Single Strand Conformation Polymorphism (SSCP) Analysis on Applied Biosystems® 3130 and 3130*xl* Capillary Electrophoresis Systems

Introduction

Increasingly in the genomics field, researchers are requiring screening and sequence variation detection tools for large numbers of samples. One widely used technique is single strand conformation polymorphism (SSCP) analysis [1]. The use of POP™ Conformational Analysis Polymer (CAP) on the Applied Biosystems® 3130 and 3130*xl* Genetic Analyzers is an efficient, convenient, and cost-effective solution for performing SSCP. This application note describes the use of CAP on the Applied Biosystems® 3130 and 3130*xl* Genetic Analyzers to perform mutation screening with SSCP.
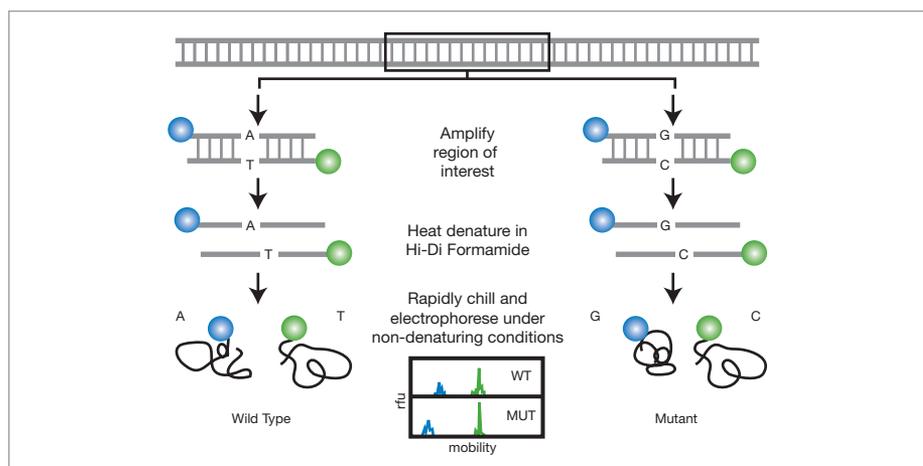
Figure 1. Schematic Overview of the SSCP Technique.

Overview of SSCP

SSCP analysis detects sequence variations (single-point mutations and other small-scale changes) through electrophoretic mobility differences. These variations can potentially cause conformational changes in the DNA molecules. Under nondenaturing conditions and often reduced temperature, single-stranded DNA molecules can assume unique conformations that vary depending on their nucleotide sequences. These conformational changes can result in detectable differences in mobility as illustrated in Figure 1. In this application note we will review SSCP analysis, a technique that is widely used for sequence variation detection because of its simplicity and ease of use.

Experimental Protocol
SSCP Requirements
- POP™ Conformational Analysis Polymer (CAP): A nondenaturing polymer (supplied at a 9% concentration), which can be diluted to a concentration of 3.0–8.1% without glycerol or 3.0–7.2% with 10% glycerol.

- GeneScan™ 600 LIZ® Size Standard: The fluorophore-labeled fragments are visualized on an Applied Biosystems® 3130 or 3130*xl* Genetic Analyzer. The GeneScan™ 600 LIZ® Size Standard is used in all samples as an internal tool to align data from different capillaries and to minimize capillary-to-capillary or run-to-run variability.

- ABI Prism® primers (oligonucleotides labeled with 5′ fluorescence): Two different labels (like 6FAM™ and VIC® dyes) can be used for the forward and reverse strand to make them more informative.

- GeneMapper® Software v4.1 for semi-automated data analysis.

- When concurrently using any Applied Biosystems® POP™ polymers for denaturing applications, the use of a dedicated 36 cm capillary array is recommended to minimize negative performance issues as a result of residual POP™ or CAP polymers within the array.

- Data collection v3.1 software contains a "ConformationAnalysis36_CAP" module that allows the SSCP application to run successfully on an Applied Biosystems® 3130 or 3130xl Genetic Analyzer.

Setting up the Instrument and Preparing the Samples
See Appendices I and II for step-by-step protocols.

Collecting and Processing the Data
Data collected during electrophoresis are analyzed with GeneMapper® Software v4.1. Migration of sample peaks is calculated and normalized relative to the internal size standard. Samples can then be aligned and compared. Results

can be validated by sequencing the region of interest and comparing sample sequences using Variant Reporter® Software v1.1 (see Appendix III).

