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Investigator[®] IDplex Plus Handbook

For multiplex amplification of 13 CODIS core
loci, plus D2S1338, D19S433, and
Amelogenin



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Kit Contents

Investigator IDplex Plus Kit	(100)	(400)
Catalog no.	381625	381627
Number of 25 μl reactions	100	400
Fast Reaction Mix*	750 μ l	2 x 1500 μ l
Primer Mix IDplex Plus	250 μ l	4 x 250 μ l
Control DNA 9948	200 μ l	200 μ l
DNA size standard 550 (BTO)	50 μ l	200 μ l
Allelic ladder IDplex Plus	25 μ l	4 x 25 μ l
Nuclease-free water	2 x 1.9 ml	5 x 1.9 ml
Quick-Start Protocol	1	1

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

Storage

The Investigator IDplex Plus Kit is shipped on dry ice. It should be stored immediately upon receipt at -20°C in a constant-temperature freezer. Avoid repeated thawing and freezing. Primer mix and allelic ladder must be stored protected from the light. DNA samples and post-PCR reagents (allelic ladder and DNA size standard) should be stored separately from the PCR reagents. Under these conditions, the components are stable until the expiration date indicated on the kit.

Once opened, the Investigator IDplex Plus Kit should be stored at $4-8^{\circ}\text{C}$ for a maximum of 2 weeks.

Intended Use

The Investigator IDplex Plus 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.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Introduction

The Investigator IDplex Plus Kit is used for multiplex PCR in forensic, human identity, and paternity testing. The kit covers the 13 CODIS (Combined DNA Index System) core loci, D2S1338, D19S433, and Amelogenin. One PCR simultaneously amplifies the 15 polymorphic STR markers (D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, CSF1PO, FGA, TH01, TPOX, and vWA) and the gender-specific Amelogenin.

The Investigator IDplex Plus Kit has been developed 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, which allows amplification in around 90 minutes and provides highly robust results through its inhibitor-resistant chemistry. The primers are fluorescence-labeled with one of the following dyes:

- 6-FAM™: Amelogenin, TH01, D3S1358, vWA, D21S11
- BTG: TPOX, D7S820, D19S433, D5S818, D2S1338
- BTY: D16S539, CSF1PO, D13S317, FGA
- BTR: D18S51, D8S1179

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 IDplex Plus 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).

Table 1. Locus-specific information of the Investigator IDplex Plus 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
D2S1338	G08202	[TGCC] ₆ [TTCC] ₁₁	2q35
D3S1358	11449919	TCTA [TCTG] ₂ [TCTA] ₁₅	3p25.3
D5S818	G08446	[AGAT] ₁₁	5q23.2
D7S820	G08616	[GATA] ₁₂	7q21.11
D8S1179	G08710	[TCTA] ₁₂	8q23.1–23.2
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
CSF1PO	X14720	[AGAT] ₁₂	5q33.1
FGA (FIBRA)	M64982	[TTTC] ₃ TTTTTCT [CTTT] ₁₃ CTCC [TTCC] ₂	4q28.2
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 material safety data sheets (MSDSs), available from the product supplier.

- Hi-Di™ Formamide, 25 ml (Applied Biosystems, cat. no. 4311320)
- Matrix Standards BT5 for single-capillary instruments, e.g., ABI PRISM 310 Genetic Analyzer (QIAGEN, cat. no. 386113)
- 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 310 Genetic Analyzer
 - ABI PRISM 3100-Avant™/3100 Genetic Analyzer
 - Applied Biosystems 3130/3130xl Genetic Analyzer
 - Applied Biosystems 3500/3500xL Genetic Analyzer
- One of the following PCR thermal cyclers:
 - QIAGEN Rotor-Gene® Q
 - GeneAmp PCR System 9700
 - Bio-Rad PTC-200
 - Biometra UNO-Thermoblock
 - Eppendorf® Mastercycler® ep
- 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 (HID) products for forensic applications. We recommend Investigator IDproof, Investigator IDproof Mixture, GeneMapper® ID, GeneMapper ID-X, or Genotyper® Software. The Investigator Template Files facilitate data analysis and are valid with the software mentioned above.

Protocol: PCR Amplification

This protocol is for PCR amplification of STR loci from forensic samples using the Investigator IDplex Plus Kit.

Important points before starting

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

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.
- 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.

Procedure

1. Thaw PCR components and template nucleic acid.

Mix thoroughly 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.

Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. This should include positive and negative control reactions.

3. Mix the master mix thoroughly, 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 μ l.

5. Prepare positive and negative controls.

Positive control: Use 5 μ l of the Control DNA.

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

Table 2. Reaction setup

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

6. Mix reactions thoroughly.

For optimal results, mix the prepared and sealed PCR plate prior to amplification using an Eppendorf Thermomixer Comfort (orbital shaker) for 5 min at 1200 rpm at room temperature. Use constant shaking without pauses.

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

Temperature	Time	Number of cycles
95°C*	5 min	–
96°C	10 s	30 cycles
61°C	120 s	
10°C	∞	–

* Hot-start to activate DNA polymerase.

8. After the cycling protocol is completed, store samples at –20°C protected from the light, or proceed directly with running the electrophoresis.

Protocol: Electrophoresis Using the ABI PRISM 310 Genetic Analyzer

For general instructions on instrument setup, matrix generation, and application of the GeneScan® or GeneMapper® ID Software, refer to the *ABI PRISM 310 Genetic Analyzer User's Manual*. Electrophoresis using the GeneScan Software is described below.

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 given in Table 4.

Table 4. Materials required for electrophoresis

Material	Specifications
Capillary	47 cm/50 μ m (green)
Polymer	POP-4™ for ABI PRISM 310 Genetic Analyzer
Buffer	10x Genetic Analyzer Buffer with EDTA

Matrix generation

Before conducting DNA fragment size analysis with the filter set G5, a matrix with the 5 fluorescent labels 6-FAM, BTG, BTY, BTR, and BTO must be generated (Table 5).

