June 2015

Investigator® ESSplex SE QS Handbook

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

Making improvements in life possible®

Sample to Insight

QIAGEN

Contents

Kit Contents

Investigator ESSplex SE QS Kit	**(100)**	**(400)**
Catalog no.	**381575**	**381577**
Number of 25 µl reactions	**100**	**400**
Fast Reaction Mix 2.0*	750 µl	4x 750 µl
Primer Mix ESSplex SE QS	250 µl	4x 250 µl
Nuclease-free water	1.9 ml	4x 1.9 ml
Control DNA 9948 (0.1 ng/µl)	200 µl	200 µl
DNA size standard 550 (BTO)	55 µl	220 µl
Allelic ladder ESSplex SE QS	25 µl	3x 25 µl

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

Storage

The Investigator ESSplex SE QS Kit is shipped on dry ice. It should be stored immediately upon receipt at –15°C to –30°C in a constant-temperature freezer. Avoid repeated freezing and thawing. The 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 ESSplex SE QS Kit can be stored at 2–8°C for a maximum of 3 months or re-frozen and stored at –15°C to –30°C in a constant-temperature freezer for longer periods of time.

Intended Use

The Investigator ESSplex SE 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.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample preparation waste.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of Investigator ESSplex SE QS Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

The Investigator ESSplex SE QS Kit is used for multiplex PCR in forensic, human identity and paternity testing. The 16 polymorphic STR markers recommended by the European Network of Forensic Science Institutes (ENFSI) and the European DNA Profiling Group (EDNAP) as the new European Standard Set of loci (D1S1656, D2S441, D2S1338, D3S1358, D8S1179, D10S1248, D12S391, D16S539, D18S51, D19S433, D21S11, D22S1045, FGA [FIBRA], TH01 [TC11], and vWA), plus SE33 [ACTBP2] and the gender-specific Amelogenin are amplified simultaneously.

The Investigator ESSplex SE QS Kit Primer Mix contains two 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 ESSplex SE QS Kit is designed specifically 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 approximately 60 minutes. It provides highly robust results with inhibitor-resistant chemistry. The primers are fluorescence-labeled with the following dyes:

- 6-FAM™: QS1, Amelogenin, TH01, D3S1358, vWA, D21S11, QS2
- BTG: D16S539, D1S1656, D19S433, SE33
- BTY: D10S1248, D22S1045, D12S391, D8S1179, D2S1338
- BTR: D2S441, D18S51, FGA

The recommended amount of DNA under standard conditions is 0.5 ng. Internal validations demonstrated robust and balanced results with 0.2–2 ng DNA, and reliable results with < 0.1 ng DNA.

The Investigator ESSplex SE QS 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 (Bär et al., 1997).

For information about known microvariants not contained in the Investigator ESSplex SE QS 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 ESSplex SE 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
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
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
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
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
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 BT5 for multi-capillary instruments, e.g., ABI PRISM 3100 and Applied Biosystems 3130 and 3500 Genetic Analyzers (QIAGEN cat. nos. 386123 or 386125)
- Pipets and pipet tips
- One of the following DNA analyzers:*
  ABI PRISM 3100-*Avant*™/3100 Genetic Analyzer
  Applied Biosystems 3130/3130*xl* Genetic Analyzer
  Applied Biosystems 3500/3500xL Genetic Analyzer
- One of the following PCR thermal cyclers:*
  GeneAmp PCR System 9700
  Biometra UNO-Thermoblock
  Eppendorf® Mastercycler® ep
- PCR tubes or plates
- Microcentrifuge for PCR tubes or plates

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

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

forensic applications. We recommend GeneMapper® *ID-X*. 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 ESSplex SE 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. Internal validations demonstrated robust and balanced results with 0.2–2 ng DNA, and reliable results with < 0.1 ng DNA.

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.

   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 besides the template (sample) DNA and nuclease-free water.

3. Mix the master mix thoroughly, centrifuge briefly, and 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 5 µl of the Control DNA (i.e., 500 pg).

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

Table 2. Master mix setup

Component	Volume per reaction
Fast Reaction Mix 2.0	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", or with a Silver 96-Well Block or Gold-plated Silver 96 Well Block, use "Max Mode". Do not use "9600 Emulation Mode".

Table 3. Standard cycling protocol, recommended for all DNA samples

Component	Time	Number of cycles
98°C*	30 s	
64°C	55 s	3 cycles
72°C	5 s	
96°C	10 s	
61°C	55 s	27 cycles
72°C	5 s	
68°C	2 min	
10°C	∞	–

* Hot-start to activate DNA polymerase.

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

Protocol: Electrophoresis using the ABI PRISM 3100-*Avant*/3100 Genetic Analyzer

For detailed instructions on instrument setup, spectral calibration, application of the ABI PRISM 3100 Data Collection software version 1.01 or 1.1 and the GeneScan® software, refer to the *ABI PRISM 3100-*Avant*/3100 Genetic Analyzer User's Manual*.

The system with 4 capillaries is the ABI PRISM 3100-*Avant* Genetic Analyzer and the system with 16 capillaries is the ABI PRISM 3100 Genetic Analyzer.

The virtual filter set G5 is used for combined application of the 5 fluorescent labels 6-FAM, BTG, BTY, BTR and BTO. This matrix standard is known as BT5.

The materials required for electrophoresis are shown in Table 4.

Table 4. Materials required for electrophoresis

Material	Specifications
Capillary	36 cm Capillary Array for ABI PRISM 3100-*Avant*/3100 Genetic Analyzer
Polymer	POP-4™ Polymer for ABI PRISM 3100-*Avant*/3100 Genetic Analyzer
Buffer	10x Genetic Analyzer Buffer with EDTA

Spectral calibration/matrix generation

Proper spectral calibration is critical for evaluation of multicolor systems with the ABI PRISM 3100-*Avant*/3100 Genetic Analyzer and should be performed before conducting fragment length analysis. The calibration procedure creates a matrix that is used to correct the overlapping of fluorescence emission spectra of the dyes.

Spectral calibration comprises the following steps:

- Preparing the spectral calibration standards
- Loading the standards to the 96-well reaction plate (one sample per capillary)
- Entering the plate composition
- Performing a spectral calibration run and checking the matrix

Preparing the spectral calibration standards

Example for 4 capillaries (ABI PRISM 3100-*Avant* Genetic Analyzer)

1. Set up a mixture of formamide and Matrix Standard BT5 according to Table 5.

Table 5. Setup of formamide and Matrix Standard BT5 mixture for 4 capillaries

Component	Volume
Hi-Di Formamide	60 µl
Matrix Standard BT5 multi cap.	5 µl

2. Load 12 µl of the mixture to the 96-well plate; e.g., positions A1–D1.
3. Denature for 3 min at 95°C.
4. Snap freeze by placing the plate on ice for 3 min.

   Alternatively, the thermal cycler set to 4°C may be used to cool the plate.

