nonspecific 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 either the pipetting during PCR setup or the PCR itself was performed incorrectly. The user could repeat the experiment for improved results.

Sensitivity experiments have 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, the analysis of the 2 internal control fragments, QS1 and QS2, and of the STR target amplification products allows for the 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. Thus, an equal ratio of QS1 and QS2, together with a ratio in favor of small STR target products, suggests the presence of sample degradation.

If inhibitors such as hematin and humic acid are present in the sample, amplification is less efficient and larger DNA fragments are amplified less than smaller ones. If the analysis of the amplification products indicates an 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.

Analysis of the presence of the two quality sensors allows the user to differentially identify the presence of PCR inhibitors or the occurrence of degradation in the forensic sample. This gives the user helpful information for data interpretation and planning the next steps. Table 17 summarizes the possible profile appearances and their meanings.

Table 17. 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	Drop/Absent	Inhibitors present
Ski-slope profile	Present	Present	Degraded DNA

Note: 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 certain samples type, and should define a regular peak height range for both QS.

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

Alleles

Table 18 shows the alleles of the allelic ladder. All analyses were performed using POP-4 polymer (Table 18 and Figure 5). Different analysis instruments, DNA size standards, or polymers may result in different fragment lengths. In addition, a visual alignment with the allelic ladder is recommended.

Scaling

- Horizontal: 70–450 bp
- Vertical: Depending on signal intensity

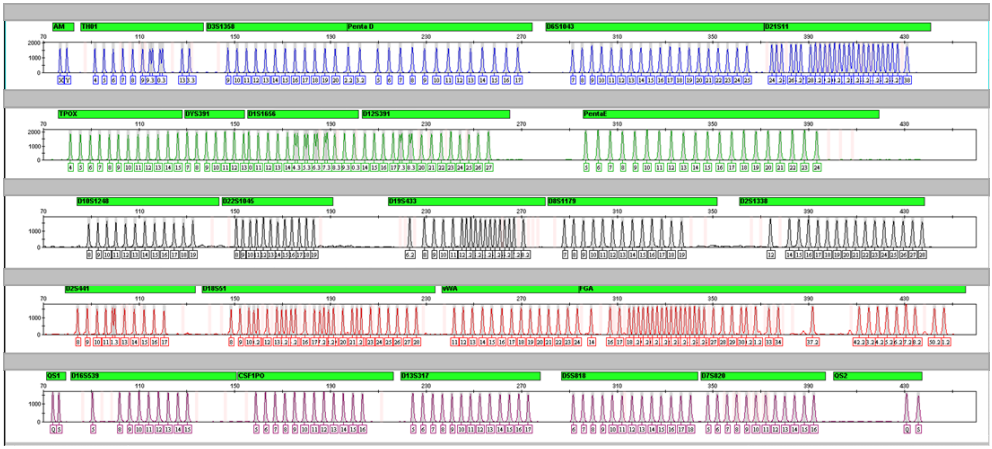

Figure 5. Electropherogram of the allelic ladder 26plex analyzed on an Applied Biosystems 3500xL Genetic Analyzer. The allelic ladder contains 2 alleles for each quality sensor (QS1 and QS2). This allows automated calling of the QS peaks for sample analysis.

Table 18. Allelic ladder fragments included in the allelic ladder 26plex

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
Penta D	6-FAM	2.2, 3.2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17
D6S1043	6-FAM	7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25
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
Penta E	BTG	5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24
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
vWA	BTR2	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24
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
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 26plex allelic ladder, see the National Institute of Standards and Technology (NIST) website (**strbase.nist.gov**).

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 at 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 the information and protocols in this handbook or about sample and assay technologies in general. For contact information, visit **www.qiagen.com**.