Table 5. 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

1. **Five electrophoresis runs should be conducted, one for each fluorescent label, under the same conditions as for the samples and allelic ladders of the Investigator IDplex Plus Kit, in order to generate suitable matrix files (Table 6).**

Table 6. Matrix setup for a single capillary instrument (ABI PRISM 310 Genetic Analyzer)

Matrix sample	Component	Volume
Matrix sample 1	Hi-Di Formamide	12.0 μ l
	Matrix standard 6-FAM	1.0 μ l
Matrix sample 2	Hi-Di Formamide	12.0 μ l
	Matrix standard BTG	1.0 μ l
Matrix sample 3	Hi-Di Formamide	12.0 μ l
	Matrix standard BTY	1.0 μ l
Matrix sample 4	Hi-Di Formamide	12.0 μ l
	Matrix standard BTR	1.0 μ l
Matrix sample 5	Hi-Di Formamide	12.0 μ l
	Matrix standard BTO	1.0 μ l

2. **Denature for 3 min at 95°C.**
3. **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.
4. **Load the samples on the tray.**
5. **Create a Sample Sheet and enter the sample designation. Table 7 shows the injection list for the matrix generation.**

Table 7. Injection list for matrix generation

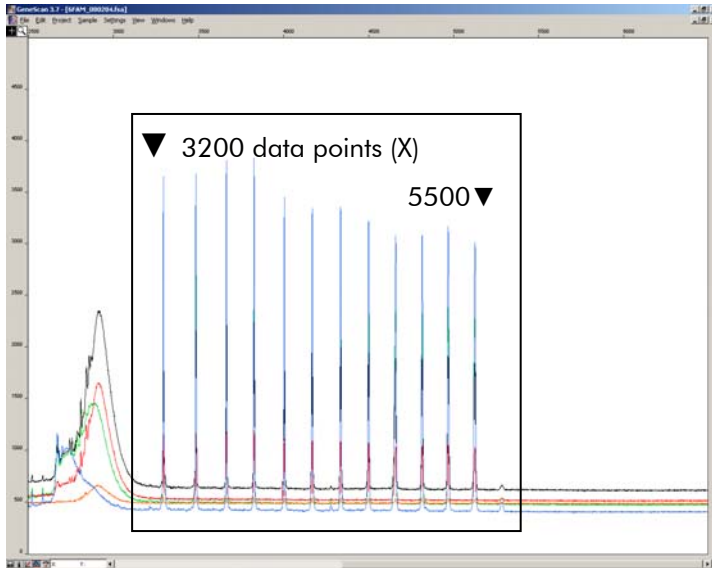
Parameter	Settings
Module File	GS STR POP-4 (1 ml) G5
Matrix File	None
Size Standard	None*
Injection Time (s)	5
Injection Voltage (kV)	15
Run Voltage (kV)	15
Run Temperature (°C)	60
Run Time (min)	24

* Always prepare matrix standards without DNA Size Standard (BTO).

Analysis of the matrix samples

- 1. Run the GeneScan Software.**
- 2. Select "New" from the File menu, and then select "Project".**
- 3. Open the folder of the current run and select "Add Sample Files".**
- 4. Select a matrix sample in the "Sample File" column.**
- 5. Click "Sample" and then "Raw Data".**
- 6. Check the matrix samples for a flat baseline. As shown in the figure (next page), there should be at least 5 peaks with peak heights of 1000–4000 RFU for each matrix sample.**

Note: The optimal range is 2000–4000 RFU.

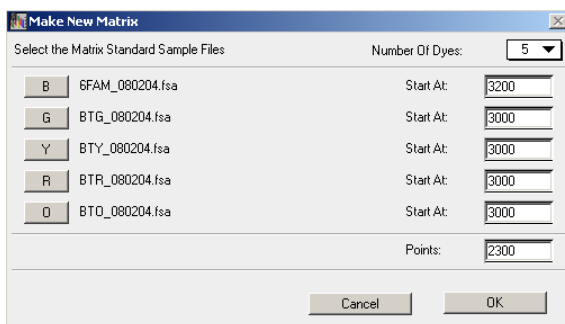


Electropherogram with raw data of the matrix standard 6-FAM.

7. Select an analysis range with a flat baseline and re-inject the matrix sample, if necessary.
8. Record start and end value (data points) of the analysis range; e.g., start value 3200, end value 5500.
9. Calculate the difference between the end and start values; e.g., $5500 - 3200 = 2300$ data points.

Generation of a matrix

1. Select "New" in the File menu, and then select "Matrix".
2. Import the matrix samples for all dyes (B, G, Y, R, and O).
3. Enter a "Start At" value, e.g., 3200.
4. Under "Points", enter the calculated difference between end and start values, e.g., 2300.
5. Click "OK" to calculate the new matrix.



Matrix sample selection.

6. Select "Save as" in the File menu to save the new matrix in the matrix folder.

The screenshot shows a window titled "Matrix BT5.mtx" with a table of values. The table has columns labeled B, G, Y, R, and O, and rows labeled B, G, Y, H, and O. The values are as follows:

	B	G	Y	R	O
B	1.0000	0.1811	0.0051	0.0418	0.0006
G	0.6891	1.0000	0.2058	0.3259	0.0017
Y	0.4687	0.8068	1.0000	0.9119	0.0029
H	0.1944	0.3619	0.5311	1.0000	0.0095
O	0.0160	0.0304	0.0477	0.2082	1.0000

New matrix BT5.

Checking the matrix

1. To check the new matrix with current samples, select "New" in the File menu, and then select "Project".
2. Open the folder of the respective run and select "Add Sample Files".
3. Select the sample(s) in the Sample File column.
4. Click "Sample" and then "Install New Matrix" to open the matrix folder and select the new matrix.
5. Re-analyze the samples.

Note: There should be no pull-up peaks between the dye panels (B, G, Y, R, O) with the new matrix.

Sample preparation

1. Set up a mixture of formamide and DNA size standard according to Table 8.

Table 8. Setup of formamide and DNA size standard mixture

Component	Volume per sample
Hi-Di Formamide	12.0 μ l
DNA Size Standard 550 (BTO)	0.5 μ l

2. Aliquot 12 μ l of the mixture to a tube for each sample to be analyzed.
3. Add 1 μ l 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.

Setting up the GeneScan Software

Create a Sample Sheet and enter sample designation.

