Preparation and Storage

**Template Preparation**

Template in the form of PCR product can be used for SNP reactions. These products should be free of non-specific products and other artifacts. Specificity can be increased by designing PCR primers with a high annealing temperature. Excess primer can lead to non-specific products. Excess PCR reagents can adversely affect final template purity if they are not completely removed during the purification process using either the combination of Exonuclease I and Shrimp Alkaline Phosphatase (Exo/SAP) or column purification. In cases where a single PCR product is not obtained, specific PCR products/bands can be cut and purified from agarose gel after electrophoresis and used as a template. It is important to verify the quality and quantity of the PCR product before attempting a SNP reaction.

Follow the manufacturer’s recommendation for Exo/SAP and column purification protocols.

Refer to the Appendices for PCR Primer Design recommendations.

**Preparation of the SNP-Primer Extension Reaction**

Refer to the Appendices for recommendations on SNP Interrogation Primer Design, Positive Control, and Troubleshooting.

- Use the recommended starting points of 0.2 μM for each SNP primer and 100 fmol template.
- If performing a multiplex SNP reaction, create a primer premix so that each primer has a final concentration of 0.2 μM.
- Raise or lower individual primer and PCR product concentrations to adjust signal balance of individual SNPs within a multiplex panel.
- Prepare each 10 μL SNP-Primer Extension reaction in a 0.2 mL thin-wall tube or microwell plate.
- Keep all components on ice while preparing the reactions and add them in the order listed below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNPStart Master Mix</td>
<td>4.0 μL</td>
</tr>
<tr>
<td>DNAse RNAse free PCR grade water</td>
<td>x.x μL</td>
</tr>
<tr>
<td>Interrogation Primer (0.1 - 1.0 μM)</td>
<td>x.x μL</td>
</tr>
<tr>
<td>PCR product or template (1-100 fmoles)</td>
<td>x.x μL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>10.0 μL</td>
</tr>
</tbody>
</table>

**CAUTION** The SNPStart Master Mix contains glycerol. Pipette this viscous solution slowly and carefully to avoid pipetting errors.

**Thermal Cycling Program**

- Use the recommended 2-step protocol: 90°C for 10 seconds followed by 45°C for 20 seconds. Cycle for 25 times and then hold at 4°C.

**NOTE** If using a thermal cycler without any ramping, add an additional step at 72°C for 30 seconds.

- Single-plex reactions can be cycled 24-30 times; multiplex reactions require 30-40 cycles.
• Use calculated temperature control, heated lid, and rapid thermal ramping between steps.

Post-reaction Sample Cleanup
Unincorporated dye labeled terminators will co-migrate with small SNP products and interfere with analysis if left untreated. To avoid this, a simple digestion by SAP is required.

This can be set up as follows:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAP 1U/µl</td>
<td>0.25 µL</td>
</tr>
<tr>
<td>SAP Buffer</td>
<td>1.30 µL</td>
</tr>
<tr>
<td>DNAse RNase free PCR grade water</td>
<td>1.45 µL</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>3.00 µL</strong></td>
</tr>
</tbody>
</table>

1. Mix the tube thoroughly and consolidate the liquid to the bottom of the tube by brief centrifugation.
2. Incubate samples for 30 minutes at 37°C to treat unincorporated dye terminators, followed by 15 minutes at 65°C to inactivate the SAP enzyme.
3. Store samples at 4°C or on ice if running the same day. Otherwise, store samples covered at -20°C.

Sample Preparation for Loading into the instrument
1. Add 0.5 µL (or as required) of purified SNP reaction to 0.5 µL Size Standard 80 (PN 608395) and 39.0 µL of Sample Loading Solution for each sample in a fresh sample plate (PN 609801). Mix and spin it briefly.

   **NOTE** Allow Size Standard 80 to thaw and equilibrate at room temperature for at least 15 minutes prior to use. Spin the tube briefly and mix thoroughly by pipetting up and down 5-10 times to ensure balanced size standard peak heights.

