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## User manual

### ChimerXplain Kit

For 8 tests

Order no. 130-095-794

### ChimerXact Kits

For 96 tests

Order no. 130-095-770

130-095-780

130-095-833

130-095-788

130-095-785

130-095-832

130-095-792

130-095-782

130-095-791

130-095-831



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**ChimerXact Kits 1–10 (singleplex PCR analysis)**

<b>ChimerXact Kit 1 (D10S2325)</b>	<b># 130-095-770</b>
<b>ChimerXact Kit 2 (D12S391)</b>	<b># 130-095-780</b>
<b>ChimerXact Kit 3 (P450CYP19)</b>	<b># 130-095-833</b>
<b>ChimerXact Kit 4 (D2S1360)</b>	<b># 130-095-788</b>
<b>ChimerXact Kit 5 (D9S1118)</b>	<b># 130-095-785</b>
<b>ChimerXact Kit 6 (MYCL1)</b>	<b># 130-095-832</b>
<b>ChimerXact Kit 7 (D7S1517)</b>	<b># 130-095-792</b>
<b>ChimerXact Kit 8 (D11S554)</b>	<b># 130-095-782</b>
<b>ChimerXact Kit 9 (D8S1132)</b>	<b># 130-095-791</b>
<b>ChimerXact Kit 10 (SE33)</b>	<b># 130-095-831</b>

**■ Components**

**1×96-well ChimerXact PCR Plate:**  
lyophilized singleplex PCR Reaction Mix

**1×0.5 mL PCR Resuspension Buffer (10×)**

**■ Capacity**

For 96 tests.

**■ Product format**

96-well plate containing 96 lyophilized single ChimerXact Reaction Mixes.

**■ Storage**

The ChimerXact PCR Plate is best stored at –20 °C, and may be stored at 2–8 °C for up to 4 weeks protected from light in the original aluminium pouch. Store the Resuspension Buffer at 2–8 °C. The expiration date is indicated on the label.

**1.2 Introduction****■ Background**

During allogeneic transplantation, donor bone marrow or stem cells are transferred to the patient to replace diseased cells. In the early post-transplant period, co-existence of the recipient and donor cells can develop, a phenomenon called mixed chimerism. Monitoring engraftment of donor cells is a key part of post-transplant treatment. Short tandem repeats (STRs) or microsatellites are tandemly repeated units of typically two to seven base pairs. The number of repeats varies among individuals and the different length of the STRs can be used to distinguish donor and recipient cells.

The ChimerXplain Kit has been developed to facilitate the identification of one or more STR markers optimally suited for the monitoring of chimerism during the post-transplant period. Ten highly polymorphic STRs (D2S1360, D7S1517, D8S1132, D9S1118, D10S2325, D11S554, D12S391, MYCL1, P450CYP19, and SE-33) have been selected, which best satisfied the criteria of the EuroChimerism consortium for the reliable differentiation between donor and recipient cells. Extensive testing of related individuals (>500 pairs) confirmed that this marker panel provides at least two informative markers which meet the stringent criteria of eligibility defined by the EuroChimerism consortium in >99% of all patient/donor constellations.<sup>1</sup> The ten microsatellite markers can be analyzed in two multiplex polymerase chain reactions (PCR) and one singleplex PCR reaction.

After the initial identification of suited STR markers, further chimerism analysis can be carried out using individual STR markers (ChimerXact Kits 1–10). These singleplex assays allow for sensitive detection of residual cells of patient or donor origin at levels ranging

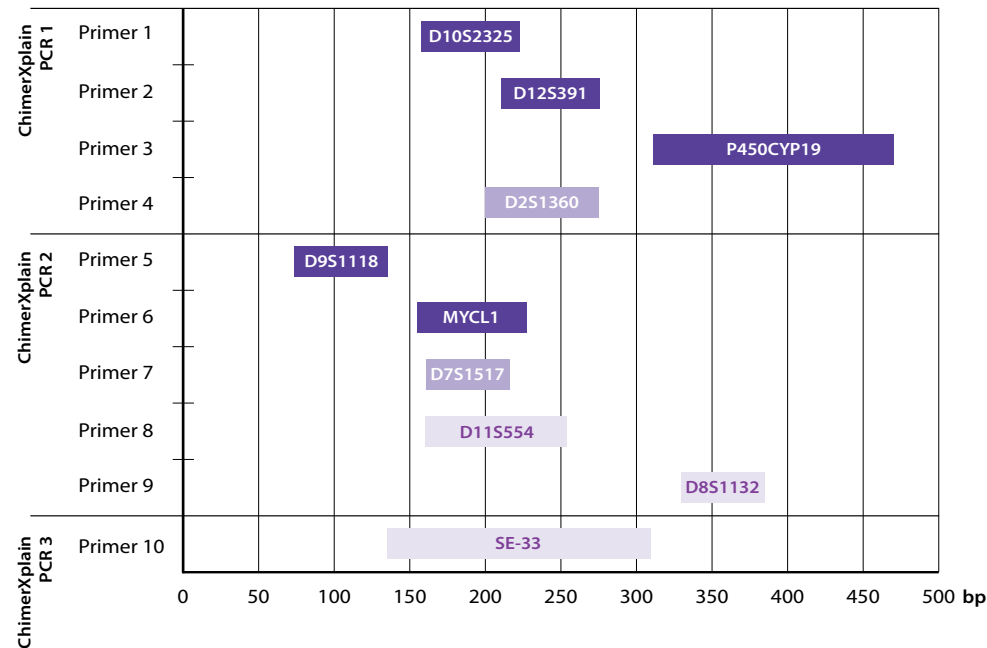
between 1.5% (3% for D10S2325) for optimal configuration of recipient and donor alleles, for example RDDR, and 3% for separated recipient and donor alleles like DDDR. In addition, ChimerXact Kits permit and facilitate accurate and reproducible quantification of donor and recipient hematopoietic cells in peripheral blood or bone marrow specimens and in specific cell lineages isolated by MACS® Technology or flow sorting (lineage-specific chimerism analysis). The use of ChimerXplain and ChimerXact Kits optimally enables the discrimination between related donors and recipients and contributes to data sharing between laboratories and international standardization of chimerism testing.

**■ Principle of the ChimerXplain Kit and ChimerXact Kits**

The ChimerXplain Kit allows the identification of appropriate markers to distinguish donor and recipient cells. Three different multiplex PCR reactions (ChimerXplain Kit, Reaction Mix 1–3) are performed to determine the length of ten highly polymorphic STR loci using both donor and recipient DNA. One PCR primer of each locus is labeled with different fluorescent dyes (FL, Joe, or TMR). After amplification of the STR loci, PCR products are separated by capillary electrophoresis. The size of each STR is determined according to a size marker added to each PCR sample (refer to figure 1). By comparing the STR sizes for every allele between donor and recipient, one or more informative STRs that differ in length can be selected. STRs that are suitable to discriminate between recipient and donor can subsequently be used as markers to monitor mixed chimerism after transplantation. Small numbers of donor- or recipient-derived cells, for example, early during

decreasing mixed donor chimerism, might not be detected accurately in multiplex PCR reactions due to interaction of the different PCR primers. For a more precise and reliable chimerism quantification, we therefore recommend to amplify the selected STRs in singleplex PCR reactions using the ChimerXact Kits after the initial identification of the discriminatory loci with the ChimerXplain Kit. For each STR that is assayed using the ChimerXplain Kit, singleplex PCR reactions (ChimerXact Kits 1–10) are available for further chimerism analysis.

Using the selected ChimerXact Kit, singleplex PCR is performed and analysed by capillary electrophoreses. The STR alleles are then assigned to recipient or donor according to the length determined previously by using the ChimerXplain Kit. The relation of donor and recipient cells is calculated using the signal intensity of the alleles measured as peak height or peak area and is usually specified as percent chimerism. Changes in the chimeric state after transplantation may be indicative for successful engraftment or potential relapse.



**Figure 1:** Overview on size range of products. Dark purple: labeled with FL; purple: labeled with Joe; light purple with purple font: labeled with TMR.