Table 9. Injection list for the ABI PRISM 310 Genetic Analyzer

Component	Settings
Module File	GS STR POP-4 (1 ml) G5
Matrix File	e.g., Matrix BT5
Size Standard	e.g., SST-BTO_60-500bp
Injection Time (s)	5*
Injection Voltage (kV)	15
Run Voltage (kV)	15
Run Temperature (°C)	60
Run Time (min)	28 [†]

* Deviating from standard settings, the injection time may range between 1 and 10 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 up to 10 s may be necessary.

[†] The run time for Investigator IDplex Plus was modified in order to be able to analyze fragments with lengths of up to 500 bp.

Analysis parameters

Table 10 lists the recommended analysis parameters.

Table 10. Recommended analysis parameters for the ABI PRISM 310 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 as high as 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 (Genotyper[®], GeneMapper *ID*, or GeneMapper *ID-X*).

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 given in Table 11.

Table 11. 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 3100/3100-Avant Genetic Analyzer and should be done before conducting fragment length analysis. The calibration procedure creates a matrix which 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 12.

Table 12. Setup of formamide and Matrix Standard BT5 mixture for 4 capillaries

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

2. Load 12 μ l of the mixture to 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 13.

Table 13. Setup of formamide and Matrix Standard BT5 mixture for 16 capillaries

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

2. Load 12 μ l of the mixture to 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 14. 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 instructions in the section "Spectral parameter" on page 20.
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 15.

Table 15. Setup of formamide and DNA size standard mixture

Component	Volume per sample
Hi-Di Formamide	12 μ l
DNA Size Standard 550 (BTO)	0.5 μ l

- 2. Aliquot 12 μ l of the mixture to a tube for each sample to be analyzed.**
- 3. Add 1 μ l 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 μ l 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 16).**
- 3. Modify the Injection Voltage to 3 kV and the Injection Time to 10 s.**
- 4. Click "Save As" and enter the name of the new module (e.g., 3kV_10s_500bp). Confirm by clicking "OK".**
- 5. Click "Close" to exit the Run Module Editor.**

Table 16. Run Module 3kV_10s_500bp for the ABI PRISM 3100-Avant/3100 Genetic Analyzer

Parameter	Setting
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)	3.0
Injection Time (s)	10*
Run Voltage (kV)	Default
Number of Steps	Default
Voltage Step Interval	Default
Data Delay Time (s)	Default
Run Time (min)	26 [†]

* Deviating from the standard settings, the injection time may range between 1 and 20 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 20 s may be necessary.

[†] The run time for Investigator IDplex Plus 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 17. 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	3kV_10s_500bp*
Analysis Module 1	DefaultAnalysis.gsp

* See Table 16, "Run Module 3kV_10s_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 18 lists the recommended analysis parameters.

Table 18. 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 (Genotyper, GeneMapper *ID*, or GeneMapper *ID-X*).

Protocol: Electrophoresis Using the Applied Biosystems 3130/3130xl 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 3130/3130xl 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 3130xl 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 given in Table 19.

Table 19. Materials required for electrophoresis

Material	Specifications
Capillary	36 cm Capillary Array for Applied Biosystems 3130/3130xl Genetic Analyzer
Polymer	POP-4 Polymer for Applied Biosystems 3130/3130xl 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 which 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 20.

Table 20. Setup of formamide and Matrix Standard BT5 mixture for 4 capillaries

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

2. Load 12 μ l of the mixture to 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 (Applied Biosystems 3130xl Genetic Analyzer)

1. Set up a mixture of formamide and Matrix Standard BT5 according to Table 21.

Table 21. Setup of formamide and Matrix Standard BT5 mixture for 16 capillaries

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

2. Load 12 μ l of the mixture to 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 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 22 and click "OK".

Table 22. 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 23 and click "OK". A new table in the Plate Editor opens automatically (Table 24).

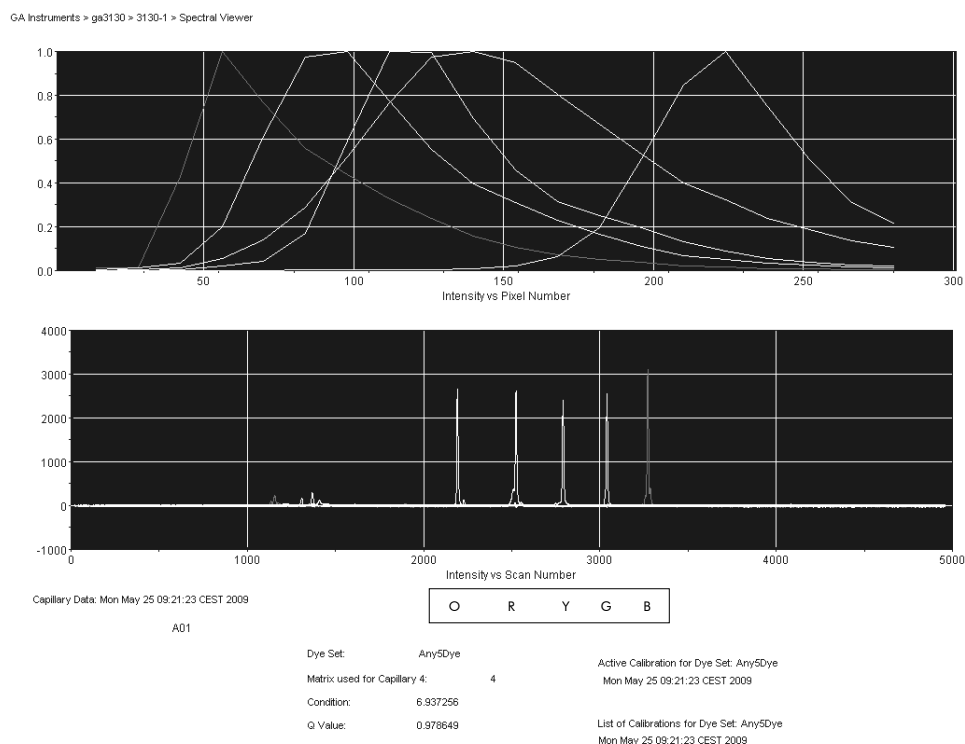
Table 23. Plate Editor for spectral calibration (I)

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

Table 24. Plate Editor for spectral calibration (II)

Parameter	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.