2. Overlay each of the diluted samples with one drop of Mineral Oil.
3. Load the sample plate into the instrument.
4. In the Sample Setup module use SNP-1 as the run method.
5. Preheat the capillary array before starting the run. Failure to do so can result in improper separation of the sample size standard.

   To do so:
   • In the Run Module, select Direct Control | Capillary Temperature.
   • Set Temperature to 50°C.
   • Select Wait for Temperature to be Reached.
   • Click on Start.

Raw Data
The recommended peak height of raw data should be between 0.5 and 1.5 times the peak height of the 13 nt size standard.

The peak heights should not exceed 80,000 rfu.

Data Analysis
The Algorithm Update must be installed for correct data analysis. If SNP locus tags have been created prior to installing the update, the apparent size of the locus may need adjusting. There can be a slight variation in the sizing of peaks (<1 nt) after installing the update.

Use the Fragment Analysis module for SNP data analysis.

1. Create a new study by selecting the sample raw data that needs to be analyzed, then select Next.
2. Select Default SNP Analysis Parameters from the drop-down menu.
   The following parameters should be modified to correctly analyze the SNPStart data:
3. Click Edit, then from the General Tab, change the Slope Threshold to 30 and Relative Peak Height Threshold to 20 (These values should be adjusted, depending on the raw data to be analyzed.) These values can be lowered for samples where the alleles signal is weaker and the alleles are not called.
4. Select the Advanced Tab and then choose the SNP ver. 2 Dye Mobility Calibration.
5. Click Save As. and then rename the new Analysis method with a unique name, e.g., “SNPStart-AnalysisParameters.”

This method is now set up to analyze the data generated using the GenomeLab SNPStart Primer Extension Kit. This method can be further modified for adjusting the threshold, or if SNP Locus Tags are added to the study.

Once created, the new analysis parameters will be available for subsequent projects, provided that they are in the “default” project within the same database. The parameters can also be selected for automatic analysis following each run. Copy and paste the new analysis parameters into the User Template Database in Data Manager to automatically populate all newly created databases with the analysis parameters.

To confirm validity of analyzed results:
• Compare the raw data with the analyzed results.
• Verify that the number of peaks (not cross-talk) is the same in raw and analyzed data. This does not take into account possible noise peaks which typically should be very small.
• During a single separation, the ratio of the peak heights in the raw data within a SNP product should remain the same in the analyzed data.

Appendices

Appendix A
PCR Primer design and template preparation:
• Design PCR primers with a high Tm (70°C-75°C) and mask the repeat sequences whenever possible. The length of the PCR primers should be between 25-32 nucleotides (nt) with 27 bp as the optimal desired length.
• Designing short PCR products (100-300 bps) reduces complementary binding and artifacts, especially in a multiplex reaction.
• For multiplex PCRs, design the primers so that the products have sufficiently different sizes to be resolved on an agarose gel. This helps to verify the presence of each template within the multiplex product before proceeding to the next step.

   **NOTE** For primer design you may use the Primer 3.0 software developed by the Whitehead Institute for Biomedical Research available at the following website:

   http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
Appendix B

Interrogation Primer Design:
When designing single and multiplex primers, consider these recommendations:

- Design primers with a high $T_m$ (70°C-75°C) and a length of 25-30 nts (complementary to the target sequence). Check primers for repeat sequences whenever possible. If necessary, a homopolymeric tail can be added to adjust the desired primer length for multiplexing.

- If designing a locus interrogation primer is problematic for one strand, design primers to anneal to the complementary strand of the DNA template. Occasionally, the sequence at the locus may make it difficult to design primers with the above mentioned criteria. In such instances, one may be forced to use suboptimal parameters. Run these extension products individually to identify artifacts before multiplexing.

- The minimum recommended spacing between primer extension products is 6 bases. The recommended range of the extension products is 26-79 nt*. To adjust the lengths of primers, add homopolymeric (poly dA, dT, dC, or dG) tails to the 5' end of the primer.