Data Normalization Using Internal Size Standards
In order to detect small changes in mobility between different samples, an internal size standard should always be used to align data from different capillaries and to minimize capillary-to-capillary variability. In the example shown in Figure 2, the standard deviation in sizing for the five main peaks of sample GM06160 injected into all 16 capillaries during one run varies between 0.03–0.09.
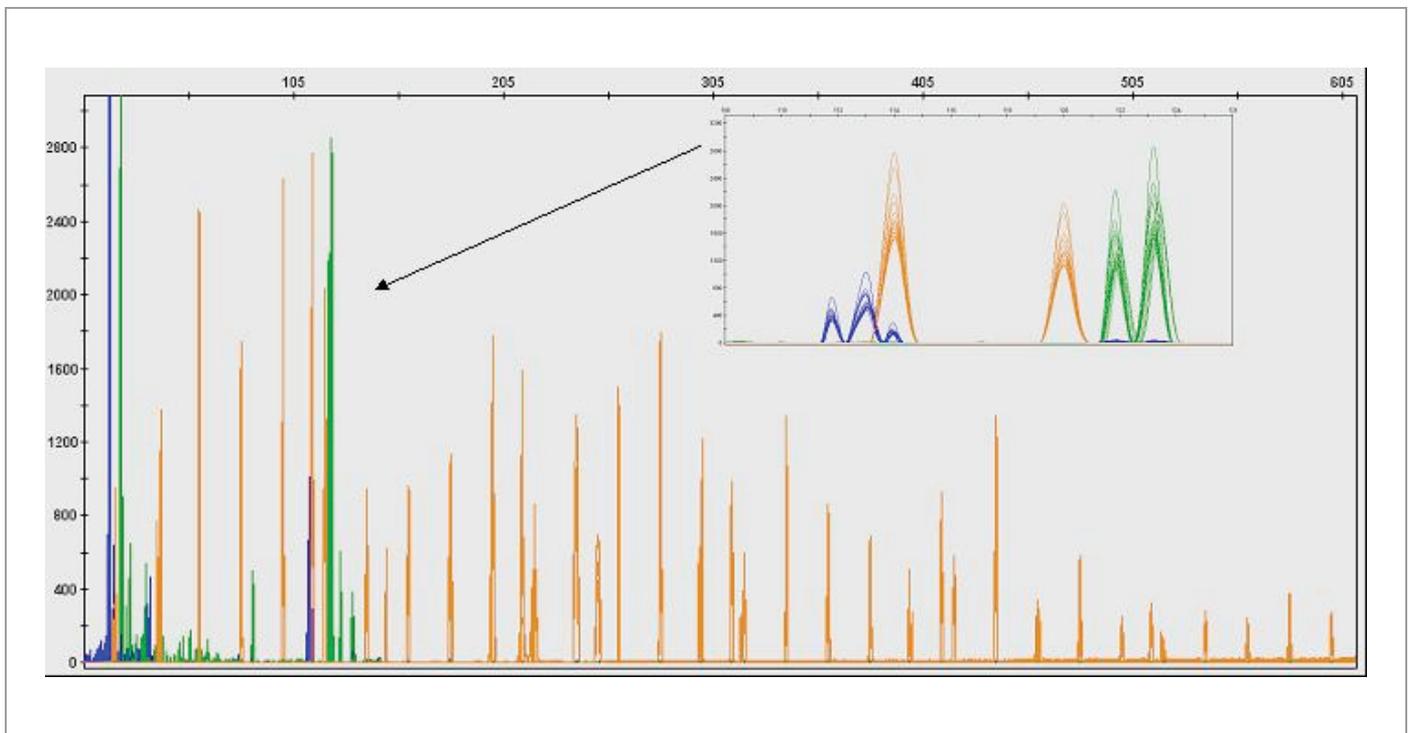
Figure 2. Data Aligned With the GeneScan™ 600 LIZ® Size Standard in the Sample Plot View of GeneMapper® Software v4.1. Data from one 16-capillary array injection. The orange trace represents the GeneScan™ 600 LIZ® Size Standard. Note that several peaks appear as split peaks reflecting different isoforms observed under SSCP conditions; the appearance and spacing may vary with different analysis temperatures. The insert shows the main peaks of the heterozygous sample GM06160, labeled with 6FAM™ and VIC® dyes.