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.
Note: The optimal range is 2000–4000 RFU.
3. 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.
4. If calibration failed, use the optimized values of the Matrix Standard BT5 and repeat the calibration run.
5. 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 25.

Table 25. Setup of formamide and DNA size standard mixture

Component	Volume per sample
Hi-Di Formamide	12.0 μ l
DNA Size Standard 550 (BTO)	0.5 μ l

2. Aliquot 12 μ l of the mixture to a tube for each sample to be analyzed.
3. Add 1 μ l 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 μ l 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 26.

2. **Modify the Injection Voltage to 3 kV and the Injection Time to 10 s (Table 26).**
3. **Click "Save As", enter a name for the new Run Module (e.g., 3kV_10s_500bp), and confirm by clicking "OK".**
4. **Click "Close" to exit the Run Module Editor.**

Table 26. Run Module 3kV_10s_500bp for the Applied Biosystems 3130/3130xl 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)	3.0
Injection Time (s)	10*
Voltage Number of Steps	Default
Voltage Step Interval	Default
Data Delay Time (s)	Default
Run Voltage (kV)	Default
Run Time (s)	1560 [†]

* Deviating from the standard settings, the injection time may range between 1 and 20 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 20 s may be necessary.

[†] The run time for Investigator IDplex Plus 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. 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 27.**
- 4. Click “OK” to exit the Protocol Editor.**

Table 27. Settings in Instrument Protocol

Protocol Editor	Settings
Name	Run36_POP4_BT5_26min
Type	REGULAR
Run Module	3kV_10s_500bp*
Dye Set	Any5Dye

* See Table 26, "Run Module 3kV_10s_500bp for the Applied Biosystems 3130/3130xl Genetic Analyzer".

- 5. 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.**
- 6. Enter the information in Table 28.**

Table 28. 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	...

- 7. Click "OK" and a new table in the Plate Editor opens automatically (Table 29).**
- 8. 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".**
- 9. 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 29. 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., IDplex_Plus_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)

10. Start the run.

11. 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.

12. 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 30 lists the recommended analysis parameters.

Table 30. Recommended settings for the Applied Biosystems 3130/3130xl Genetic Analyzer

Parameter	Settings
Peak Detection Algorithm	Advanced
Ranges	Analysis: Partial Range Start Point: 2000; Stop Point: 10,000 Sizing: All Sizes
Smoothing and Baselineing	Smoothing: Light Baseline Window: 51 pts
Size Calling Method	Local Southern Method
Peak Detection	Peak Amplitude Thresholds B:* Y:* G:* R:* 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* 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 (Genotyper, GeneMapper *ID*, or GeneMapper *ID-X*).

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

For detailed instructions on instrument setup, spectral calibration, or application of the Applied Biosystems 3500 Series Data Collection Software version 1.0 and the GeneMapper ID-X Software version 1.2, refer to the *Applied Biosystems 3500/3500xL Genetic Analyzers User Guide*.

The system with 8 capillaries is the Applied Biosystems 3500 Genetic Analyzer and 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 given in Table 31.

Table 31. 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, it is necessary to perform a spectral calibration with the 5 fluorescent labels 6-FAM, BTG, BTY, BTR, and BTO for each analyzer (Table 32). The calibration procedure creates a matrix which is used to correct the overlapping of fluorescence emission spectra of the dyes.

IMPORTANT: Spectral calibration must be performed for each new capillary array.

Spectral calibration is comprised of the following steps:

- Preparation of the instrument
- Preparation of dye set BT5
- Preparation of the standard calibration plate
- Plate assembly and loading the plate in the instrument

- 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*.

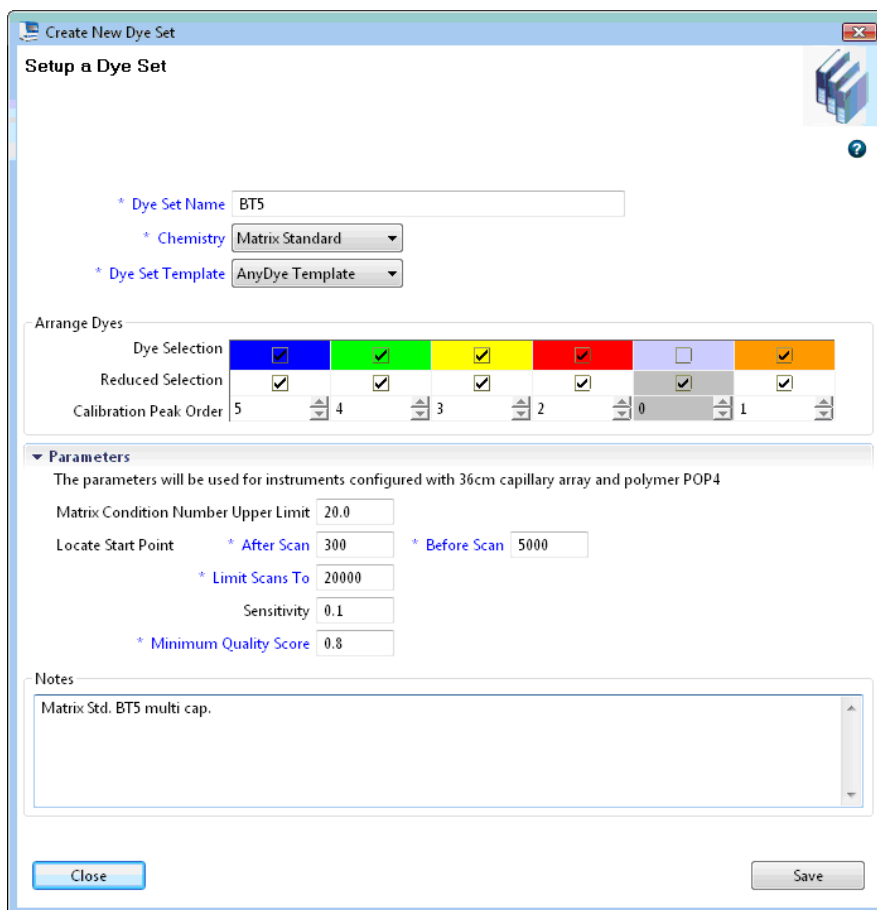
Preparation of dye set BT5

Table 32. 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

Prior to the spectral calibration, a dye set for the Matrix Standard BT5 must be set up.