Example: If the primer sequence is 5’-cgccaggttttcagcacaag-3’ (25 nt) and the desired SNP product length is 36 nt, the primer sequence should be 5’-AAAAAAAAAAAAAAAAAAccaggttttcagcacaag-3’ (35 nt). Here the poly dA is not complementary to the target sequence but is added to get the desired length. After the primer extension reaction, one base is added to the primer making the length 36 nt.

- The target sequence should not contain the complementary homopolymeric tail sequence as the interrogation primer. In a multiplex reaction, other target sequences should be screened for complementarities.

- For multiplex reactions, do not mix the base used to create the tail, such as poly dA and poly dT. The complementation of poly dA and poly dT will lead to dimers and artifact extension products.

- Interrogation primers should be purified using high-performance liquid chromatography (HPLC) or polyacrylamide gel electrophoresis (PAGE) to minimize the presence of na1 primer products, which give rise to shoulder peaks.

- Primers must be free of hairpin structures and must not form primer dimers. These structures can be extended with labeled terminators during the extension reaction. Check primer sequences with suitable primer design software.

- Due to effects of base composition and incorporated dyes, migration times of fragments can differ from actual sizes.

*The spacing can be 5 bp for products > 70 nt. These are general recommendations. Allele combinations and individual SNP migration should be taken into consideration when designing the Primers. If there are less than 10 SNPs in a multiplex panel, increase the spacing to 8bp or 10bp.

Appendix C

Locus Tags:
Create locus tags using the SNP Locus Tag Editor.

When creating locus tags, remove the unused bases from the tags to avoid non-specific and noise peak identification. For example, you may remove the C and G bases from an A/T SNP.

Select only the appropriate tags when analyzing data. Tags that do not have the representative peaks in the raw data may lead to wrong allele calling and additional fragment list entries in the analyzed data.

Appendix D

Positive Control:
- A positive control is provided to verify that the SNPStart reagents are functional.

Preparation of the Positive Control:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNPStart Master Mix</td>
<td>4.0 μL</td>
</tr>
<tr>
<td>Positive Control</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>DNase/RNase Free PCR Grade Water</td>
<td>5.0 μL</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>10.0 μL</strong></td>
</tr>
</tbody>
</table>

- For thermal cycling (25 cycles) and post extension steps, follow the same protocol as sample preparation.

- A successful reaction will contain two homozygous and a heterozygous locus.

The following alleles are observed in the positive control:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ex Base</th>
<th>Homozygous/Heterozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>C</td>
<td>Homozygous</td>
</tr>
<tr>
<td>Control 2</td>
<td>A</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>Control 2</td>
<td>G</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>Control 3</td>
<td>T</td>
<td>Homozygous</td>
</tr>
</tbody>
</table>