### 1.3 Reagent and instrument requirements

- 0.2 mL PCR tubes or 96-well plates
- 0.2 mL tubes, 8-tube strips, or 96-well plates without caps to be used with capillary sequencer
- Pipette tips
- Internal Lane Standard (ILS) 600 (Promega® Cat. # DG1071) or equivalent size standard
- Hi-Di™ formamide (Applied Biosystems® Cat. # 4311320)
- 310 Running Buffer 10× (Applied Biosystems Cat. # 402824)
- Performance optimized polymer 4 POP-4™ Polymer for 3100/3100-Avant™ Genetic Analyzers (Applied Biosystems Cat. # 402838)
- Matrix Standard [Fluorescein, JOE A, JOE B, TMR, and CXR] (Promega® PowerPlex® Matrix Standards, 310/377 Cat. # DG3640)
- Kits for genomic DNA isolation, e.g., Blood and cell culture DNA Maxi Kit (Qiagen® # 13362), Chemagic DNA Blood Kit (Chemagen AG), or DNA Isolation Kit for Mammalian Blood (Hoffmann-La Roche AG)
- Genetic Analyzer 96 Sample Tray Adapter (Applied Biosystems Cat. # 4305051)
- Genetic Analyzer Micro Amp Tray and Retainer (Applied Biosystems Cat. # 403081)
- Genetic Analyzer Retainer Clip (Applied Biosystems Cat. # 402866)
- Genetic Analyzer Septa Strip (Applied Biosystems Cat. # 4305547)
- Capillary sequencer, e.g., Applied Biosystems 310 Genetic Analyzer, 3100 Genetic Analyzer, 3130 Genetic Analyzer, 3500 Genetic Analyzer
- Spectrophotometer, e.g., NanoDrop™ ND1000 (Thermo Fisher Scientific®)
- Thermocycler for 96-well plates or 0.2 mL tubes, with heatable lid
- Microcentrifuge suitable for 0.2 mL tubes
- (Optional) Centrifuge for 96-well plates

### 1.4 Related products

- MACS® Whole Blood MicroBeads
- KIR Typing Kit (#130-092-551, #130-092-584)

For more information about MACS Products for cell separation refer to [www.miltenyibiotec.com](http://www.miltenyibiotec.com).

## 2. Protocol for the ChimerXplain Kit (multiplex PCR)

▲ The amounts and conditions given in this manual have been extensively validated on an Applied Biosystems 310 Genetic Analyzer. Parameters might need to be adapted

depending on the model of the capillary sequencer used. The protocol can also be modified according to individual workflow requirements.

### 2.1 Sample preparation

#### ■ Genomic DNA isolation

▲ Blood samples should be collected in a buffer containing EDTA; buffers with heparin may inhibit DNA amplification.

▲ Genomic DNA isolation can be performed by any protocol that produces high-purity DNA, for example, Blood and cell culture DNA Maxi Kit (Qiagen), Chemagic DNA Blood Kit (Chemagen AG), or DNA Isolation Kit for Mammalian Blood (Hoffmann-La Roche AG).

▲ Dilute genomic DNA in 10 mM Tris / HCl pH 8 to a final concentration of 0.8 ng/μL.

The positive control, genomic DNA of WT47 cells, can be dissolved in 120 μL 10 mM Tris/HCl, pH 8 to a final concentration of 0.8 ng/μL.

▲ **Note:** The recommended amount of genomic DNA has been validated to provide analyzable data in most instances. Depending on the workflow requirements, other standard amounts can be established, such as a given volume of isolated genomic DNA taken from a certain amount of blood or cells.

### 2.2 PCR

#### ■ Set up PCR experiment

▲ The reactions can be set up at room temperature.

1. Take one ChimerXplain PCR Plate from refrigerator. Ensure that the lyophilized Reaction Mix is at the bottom of each well. To place the lyophilizate at the bottom of the well, gently slap the plate on to the bench.

▲ **Note:** For correct orientation, PCR plate has to be positioned with label away from you.

2. One row comprising three different PCR reactions, Reaction Mix 1, 2, and 3 is used for each sample (refer to table 1).

▲ **Note:** The covering foil can easily be pierced using a pipette tip or cut with a scalpel and peeled off by hand wearing gloves. The dissolved PCR mixes can then be transferred to 0.2 mL PCR tubes or 96-well PCR plates. Alternatively, the ChimerXplain PCR plates can directly be used for PCR. To do so, cut the number of rows according to the number of samples to be analyzed from the ChimerXplain PCR plate and peel off the cover foil. For instance, in case 4 samples are to be processed, cut the ChimerXplain PCR plate in two pieces each comprising 4 rows.

	Column 1 (ChimerXact primer 1–4)	Column 2 (ChimerXact primer 5–9)	Column 3 (ChimerXact primer 10)
A	Reaction Mix 1	Reaction Mix 2	Reaction Mix 3
B	Reaction Mix 1	Reaction Mix 2	Reaction Mix 3
C	Reaction Mix 1	Reaction Mix 2	Reaction Mix 3
D	Reaction Mix 1	Reaction Mix 2	Reaction Mix 3
E	Reaction Mix 1	Reaction Mix 2	Reaction Mix 3
F	Reaction Mix 1	Reaction Mix 2	Reaction Mix 3
G	Reaction Mix 1	Reaction Mix 2	Reaction Mix 3
H	Reaction Mix 1	Reaction Mix 2	Reaction Mix 3

**Table 1:** 24-well ChimerXplain PCR Plate, for 8 tests (3 wells per test). All wells contain lyophilized Reaction Mix. PCR wells in column 1 contain Reaction Mix 1 with ChimerXact primer 1–4 for the amplification of loci D10S2325, D12S319, P450CYP19, and D2S1360. PCR wells in column 2 contain Reaction Mix 2 with ChimerXact primer 5–9 for the amplification of loci D9S1118, MYCL1, D7S1517, D11S554, and D8S1132. PCR wells in column 3 contain Reaction Mix 3 with ChimerXact primer 10 for the amplification of locus SE33.

- Place the plate in a plate holder, e.g., an empty holder for pipette tips, and carefully pierce or remove the red cover foil.
- For resuspension solution 1 (RS1) combine 4.8 ng genomic DNA and 5.5 μL 10× PCR Resuspension Buffer and adjust with water to a final volume of 55 μL. For resuspension solution 2 (RS2), combine 0.8 ng genomic DNA and 2.5 μL 10× PCR Resuspension Buffer and adjust with water to a final volume of 25 μL.
 

▲ **Note:** It is recommended to run a negative control reaction using just buffer instead of genomic DNA to monitor for potential contaminations. In addition, the positive control DNA can be used to ensure the principal functioning of the assay.
- Dissolve lyophilized Reaction Mix 1 and Reaction Mix 3 each with 25 μL RS1 containing 2.2 ng genomic DNA as prepared above. To dissolve Reaction Mix 2 (column 2) use 25 μL RS2 containing 0.8 ng genomic DNA.
- In case the original ChimerXplain plates should directly be used for PCR, cut the provided sealing foil according to the number of rows prepared for the PCR and carefully seal the plate.
 

▲ **Note:** Avoid prolonged storage of the reaction mixes at room temperature. Put mixes on ice in case PCR cannot be started immediately.
- Centrifuge shortly or gently shake down the liquid to place it at the bottom.
- Begin thermal cycling as specified below.

### ■ Run PCR program for ChimerXplain Kit

1. Place tubes or ChimerXplain PCR Plate in a thermal cycler and run PCR program as indicated below:

#### Cycling conditions

LID=105 °C

T=95 °C	11 minutes		
T=96 °C	1 minute		
T=94 °C	30 seconds		} 9 cycles
T=60 °C	30 seconds	R (RAMP) = 1 °C/second	
T=70 °C	45 seconds	R (RAMP) = 1 °C/second	
T=90 °C	30 seconds		} 21 cycles
T=60 °C	30 seconds	R (RAMP) = 1 °C/second	
T=70 °C	45 seconds	R (RAMP) = 1 °C/second	
T=60 °C	30 minutes		
Hold 4 °C			

The thermal cycler profile is optimized for Eppendorf and MJ Research cyclers.

▲ If other instruments are used, optimization of the thermal cycler profile may be necessary.

2. After amplification, centrifuge shortly or gently shake down the liquid to place it at the bottom.
3. Proceed with capillary electrophoresis (section 2.3) or store amplified DNA at 4 °C.

## 2.3 Capillary electrophoresis

### ■ Sample preparation

1. Prepare electrophoresis mastermix containing Hi-Di formamide and a suitable size standard. Use 1.5 µL size standard and 23.5 µL Hi-Di formamide per sample. Mix by vortexing.

▲ Note: The amount of size standard used in the electrophoresis mastermix might have to be adapted to get optimal peak signals (typical range: 0.25 to 2 µL).

2. Vortex and briefly spin down the liquid in a microcentrifuge.
3. For each sample add 25 µL electrophoresis mastermix to either a 0.2 mL tube, one well of an 8-tube strip, or one well of a 96-well plate.
4. Add 1 µL of ChimerXplain PCR product.