1. To create a new dye set, go to "Library" and select "Analyze", followed by "Dye Sets" and click "Create".
2. Enter a "Dye Set Name", e.g., BT5.
3. Select "Matrix Standard" as a chemistry and "AnyDye Template" as a dye set 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.



Setup of dye set BT5.

Preparation of the standard calibration plate

Example for 8 capillaries (Applied Biosystems 3500 Genetic Analyzer)

1. Set up a mixture of formamide and Matrix Standard BT5 according to Table 33.

Table 33. Setup of formamide and Matrix Standard BT5 mixture for 8 capillaries

Component	Volume
Hi-Di Formamide	90 μ l
Matrix Standard BT5 multi cap.	10 μ l

2. Load 10 μl of the mixture to a 96-well plate, e.g., positions A1–H1.
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 24 capillaries (Applied Biosystems 3500xL Genetic Analyzer)

1. Set up a mixture of formamide and Matrix Standard BT5 according to Table 34.

Table 34. 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

2. Load 10 μl of the mixture to a 96-well plate, e.g., positions A1–H1, A2–H2, and A3–H3.
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.

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*.

Performing a spectral calibration run

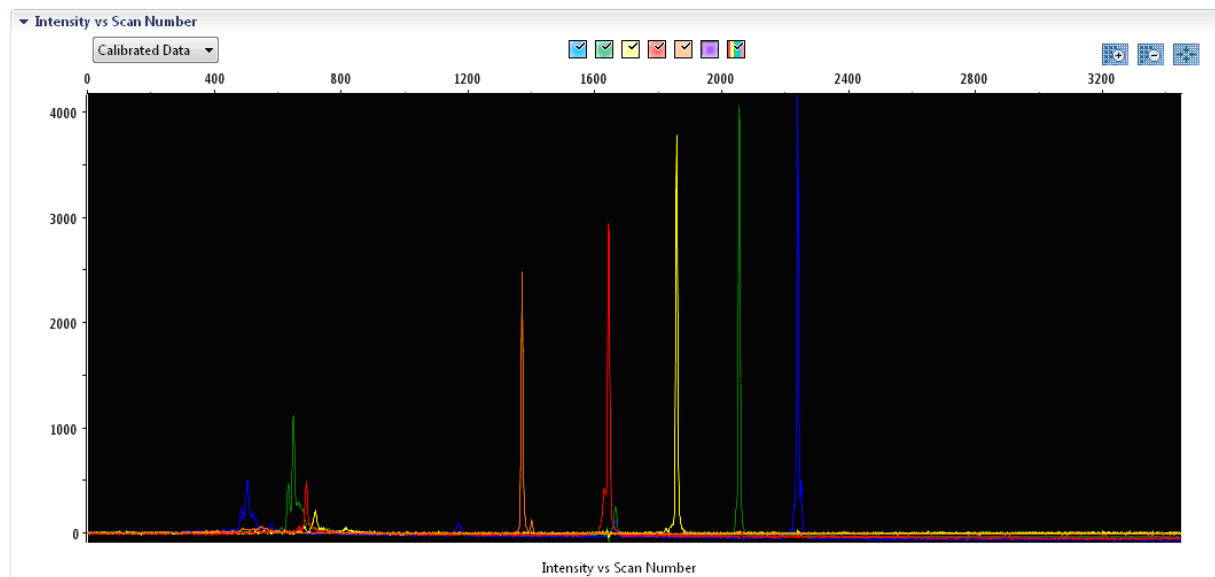
Once the multiwell plates containing the spectral calibration mixture are placed in the autosampler tray, the spectral calibration process can be started.

1. To access the Spectral Calibration screen, select “Maintenance” on the Dashboard of the 3500 Series Data Collection software.
2. The number of wells in the spectral calibration plate and their location in the instrument must be specified.
3. Select “Matrix Standard” as a chemistry standard and “BT5” for dye set.
4. (Optional) Enable “Allow Borrowing”.
5. Click “Start Run”.

Checking the matrix

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

- 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, 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).



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*.

▼ Capillary Run Data								
Capillary	1	2	3	4	5	6	7	8
Run 1	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Run 2	Not Calibrated	Not Calibrated	Not Calibrated	Not Calibrated	Not Calibrated	Not Calibrated	Not Calibrated	Not Calibrated
Run 3	Not Calibrated	Not Calibrated	Not Calibrated	Not Calibrated	Not Calibrated	Not Calibrated	Not Calibrated	Not Calibrated
Overall	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed

■ Passed
 ■ Failed
 ■ Borrowed
 Not Calibrated

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 appear in the raw data profile
- Peak morphology in the spectral profile shows 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 Results". If any capillary data does not meet the criteria above, click "Reject Results", and refer to the "spectral calibration troubleshooting" section of the *Applied Biosystems 3500/3500xL Genetic Analyzers User Guide*.

Sample preparation

1. **Set up a mixture of formamide and DNA size standard according to Table 35.**

Table 35. Setup of formamide and DNA size standard mixture

Component	Volume per sample
Hi-Di Formamide	12.0 μ l
DNA Size Standard 550 (BTO)	0.5 μ l

2. **Aliquot 12 μ l of the mixture to a tube for each sample to be analyzed.**
3. **Add 1 μ l 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, 8 or 24 samples must be pipetted onto the plate of multi-capillary analyzers. If fewer samples are analyzed, the empty positions must be filled with 12 μ l 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 1 injection

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 a run

If you are using the Investigator IDplex Plus 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.

- 1. To set up the Instrument Protocol, go to “Library” and select “Analyze”, followed by “Instrument Protocols” and click “Create”.**

Note: Modify the Run Module Default settings from “HID36_POP4” as shown in Table 36.

- 2. The parameters in Table 36 must be entered or selected.**

Table 36. Instrument Protocol parameters for the Applied Biosystems 3500/3500xL 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 IDplex Plus
Oven Temperature (°C)	Default
Run Voltage (kV)	Default
PreRun Voltage (kV)	Default
Injection Voltage (kV)	3.0
Run Time (s)	1300
PreRun Time (s)	Default
Injection Time (s)	8.0*
Data Delay (s)	Default
Advanced Options	Default

* Deviating from the standard settings, the injection time may range between 1 and 20 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 20 s may be necessary.