The illustration below represents a positive control sample. Your fragment sizes may vary from this example.
<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Allele Signals.</td>
<td>Insufficient primer concentration.</td>
<td>Increase the primer and/or PCR template concentration.</td>
</tr>
<tr>
<td></td>
<td>Insufficient PCR template.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poor template quality.</td>
<td>Run templates on an agarose slab gel to verify template quality and quantity.</td>
</tr>
<tr>
<td></td>
<td>Incomplete inactivation of Exo/SAP after PCR.</td>
<td>Follow the manufacturer’s recommendations.</td>
</tr>
<tr>
<td></td>
<td>Non-standard PCR buffers or additives are carried over along with PCR template.</td>
<td>Use Column purification to remove the reagents.</td>
</tr>
<tr>
<td>Extra peaks.</td>
<td>PCR artifacts.</td>
<td>Optimize PCR condition to get a clean single amplification product. Raising the annealing temperature helps on many occasions. Redesign of PCR primers may be necessary if artifact bands persist. Visualisation of artifact bands is dependent on the staining method used. Consider the sensitivity of the staining dye before deciding on the loading volume.</td>
</tr>
<tr>
<td></td>
<td>Incomplete Exo/SAP digestion of PCR product template. Residual dNTPs from PCR reaction will create multiple allele peaks.</td>
<td>Ensure that fresh Exo/SAP is being used. Raising the enzyme concentrations or extending the time for the 37°C digestion step should yield cleaner PCR products. Residual PCR primers may cause extra allele signals.</td>
</tr>
<tr>
<td></td>
<td>Incomplete SAP digestion of unincorporated terminators after primer extension. Dye terminators will co-migrate with short SNP products to create false allele signals.</td>
<td>Ensure that fresh SAP is being used. Raising the enzyme concentrations or extending the time for the 37°C digestion step should yield cleaner SNP products.</td>
</tr>
<tr>
<td></td>
<td>Over-ranged peaks.</td>
<td>Cross talk being identified as peaks. Reanalyze the data using the system dye spectra. If the raw data peaks are over ranged (above 80,000 rfu) reload the sample after appropriate dilution.</td>
</tr>
<tr>
<td></td>
<td>Allele peaks are too close together.</td>
<td>Redesign interrogation primers and increase spacing.</td>
</tr>
<tr>
<td></td>
<td>Size standard not being identified correctly. Sample peaks close to either of the size standard or the peaks are too tall compared to the size standard peaks.</td>
<td>Design primers further away from the size standard, especially for samples having an &quot;A&quot; base allele. Reload the sample after appropriate dilution.</td>
</tr>
<tr>
<td>Fragment list contains entries with zero for migration time-peak height but has entries for locus tags.</td>
<td>Listed locus tags are not found in the sample.</td>
<td>Do not use tags for fragments known to be missing in the sample.</td>
</tr>
<tr>
<td>Data Analysis errors.</td>
<td>Size standard is not identified correctly.</td>
<td>Use appropriate heights when loading a volume of SNP product to get an equal proportion in peak height of size standard and sample. Presence of additional (artifact) peaks close to the size standard peaks will lead to analysis errors. Optimize SNP reaction to reduce artifact peaks.</td>
</tr>
<tr>
<td></td>
<td>Size standard 88 peak does not appear before the end of the run.</td>
<td>Preheat capillaries to 50°C prior to the start of the run. Extend the run time to 17 minutes and reload the samples.</td>
</tr>
</tbody>
</table>

**GHS HAZARD CLASSIFICATION**

**GenomeLab SNPStart Master Mix**
- H302 Harmful if swallowed.
- H315 May be harmful in contact with skin.
- H370 Causes damage to organs.
- P260 Do not breathe vapors.
- P264 Wash hands thoroughly after handling.
- P270 Do not eat, drink or smoke when using this product.
- P301+P312 IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell.
- P308+P311 IF EXPOSED OR CONCERNED: Get medical advice/attention.

**CEQ™ Sample Loading Solution (SLS)**
- H360 May damage fertility or the unborn child.
- P201 Observe special instructions before use.
- P208 Wear protective gloves, protective clothing and eye/face protection.
- P301+P313 IF exposed or concerned: Get medical advice/attention.

**EUROPEAN HAZARD CLASSIFICATION**

**GenomeLab SNPStart Master Mix**
- R18 Flammable.
- R20/21/22 Harmful by inhalation, in contact with skin and if swallowed.
- R36/37/38 Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed.
- S16 Keep away from sources of ignition - No smoking.
- S19 Keep away from sources of ignition - No smoking.
- S27 Take off immediately all contaminated clothing.
- S36 S37 S38 Wear suitable protective clothing and gloves. In case of insufficient ventilation, use suitable protective face equipment. In case of accident or if you feel unwell, seek medical advice (show the label where possible).

**CEQ Sample Loading Solution (SLS)**
- S36 Wear suitable protective clothing.
- S45 In case of accident or if you feel unwell, seek medical advice (show the label where possible).