Note: the sizes of the sample peaks are relatively small. In such cases the collection time can be shortened, thereby increasing the throughput. Please note that the size standard definition will need to be adjusted accordingly to only include the size standard peaks that will be collected in this time frame.
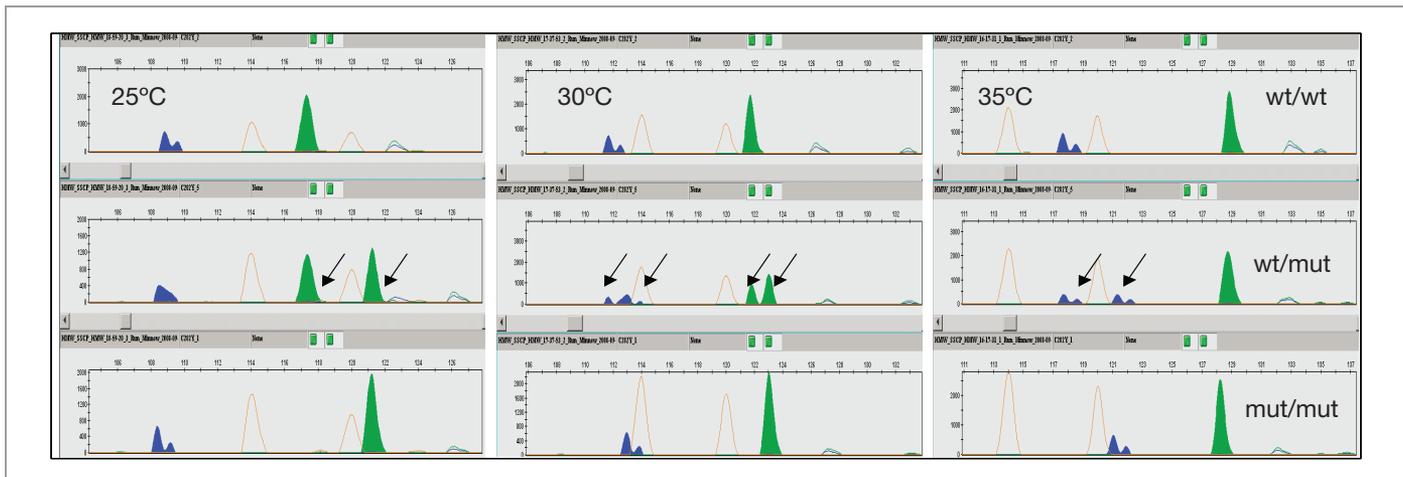
Figure 3. The Effect of Temperature on the Resolution of SSCP Fragments. Three DNA samples that are either homozygous for the wild type (GM13591, top), homozygous for the mutation C282Y (GM14631, bottom), or heterozygous (GM06160, middle) for codon position 282 of the HFE gene [2] were analyzed at 25°C, 30°C, and 35°C (samples were obtained through the Coriell Institute for Medical Research, Camden, NJ, USA). The forward strand was labeled with 6FAM™ dye (blue peaks), the reverse strand with VIC® dye (green peaks). GeneScan™ 600 LIZ® Size Standard peaks are shown in orange. Polymer conditions used were 5% CAP and 10% glycerol in 1X TBE.

Impact of Electrophoresis Temperature

For successful identification of mutations by SSCP, it may be necessary to optimize electrophoretic run conditions. The most critical condition for successful variant detection is the run temperature. Figure 3 shows an example of how the two strands of a PCR product (for detecting changes in codon position 282 of the HFE gene [2]) react to changes in electrophoresis temperature. It is evident that the two strands labeled with two different fluorophores have different reactions to changes in temperature. While at 25°C the VIC® dye–labeled (green) strands of the heterozygous sample GM06160 are well separated (middle) and allow for easy identification of either a homozygous wild type sample (top) or homozygous mutant sample (bottom), at 35°C both main peaks of the heterozygous sample merge, and cannot be distinguished with certainty.

However, a comparison of the 6FAM™ dye–labeled (blue) strands of the heterozygous sample demonstrate a significant separation at 35°C, but hardly any separation at 25°C. At 30°C both the VIC®- and 6FAM™ dye–labeled strands of the heterozygous sample are sufficiently separated and allow the identification of either a homozygous wild type or mutant sample. Note that a user might notice additional peaks for a homozygous sample for either the forward or reverse strands (in the example, the strands labeled with 6FAM™ dye). These additional peaks represent multiple fragment conformations (isoforms) and can, together with mobility shifts, be used to increase the information content of this assay.