- 3. Click "Save" to confirm the changes.**
- 4. To set up the Size Standard, go to "Library", select "Analyze", followed by "Size Standards", and click "Create".**
- 5. The parameters in Table 37 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.

Table 37. Size standard parameters

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

6. Click **“Save”** to confirm the changes.
7. To set up the QC Protocol, go to **“Library”** and select **“Analyze”**, followed by **“QC Protocols”**, and click **“Create”**.
8. The parameters in Table 38 must be entered or selected.

Table 38. QC Protocol parameters

Parameter	Setting
Protocol Name	e.g., BTO_550
Size Standard	SST-BTO_60-500bp (from step 4)
Sizecaller	SizeCaller v1.1.0

9. 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 41. All other settings should remain as **“Default”**.
10. Click **“Save”** to confirm the changes.
11. To set up an Assay, go to **“Library”** and select **“Manage”**, followed by **“Assays”**, and click **“Create”**.
12. To analyze Investigator IDplex Plus fragments, the parameters in Table 39 must be selected.

Table 39. Assay parameters

Parameter	Setting
Assay Name	e.g., Investigator IDplex Plus
Color	Default
Application Type	HID
Instrument Protocol	e.g., Investigator IDplex Plus (from step 1)
QC Protocols	e.g., BTO_550 (from step 7)

13. Click “Save” to confirm the changes.

Starting the run

- 1. In the Dashboard, click “Create New Plate”.**
- 2. Go to “Define Plate Properties” and select “Plate Details”. Select or enter the parameters in Table 40.**

Table 40. Plate properties

Property	Setting
Name	e.g., Investigator IDplex Plus
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. Choose the correct Assay for the analysis. If you followed the steps under “Setting up the Run”, this would be Investigator IDplex Plus from step 11. All named wells on the plate must have an assigned assay.**
- 6. Select the wells for which to specify an assay. Check the box next to the assay name to assign it to the selected wells.**

7. (Optional) Repeat for file name conventions and results group.
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 41 lists the recommended analysis parameters in the worksheet Peak Detector.

Table 41. Recommended settings for the Applied Biosystems 3500/3500xL Genetic Analyzer

Parameter	Settings
Peak Detection Algorithm	Advanced
Ranges	Analysis: Partial Range Start Point: 1000; Stop Point: 20,000 Sizing: All Sizes
Smoothing and Baselineing	Smoothing: Light Baseline Window: 51 pts
Size Calling Method	Local Southern Method
Peak Detection	Peak Amplitude Thresholds B:* Y:* G:* R:* 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 version 1.2. 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 defaults from Applied Biosystems for HID analysis.

Protocol: Analysis

For general instructions on automatic sample analysis, refer to the appropriate User and/or Workflow Guides for Investigator IDproof Software, Investigator IDproof Mixture Software, GeneScan, 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) 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.

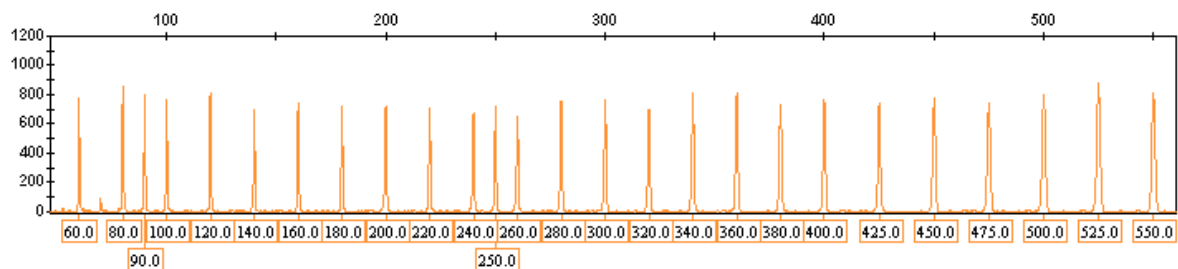


Figure 1. Electropherogram of the DNA Size Standard 550 (BTO), fragments with lengths in bp.

Analysis software

Allele allocation should be carried out with suitable analysis software, e.g., QIAGEN Investigator IDproof or IDproof Mixture Software, or Genotyper, GeneMapper *ID*, or GeneMapper *ID-X* Software in combination with the Investigator Template Files available as a download from www.qiagen.com or on a CD-ROM (cat. no. 389900), see Table 42 and Table 43.

The recommended Investigator Template File for Genotyper Software is the IDplex.

Table 42. Recommended Investigator Template Files for GeneMapper ID

File type	File name
Panels	IDplex_Plus_Panels
BinSets	IDplex_Plus_Bins
Size standard	SST-BTO_60–500bp
Analysis Method	Analysis_HID_310 Analysis_HID_3130 Analysis_HID_310_50rfu Analysis_HID_3130_50rfu
Plot Settings	Plots_5dyes
Table Settings	Table for 2 alleles Table for 10 alleles

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

Table 43. Recommended Investigator Template Files for GeneMapper ID-X

File type	File name
Panels	IDplex_Plus_Panels_x
BinSets	IDplex_Plus_Bins_x
Stutter	IDplex_Plus_Stutter_x
Size standard	SST-BTO_60–500bp
Analysis Method	Analysis_HID_310 Analysis_HID_3130 Analysis_HID_310_50rfu Analysis_HID_3130_50rfu Analysis_HID_3500
Plot Settings	Plots_5dyes
Table Settings	310 Data Analysis/31xx Data Analysis

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

Controls

The alleles listed in Table 44 represent the Control DNA 9948 (included in the Investigator IDplex Plus Kit) and DNA from other commercially available standard cell lines.