Example for 16 capillaries (ABI PRISM 3100 Genetic Analyzer)

1. Set up a mixture of formamide and Matrix Standard BT5 according to Table 6.

Table 6. Setup of formamide and Matrix Standard BT5 mixtures for 16 capillaries

Component	Volume
Hi-Di Formamide	204 µl
Matrix Standard BT5 multi cap.	17 µl

2. Load 12 µl of the mixture to the 96-well plate; e.g., positions A1–H1 and A2–H2.
3. Denature for 3 min at 95°C.
4. Snap freeze by placing the plate on ice for 3 min.

   Alternatively, the thermal cycler set to 4°C may be used to cool the plate.

Performing a spectral calibration run

The parameter file for DyeSetG5 must be modified once to achieve successful calibration with the Data Collection Software version 1.0.1 or 1.1.

Spectral parameter

1. To change settings in the parameter file, go to the following path:

   D:\AppliedBio\Support Files\Data Collection SupportFiles\CalibrationData\Spectral Calibration\ParamFiles
2. Select "MtxSTD{Genescan_SetG5} to open the PAR file.
3. Change "Condition Bounds Range" to [1.0, 20.0].
4. If the calibration was unsuccessful, also change Sensitivity to 0.1 and Quality to 0.8.
5. Select "Save As" in the File menu and save the parameter file under a new name; e.g., MtxStd{Genescan_SetG5_BT5}.par.

   Note: Always use this parameter file for spectral calibration runs using QIAGEN Matrix Standard BT5.

Plate Editor for spectral calibration

1. Place the 96-well plate on the autosampler tray.
2. Run the ABI PRISM 3100 Data Collection Software.
3. In Plate View, click "New" to open the Plate Editor dialog box.
4. Enter a name for the plate.
5. Select a Spectral Calibration.
6. Select "96-Well" as plate type, and click "Finish".

Table 7. Plate Editor for spectral calibration

Parameter	Settings
Sample Name	Enter name for the matrix samples
Dye Set	G5
Spectral Run Module	Default (e.g., Spect36_POP4)
Spectral Parameters	MtxStd{GeneScan_SetG5_BT5}.par (parameters created before)

7. Click the column header to select the entire column, and select "Fill Down" from the Edit menu to apply the information to the selected samples. Confirm by clicking "OK".
8. Link the reaction plate on the autosampler tray with the created plate ID and start the run.
9. Upon completion of the run, check in the Spectral Calibration Result dialog box that all capillaries have successfully passed calibration (label A).

   If individual capillaries are labeled X, refer to the *ABI PRISM 3100*-Avant*/ 3100 Genetic Analyzer User's Manual.*
10. Click "OK" to confirm completion of the run.

Checking the matrix

1. Select "Display Spectral Calibration" from the Tools menu, then "Dye Set" and "G5" to review the spectral calibration profile for each capillary.

2. The quality value (Q value) must be greater than 0.95 and the condition number (C value) must be between 1 and 20. Both values must be within the predetermined range.
3. Check for a flat baseline in the matrix samples. There should be 5 peaks with heights of 1000–5000 RFU in each matrix sample.

   Note: The optimal range is 2000–4000 RFU.
4. Check the new matrix with the current samples. There should be no pull-up peaks between the dye panels (B, G, Y, R and O) with the new matrix.
5. If the calibration failed, follow the instructions in the section "Spectral parameter" on page 17.
6. If all capillaries have passed the calibration, the last calibration file for Dye Set G5 must be activated manually. Click "Set Active Spectral Calibration" under the Tools menu.
7. Rename the calibration file under Set Matrix Name (e.g., BT5_Date of calibration).

Sample preparation

1. Set up a mixture of formamide and DNA size standard according to Table 1.

Table 8. Setup of formamide and DNA size standard mixture

Component	Volume
Hi-Di Formamide	12 µl
Matrix Standard BT5 multi cap.	0.5 µl

2. Aliquot 12 µl of the mixture into a tube for each sample to be analyzed.
3. Add 1 µl PCR product or allelic ladder (diluted, if necessary).
4. Denature for 3 min at 95°C.
5. Snap freeze by placing the plate on ice for 3 min.

   Alternatively, the thermal cycler set to 4°C may be used to cool the plate.
6. Load the samples on the tray.

Since injections take place simultaneously on all capillaries, 4 or 16 samples 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, several ladders should be run.

Room temperature may influence the performance of PCR products on multi-capillary instruments, so that shoulder peaks or split peaks occur especially at low temperatures. Ensure ambient conditions are kept as recommended by the instrument manufacturer.

Setting up the GeneScan Software

1. Edit the default run module in Dye Set G5 once for the first run. Select "Module Editor" to open the dialog box.
2. Select the appropriate Run Module as template from the GeneScan table (see Table 9).
3. Modify the Injection Voltage to 2.5 kV, the Injection Time to 30 s, the Run Voltage to 13 kV and the Run Time to 30 min.
4. Click "Save As" and enter the name of the new module (e.g., 2.5kV_30s_500bp). Confirm by clicking "OK".
5. Click "Close" to exit the Run Module Editor.

Table 9. Run Module 2.5kV_30s_500bp for the ABI PRISM 3100-*Avant*/3100 Genetic Analyzer

Parameter	Settings
Run Temperature (°C)	Default
Cap Fill Volume	Default
Maximum Current (A)	Default
Current Tolerance (A)	Default
Run Current (A)	Default
Voltage Tolerance (kV)	Default
Pre-Run Voltage (kV)	Default
Pre-Run Time (s)	Default
Injection Voltage (kV)	2.5
Injection Time (s)	30*
Run Voltage (kV)	13
Number of Steps	Default
Voltage Step Interval	Default
Data Delay Time (s)	Default
Run Time (min)	30†

* 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.

† The run time for Investigator ESSplex SE QS was modified in order to be able to analyze fragments with lengths of up to 500 bp.

Starting the run

1. Place the prepared 96-well plate on the autosampler tray.
2. Run the ABI PRISM 3100 Data Collection Software.
3. In Plate View, click “New” to open the Plate Editor dialog box.
4. Enter a name for the plate.
5. Select “GeneScan” as the application type.
6. Select “96-Well” as plate type, and click “Finish”.

Table 10. Settings in Plate Editor

Parameter	Settings
Sample Name	Enter name for the matrix samples
Dyes	O
Color Info	Ladder or sample
Project Name	E.g., 3100_Project1
Dye Set	G5
Run Module	2.5kV_30s_500bp*
Analysis Module 1	DefaultAnalysis.gsp

* See

Table 9., "Run Module 2.5kV_30s_500bp for the ABI PRISM 3100-*Avant*/3100 Genetic Analyzer".

7. Complete the table in the Plate Editor and click "OK".
8. Click the column header to highlight the entire column and select "Fill Down" from the Edit menu to apply the information to the selected samples.
9. Link the reaction plate on the autosampler tray to the created plate ID and start the run.
10. Upon completion of the run, view the data as Color Data in the Array View of the 3100 Data Collection Software or as Analyzed Sample Files under D:/AppliedBio/3100/DataExtractor/ExtractRuns.