▲ Note: Since instrument detection limits vary, the amount of PCR product mixed with electrophoresis mastermix may need to be adapted. Alternatively, the injection time can be adjusted. Use the Module Editor in the Tools Menu to modify injection time or voltage in the run module.

5. Put tubes, stripes, or 96-well plate into the sample tray and close the tray using the retainer.
6. Cover wells with appropriate septa strips.
7. Just prior to sample loading, denature samples at 95 °C for 3 minutes and snap cool in an ice-water bath for 5 minutes.
8. Fix septa strips with retainer clips.
9. Dispense well by vortexing, briefly spin down liquid in a centrifuge, or gently shake down the liquid to remove air bubbles if necessary.

### ■ Instrument preparation

▲ Please also refer to your Applied Biosystems Genetic Analyzer User's Manual on how to set up the instrument and for further details

#### GeneScan software version 3.7 or higher

##### Prepare a matrix

To compensate for crosstalk between the different fluorescence channels, it is necessary to set up a matrix file. Prepare a matrix file using the Promega PowerPlex Matrix Standards (Fluorescein, JOE A, JOE B, TMR, and CXR) according to the description of the Applied Biosystems Genetic Analyzer User's Manual.

##### Prepare a sample sheet

1. Open the ABI PRISM® 310 data collection software.
2. Prepare a GeneScan sample sheet:
  1. From the "File" menu, choose "New" to set up a new sample sheet.
  2. In the dialog box, click on the appropriate icon "GeneScan Sample Sheet 48" or "GeneScan Sample Sheet 96 tube". By clicking on the respective icon, a sample sheet will open.
  3. Select "4 Dyes" from the pull-down menu in the upper right corner of the window.
  4. Type the name of the first sample in the "sample" column.
  5. Mark the size standard color by clicking on the respective "Std" column. A diamond appears next to the color indicating the color used for the size standard.

6. Indicate the colors present in the sample by checking boxes in the “Pres” column.

7. You can enter sample information (sample, ladder, negative control, or positive control) into the “sample info” column, and any comments in the “comments” column.

▲ **Note:** The Genotyper™ software requires that you fill in the “sample information” column. Refer to Genotyper DNA Fragment Analysis Software User’s Manual.

8. Repeat this process for all samples.

9. When finished choose from the file menu “save” or “save as”. We recommend to choose an informative sample sheet name including the date and to save the sample sheet to the default sample sheet folder.

10. The sample sheet can now be closed.

#### Prepare a GeneScan® injection list

1. From the “File” menu, choose “New” to set up a new injection list.
2. In the dialog box, click the appropriate icon “Genescan injection list”. By clicking on the respective icon, a new injection list will open.
3. Select the appropriate sample sheet (see above) by using the pull-down menu.
4. In the “Module” column select the “GS STR POP4 (1 mL) A” module using the pull-down menu.
5. In the following columns set
  - the injection time “inj. Secs” to 5
  - the injection voltage “inj. kV” to 15.0
  - the run voltage “Run kV” to 15.0
  - the gel temperature “run °C” to 60
  - the run time “Run Time” to 30

▲ **Note:** It may be necessary to optimize the injection time for individual instruments. Injection times are typically in a range of 2–5 seconds.

6. Select the appropriate matrix file by using the pull-down menu. Refer to the ABI PRISM 310 Genetic Analyzer User’s Manual for instructions on how to create matrix files.

▲ **Note:** The matrix file is not necessary for data acquisition. A matrix file can also be selected just prior to data analysis.

7. To analyze data automatically, select the autoanalysis checkbox.

▲ **Note:** The autoanalyses function requires an appropriate matrix file, analysis parameters and size standard.

8. Repeat this process for all samples.
9. The injection list can then be used to start the electrophoresis. Load the denatured samples onto the sample tray and close the doors. Then select “Run” to start the capillary electrophoresis system and confirm by pressing “Continue”.
10. To control the electrophoresis choose from the window drop-down menu “Status”. A window showing the electrophoresis parameters as well as the live electropherogram will appear.

▲ **Note:** Processing of each sample will take approximately 40 minutes, including syringe pumping, sample injection and sample electrophoresis.

11. Once the electrophoresis is finished, a data analysis window “analysis control” opens automatically if the autoanalysis checkbox has been activated in the injection list (refer to above).
12. Mark each sample and press the “Analyse” button. The “results control” window opens automatically. Continue according to section 2.5 (Select STR peaks).

## 2.4 Data analysis

### ■ Applied Biosystems GeneScan Software

#### Set up analysis parameters

1. In case the autoanalysis checkbox has not been activated in the injection list (see above), open the GeneScan Analysis Software.

2. From the “File” menu, choose “New” to set up a new analysis.
3. Click the “Analysis Parameters” icon and specify the settings:

<b>Analysis range</b> <input type="radio"/> Full range <input checked="" type="radio"/> This range (data points) Start: 3100 Stop: 12,000	<b>Size call range:</b> <input checked="" type="radio"/> Full range <input type="radio"/> This range (base pairs)
<b>Smooth options:</b> <input type="radio"/> None <input checked="" type="radio"/> Light <input type="radio"/> Heavy	<b>Size calling method</b> <input type="radio"/> 2nd order least squares <input type="radio"/> 3rd order least squares <input type="radio"/> Cubic spline interpolation <input checked="" type="radio"/> Local southern method <input type="radio"/> Global southern method
<b>Peak detection</b> Peak amplitude thresholds B: 30      Y: 30 G: 30      R: 30  Min. peak half width: 2 Pts Peak window size: 10 Pts Slope threshold for peak start: 0.0 Slope threshold for peak end: 0.0	<b>Baselining</b> BaseLine window size 31 Pts  Auto analysis only Size standard:

**Table 2:** Example of analysis parameters for the Applied Biosystems GeneScan Software.

▲ The analysis range should start immediately after the primer peak. The primer peak is usually detected in the 2600–3000 scan number range. To check for the position of the primer peak examine the raw data file.

4. From the file menu, choose “Save As” and save the analysis parameters, e.g., as Chimer STR analysis in the Param folder (Applied Bio/Shared/Analysis/Sizecaller/Params) of your GeneScan™ software.
5. Close the “Analysis Parameter” window.

#### Set up a size standard

1. In case the autoanalysis checkbox has not been activated in the injection list (see above), open the GeneScan Analysis Software .
2. From the “File” menu, choose “New” to set up a size standard.
3. Click the “Size Standard” icon and a “Select Sample File” dialog box appears.
4. From the “Look in” drop-down menu, choose the folder containing your sample files.
5. Select the sample file containing your standard and click “Open”.
6. The “Select Dye and Analysis Parameters” dialog box appears.
7. From the “Dye” drop-down menu choose the code that represents the dye label of the size standard.
8. From the “Analysis Parameters” drop-down menu choose the analysis parameters and click “OK”.
9. A new window appears showing the electropherogram and a table of peaks for the selected dye color and sample.

10. The peaks have to be related to the corresponding theoretical sizes as given in the specifications of the size standard. To do so, click on a peak in the electropherogram or a number in the table to select a peak. Type the value for the selected peak in the corresponding “Size” field in the table.

▲ **Note:** To ignore a peak for the size standard definition leave zero in the “Size” field.

11. Once all peaks of the size standard are assigned to the appropriate sizes, choose from the “File” menu “Save as” to save the size standard definition in the size standard folder (Applied Bio/Shared/Analysis/Sizecaller/Params).

12. Close the “Size Standard” window.

#### Set up a new project for sample analysis

▲ To analyze multiple samples, the sample files can be added to a project for analysis.

▲ For single sample analysis, sample files can be opened as separate files outside of projects. For details on analyzing single sample files refer to the GeneScan user manual.

▲ You can create a new project and add any combination of sample files, allowing you to analyze and display samples from different runs. Adding a sample file to the project sets up a link between the project and the sample file. The file itself is not imported into the project.

1. In case the *autoanalysis checkbox* has not been activated in the injection list (see above), open the GeneScan Analysis Software .
2. From the “File” menu, choose “New” to set up a new project.
3. Click the “Project” icon and an “untitled - Analysis Control” window appears.

4. To add samples to the project, choose from the “Project” menu “Add Sample Files...” and an “Add Sample Files” window appears.
5. From the “Look in” drop-down menu, choose the folder containing your sample files.
6. Select the sample files to be added and press “Add” or choose “Add all” to import all sample files to the project.
7. After all sample files have been added, click “Finish”.
8. From the “File” menu, choose “Save As” to save the project.