Table 44. Allele assignment of the Investigator IDplex Plus Kit

Locus	CCR 9948	CCR 9947A	ATCC K-562
Amelogenin	X/Y	X/X	X/X
D2S1338	23/23	19/23	17/17
D3S1358	15/17	14/15	16/16
D5S818	11/13	11/11	11/12
D7S820	11/11	10/11	9/11
D8S1179	12/13	13/13	12/12
D13S317	11/11	11/11	8/8
D16S539	11/11	11/12	11/12
D18S51	15/18	15/19	15/16
D19S433	13/14	14/15	14/14.2
D21S11	29/30	30/30	29/30/31
CSF1PO	10/11	10/12	9/10
FGA	24/26	23/24	21/24
TH01	6/9.3	8/9.3	9.3/9.3
TPOX	8/9	8/8	8/9
vWA	17/17	17/18	16/16

For further confirmation, the table above displays the alleles of the reference DNA purchased from Coriell Cell Repositories (CCR), as well as 2 reference DNAs purchased from CCR and ATCC up to the standard of Szibor et al. (2003).

Alleles

Table 45 shows the alleles of the allelic ladder. All analyses were performed using POP-4 polymer (Figure 2 and Figure 3). 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: 70–480 bp
- Vertical: Depending on signal intensity

Table 45. Allelic ladder fragments included in the Investigator IDplex Plus Kit

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, 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
D21S11	6-FAM	24, 24.2, 25, 26, 26.2, 27, 28, 28.2, 29, 29.2, 30, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 36, 36.2, 37
TPOX	BTG	4, 6, 8, 9, 10, 11, 12, 13, 15
D7S820	BTG	5, 7, 8, 9, 10, 11, 12, 13, 14, 15
D19S433	BTG	6.2, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2
D5S818	BTG	6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17
D2S1338	BTG	14, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28
D16S539	BTY	8, 9, 10, 11, 12, 13, 14, 15
CSF1PO	BTY	6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16
D13S317	BTY	5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16
FGA	BTY	14, 16, 17, 18, 19, 20, 21, 21.2, 22, 23, 23.2, 24, 25, 26, 27, 28, 29, 30, 31.2, 33, 34, 37.2, 42.2, 44.2, 45.2, 47.2, 50.2
D18S51	BTR	8, 9, 10, 10.2, 11, 12, 13, 14, 15, 16, 17, 17.2, 18, 18.2, 19, 20, 21, 21.2, 22, 23, 24, 25, 26, 27, 28
D8S1179	BTR	7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19

For information about known microvariants not contained in the Investigator IDplex Plus Allelic Ladder, see the National Institute of Standards and Technology (NIST) web site (www.cstl.nist.gov/biotech/strbase/).

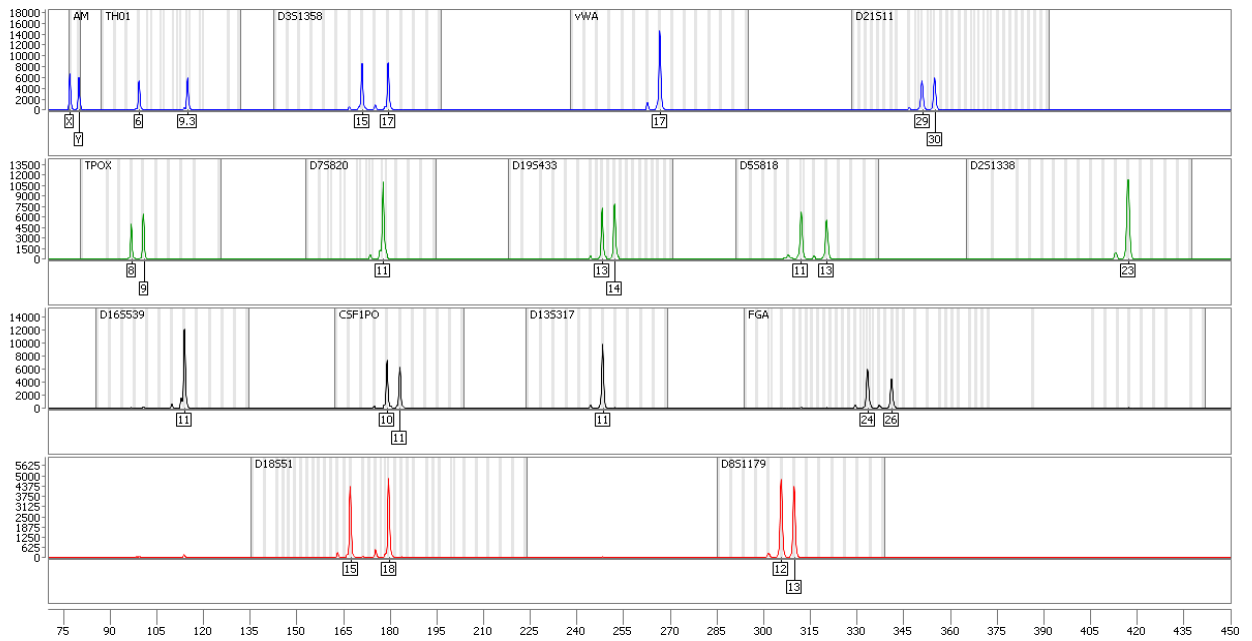


Figure 2. Electropherogram of the Investigator IDplex Plus Kit using 500 pg Control DNA 9948. Analysis was performed on an Applied Biosystems 3500 Genetic Analyzer. Allele assignment was performed using Investigator IDproof Software.