Analysis parameters

Table 11 lists the recommended analysis parameters.

Table 11. Recommended analysis parameters for the ABI PRISM 3100-*Avant*/3100 Genetic Analyzer

Parameter	Settings
Analysis Range	Start: 2000
Stop: 10,000	
Data Processing	Baseline: Checked
	Multi-component: Checked
	Smooth options: Light
Peak Detection	Peak Amplitude Thresholds
	B:* Y:*
	G:* R:*
	O:*
	Min. Peak Half Width: 2 pts
	Polynomial Degree: 3
	Peak Window Size: 11 pts†
Size Call Range	Min: 60
	Max: 550
Size Calling Method	Local Southern Method
Split Peak Correction	None

* The peak amplitude threshold (cutoff value) corresponds to the minimum peak height that will be detected by the GeneScan or GeneMapper *ID* Software. 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 defaults from Applied Biosystems for HID analysis.

Note: For information on the use of the recommended Template Files (as analysis parameters), refer to the appropriate Investigator Template Files User Guide (GeneMapper *ID*, or GeneMapper *ID-X*).

Protocol: Electrophoresis Using the Applied Biosystems 3130/3130*xl* Genetic Analyzer

For detailed instructions on instrument setup, spectral calibration, or application of the ABI PRISM Data Collection Software version 3.0 and the GeneMapper *ID* Software, refer to the *Applied Biosystems 3130/3130*xl *Genetic Analyzers Getting Started Guide*.

The system with 4 capillaries is the Applied Biosystems 3130 Genetic Analyzer and the system with 16 capillaries is the Applied Biosystems 3130*xl* Genetic Analyzer.

The virtual filter set ''Any5Dye'' is used for combined application of the 5 fluorescent labels 6-FAM, BTG, BTY, BTR and BTO. This matrix standard is known as BT5.

The materials required for electrophoresis are listed in Table 12.

Table 12. Materials required for electrophoresis

Material	Specifications
Capillary	36 cm Capillary Array for Applied Biosystems 3130/3130*xl* Genetic Analyzer
Polymer	POP-4 Polymer for Applied Biosystems 3130/3130*xl* Genetic Analyzer
Buffer	10x Genetic Analyzer Buffer with EDTA

Spectral calibration/matrix generation

Before conducting DNA fragment size analysis, it is necessary to perform a spectral calibration with the 5 fluorescent labels 6-FAM, BTG, BTY, BTR and BTO for each analyzer. The calibration procedure creates a matrix that is used to correct the overlapping of fluorescence emission spectra of the dyes.

Spectral calibration is comprised of the following steps:

- Preparing the spectral calibration standards
- Loading the standards to the 96-well reaction plate (one sample per capillary)
- Creating the instrument protocol for spectral calibration (Protocol Manager)
- Defining the plate composition in the plate editor (Plate Manager)
- Performing a spectral calibration run and checking the matrix

Preparing the spectral calibration standards

Example for 4 capillaries (Applied Biosystems 3130 Genetic Analyzer)

1. Set up a mixture of formamide and Matrix Standard BT5 according to Table 13.

Table 13. Setup of formamide and Matrix Standard BT5 mixture for 4 capillaries

Component	Volume
Hi-Di Formamide	60 µl
Matrix Standard BT5 multi cap.	5 µl

2. Load 12 µl of the mixture to the 96-well plate, e.g., positions A1–D1.
3. Denature for 3 min at 95°C.
4. Snap freeze by placing the plate on ice for 3 min.
5. Alternatively, use the thermal cycler set to 4°C to cool the plate.

Example for 16 capillaries (Applied Biosystems 3130*xl* Genetic Analyzer)

1. Set up a mixture of formamide and Matrix Standard BT5 according to Table 14..

Table 14. Setup of formamide and Matrix Standard BT5 mixture for 16 capillaries

Component	Volume
Hi-Di Formamide	204 µl
Matrix Standard BT5 multi cap.	17 µl

2. Load 12 µl of the mixture to the 96-well plate, e.g., positions A1–H1 and A2–H2.
3. Denature for 3 min at 95°C.
4. Snap freeze by placing the plate on ice for 3 min.

   Alternatively, use the thermal cycler set to 4°C may be used to cool the plate.

Performing the spectral calibration run

1. Place the 96-well plate on the autosampler tray.
2. In the Protocol Manager of the Data Collection Software, open the Instrument Protocol window.
3. Click "New" to open the Protocol Editor dialog box.
4. Complete the dialog box with information from Table 15 and click "OK".

Table 15. Instrument protocol for spectral calibration

Protocol Editor	Settings
Name	User (e.g., Spectral36_POP4_BT5)
Type	SPECTRAL
Dye Set	Any5Dye
Polymer	User (e.g., POP4)*
Array Length	User (e.g., 36cm)*
Chemistry	Matrix Standard
Run Module	Default (e.g., Spect36_POP4_1)*

* Depends on the type of polymer and length of capillary used.

5. Click “New” in the Plate Manager of the Data Collection Software to open the New Plate Dialog box.
6. Enter information from Table 16 and click “OK”. A new table in the Plate Editor opens automatically (Table 17).

Table 16. Plate Editor for spectral calibration (I)

New plate dialog	Settings
Name	e.g., Spectral_BT5_date
Application	Spectral Calibration
Plate Type	96-well
Owner Name/ Operator Name	…

Table 17. Plate Editor for spectral calibration (II)

New plate dialog	Settings
Sample Name	Enter name for the matrix samples
Priority	E.g., 100
Instrument Protocol 1	Spectral36_POP4_BT5 (setting described before)

7. Click the column header to select the entire column, and select “Fill Down” from the Edit menu to apply the information to the selected samples. Confirm by clicking “OK”.
8. Link the reaction plate on the autosampler tray with the created plate ID (position A or B) and start the run.

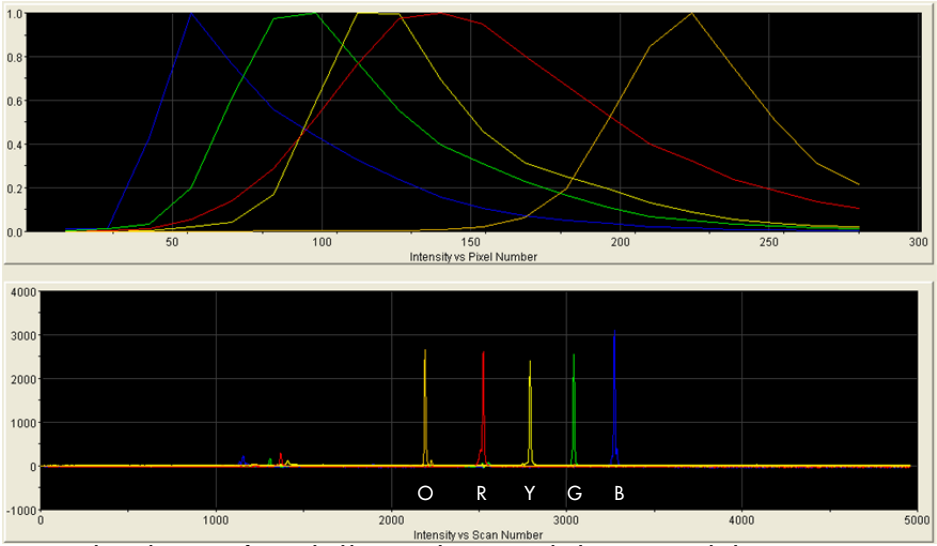

Figure 1. Electropherogram of spectral calibration with matrix standard BT5 on an Applied Biosystems 3130 Genetic Analyzer.