Assay Validation

Following the SSCP screening assay, sequencing should be used to verify that the mobility variation observed is indicative of a sequence variant in the region of interest. The sequences generated can be analyzed with Variant Reporter® Software v1.1. Figure 4 shows sequencing results for the two DNA samples used in this study that are either homozygous or heterozygous for the G mutation, and confirms the expected nucleotide changes.

Conclusion

This application note demonstrates the use of POP™ Conformational Analysis Polymer and GeneScan™ 600 LIZ® Size Standard for variant detection in PCR amplicons by SSCP on the Applied Biosystems® 3130 and 3130xl Genetic Analyzers. The use of the size standard helps to minimize capillary-to-capillary and run-to-run sizing variability. The application of different electrophoresis temperatures can maximize the success rate of sequence variation detection.
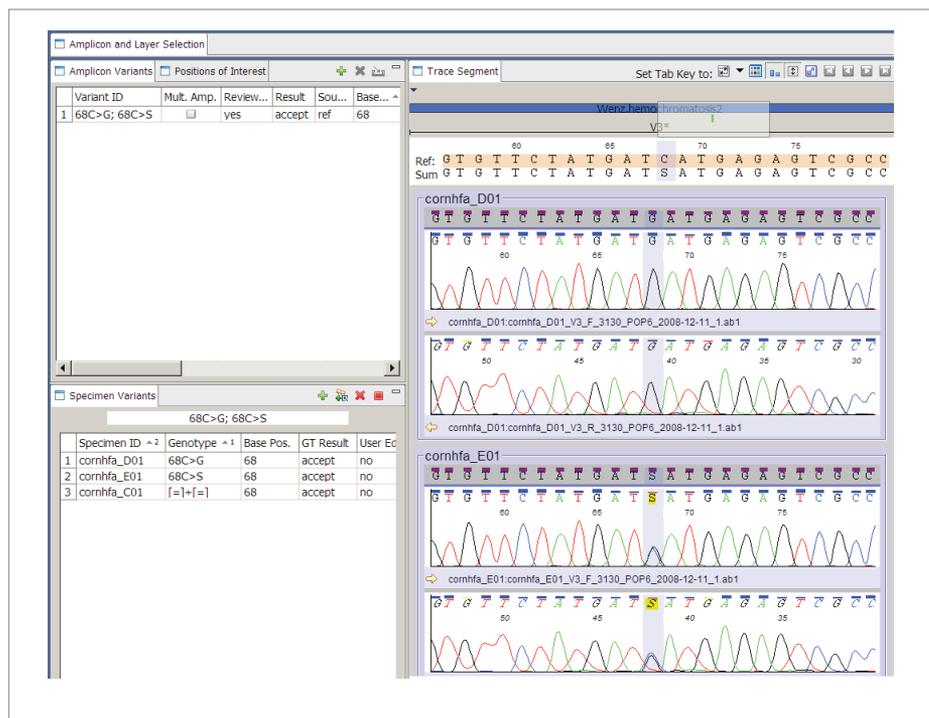
Figure 4. Confirmation of Base Mutations During Assay Validation Using Variant Reporter® Software v1.1. Consensus sequences are generated from the forward and reverse sequences of samples. Alignment of the consensus to a reference sequence confirms the correlation between the mobility shift and a single base change in the sequence.

Appendix I: Preparing the 3130 or 3130*xl* Genetic Analyzer for SSCP Runs

Please complete the following steps for SSCP analysis on the 3130 or 3130*xl* Genetic Analyzer:

1. Prepare the polymer, e.g., the formulation for 5 g of polymer (5% CAP, 10% glycerol, 1X TBE) would be:

- 9% Conformational Analysis Polymer: 2.78 g

- 10X TBE* (running buffer): 0.5 g

- 100% molecular biology grade glycerol: 0.5 g

- Molecular biology grade H_2O: bring up to 5 g

  * TBE in combination with glycerol is recommended as a running buffer for this particular application [3].

Note: Mix for ~3 hr at room temperature. Before use, filter through a 0.45 μm filter. It might be necessary to quick-spin the filtered solution to remove air bubbles. The prepared polymer solution is stable at 4°C for at least two weeks. Once on the instrument, the polymer should be replaced after 50 injections, or one week, whichever comes first.