#### Analyzing sample file projects

1. From the “File” menu, choose “Open” to open a project for analysis.
2. In the “*project name* - Analysis Control” window all samples of the project are shown. The first columns of the table named by

single characters indicate the different dyes. The “Sample File” column gives the names of the sample files. The “Size Standard” column indicates the size standard definition file to be used for analysis. The “Parameters” column gives the name of the analysis parameter file.

3. From the “Size Standard” drop-down menu, choose the size standard file.
4. From the “Parameters” drop-down menu, choose the analysis parameter file.
5. In case no matrix has been specified for data acquisition, select the samples to be analyzed and choose from the “Sample” menu “Install New Matrix”.
6. Click “Analyze” and the “*project name* - Results Control” window opens.

▲ **Note:** Triangles in the “*project name* - Analysis Control” window indicate analyzed samples.

### ■ Applied Biosystems GeneMapper Software

▲ The analysis method described below is based on the sizing-only application.

#### Set up analysis parameters

1. Open the GeneMapper software .
2. From the “Tools” menu, choose “GeneMapper Manager” to open the GeneMapper Manager window and select the “Analysis Methods” tab.
3. Click the “New...” button to create a custom analysis method.
4. A dialog box will open. Select “Microsatellite” and press “OK” and the “Analysis Method Editor” window will open.

Ranges		Peak detection	
Analysis	Sizing	Peak amplitude thresholds	
<input type="radio"/> Full range	<input checked="" type="radio"/> All sizes	B: 30	R: 30
<input checked="" type="radio"/> Partial range	<input type="radio"/> Partial sizes	G: 30	O: 50
Start: 3100	(Base pairs)	Y: 30	
Stop: 12,000			
Data processing		Min. peak half width: 3 Pts	
<input checked="" type="checkbox"/> Baseline			
<input checked="" type="checkbox"/> MultiComponent		<input type="radio"/> No peak correction	
<input type="radio"/> None		<input checked="" type="radio"/> GS2500	
<input type="radio"/> Light		<input type="radio"/> Left most	
<input type="radio"/> Heavy		<input type="radio"/> Right most	
		Correction limit 30 Pts	
Size calling method			
<input type="radio"/> 2nd order least squares			
<input type="radio"/> 3rd order least squares			
<input type="radio"/> Cubic spline interpolation			
<input checked="" type="radio"/> Local southern method			
<input type="radio"/> Global southern method		Factory defaults	

Table 3: Example of peakdetector parameters for the Applied Biosystems GeneMapper Software.

5. In the “General” tab enter a name and optionally a short description.
6. In the “Allele” tab select “None” as been set. Marker Repeat Type: Uncheck “Use marker-specific stutter ratio if available”
7. In the “Peak Detector” tab select the “Classic” Peak Detector Algorithm and specify the settings.
  - ▲ Note: The Peak Detector Algorithm has to be the same as used for the size standard. In case different Peak Detector Algorithm have been used, the software issues the following alert when pressing the “Analyze” button: “There are sample(s) that do not meet analysis requirements. Please see Error Message in the info view of each sample. Do you want to continue?”

▲ The analysis range should start immediately after the primer peak. The primer peak is usually detected in the 2600–3000 scan number range. To determine the position of the primer peak examine the raw data file.

8. In the “Peak Quality” tab use “Factory Defaults” settings.
9. In the “Quality Flags” tab use “Factory Defaults” settings.
10. Click “OK” to close the “Analysis Method Editor” window.
11. Click “Done” to close the GeneMapper Manager window or directly continue to set up a size standard.

#### Set up a size standard:

1. Open the GeneMapper software .
2. From the “Tools” menu, choose “GeneMapper Manager” to open the GeneMapper Manager window and select the “Size Standards” tab.
3. Click the “New...” button to open the “Select Dye and Analysis Method” dialog box.
4. Select “Classic” and from the “Dye” drop down menu select “Red”, from the “Analysis Method” drop-down menu select “Microsatellite Default”.
5. Click the “Select Sample” button and the “Select Sample” window will open.
6. From the “Look in” drop-down menu, choose the folder containing your sample files.
7. Select the sample file containing your size standard and click “Select Sample”.
8. Click “OK” to open the “Size Standard Editor” window.

9. Enter a name and optionally a short description.
10. The peaks of the size standard are displayed in the electropherogram. In the table below the electropherogram, all peaks are listed.
11. Assign the sizes of the peaks according to the definition of your size standard. You can select the peaks either in the electropherogram or from the table.
  - ▲ Note: Peaks not corresponding to the fragments of the size standard can be kept as 0 and will be ignored.
12. Click “OK” to save the size standard and close the “Size Standard Editor” window.
13. Click “Done” to close the GeneMapper Manager window.

#### Set up a new project for sample analysis

▲ You can create a new project and add any combination of sample files, allowing you to analyze and display samples from different runs. Adding a sample file to the project sets up a link between the project and the sample file. The file itself is not imported into the project.

1. Open the GeneMapper software .
2. To add samples to the project, choose from the “File” menu “Add Samples to Project” and an “Add Samples to Project” window appears.
3. In the “Files” tab, navigate to the folder containing your sample files.
4. Select either the folder or a GeneScan Analysis Data File (.fsa) to be added and press “Add To List” and then “Add”. To add more than one file, keep the control key pressed when selecting the files.
5. The added samples are then displayed in the “Sample” table of the “Project” window.

### Analyzing sample file projects

1. Add samples to the project as described above or choose from the “File” menu “Open” to open an existing project for analysis.
  2. All samples of the project are shown in the “Samples” table of the “Project” window. For the default settings, the table contains the following columns:
    - The first column of the table indicates the status of the sample, e.g., whether it has already been analyzed.
    - The “Sample File” column gives the names of the sample files.
    - The “Sample Name” column gives the names of the samples.
    - The “Comments” column allows additional informations for the samples to be added.
    - In the “Sample Type” column, samples can be marked as “Sample”, “Positive Control”, “Negative Control”, or “Allelic Ladder”.
    - The “Analysis Method” column gives the name of the analysis parameter file.
    - For the “Panel” column keep “None” as there is no allelic ladder for the ChimerXplain Kit.
    - The “Size Standard” column indicates the size standard definition file to be used for analysis.
    - The “Matrix” column indicates the matrix file to be used for analysis.
    - “Run Name”
      - ▲ Note: From sample sheet; not editable.
  - “Instrument Type”
    - ▲ Note: From sample sheet; not editable.
  - “Instrument ID”
    - ▲ Note: From sample sheet; not editable.
  - “Run Date and Time”
    - ▲ Note: From sample sheet; not editable.
  - “REF”
    - ▲ Note: Reference data; indicates that the selected sample is defined as reference data in the Panel Manager.
  - “SQO”
    - ▲ Note: Sizing quality invalidated; checkmark indicates that sizing quality value is 1.0.
  - “SFNF”
  - “MNF”
  - “OS”
  - “SQ”
3. From the “Analysis Method” drop-down menu, choose the analysis method.
  4. From the “Size Standard” drop-down menu, choose the size standard used with your data.
  5. In case no matrix has been specified for data acquisition, select from the “Matrix” drop-down menu an appropriate matrix file.
  6. Click the green triangle analyze button and the “Save Project” dialog box will open.
  7. Enter a project name and click “OK” to save the project.
  8. The data sets are analyzed and the “to be analyzed” icon in the status column will disappear for all successfully analyzed data sets.

### 2.5 Select STR peaks

#### ■ Applied Biosystems GeneScan Software

▲ To assign detected peaks to STR loci for recipient and donor, we recommend using the “Results Control” function when working with the GeneScan software. The “*project name* - Results Control” window opens automatically after the analysis of all samples of one project.

▲ To accurately compare allele sizes of different samples, all samples have to be analyzed using the same size marker.