Checking the matrix

1. 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 20.
2. Check for a flat baseline in the matrix samples. As shown in the figure on the previous page, there should be 5 peaks with peak heights of about 1000–5000 RFU in each matrix sample.
3. Note: The optimal range is 2000–4000 RFU.
4. Check the new matrix with the current samples. There should be no pull-up peaks between the dye panels (B, G, Y, R, O) with the new matrix.
5. If calibration failed, use the optimized values of the Matrix Standard BT5 and repeat the calibration run.
6. If all capillaries have passed the test, the last calibration file for the Dye Set Any5Dye is activated automatically in the Spectral Viewer. Rename the calibration file (e.g., BT5_Date of calibration).

Sample preparation

1. Set up a mixture of formamide and DNA size standard according to Table 18.

Table 18. Setup of formamide and DNA size standard mixture

Component	Volume
Hi-Di Formamide	12.0 µl
DNA Size Standard 550 (BTO)	0.5 µl

2. Aliquot 12 µl of the mixture to a tube for each sample to be analyzed.
3. Add 1 µl PCR product or allelic ladder (diluted, if necessary).
4. Denature for 3 min at 95°C.
5. Snap freeze by placing the plate on ice for 3 min.

   Alternatively, use the thermal cycler set to 4°C to cool the plate.
6. Load the samples on the tray.

Since injections take place simultaneously on all capillaries, 4 or 16 samples 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, several ladders should be run.

Room temperature may influence the performance of PCR products on multi-capillary instruments, so that shoulder peaks or split peaks occur especially at low temperatures. Ensure ambient conditions are kept, as recommended by the instrument manufacturer.

Setting up the Data Collection Software

1. Edit the Run Module once for the first run. In the Module Manager of the Data Collection Software, click "New" to open the Run Module Editor dialog box.

   Note: Modify the Run Module Default settings from "HIDFragmentAnalysis36_POP4_1" to those shown in Table 19.

2. Modify the Injection Voltage to 2.5 kV, the Injection Time to 30 s, the Run Voltage to 13 kV and the Run Time to 1800 s (Table 19).
3. Click "Save As", enter a name for the new Run Module (e.g., 2.5kV_30s_500bp), and confirm by clicking "OK".
4. Click "Close" to exit the Run Module Editor.

Table 19. Run Module 2.5kV_30s_500bp for the Applied Biosystems 3130/3130*xl* Genetic Analyzer

Parameter	Settings
Oven Temperature (°C)	Default
Poly Fill Volume	Default
Current Stability (µA)	Default
Pre-Run Voltage (kV)	Default
Pre-Run Time (s)	Default
Injection Voltage (kV)	2.5
Injection Time (s)	30*
Voltage Number of Steps	Default
Voltage Step Interval	Default
Data Delay Time (s)	Default
Run Voltage (kV)	13 kV
Run Time (s)	1800†

* 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.

† The run time for Investigator ESSplex SE QS Kit was modified enable analysis of fragments with lengths of up to 500 bp.

Starting the run

1. Place the prepared 96-well plate on the autosampler tray.
2. Open the Protocol Manager of the Data Collection Software.
3. Click "New" in the Instrument Protocol window to open the Protocol Editor dialog box and enter the information in Table 20.
4. Click "OK" to exit the Protocol Editor.

Table 20. Settings in Instrument Protocol

Protocol Editor	Settings
Name	Run36_POP4_BT5_26min
Type	REGULAR
Run Module	2.5kV_30s_500bp*
Dye Set	Any5Dye

* See Table 19, "Run Module 2.5kV_30s_500bp for the Applied Biosystems 3130/3130xl Genetic Analyzer".

1. Before each run, it is necessary to create a plate definition. In the Plate Manager of the Data Collection Software, click "New" to open the New Plate Dialog box.
2. Enter the information in Table 21.

Table 21. GeneMapper Plate Editor (I)

Protocol Editor	Settings
Name	E.g., Plate_BT5_Date
Application	Select GeneMapper Application
Plate type	96-Well
Owner Name/ Operator Name	…

3. Click "OK" and a new table in the Plate Editor opens automatically (Table 22).
4. Click the column header to select the entire column. Select "Fill Down" from the Edit menu to apply the information to all selected samples. Click "OK".
5. In the Run Scheduler, click "Find All", and select "Link" to link the reaction plate on the autosampler tray to the newly created plate record (position A or B).

Table 22. GeneMapper Plate Editor (II)

Parameter	Settings
Sample Name	Enter the name for the samples
Priority	E.g., 100 (Default)
Sample Type	Sample or Allelic Ladder
Size Standard	E.g., SST-BTO_60-500bp
Panel	E.g., ESSplex_SE_QS_Panels
Analysis Method	E.g., Analysis_HID_3130
Snp Set	–
User-defined 1-3	–
Results Group 1	(Select results group)
Instrument Protocol 1	Run36_POP4_BT5_26min (setting described before)

6. Start the run.
7. During the run, view Error Status in the Event Log or examine the quality of the raw data for each capillary in the Capillaries Viewer or the Cap/Array Viewer.
8. View data as an overview in Run History or Cap/Array Viewer of the Data Collection Software.

   Run data are saved in the Run Folder of the previously chosen Result Group.

Analysis parameters/analysis method

Table 23 lists the recommended analysis parameters.

Table 23. Recommended settings for the Applied Biosystems 3130/3130*xl* Genetic Analyzer

Parameter	Settings
Peak Detection Algorithm	Advanced
Ranges	Analysis: Partial Range
	Start Point: 2000; Stop Point: 10,000
	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:*
	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* or *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 defaults from Applied Biosystems for HID analysis.

Note: For information on the use of the recommended Template Files (as analysis parameters), refer to the appropriate Investigator Template Files User Guide (GeneMapper *ID*, or GeneMapper *ID-X*).

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

The Investigator ESSplex SE QS Kit is validated for use on the 3500/3500xL Genetic Analyzer, which requires the following software:

- 3500 Data Collection Software v1 or v2
- HID Updater 3500 Data Collection v2.0

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 Analyzers 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 5 fluorescent labels 6-FAM, BTG, BTY, BTR and BTO. This matrix standard is known as BT5.