2. Prepare the run buffer, e.g., the formulation for 50 g of buffer (10% glycerol, 1X TBE) would be:

- 10X TBE: 5g

- 100% molecular biology grade glycerol: 5 g

- Molecular biology grade H_2O: bring up to 50 g
  Note: Mix for ~3 hr at room temperature. The prepared buffer solution is stable at

4°C for at least two weeks. Once on the instrument, the buffer should be replaced after 50 injections, or one week, whichever comes first.

3. Install a 4- or 16-capillary array. To minimize negative performance issues as a result of residual POP™ or CAP polymers within an array, it is strongly recommended that a dedicated array is used for the SSCP application. Use the "Install Array and Change Polymer Wizard" available in Data Collection v3.1 to change the array and switch to CAP polymer.

   Note: We recommend using 36 cm arrays. We have not observed any significant benefits from using a longer array.

4. Create a new run module, such as "SSCP_CAP_x", where x is the oven temperature. Use the default "ConformationAnalysis36_
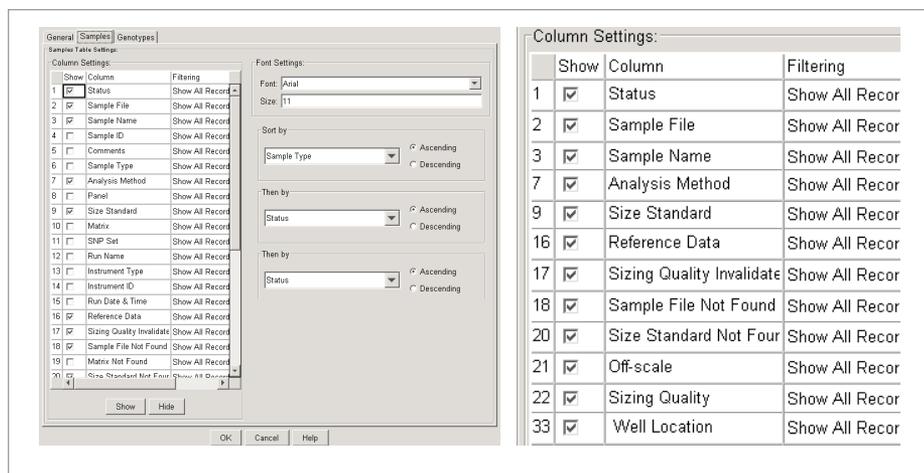
Figure 5. Selecting the Column Settings in the Samples Tab.
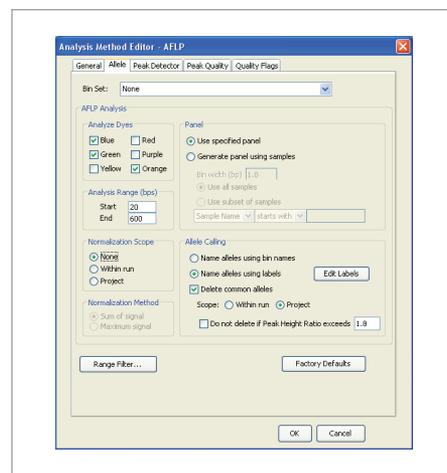
Figure 7. Configuring the Settings in the Allele Tab.
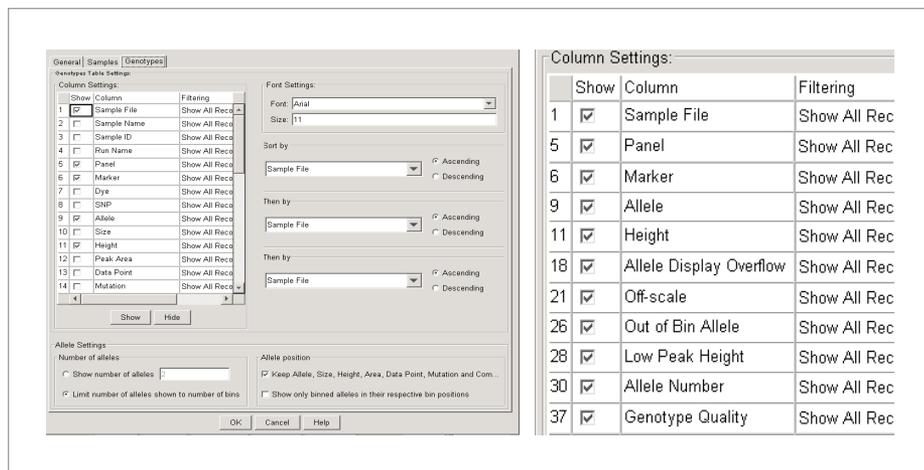
Figure 6. Selecting the Column Settings in the Genotypes Tab.
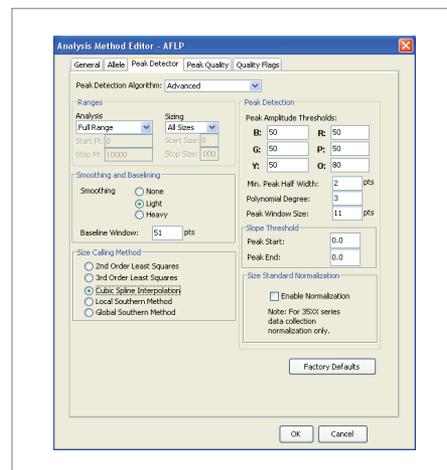
Figure 8. Configuring the Settings in the Peak Detector Tab.