1. In the “*project name* - Results Control” window all analyzed samples of the project are shown in a table. The first columns of the table named by single characters indicate the different dyes. The “Sample File” column gives the names of the sample files.
2. From the “# of Panels” drop-down menu on the right site of the window, choose 4.
3. Click the first of the numbered buttons in the vertical row right aside of the sample table.
4. Choose the red channel of the size marker of the first sample, e.g., recipient sample by clicking the button in the “R” column of the first sample.
5. Click the second of the numbered buttons in the vertical row right aside of the sample table.
6. Choose the blue channel of the first sample by clicking the button in the “B” column of the first sample.
7. Click the third of the numbered buttons in the vertical row right aside of the sample table.
8. Choose the green channel of the first sample by clicking the button in the “G” column of the first sample.
9. Click the fourth of the numbered buttons in the vertical row right aside of the sample table.
10. Choose the yellow channel of the first sample by clicking the button in the “Y” column of the first sample.
11. Select “off” for the “Quick Tile” function.
12. Click “Display” to open a new “*project name* - Display” window showing the electropherograms of the selected samples and the corresponding table.
  - ▲ Note: We recommend to control the size calling of the size marker in the electropherogram. By clicking of one of the peaks, the data for this peak including the size is highlighted in the table.
13. For each locus, defined by the size range and dye (given in table 4, section 2.6) select the highest peak by clicking on the peak while keeping the control key pressed. In most cases, there will be a second peak of similar height in the same size range due to heterozygous alleles of the locus. Still keeping the control key pressed also select this second peak for a given STR. In case of homozygous alleles only one single dominant peak appears which should be selected.
  - ▲ Note: The height of the two peaks of heterozygous loci typically deviates by less than 30 %.
  - ▲ Note: Ambiguous peaks can be selected temporarily to keep them in the list for further analysis.
14. After having selected all relevant peaks, click from the “View” menu “Show only selected rows”.

15. A condensed table comprising the selected STR alleles will be displayed.
16. The condensed table can be saved by clicking from the “File” menu the “Save As...” function.
17. The table can also be exported by clicking from the “File” menu the “Export Table” function. In addition, the results can be printed by clicking from the “File” menu “Print”.
18. Repeat the procedure for the next sample, e.g. the donor sample.
19. To compare recipient and donor values, table 4 can be used as template.

#### ■ Applied Biosystems GeneMapper Software

▲ To assign detected peaks to STR loci for recipient and donor we recommend using the “Samples Plot” function when working with the GeneMapper software.

▲ To accurately compare allele sizes of different samples, all samples have to be analyzed using the same size marker.

1. From the “View” menu select “Samples” to display the “Samples” tab.
2. Select the first three samples, e.g., the recipient samples resulting from the ChimerXplain reactions 1 to 3 by keeping the control (ctrl) key of your keyboard pressed and selecting the appropriate rows.
3. From the “Analysis” menu select “Display Plots” to display the electropherogram for each selected sample.
4. From the “Plot Setting” drop-down menu select “Microsatellite Default”.

▲ **Note:** We recommend to control the size calling of the size marker in the electropherogram. By clicking on one of the peaks, the data for this peak including the size is highlighted in the table.

5. For each locus, defined by the size range and dye (given in table 4) select the highest peak by clicking on the peak while keeping the control key pressed. In most cases, there will be a second peak of similar height in the same size range due to heterozygous alleles of the locus. Still keeping the control key pressed also select this second peak for a given STR. In case of homozygous alleles only one single dominant peak appears which should be selected.

▲ **Note:** The height of the two peaks of heterozygous loci typically deviates by less than 30 %.

▲ **Note:** Ambiguous peaks can be selected temporarily to keep them in the list for further analysis.

6. From the “View” menu select “show selected rows”. Only the selected STR alleles will be displayed in the table.
7. The condensed table can be exported by choosing the “Export Table” function from the “File” menu. In addition, the results can

be printed by selecting “Print” from the “File” menu.

8. Repeat the procedure for the second sample, e.g., the donor sample.
9. To compare recipient and donor values, table 4 can be used as template.

### 2.6 Select informative STR markers

#### ChimerXplain PCR 1:

No.	Locus ID	Chromosome	Dye	Code	Length range	Recipient allele 1	Recipient allele 2	Donor allele 1	Donor allele 2
1	D10S2325	10	FL	B	163–213				
2	D12S391	12	FL	B	213–269				
3	P450CYP19	15	FL	B	314–464				
4	D2S1360	2	Joe	G	200–273				

#### ChimerXplain PCR 2:

No.	Locus ID	Chromosome	Dye	Code	Length range	Recipient allele 1	Recipient allele 2	Donor allele 1	Donor allele 2
5	D9S1118	9	FL	B	80–128				
6	MYCL1	1	FL	B	156–225				
7	D7S1517	7	Joe	G	164–212				
8	D11S554	11	TMR	Y	166–253				
9	D8S1132	8	TMR	Y	330–379				

#### ChimerXplain PCR 3:

No.	Locus ID	Chromosome	Dye	Code	Length range	Recipient allele 1	Recipient allele 2	Donor allele 1	Donor allele 2
10	SE-33	6	TMR	Y	138–305				

**Table 4:** Template for comparison of donor and recipient allele length.

▲ For a detailed description of the different allele combinations refer also to Watzinger *et al.*<sup>1</sup> and Thiede *et al.*<sup>2</sup>

1. Exclude all STRs with identical length of both alleles for heterozygous loci or with identical homozygous allele length.
2. From the remaining STRs, preferably select heterozygous STRs with both alleles differing between recipient and donor. A minimal distance of 8 base pairs is recommended. A distance of one STR repeat, typically 4 base pairs should be avoided as stutter peaks might impair the quantification. In case of several STRs with both alleles differing between recipient and donor, prefer an allelic pattern with the two, e.g., donor peaks surrounding the two recipient peaks (DRRD or RDDR)<sup>1</sup>. Also alternating configurations like DRDR or RDRD can be useful to avoid inexact chimerism estimates due to different PCR efficiencies between shorter and longer STRs.
3. If no heterozygous STRs with both alleles differing between recipient and donor are available, two homozygous loci differing between recipient and donor should be preferred. A minimal distance of 8 base pairs is recommended. A distance of one STR repeat, typically 4 base pairs should be avoided as stutter peaks might impair the quantification.
4. In the case where neither heterozygous STRs nor homozygous STRs differing between recipient and donor are available, check for heterozygous STRs which differ in only one allele for recipient and donor. In this case, the overlapping allele cannot be used for quantification. Again, a minimal distance of 8 base pairs is recommended. A distance of one STR repeat, typically 4 base pairs should be avoided as stutter peaks might impair the quantification.
5. In case recipient and donor differ only by an STR heterozygous in one case and homozygous in the other case, such an STR, although not optimal, can be used to distinguish recipient and donor cells.

▲ **Note:** Allele length can vary depending on electrophoretic conditions like Pop4 lot, temperature, etc.

### 3. Protocol for the ChimerXact Kits (singleplex PCR)

#### 3.1 Sample preparation

##### Genomic DNA isolation

▲ Blood samples should be collected in a buffer containing EDTA; buffers with heparin may inhibit DNA amplification.

▲ Genomic DNA isolation can be performed by any protocol that produces high-purity DNA, for example, Blood and cell culture DNA Maxi Kit (Qiagen), Chemagic DNA Blood Kit (Chemagen AG), or DNA Isolation Kit for Mammalian Blood (Hoffmann-La Roche AG).

▲ Dilute genomic DNA in 10 mM Tris/HCl, pH 8 to a final concentration of 0.8 ng/μL.

▲ **Note:** The recommended amount of genomic DNA has been validated to provide analyzable data in most instances. Depending on the workflow requirements, other standard amounts can be established, such as a given volume of isolated genomic DNA taken from a certain amount of blood or cells.

#### 3.2 PCR

##### Set up PCR experiment

▲ The reactions can be set up at room temperature.

1. Take ChimerXact PCR Plate from the refrigerator. Be sure that the lyophilized Reaction Mix is on the bottom of each well. To place the lyophilizate at the bottom of the well, gently slap the plate on to the bench.
2. The cover foil sealing the lyophilized Reaction Mix can either be peeled off by hand or pierced with a pipette tip. For peeling off, it is recommended to first cut the cover of an individual well or the appropriate number of wells used for an experiment with a scalpel. Then, peel off the film by hand wearing gloves. To pierce the cover, we recommend to use a fresh pipette tip and to take another fresh pipette tip for resuspending the mix.
3. For each sample to be analyzed add 2.2 ng and 2.5 μL 10× PCR Resuspension Buffer and adjust to a final volume of 25 μL.

4. Dissolve one lyophilized ChimerXact PCR Mix using 25 μL of the prepared PCR Resuspension Buffer containing 2.2 ng genomic DNA.

▲ **Note:** It is recommended to run a negative control reaction using just buffer instead of genomic DNA to monitor for potential contaminations.

5. The dissolved PCR mixes can be transferred to 0.2 mL PCR tubes or 96-well PCR plates. Alternatively, ChimerXact PCR Plates can directly be used for PCR. Close tubes or cut sealing foil according to the number of rows prepared for the PCR and seal plate.