The materials required for electrophoresis are listed in Table 24.

Table 24. 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 5 fluorescent labels 6-FAM, BTG, BTY, BTR and BTO for each analyzer (Table 25). 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 BT5
- 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 25. The 5 fluorescent labels of BT5

Color	Matrix standard
Blue (B)	6-FAM
Green (G)	BTG
Yellow (Y)	BTY
Red (R)	BTR
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 BT5 according Table 26.

Table 26. Setup of formamide and Matrix Standard BT5 mixture for 8 capillaries

Component	Volume
Hi-Di Formamide	90 µl
Matrix Standard BT5 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.

   Alternatively, 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 BT5 according to Table 27.

Table 27. Setup of formamide and Matrix Standard BT5 mixture for 24 capillaries

Component	Volume
Hi-Di Formamide	225 µl
Matrix Standard BT5 multi cap.	25 µl

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.

   Alternatively, a thermal cycler set to 4°C may be used to cool the plate instead.

Plate assembly and loading the plate into the instrument

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

Software setup of dye set BT5

Prior to the spectral calibration, a dye set for the Matrix Standard BT5 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, BT5.
3. Under “Chemistry” select “Matrix Standard” and as a “dye set template” select “AnyDye Template”
4. Disable “Purple” in the field “Arrange Dyes”. Ensure that all other colors are enabled.
5. Under “Calibration Peak Order” the colors need to be arranged as follows: 5 – blue, 4 – green, 3 – yellow, 2 – red, and 1 – orange.
6. Do not alter the “Parameter” settings.
7. Click “Save” to confirm the changes.

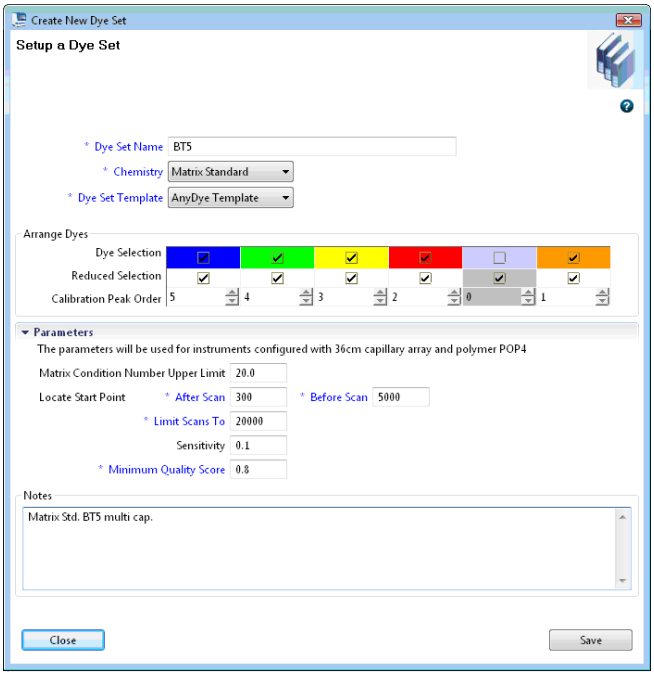

Figure 2. Setup of dye set BT5.

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, e.g., the previously created BT5 (from step 2).
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 0.8 and the number range (C value) must be between 1 and 20.
- Check the matrix samples for a flat baseline. As shown in the Figure 3, there should be 5 peaks with peak heights of about 1000–5000 RFU for each matrix sample (Note: The optimal range is 2000–4000 RFU).

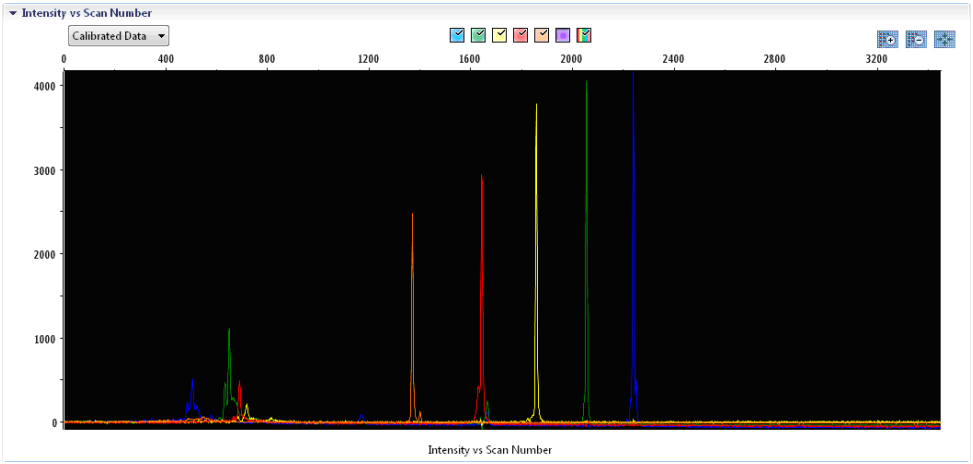

Figure 3. Electropherogram of spectral calibration of the matrix standard BT5 on an Applied Biosystems 3500 Genetic Analyzer.

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 Analyzers User Guide*.

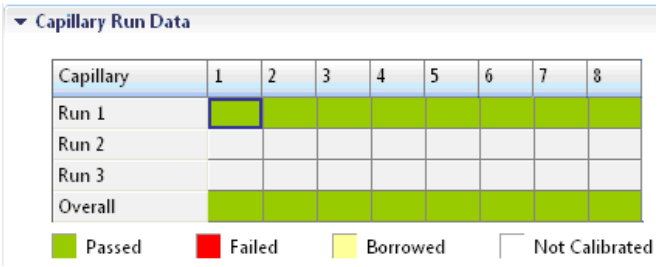

Figure 4. Example of successful spectral calibration of the matrix standard BT5 for all capillaries on an Applied Biosystems 3500 Genetic Analyzer.

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 read orange-red-yellow-green-blue
- 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 28.
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 28. Setup of formamide and DNA size standard mixture

Component	Volume
Hi-Di Formamide	12.0 µl
DNA Size Standard 550 (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 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 ESSplex SE 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 go to “Analyze” under “Instrument Protocols”. Click “Create”.

   Note: Modify the Run Module Default settings from “HID36_POP4” as shown in Table 29.

2. For Applied Biosystems 3500 Genetic Analyzer the parameters from Table 29 must be entered or selected. The parameters from Table 30 must be entered or selected for the Applied Biosystems 3500xL Genetic Analyzer.

3. Click “Save” to confirm the changes.

Table 29. Instrument Protocol parameters for Applied Biosystems 3500 Genetic Analyzer

Parameter	Setting
Application Type	HID
Capillary Length	36 cm
Polymer	POP4
Dye Set	E.g., BT5
Run Module	HID36_POP4
Protocol Name	E.g., Investigator ESSplex SE QS
Oven Temperature (°C)	Default (60)
Run Voltage (kV)	13.0
PreRun Voltage (kV)	Default (15)
Injection Voltage (kV)	1.2
Run Time (s)	1550
PreRun Time (s)	Default (180)
Injection Time (s)	30.0*
Data Delay (s)	Default (1)
Advanced Options	Default

* If 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.