CAP™ module as the template. This run module is available in Data Collection v3.1. Selecting run temperatures between 25°C and 35°C is recommended, as temperatures outside of this range might result in inconsistent run data. The default collection time will collect all fragments of the GeneScan™ 600 LIZ® Size Standard at 30°C. Depending on the temperature setting selected or the size of the amplicon of interest, the collection time may need to be increased or decreased. The default settings for a 5% CAP, 10% glycerol, and 1X TBE formulation at a 30°C run are:

• Injection voltage: 5.0 kV

• Injection time: 12 sec

• Run time: 3,840 sec

• Run voltage: 9.5 kV
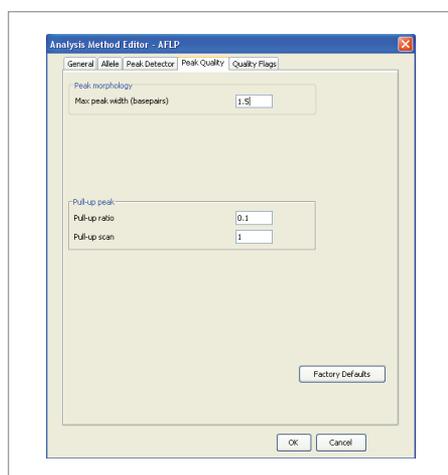
Note: When using TBE as the run buffer, run voltages above 9.5 kV may have a detrimental effect on performance.

5. Create instrument protocols for the spectral and regular run types. Use the G5 set with GeneScan™ 600 LIZ® Size Standard.

6. Prepare and run a spectral standard plate, using the "Spect36_CAP" module available in Data Collection v3.1. Use the Matrix Standard Set DS-33 if using GeneScan™ 600 LIZ® Size Standard and 6FAM™ and VIC® dyes as labels for the amplicons.

Appendix II: Preparing the Samples
• Sample—1 µL

Note: Test different dilutions for any given sample in order to optimize signal intensity. Ensure that the signals for the

individual strands of the amplicons are within the intensity range of the system.

• GeneScan™ 600 LIZ® Size Standard— 1 µL (Due to potential splitting of peaks, a higher volume of size standard is recommended.)

• Hi-Di™ Formamide—10 µL

• Add sample, size standard, and Hi-Di™ formamide into each well of the sample plate.

• Denature the double-stranded PCR product for 3 min at 95°C.

• Cool on ice for 2 min to avoid re-annealing of the complementary strands.

Run the sample plate using the newly created run module, and then analyze the results with GeneMapper® Software v4.1 (see Appendix III).

Appendix III: GeneMapper® Software Analysis
The following are guidelines for setting up SSCP analysis in GeneMapper® Software v4.1. The alignment of sample peaks for comparison is facilitated by using the GeneScan™ 600 LIZ® Size Standard. Other settings, such as peak detection thresholds, polynomial degree, peak window size, or size calling method can be customized to meet user/application requirements. The following settings and workflow can be used as a starting point.

Setting up the Software
Open GeneMapper® software.
1. Click File>New Project to create a new project. In the New Project dialog box, select AFLP, and click OK.