▲ **Note:** Avoid prolonged storage of the Reaction Mixes at room temperature. Put mixes on ice in case PCR cannot be started immediately.

6. Centrifuge shortly or gently shake the liquid down to place it at the bottom.
7. Begin thermal cycling as specified below.

**Run PCR program for ChimerXact Kit**

- Place the tubes or PCR plate in a thermocycler and run PCR program as indicated below:

**Cycling conditions**

LID=105 °C

T=95 °C	11 minutes		
T=96 °C	1 minute		
T=94 °C	30 seconds		} 9 cycles
T=60 °C	30 seconds	R (RAMP) = 1 °C/ Second	
T=70 °C	45 seconds	R (RAMP) = 1 °C/ Second	
T=90 °C	30 seconds		} 21 cycles
T=60 °C	30 seconds	R (RAMP) = 1 °C/ Second	
T=70 °C	45 seconds	R (RAMP) = 1 °C/ Second	
T=60 °C	30 minutes		
Hold 4 °C			

The thermal cycler profile is optimized for Eppendorf and MJ Research cyclers.

▲ If other instruments are used, optimization of the thermal cycler profile may be necessary.

- After amplification, centrifuge shortly or gently shake down the liquid to place it to the bottom.
- Proceed with capillary electrophoresis (section 3.3) or store amplified DNA at 4°C.

**3.3 Capillary electrophoresis****Sample preparation**

- Prepare electrophoresis mastermix containing Hi-Di formamide and a suitable size standard. Use 1.5 µL size standard and 23.5 µL Hi-Di formamide per sample. Mix by vortexing.
 

▲ Note: The amount of size standard used in the electrophoresis mastermix might have to be adapted to get optimal peak signals (typical range: 0.25 to 2 µL).
- Vortex and briefly spin down the liquid in a microcentrifuge
- For each sample add 25 µL electrophoresis mastermix into either a 0.2 mL tube, one well of 8-tube stripes, or one well of a 96-well plate.

- Add 1 µL of ChimerXact PCR product.
 

▲ Note: Since instrument detection limits vary, the amount of PCR product mixed with electrophoresis mastermix may need to be adapted. Alternatively, the injection time can be adjusted. Use the Module Editor in the Tools Menu to modify injection time or voltage in the run module.

▲ Note: ChimerXact PCR products of non overlapping length range or labeled with different dyes can be combined and run together to save analysis time. Refer to figure 1.
- Put tubes, stripes, or 96-well plate into the sample tray and close the tray using the retainer.
- Cover wells with appropriate septa strips.

- Just prior to sample loading, denature samples at 95 °C for 3 minutes, and snap cool in an ice-water bath for 5 minutes.
- Fix septa strips with retainer clips.
- Dispense well by vortexing, briefly spin down liquid in a microcentrifuge, or gently shake down the liquid to remove air bubbles if necessary.

**Instrument preparation**

▲ Please also refer to your Applied Biosystems Genetic Analyzer User's Manual on how to set up the instrument and for further details

**GeneScan software versions 3.7 or higher****Prepare a matrix**

▲ To compensate for crosstalk between the different fluorescence channels, it is necessary to set up a matrix file. Prepare a matrix file using the Promega PowerPlex® Matrix Standards (Fluorescein, JOE A, JOE B, TMR, and CXR) according to the description of the Applied Biosystems Genetic Analyzer User's Manual.

**Prepare a sample sheet**

- Open the ABI PRISM® 310 data collection software.
- Prepare a GeneScan sample sheet:
  - From the "File" menu, choose "New" to set up a new sample sheet.
  - In the dialog box, click on the appropriate icon "Genescan Sample Sheet 96 tube". By clicking on the respective icon, a sample sheet will open.
  - Select "4 Dyes" from the pull-down menu in the upper right corner of the window.
  - Type the name of the first sample in the "sample" column.

5. Mark the size standard color by clicking in the respective "Std" column. A diamond appears next to the color indicating the color used for the size standard.

6. Indicate the colors present in the sample by checking boxes in the "Pres" column.

7. You can enter sample information (sample, ladder, negative control, or positive control) into the "sample info" column, and any comments in the "comments" column.

▲ Note: The Genotyper™ software requires that you fill in the "sample information" column. Refer to Genotyper DNA Fragment Analysis Software User's Manual.

8. Repeat this process for all samples.

9. When finished, choose from the file menu "save" or "save as". We recommend to choose an informative sample sheet name including the date and to save the sample sheet to the default sample sheet folder.

10. The sample sheet can now be closed.

**Prepare a GeneScan injection list**

- From the "File" menu, choose "New" to set up a new injection list.
- In the dialog box, click the appropriate icon "GeneScan injection list". By clicking on the respective icon, a new injection list will open.
- Select the appropriate sample sheet (see above) by using the pull-down menu.
- In the "Module" column select the "GS STR POP4 (1 mL) A" module using the pull-down menu.

5. In the following columns set
  - the injection time “inj. Secs” to 5
  - the injection voltage “inj. kV” to 15.0
  - the run voltage “Run kV” to 15.0
  - the gel temperature “run °C” to 60
  - the run time “Run Time” to 30

▲ **Note:** It may be necessary to optimize the injection time for individual instruments. Injection times are typically in a range of 2–5 seconds.
6. Select the appropriate matrix file by using the pull-down menu. Refer to the ABI PRISM® 310 Genetic Analyzer User’s Manual for instructions on how to create matrix files.
 

▲ **Note:** The matrix file is not necessary for data acquisition. A matrix file can also be selected just prior to data analysis.
7. To analyze data automatically, select the autoanalysis checkbox.
 

▲ **Note:** The autoanalyses function requires an appropriate matrix file, analysis parameters and size standard.
8. Repeat this process for all samples.
9. The injection list can then be used to start the electrophoresis. Load the denatured samples onto the sample tray and close the doors. Then select “Run” to start the capillary electrophoresis system and confirm by pressing “Continue”.
10. To control the electrophoresis choose from the window drop down menu “Status”. A window showing the electrophoresis parameters as well as the live electropherogram will appear.
 

▲ **Note:** Processing of each sample will take approximately 40 minutes, including syringe pumping, sample injection, and sample electrophoresis.
11. Once the electrophoresis is finished, a data analysis window “analysis control” opens automatically if the autoanalysis checkbox has been activated in the injection list (see above).
12. Mark each sample and press the “Analyse” button. The “results control” window opens automatically. Continue according to section 2.5 (Select STR peaks).

### 3.4 Data analysis

#### ■ Applied Biosystems GeneScan Software

▲ Please refer to the instruction for data analysis on page 13 for more information.

▲ To assign detected peaks to STR loci for recipient and donor, we recommend to use the “Results Control” function when working with the GeneScan software. The “*project name* - Results Control” window opens automatically after the analysis of all samples of one project.

1. In the “*project name* - Results Control” window all analyzed samples of the project are shown in the table on the left. The first columns of the table named by single characters indicate the different dyes. The “Sample File” column gives the names of the sample files.

2. From the “# of Panels” drop-down menu, choose 2–8 depending on the number of different STRs to be analyzed.
3. Click the first of the numbered buttons in the vertical row right aside of the sample table.
4. Optionally, choose the red channel of the size marker of the first sample by clicking the button in the “R” column of the first sample.
5. Click the second of the numbered buttons in the vertical row right aside of the sample table.
6. Choose the channel of the first STR sample by clicking the button of the respective color of the first sample.

7. Repeat steps 5 and 6 by selecting the next numbered buttons in the vertical row right aside of the sample table for the remaining STRs.

8. Select “off” for the “Quick Tile” function.

9. Click “Display” to open a new “*project name* - Display” window showing the electropherograms of the selected samples and the corresponding table.

▲ **Note:** We recommend to control the size calling of the size marker in the electropherogram. By clicking on one of the peaks, the data for this peak including the size is highlighted in the table.

10. For each of the STRs, select the peaks corresponding to the size of the recipient or donor alleles as determined in the ChimerXplain PCR by clicking on each peak while keeping the control key pressed.

▲ **Note:** Determined allele sizes might slightly vary between different runs, e.g., due to variable electrophoresis conditions. Therefore, the size can deviate from the one previously determined in the ChimerXplain PCR by up to 5 base pairs.

11. After having selected all relevant peaks, click from the “View” menu “Show only selected rows”.
12. A condensed table comprising the selected STR alleles will be displayed.
13. The condensed table can be saved by clicking from the “File” menu the “Save As...” function.
14. The table can also be exported by clicking from the “File” menu the “Export Table” function. In addition, the results can be printed by clicking from the “File” menu “Print”.
15. The peak height or peak area of the recipient and donor STRs can then be used for chimerism quantification.