	Comments and suggestions
Unbalanced profiles, low signals	
a) Incorrect volume of fast reaction mix or primer mix	Check reaction setup and repeat amplification.
b) Master Mix not vortexed before distribution	Vortex Master Mix thoroughly and centrifuge briefly.
Reduced peak heights of QS1 and/or QS2 in standard experiments	
A slight peak height scattering of the quality sensors is usual and is not dependent on inhibitor influence.	During the validation, the analyst should evaluate the usual variation spectrum in relation to their certain sample types and define a regular peak height range for both QSs. A drop of the QS2 signal below 20% of the QS1 signal indicates inhibition of the PCR reaction.
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.
Sample preparation	
Sample signal intensity must be increased.	Reduce the volume of the DNA Size Standard (BTO) to peak heights of about 500 RFU. Purify the PCR products before starting the analysis. We recommend the MinElute® PCR Purification Kit (cat. nos. 28004 and 28006) for rapid and effective purification.
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.

	Comments and suggestions
Many peaks are labeled as off-ladder (OL) alleles in the samples	
a) DNA Size Standard 24plex (BTO) or DNA Size Standard (BTO) 450 was not defined or identified correctly.	Click the orange "Size Match Editor" icon in the upper toolbar of the GeneMapper *ID* or GeneMapper *ID-X* Software. Mark the orange fragments of all samples. Always use DNA Size Standard 24plex (BTO) or DNA Size Standard (BTO) 450 for Investigator Human Identification PCR Kits.
b) Signal intensities are too high. If the peak heights of the samples are outside the linear detection range using Applied Biosystems 3500/3500xL Genetic Analyzers, the occurrence of stutters, split peaks, and artifacts may increase.	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"), resulting 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 multicapillary analyzer may result in allelic assignment shift.	For reliable allelic assignment on multicapillary analyzers, a number of allelic ladders should be run.
e) Low room temperature or low CE buffer temperature may result in fragment migration shifts or OL peaks.	Ensure that ambient conditions are maintained as recommended by the instrument manufacturer. Ensure that buffers are equilibrated to ambient conditions. 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 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 DNA Size Standard 24plex (BTO) or DNA Size Standard (BTO) 450 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, the allelic ladder data can be analyzed with a lower peak detection value in the analysis software.
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 ISFH regarding the use of short tandem repeat systems. Int. J. Legal Med. **110**, 175–176.

Appendix A: Interpretation of Results

Post-PCR analysis and automatic allele assignment with suitable analysis software ensure a 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 and negative controls.
4. 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") or if an incorrect matrix was applied. They appear at positions of specific peaks in other color channels, typically with lower signal intensities. To prevent pull-up peaks, peak heights should not exceed thresholds.

Stutter peaks

The occurrence of stutter peaks depends on the sequence of the repeat structure and the number of alleles. The n – 4 (n minus 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. The n – 3 peaks appear 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 26plex QS Kit are designed

to minimize these artifacts. The 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 multicapillary instruments, so shoulder peaks or split peaks might occur. If shoulder or split peaks appear, we recommend injecting the sample again. Ensure that ambient conditions are maintained as recommended by the instrument manufacturer. Ensure that buffers are equilibrated to the ambient conditions.

Appendix B: Varying PCR Volumes Using Investigator 26plex QS Kit

The Investigator 26plex QS 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.

Ordering Information

Product	Contents	Cat. no.
Investigator 26plex QS Kit (100)	Primer mix, fast reaction mix 3.0, control DNA, allelic ladder 26plex, and nuclease-free water	382615
Investigator 26plex QS Kit (400)	Primer mix, fast reaction mix 3.0, control DNA, allelic ladder 26plex, and nuclease-free water	382617
Related products		
Matrix Standard BT6 (50)	Matrix standard for 6-FAM, BTG, BTY, BTR2, BTP, and BTO, for Applied Biosystems 3500 Genetic Analyzers	386224
DNA Size Standard 450 (BTO) (100)	DNA size standard with 13 fragments for 100 reactions	386045
DNA Size Standard 24plex (BTO) (100)	DNA size standard with 26 fragments for 100 reactions	386035
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 RGQ Kit (200)	For use on QIAGEN RotorGene Q Real-Time Systems: Quantiplex Pro RGQ Reaction Mix, Quantiplex Pro RGQ Primer Mix, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387316

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
08/2019	Initial release
06/2021	Revised Table 3. Updated the Ordering information section. Added “Veriti 96-Well Thermal Cycler”, “ProFlex 96-well PCR System”, and “QIAamplifier 96” in the “Equipment and Reagents to Be Supplied by User” section. Editorial and layout changes.

Notes