Table 30. Instrument Protocol parameters for Applied Biosystems 3500xL Genetic Analyzer

Parameter	Setting
Application Type	HID
Capillary Length	36 cm
Polymer	POP4
Dye Set	E.g., BT5
Run Module	HID36_POP4
Protocol Name	E.g., Investigator ESSplex SE QS
Oven Temperature (°C)	Default (60)
Run Voltage (kV)	13.0
PreRun Voltage (kV)	Default (15)
Injection Voltage (kV)	1.6
Run Time (s)	1550
PreRun Time (s)	Default (180)
Injection Time (s)	25.0*
Data Delay (s)	Default (1)
Advanced Options	Default

* If deviating from the standard settings, the injection time may range between 1 and 30 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 30 s may be necessary.

Size Standard

1. To set up the Size Standard, select "Library", go to "Analyze" under "Size Standards", and click "Create".
2. The parameters in Table 30 must be entered or selected.

   The DNA Size Standard 550 (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.
3. Alternatively, import the DNA Size Standard 550 (BTO) parameters using the recommended Investigator template file "SST-BTO_60-500bp" (Table 31).

4. Click “Save” to confirm the changes.

Table 31. Size standard parameters

Parameter	Settings
Size Standard	E.g., SST-BTO_60-500bp
Dye Color	Orange

QC Protocol

1. To set up the QC Protocol, select “Library”, go to “Analyze” under “QC Protocols”, and click “Create”.
2. The parameters in Table 32 must be entered or selected.

Table 32. QC Protocol parameters

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

3. Go to “Analysis Settings”, followed by “Peak Amplitude Threshold” and disable “Purple”. Ensure that all other colors are enabled.

   Check the recommended analysis settings in Table 35 on page 50. 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”, select “Assays”, and click “Create”.

To analyze Investigator ESSplex SE QS Kit fragments, the parameters in Table 33 must be selected.

2. Click "Save" to confirm the changes.

Table 33. Assay parameters

Parameter	Settings
Assay Name	E.g., Investigator ESSplex SE QS
Color	Default
Application Type	HID
Instrument Protocol	E.g., Investigator ESSplex SE QS
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 34.

Table 34. Plate properties

Parameter	Settings
Name	e.g., Investigator ESSplex SE QS
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 ESSplex SE QS 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. 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 to 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 35 lists the recommended analysis parameters in the worksheet Peak Detector.

Table 35. 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:*
	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 three 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 Guides for GeneMapper *ID* or 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 550 (BTO; Figure 5) 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.

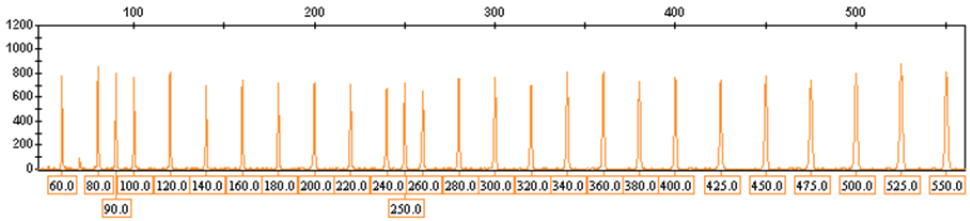

Figure 5. Electropherogram of the DNA Size Standard 550 (BTO), fragments with lengths in bp.

Analysis software

Allele allocation should be carried out using suitable analysis software, e.g. GeneMapper *ID* or GeneMapper *ID-X* Software in combination with the Investigator Template Files, which are available for download from **www.qiagen.com**, see Table 36 and Table 37.

Table 36. Recommended Investigator Template Files for GeneMapper *ID*

File type	File name
Panels	ESSplex_SE_QS_Panels
BinSets	ESSplex_SE_QS_Bins
Size standard	SST-BTO_60–500bp
Analysis Method	Analysis_HID_3130_50rfu Analysis_HID_3130_200rfu
Plot Settings	Plots_5dyes

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

Table 37. Recommended Investigator Template Files for GeneMapper *ID-X*

File type	File name
Panels	ESSplex_SE_QS_Panels_x
BinSets	ESSplex_SE_QS_Bins_x
Stutter	ESSplex_SE_QS_Stutter_x
Size standard	SST-BTO_60–500bp
Analysis Method	Analysis_HID_3130_50rfu Analysis_HID_3130_200rfu Analysis_HID_3500_50rfu Analysis_HID_3500_200rfu
Plot Settings	Plots_5dyes

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

Controls

The alleles listed in Table 38 represent the control DNA 9948 (included in the Investigator ESSplex SE QS Kit) and DNA from other commercially available standard cell lines.

Table 38. Allele assignment of the Investigator ESSplex SE QS Kit

Locus	CCR 9948	CCR 9947A
Amelogenin	X/Y	X/X
D1S1656	14/17	18.3/18.3
D2S441	11/12	10/14
D2S1338	23/23	19/23
D3S1358	15/17	14/15
D8S1179	12/13	13/13
D10S1248	12/15	13/15
D12S391	18/24	18/20
D16S539	11/11	11/12
D18S51	15/18	15/19
D19S433	13/14	14/15
D21S11	29/30	30/30
D22S1045	16/18	11/14
FGA	24/26	23/24
SE33	23.2/26.2	19/29.2
THO1	6/9.3	8/9.3
vWA	17/17	17/18

For further confirmation, the table above displays the alleles of the reference DNA purchased from Coriell Cell Repositories (CCR), as well as 3 reference DNAs purchased from CCR and ATCC up to the standard of Szibor et al. (2003).

Quality Sensor

The Investigator ESSplex SE QS Kit contains two 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 enclosed in the Primer Mix and are amplified simultaneously with the polymorphic STR markers. The Quality Sensors are labeled with FAM and appear at fragment sizes of 71 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 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 pipetting of the PCR setup or the PCR itself were performed incorrectly. This indicates that the user could repeat the experiment with more care around the setup in the hope of getting results.

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, the analysis of the two internal control fragments, QS1 and QS2, and of the STR target amplification products allow 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 (Figure 6). 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.