#### ■ Applied Biosystems GeneMapper Software

▲ To assign detected peaks to STR loci for recipient and donor, we recommend using the “Samples Plot” function when working with the GeneMapper software.

1. From the “View” menu select “Samples” to display the “Samples” tab.

2. Select the sample of the ChimerXact PCR to be analyzed.

3. From the “Analysis” menu select “Display Plots” to display the electropherogram for the selected sample.

4. From the “Plot Setting” drop-down menu select “Microsatellite Default”.

▲ **Note:** We recommend to control the size calling of the size marker in the electropherogram. By clicking on one of the peaks, the data for this peak including the size is highlighted in the table.

5. Select the peaks according to the sizes determined in the ChimerXplain PCR for recipient and donor. To do so, click on the peaks while keeping the control key pressed.

▲ **Note:** In case of small peaks, only one of the two peaks of heterozygous loci might be detected. Select the single peak and use this peak for chimerism analysis.

6. From the “View” menu select “show selected rows”. Only the selected STR alleles will be displayed in the table.

7. The condensed table can be exported by clicking from the “File” menu the “Export Table” function. In addition, the results can be printed by selecting “Print” from the “File” menu.

8. The peak height or peak area of the recipient and donor STRs can then be used for chimerism quantification.

### 3.5 Chimerism quantification

#### ■ Donor and recipient are heterozygous and have no overlapping alleles

$$\% \text{ Chimerism} = (D1+D2) / (D1+D2+R1+R2) * 100$$

D = peak height (or area) of donor alleles

R = peak height (or area) of recipient alleles

▲ **Note:** In case of two short alleles, e.g., for the donor and two long alleles for the recipient, the donor allele might be amplified more efficiently leading to an overestimation of the donor population.

▲ **Note:** In case of heterozygous loci for recipient and donor, but only one allele detected for the minor population, an adapted formula can be used for chimerism estimation:

$$\% \text{ Chimerism} = D/[D+(R1+R2)/2] * 100$$

or

$$\% \text{ Chimerism} = [(D1+D2)/2] / [(D1+D2)/2+R] * 100$$

▲ In addition to the percentage of chimerism, quality measures can be determined according to suggestions of Kristt *et al.*<sup>3</sup>:

The DNA measurement error (ME) determines the imbalance of the two peaks from a heterozygous locus. Ideally, the measured peak area or height should be equal for both peaks as they represent two genomic copies. In such an ideal case, the difference in the peak area or peak height between the peak representing the longer allele and peak of the short allele would be zero. The relative deviation from such an ideal configuration can be calculated for recipient and donor alleles according to:

$$\% \text{ ME} = (R1-R2) / (R1+R2) * 100$$

$$\% \text{ ME} = (D1-D2) / (D1+D2) * 100$$

D = Peak height (or area) of donor alleles

R = Peak height (or area) of recipient alleles

D1 and R1 being the shorter alleles compared to D2 and R2, respectively.

The DNA measurement error can also be calculated to account for recipient and donor errors together:

$$\% \text{ ME} = [(R1-R2)+(D1-D2)] / (R1+D1+R2+D2) * 100$$

As one advantage, this single error estimate will be lower in cases with contrary differences for recipient and donor. Thereby, the error estimate takes into account that opposing differences might at least partially outbalance each other.

If more than one locus has been used for quantification, it is recommended to calculate the chimerism based on all available STRs. The mean chimerism of the determined chimerism for the different loci is then used to monitor chimerism. Also additional basic statistical quality measures can be determined:

Standard deviation:

$$SD = \sqrt{[(\text{Chimerism STR A} - \text{Mean Chimerism})^2 + (\text{Chimerism STR B} - \text{Mean Chimerism})^2 + (\text{Chimerism STR C} - \text{Mean Chimerism})^2 / 2]}$$

Relative deviation of the different STRs:

$$RD = |(\text{Chimerism STR A} - \text{Mean Chimerism})| / \text{Mean Chimerism}$$

#### ■ Either recipient or donor are homozygous

$$\% \text{ Chimerism} = D/[D+(R1+R2)] * 100$$

or

$$\% \text{ Chimerism} = (D1+D2)/[(D1+D2)+R] * 100$$

D = Peak height (or area) of donor alleles

R = Peak height (or area) of recipient alleles

#### ■ Recipient and donor are homozygous

This calculation can also be used in case of heterozygous loci for recipient and donor with one overlapping allele. The overlapping allele cannot be used for quantification and therefore the remaining single alleles are used for chimerism calculation.

▲ **Note:** Depending on the configuration of the overlapping alleles, the remaining alleles can lead to an inaccurate chimerism determination. For example, if the remaining donor allele is much shorter than the remaining recipient allele, the donor allele might be amplified more efficiently leading to an overestimation of the donor population.

$$\% \text{ Chimerism} = D / (D+R) * 100$$

#### 4. Short instructions

The amounts and conditions given are based on extensive optimization and validation using an Applied Biosystem 310 Genetic analyzer.

Parameters and DNA amounts might need to be adapted for other capillary sequencer models.

#### 4.1 ChimerXplain Kit (multiplex PCR)

##### ■ Genomic DNA isolation

1. Isolate genomic DNA by any protocol that yields high-purity DNA.
2. Dilute sample DNA and positive control DNA (WT47 cells) in 10 mM Tris/HCl, pH 8 to a final concentration of 0.8 µg/µL.

##### ■ Set up PCR experiment

1. For resuspension solution 1 (RS1) combine 4.8 ng genomic DNA and 5.5 µL 10× PCR Resuspension Buffer and adjust with water to a final volume of 55 µL.
2. For resuspension solution 2 (RS2) combine 0.8 ng genomic DNA and 2.5 µL 10× PCR Resuspension Buffer and adjust with water to a final volume of 25 µL.

Column 1 (ChimerXact primer 1–4)	Column 2 (ChimerXact primer 5–9)	Column 3 (ChimerXact primer 10)
Reaction Mix 1	Reaction Mix 2	Reaction Mix 3

Scheme of the ChimerXplain PCR Plate.

▲ **Note:** For correct orientation, PCR plate has to be positioned with label away from you.

3. Dissolve lyophilized Mix 1 and 3 each with 25 µL RS1 containing 2.2 ng genomic DNA.
4. Dissolve lyophilized Mix 2 using 25 µL RS2 containing 0.8 ng genomic DNA.
5. Run PCR (see program page 10).

##### ■ Capillary electrophoresis

###### Sample preparation

Electrophoreses mastermix: Mix 1.5 µL size standard and 23.5 µL Hi-Di formamide per sample. Add 1 µL of ChimerXplain PCR product to one 25 µL mastermix aliquot. Denature samples at 95 °C for 3 minutes and snap cool.

###### Prepare a sample sheet

Prepare a GeneScan sample sheet for 4 dyes. Type sample name, mark size standard column, and enter sample information for all samples to be analyzed.

###### Prepare a GeneScan injection list

Set up a new injection list and select the appropriate sample sheet. For all samples select

“GS STR POP4 (1 mL) A”

“inj. Secs” to 5

“inj. kV” to 15.0

“Run kV” to 15.0

“run °C” to 60

“Run Time” to 30

matrix file

Optionally activate the autoanalysis checkbox.

▲ **Note:** The injection parameters might have to be adapted depending on the signal intensities. Lowering the injection time or voltage will decrease the peak signals.

Load the denatured samples onto the sample tray, close the doors, and start electrophoresis.

##### ■ Data analysis

###### Set up analysis parameters

Define the analysis parameters for peak detection.

###### Set up a size standard

Assign sizes to the respective peaks of a representative run containing the size marker.

###### Set up a project for sample analysis

Define samples to be analyzed. Choose appropriate analysis parameters, a size standard, and a matrix for the analysis. Perform data analysis.

##### ■ Select STR peaks

Display the electropherograms and the corresponding table of the three ChimerXplain samples. Select the peak(s) for each STR according to the size range and dye (for an overview refer to figure 1).

#### 4.2 ChimerXact Kits (singleplex PCR)

##### ■ Genomic DNA isolation

1. Isolate genomic DNA by any protocol that yields high-purity DNA.
2. Dilute sample DNA in 10 mM Tris/HCl, pH 8 to a final concentration of 0.8 µg/µL.

##### ■ Set up PCR experiment

1. Combine 2.2 ng genomic DNA and 2.5 µL 10× PCR Resuspension Buffer and adjust with water to a final volume of 25 µL.
2. Dissolve lyophilized ChimerXact PCR Mix using 25 µL PCR-Resuspension Buffer containing 2.2 ng genomic DNA.
3. Run PCR (see program page 24).