2. Click File>Add Samples to Project.

3. In the Add Samples to Project dialog box, select the Files tab.

4. Navigate to the data folder and import the sample files.

5. Open the GeneMapper® Manager (Tools>GeneMapper Manager).

Figure 9. Configuring the Settings in the Peak Quality Tab.

Figure 10. Configuring the Settings in the Quality Flags Tab.
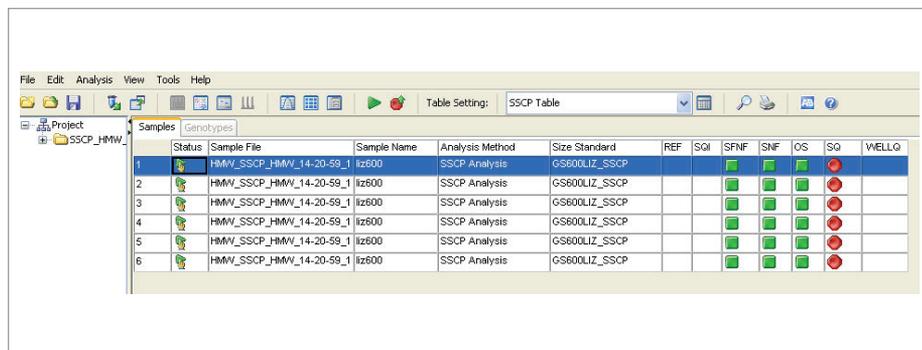
Figure 11. Applying the SSCP-Specific Parameters to the Sample Files in the Project.
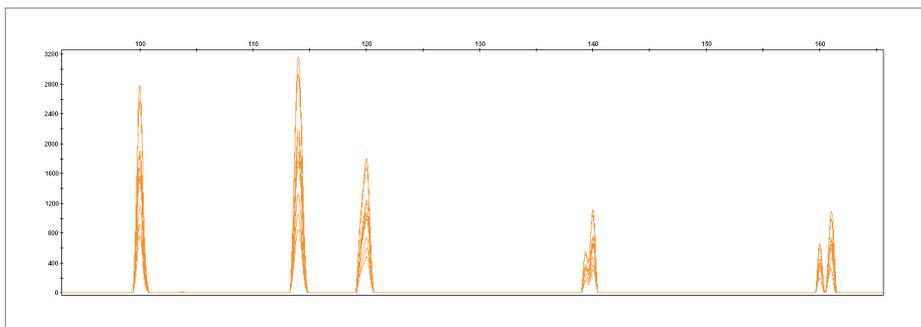
Figure 12. Example of Size Standard Peaks in Alignment.
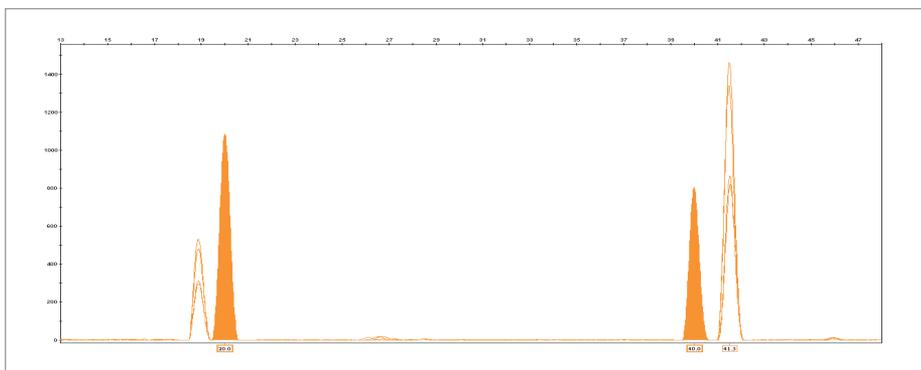
Figure 13. Example of Non-Aligned Size Standard Peaks.
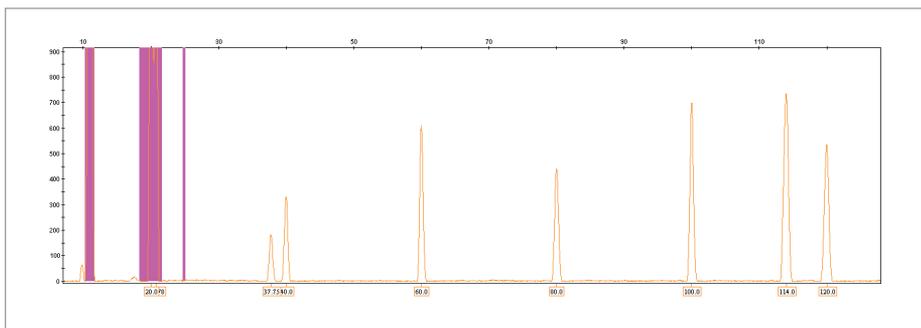
Figure 14. Example of Small Size Standard Peaks Being Hidden by Off-Scale Primer Peak Regions.