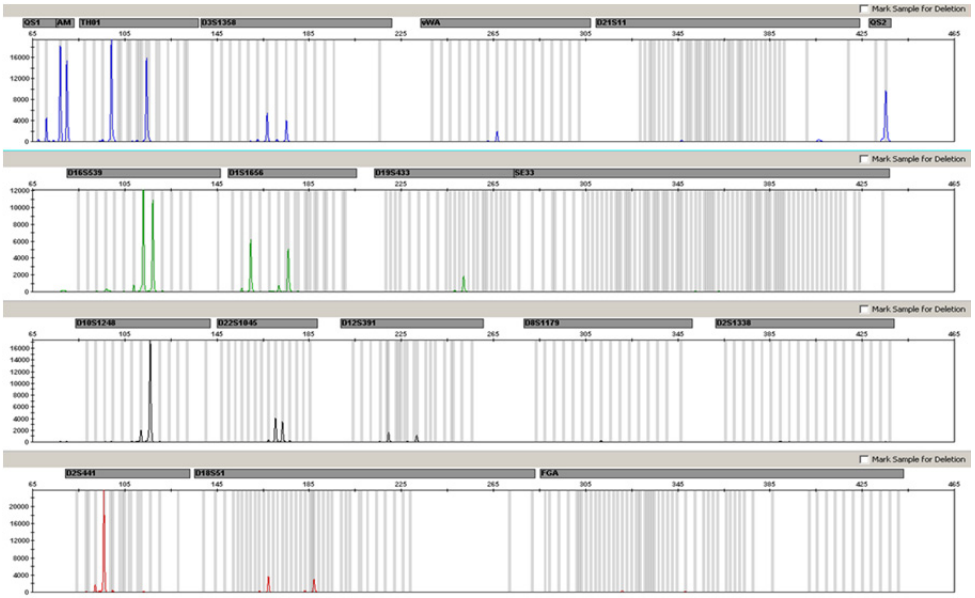

Figure 6. Electropherogram of STR analysis in the presence of degraded DNA (fragments of 150 bp). Genomic DNA was sheared to fragments of 150 bp using ultrasound. The large STR fragments were amplified with a very low PCR yield, but QS1 and QS2 were amplified normally with equal peak heights. The markers are shown at the top of the electropherogram. The Quality Sensors are labeled with FAM (panel 1) and appear at fragment sizes of 71 bp (QS1) and 435 bp (QS2).

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 (Figure 7).

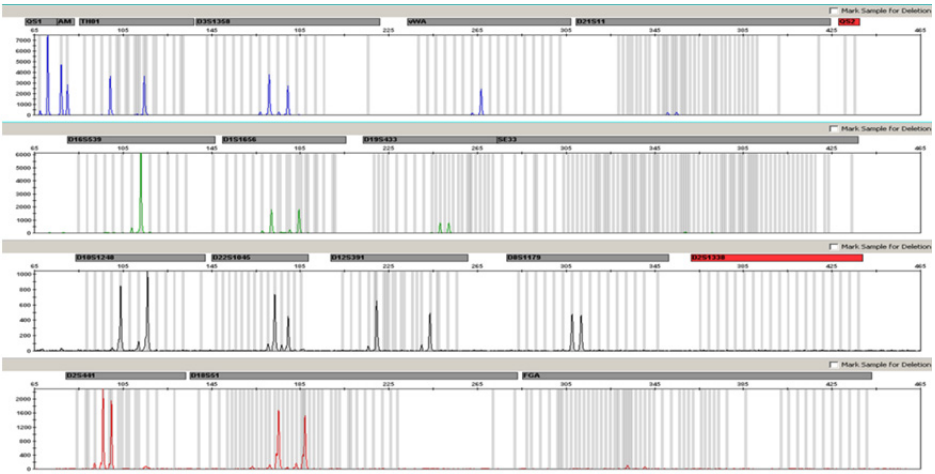

Figure 7. Electropherogram of STR analysis in the presence of hematin. 16 STR markers, Amelogenin and the two Quality Sensors were amplified in the presence of 1000 µM hematin and analyzed using capillary electrophoresis. The amplification of high molecular weight fragments, including STR makers of more than 250 bp and QS2, was inhibited by the high hematin content. The markers are presented at the top of the electropherogram. The Quality Sensors are labeled with FAM (panel 1) and appear at fragment sizes of 71 bp (QS1) and 435 bp (QS2, not visible).

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 provides the user with helpful information for data interpretation and planning the next steps. Table 39 summarizes the possible profile appearances and their meanings.

Table 39. 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 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-down of the QS2 signal below 20% of the QS1 signal indicates inhibition of the PCR reaction.

AllelesTable 40. shows the alleles of the allelic ladder. All analyses were performed using POP-4 polymer (Table 40 and Figure 8). 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: 65–465 bp
- Vertical: Depending on signal intensity

Table 40. Allelic ladder fragments included in the Investigator ESSplex SE QS Kit

Locus	Dye label	Repeat numbers of allelic ladder
QS1	6-FAM	S, Q
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
QS2	6-FAM	Q, S
D16S539	BTG	5, 8, 9, 10, 11, 12, 13, 14, 15
D1S1656	BTG	9, 10, 11, 12, 13, 14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18, 18.3, 19.3, 20.3
D19S433	BTG	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
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
D12S391	BTY	14, 15, 16, 17, 17.3, 18, 18.3, 19, 20, 21, 22, 23, 24, 25, 26, 27
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	BTR	8, 9, 10, 11, 11.3, 12, 13, 14, 15, 16, 17
D18S51	BTR	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	BTR	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, 31.2, 33, 34, 37.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2

* Alleles are heightened within the allelic ladder for better orientation.

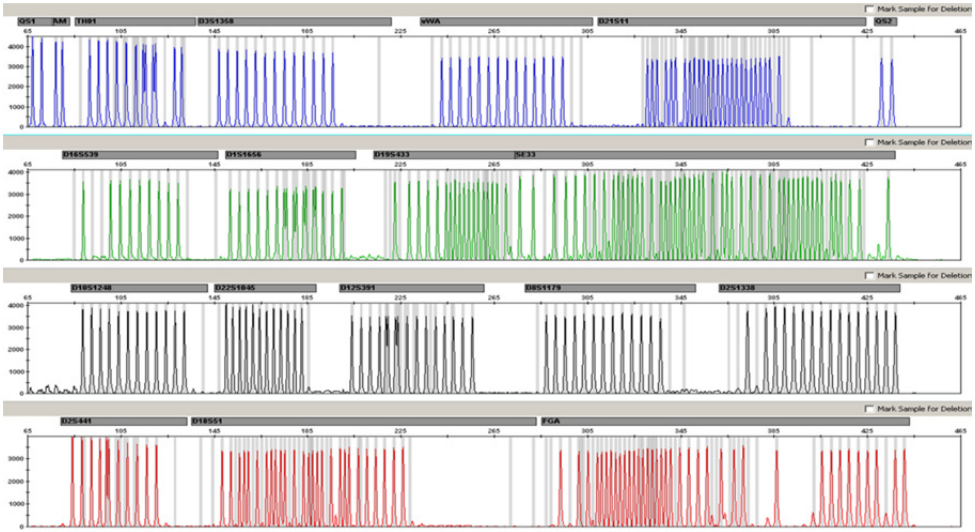

Figure 8. Electropherogram of the allelic ladder ESSplex SE QS analyzed on an Applied Biosystems 3500 Genetic Analyzer. The allelic ladder contains two alleles for each Quality Sensor (QS1 and QS2). This allows an automated calling of the QS peaks for sample analysis.