##### ■ Select informative STR markers

1. Exclude all STRs with identical lengths.
2. Prefer an allelic pattern with the two, e.g., donor peaks surrounding the two recipient peaks (DRRD or RDDR) or alternating configurations like DRDR or RDRD.
3. Alternatively, choose two homozygous loci.
4. In addition, heterozygous STRs differing in only one allele can be used.
5. Finally, a STR heterozygous in one case and homozygous in the other case can be used.

**Prepare a GeneScan injection list**

Set up a new injection list and select the appropriate sample sheet. For all samples select

“GS STR POP4 (1 mL) A”

“inj. Secs” to 5

“inj. kV” to 15.0

“Run kV” to 15.0

“run °C” to 60

“Run Time” to 30

matrix file

Optionally activate the autoanalysis checkbox.

▲ **Note:** The injection parameters might have to be adapted depending on the signal intensities. Lowering the injection time or voltage will decrease the peak signals.

Load the denatured samples onto the sample tray, close the doors, and start electrophoresis.

**Data analysis****Set up analysis parameters**

Define the analysis parameters for peak detection.

**Set up a size standard**

Assign sizes to the respective peaks of a representative run containing the size marker.

**Set up a project for sample analysis**

Define samples to be analyzed. Choose appropriate analysis parameters, a size standard, and a matrix for the analysis. Perform data analysis.

**Select STR peaks**

Display the electropherograms and the corresponding table of the ChimerXact samples. Select the peak(s) for each STR according to the dye and the sizes for recipient and donor as determined during the ChimerXplain analysis.

**Chimerism quantification**

$\% \text{ Chimerism} = (D1+D2) / (D1+D2+R1+R2) * 100$

D = Peak height (or area) of donor alleles

R = Peak height (or area) of recipient alleles

**5. Tips & hints****Lyophilized mixes**

The foils sealing the plates containing the lyophilized reaction mixes can either be peeled off by hand or pierced with a pipette tip. For peeling off, it is recommended to first cut the cover of an individual well with a scalpel. Then, peel off the film by hand wearing gloves.

To pierce the plastic film, we recommend using a fresh pipette tip; take another fresh pipette tip to dissolve the mix.

For resuspension of lyophilized mixes, add the resuspension buffer into the well and dissolve by gently pipetting up and down till a clear solution is obtained. Avoid foam formation.

## 6. Troubleshooting

▲ Please also refer to your Applied Biosystems Genetic Analyzer User's Manual for instrument related troubleshooting.

▲ The included positive control DNA can be used to check the principal function of the ChimerXplain PCR assay (refer to table 5 for comparison). As the positive control DNA is derived from the WT47 cell line, it will show allelic imbalance and imbalances between STR loci. Therefore, the positive control DNA can be used to confirm that the correct STR profile is obtained, but will not show a balanced profile. The positive control DNA is not suitable to determine sensitivity or verify balance.

### ChimerXplain PCR 1:

No.	Locus ID	Chromosome	Dye	Code	Length range	Allele 1	Allele 2
1	D10S2325	10	FL	B	163–213	184	
2	D12S391	12	FL	B	213–269	227	235
3	P450CYP19	15	FL	B	314–464	342	369
4	D2S1360	2	Joe	G	200–273	214	222

### ChimerXplain PCR 2:

No.	Locus ID	Chromosome	Dye	Code	Length range	Allele 1	Allele 2
5	D9S1118	9	FL	B	80–128	84	114/117*
6	MYCL1	1	FL	B	156–225	188	198
7	D7S1517	7	Joe	G	164–212	169	181
8	D11S554	11	TMR	Y	166–253	209	218
9	D8S1132	8	TMR	Y	330–379	340	348

\*The positive control DNA in some instances shows two peaks for the second allele of D9S1118. The expected length is 117 Bp.

### ChimerXplain PCR 3:

No.	Locus ID	Chromosome	Dye	Code	Length range	Allele 1	Allele 2
10	SE-33	6	TMR	Y	138–305	233	257

**Table 5:** Allele pattern of positive control WT 47 cell line genomic DNA

Observation	Possible causes	Possible actions
No current	<ul style="list-style-type: none"> <li>· Too little buffer in anode buffer reservoir</li> <li>· Too little buffer in autosampler position 1</li> <li>· Unfilled capillary, bubbles in capillary and block</li> <li>· Pump block is plugged with urea or crystallized buffer</li> <li>· Old, defective, or incorrectly made buffer or polymer</li> </ul>	<ul style="list-style-type: none"> <li>· Fill buffer reservoir.</li> <li>· Refill buffer in position 1 of autosampler.</li> <li>· Examine the instrument for leaks.</li> <li>· Remove and clean block.</li> <li>· Replace buffer or polymer.</li> </ul>
No signal	<ul style="list-style-type: none"> <li>· Sample not at bottom of tube or air bubble at bottom of sample tube</li> <li>· Volume of sample too low</li> <li>· Insufficient injection time</li> </ul>	<ul style="list-style-type: none"> <li>· Spin sample tube in microcentrifuge.</li> <li>· Sample volume must be at least 10 <math>\mu</math>L.</li> <li>· Increase the electrokinetic injection time or voltage.</li> </ul>
Weak signals	<ul style="list-style-type: none"> <li>· Insufficient injection time</li> <li>· Insufficient PCR yield</li> <li>· Insufficient sample amount</li> </ul>	<ul style="list-style-type: none"> <li>· Increase the electrokinetic injection time or voltage.</li> <li>· Amount of used genomic DNA too low. Use the recommended amount of template DNA.</li> <li>· Increase the sample amount added to the electrophoresis mastermix, e.g., 2 <math>\mu</math>L instead of 1 <math>\mu</math>L ChimerXplain PCR products and 2 <math>\mu</math>L instead of 1 <math>\mu</math>L ChimerXact PCR products.</li> </ul>
High baseline	<ul style="list-style-type: none"> <li>· Dirty capillary window or dirty detector window</li> <li>· Dirty syringe</li> <li>· Dirty pump block</li> <li>· Capillary moved out of position in front of detector window</li> <li>· Precipitate in polymer</li> </ul>	<ul style="list-style-type: none"> <li>· Clean capillary window using 95 % ethanol and lint free paper. Clean the detector window carefully using lense paper.</li> <li>· Clean the syringe with warm water.</li> <li>· Clean the pump block with warm water.</li> <li>· Position capillary in front of laser window.</li> <li>· Allow polymer to equilibrate to room temperature before use.</li> </ul>
Extra peaks	<ul style="list-style-type: none"> <li>· Samples not fully denatured or renaturation of denatured samples</li> </ul>	<ul style="list-style-type: none"> <li>· Make sure the samples are heated at 95 °C for three minutes prior to loading onto autosampler. Load samples immediately following denaturation, or store on ice until you are ready to load.</li> </ul>
Size-standard peaks not recognized when defining size standard (GeneScan)	<ul style="list-style-type: none"> <li>· Height of a size-standard peak less than the Peak Amplitude Threshold for the size-standard color (in Analysis Parameters) ▲ Note: 50 RFU is the default threshold.</li> <li>· Primer peaks wrongly assigned as size standard peaks.</li> </ul>	<ul style="list-style-type: none"> <li>· Lower the value for the size-standard color in the Peak Amplitude Threshold field to 30 RFU. Re-run sample adding a higher amount of size standard.</li> <li>· Select partial range for data analysis to exclude primer region.</li> </ul>

### Hazard identification

Formamide: T, R61; *Formamide* causes irritation to the gastrointestinal tract. Affects the central nervous system. May cause headache, dizziness, nausea, vomiting, abdominal pain, and unconsciousness. May affect the reproductive system.

### 7. References

1. Watzinger, F. *et al.* (2006) The RSD code: proposal for a nomenclature of allelic configurations in STR-PCR-based chimerism testing after allogeneic stem cell transplantation. *Leukemia* 20: 1448–1452.
2. Thiede, C. *et al.* (2004) Evaluation of STR informativity for chimerism testing-comparative analysis of 27 STR systems in 203 matched related donor recipient pairs. *Leukemia* 18: 248–254.
3. Kristt, D. and Klein, T. (2006) Reliability of quantitative chimerism results: assessment of sample performance using novel parameters. *Leukemia* 20: 1169–1172.

All protocols and data sheets are available at [www.miltenyibiotec.com](http://www.miltenyibiotec.com).

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