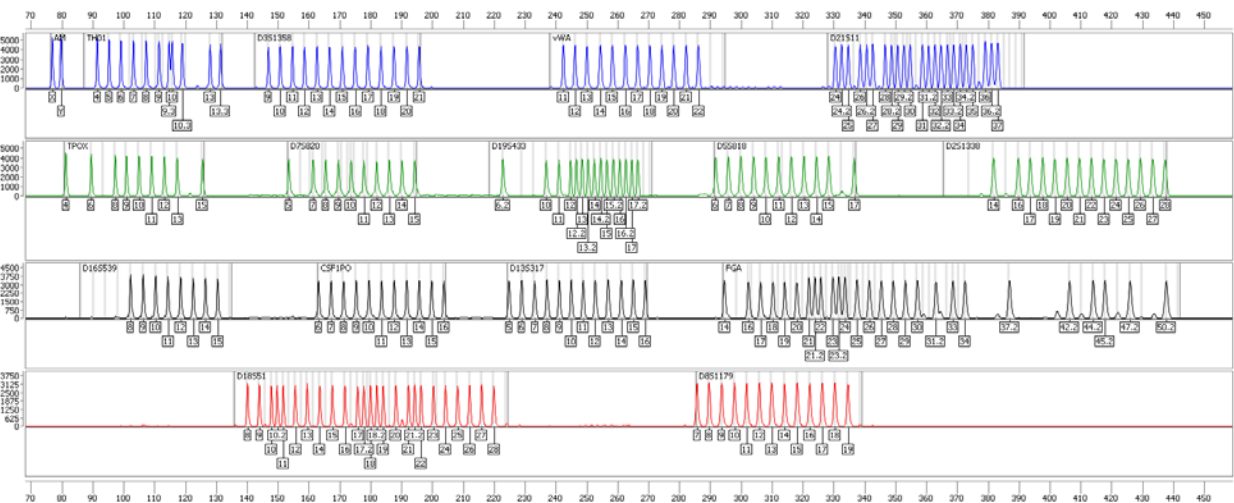


Figure 3. Electropherogram of the allelic ladder IDplex Plus analyzed on an Applied Biosystems 3500 Genetic Analyzer. Allele assignment was performed using Investigator IDproof Software.

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 54), 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 3000 RFU 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 Genotyper, 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 IDplex Plus 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.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise.

Comments and suggestions

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 for rapid and effective purification (see Ordering Information).
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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.
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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 or the GeneMapper <i>ID</i> or GeneMapper <i>ID-X</i> Software. Mark the orange fragments of all samples.

Always use the DNA Size Standard 550 included in Investigator Human Identification PCR Kits. |
| b) Signal intensities are too high. If the peak heights of the samples are outside the linear detection range (>4000 RFU/>5000 RFU)*, stutters, split peaks, and artifacts may be increased | Reduce the injection time in increments to a minimum of 1 s, reduce the amount of the PCR amplification product for analysis, or reduce the quantity of DNA for PCR. |

* >4000 RFU for the ABI PRISM 310 Genetic Analyzer; >5000 RFU for the ABI PRISM 3100 and Applied Biosystems 3130/3500 Genetic Analyzers.

Comments and suggestions

- | | |
|--|--|
| 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. |
| 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. |

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 samples to be analyzed.

Alternatively, allelic ladder data can be analyzed with a lower peak detection value in 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

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.

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Cited references

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.

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.

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

Ordering Information

Product	Contents	Cat. no.
Investigator IDplex Plus Kit (100)	Primer mix, Fast Reaction Mix including HotStarTaq <i>Plus</i> DNA Polymerase, Control DNA, allelic ladder IDplex Plus, DNA size standard 550 (BTO), and nuclease-free water	381625
Investigator IDplex Plus Kit (400)	Primer mix, Fast Reaction Mix including HotStarTaq <i>Plus</i> DNA Polymerase, Control DNA, allelic ladder IDplex Plus, DNA size standard 550 (BTO), and nuclease-free water	381627
Related products		
Investigator Human Identification PCR Kits		
Investigator ESSplex Plus Kit (100)*	Primer mix, Fast Reaction Mix including HotStarTaq <i>Plus</i> 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 <i>Plus</i> DNA Polymerase, Control DNA, allelic ladder ESSplex SE Plus, DNA size standard 550 (BTO), and nuclease-free water	381545
Investigator Nonaplex ESS Kit (100)*	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	381315
Investigator HDplex Kit (25)*	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	381213
Investigator Triplex AFS QS Kit (100)*	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	380315
Investigator Triplex DSF Kit (100)*	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	380325
Investigator Argus X-12 Kit (25)*	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	383213

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 (25)*	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	384013
Investigator Quantification Kits		
Investigator Quantiplex Kit (200)	Primer mix IC FQ, reaction mix FQ, control DNA Z1, dilution buffer	387016
Investigator Quantiplex HYres Kit	Primer mix IC YQ, reaction mix FQ, control DNA Z1, dilution buffer	387116
Investigator Human Identification PCR Kit Accessories		
Matrix Standard BT5 single cap. (5 x 25)	Matrix standard 6-FAM, BTG, BTY, BTR, and BTO	386113
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
Analysis software		
Investigator IDproof Software	Software package on CD including installation files for the Desktop, Server and Client versions of IDproof Software	9020775
Investigator IDproof Demo Key	Free use of the IDproof Desktop version of the software for 30 days after installation	389001
Investigator IDproof Single Key	Allows the unlimited use of the Desktop version of the software; to be installed on a single workstation with a local database	389002
Investigator IDproof Server Key	Allows for setup of a server that maintains the database and various workstations to access that database. Must be purchased in conjunction with the Client Key	389003

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
Investigator IDproof Client Key	Must be purchased in conjunction with the Server Key	389004
Investigator IDproof Mixture Software	Software package on CD including installation files for the Desktop, Server and Client versions of IDproof Mixture Software	9020777
Investigator IDproof Mixture Demo Key	Free use of the IDproof Mixture Desktop version of the software for 30 days after installation	389401
Investigator IDproof Mixture Single Key	Allows the unlimited use of the Desktop version of the software; to be installed on a single workstation with a local database	389402
Investigator IDproof Mixture Server Key	Allows for setup of a server that maintains the database and various workstations to access that database. Must be purchased in conjunction with the Client Key	389403
Investigator IDproof Mixture Client Key	Must be purchased in conjunction with the Server Key	389404
Investigator Template Files	All template files for Investigator Human Identification PCR Kits for use with GeneMapper <i>ID</i> , GeneMapper <i>ID-X</i> , and Genotyper Software, as well as DIPSorter freeware (CD-ROM)	389900
DNA extraction and purification		
QIAamp® DNA Investigator Kit (50)	50 QIAamp MinElute Columns, Proteinase K, Carrier RNA, Buffers, Collection Tubes (2 ml)	56504
EZ1® DNA Investigator Kit (48)	Reagent Cartridges (DNA Investigator), Disposable Filter-Tips, Disposable Tip-Holders, Sample Tubes (2 ml), Elution Tubes (1.5 ml), Buffer G2, Proteinase K, Carrier RNA	952034
MinElute PCR Purification Kit (50)*	50 MinElute Spin Columns, Buffers, Collection Tubes (2 ml)	28004

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Notes

Notes

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