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 either the information and/or protocols in this handbook or sample and assay technologies (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.
Peak heights of QS1 and/or QS2 drop-down 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 QS. A drop down of the QS2 signal below 20% of the QS1 signal indicates inhibition of the PCR reaction.
Sample preparation		
	Sample signal intensity must be increased	Reduce the volume of the DNA Size Standard 550 (BTO) to peak heights of about 500 RFU. Purify the PCR products before starting the analysis. We recommend the MinElute® PCR Purification Kit (QIAGEN, cat. nos. 28004 and 28006) for rapid and effective purification.

		Comments and suggestions
Matrix/spectral calibration is not appropriate		
	There are pull-up peaks between the dye panels (B, G, Y, R, 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 550 (BTO) 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 550 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 (>5000 RFU/>20000 RFU)*, the occurrence of stutters, split peaks, and artifacts may increase.	Reduce the injection time in increments to a minimum of 1 second, 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. Setup 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.

		Comments and suggestions
e)	Low room temperature or low CE buffer temperature may result in fragment migration shifts or OL peaks.	Ensure ambient conditions are maintained as recommended by the instrument manufacturer. Ensure buffers are equilibrated to ambient conditions. Preheating of the CE instrument (~30 minutes) is recommended by the instrument manufacturer.

* >5000 RFU for the 3100 and 3130 Genetic Analyzer; >20000 RFU for the Applied Biosystems 3500 Genetic Analyzer.

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 the DNA Size Standard 550 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.
e)	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.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, contact QIAGEN Technical Services.

Cited 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. Hill, C.R., Kline, M.C., Coble, M.D., and Butler, J.M. (2008) Characterization of 26 miniSTR loci for improved analysis of degraded DNA samples. J. Forensic Sci., **53**, 73.

3. Szibor, R., et al. (2003) Cell line DNA typing in forensic genetics – the necessity of reliable standards. Forensic Sci. Int. **138**, 37.

Appendix: 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 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 60), 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. 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* and 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 ESSplex SE QS 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.

Ordering Information

Product	Contents	Cat. no.
Investigator ESSplex SE QS Kit (100)	Primer Mix, Fast Reaction Mix 2.0 including *Taq* DNA Polymerase, Control DNA, allelic ladder ESSplex SE QS, DNA size standard 550 (BTO) and Nuclease-Free Water	381575
Investigator ESSplex SE QS Kit (400)	Primer Mix, Fast Reaction Mix 2.0 including *Taq* DNA Polymerase, Control DNA, allelic ladder ESSplex SE QS, DNA size standard 550 (BTO) and Nuclease-Free Water	381577
Related product		
Matrix Standard BT5 multi cap. (25)	Matrix standard 6-FAM, BTG, BTY, BTR and BTO	386123
Matrix Standard BT5 multi cap. (50)	Matrix standard 6-FAM, BTG, BTY, BTR and BTO	386125
Investigator Human Identification PCR Kits		
Investigator 24plex QS Kit (100)*	Primer mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder 24plex, DNA size standard 550 (BTO) and Nuclease-Free Water	382415
Investigator 24plex GO! Kit (200)*	Primer mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder 24plex, DNA size standard 550 (BTO) and Nuclease-Free Water	382426

* Larger kit sizes are available; please inquire.

Product	Contents	Cat. no.
Investigator ESSplex Plus Kit (100)*	Primer mix, Fast Reaction Mix including HotStarTaq® *Plus* DNA Polymerase, Control DNA, allelic ladder ESSplex Plus, DNA size standard 550 (BTO) and Nuclease-Free Water	381535
Investigator ESSplex SE Plus Kit (100)*	Primer mix, Fast Reaction Mix including HotStarTaq *Plus* DNA Polymerase, Control DNA, allelic ladder ESSplex SE Plus, DNA size standard 550 (BTO) and Nuclease-Free Water	381545
Investigator IDplex Plus Kit (100)*	Primer mix, Fast Reaction Mix including HotStarTaq *Plus* DNA Polymerase, Control DNA, allelic ladder IDplex Plus, DNA size standard 550 (BTO) and Nuclease-Free Water	381625
Investigator HDplex Kit (100)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard and Nuclease-Free Water	381215
Investigator Triplex AFS QS Kit (400)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard and Nuclease-Free Water	380317
Investigator Triplex DSF Kit (400)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard and Nuclease-Free Water	380327
Investigator Argus X-12 Kit (25)*	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard and Nuclease-Free Water	383213

* Larger kit sizes are available; please inquire.

Product	Contents	Cat. no.
Investigator Argus Y-12 QS Kit (100)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard and Nuclease-Free Water	383615
Investigator DIPplex Kit (100)*	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard and Nuclease-Free Water	384015
Investigator Quantiplex HYres Kit (200)	Reaction Mix FQ, Primer Mix IC YQ, Control DNA Z1, QuantiTect® Nucleic Acid Dilution Buffer	387116
Investigator Quantiplex Kit (200)	Reaction Mix FQ, Primer Mix IC FQ, Control DNA Z1, QuantiTect Nucleic Acid Dilution Buffer	387016
DNA extraction and purification		
QIAamp® DNA Investigator Kit (50)	50 QIAamp MinElute Columns, Proteinase K, Carrier RNA, Buffers, Collection Tubes (2 ml)	56504
MinElute Reaction Cleanup Kit (50)*	50 MinElute Spin Columns, Buffers, Collection Tubes (2 ml)	28004
Rotor-Gene® Q		
Rotor-Gene Q 2plex	Real-time PCR cycler with 2 channels, laptop computer, software, accessories, 1-year warranty on parts and labor	Inquire

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
Rotor-Gene Q 2plex HRM®	Real-time PCR cycler and High Resolution Melt analyzer with 2 channels, plus HRM channel, laptop, software, accessories, 1-year warranty on parts and labor	Inquire
Rotor-Gene Q 5plex	Real-time PCR cycler with 5 channels (green, yellow, orange, red, crimson), laptop computer, software, accessories, 1-year warranty on parts and labor	Inquire
Rotor-Gene Q 5plex HRM	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels, plus HRM channel, laptop, software, accessories, 1-year warranty on parts and labor	Inquire
Rotor-Gene Q 6plex	Real-time PCR cycler with 6 channels, including laptop, software, accessories, 1-year warranty on parts and labor	Inquire

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.

Limited License Agreement for Investigator ESSplex SE QS Kit

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this handbook and for use with components contained in the kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this kit with any components not included within this kit except as described in the protocols provided with the product, this handbook, and additional protocols available at www.qiagen.com. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this kit and/or its use(s) do not infringe the rights of third-parties.
3. This kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the kit agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the kit and/or its components.

For updated license terms, see **www.qiagen.com.**

1095534 HB-1963-001 06/2015 © 2015

Notes

Notes

1095534 HB-1963-001 06/2015