6. Select the Table Settings tab and select "New" to create a new table setting.

7. In the Table Setting Editor, select the General tab and type "SSCP_Table" in the Name field.

8. In the Table Setting Editor, select the Samples tab and check the Column Settings indicated in Figure 5.

9. In the Table Setting Editor, select the Genotypes tab and check the Column Settings indicated in Figure 6.

10. Click OK to save the new table setting.

11. In the GeneMapper® Manager, select the Analysis Method tab.

12. Click "New" to create a new AFLP analysis method.

13. In the Analysis Method Editor, select the General tab and type "SSCP Analysis" in the Name field.

14. Select the Allele tab and configure the settings as shown in Figure 7.

15. Select the Peak Detector tab and configure the settings as shown in Figure 8.

16. Select the Peak Quality tab and configure the settings as shown in Figure 9.

17. Select the Quality Flags tab and configure the settings as shown in Figure 10.

18. Click OK to save the analysis method.

19. In the GeneMapper® Manager, select the Size Standards tab.

20. Click "New" and select Basic or Advanced in the Dye and Analysis Method dialog box.

21. Click OK to open the Size Standard Editor.

22. In the Size Standard Editor, type "GS600LIZ_SSCP" in the Name field and choose "Orange" in the Size Standard Dye pull-down menu.

23. In the "Size Standard Table" section, add the following values to the "Size in Basepairs" column: 20, 40, 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, 460, 480, 500, 514, 520, 540, 560, 580, 600.

24. Click OK to save the size standard definition.

25. Apply the SSCP-specific parameters just created to the sample files in the project, as shown in Figure 11.

26. Analyze samples.

Reviewing the Results

1. Review the Size Standard:

Manually control mobility correction by overlapping all size standard peaks in one plot view (Use "Overlay LIZ" default plot display settings).

All size standard peaks should be aligned (Figure 12), if the mobility correction is accurate.

2. If some peaks are not aligned (Figure 13) and adjustments are required, open the Size Match editor to edit size standard peak labels and re-analyze sample(s).

   Note: Depending on the electrophoresis temperature, there may be split size standard peaks (for example, the 20 and 40 bp peaks often display as two peaks instead of one; see Figure 13). Review these peaks carefully to make sure the label assignment is consistent within the project. If this is not the case, re-assign the labels manually (using the Size Match Editor) and re-analyze if necessary.

   Note: The smallest size standard peak(s) may be masked by the off-scale primer peak region (see Figure 14). To increase the mobility correction success rate, remove these peaks from analysis (for example, edit the size standard definition by removing the 20 and 40 peaks), and adjust the sizing range in the Allele tab of the Analysis Method Editor.

3. After the size standard peak alignment is satisfactory, fragments of interest can be compared to identify mobility shifts.

   Note: Refer to the instrument User Guide or contact the local Applied Biosystems Support group for additional information.

   Note: Because laboratories have optimized procedures and protocols to suit individual needs, be aware that separation temperature or polymer formulation may change. Researchers are encouraged to optimize these procedures and protocols to meet these specific study requirements.

References

1. Orita M, Iwahana H, Kanazawa H et al. (1989) *Proc Natl Acad Sci U S A* 86:2766–2770.

2. Wenz HM, Baumhueter S, Ramachandra S et al. (1999) *Hum Genet* 104:29–35.

3. Andersen PS, Jespersgaard C, Vuust J et al. (2003) *Hum Mutat* 21:455–465.

ORDERING INFORMATION

Description	Size	Part Number
POP™ Conformational Analysis Polymer	25 mL	4340379
GeneScan™ 600 LIZ® Size Standard	800 rxns	4366589
3130 Genetic Analyzer Capillary Array, 36 cm	Qty 1	4333464
3130*xl* Genetic Analyzer Capillary Array, 36 cm	Qty 1	4315931
GeneMapper® Software v4.1	Full	4366925

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