P/N 390315-AD
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GenomeLab™ SNP-Primer Extension Kit

FOR RESEARCH USE ONLY

Storage of the SNP-Primer Extension Kit and all components must be in a -20°C **non-frost free** freezer. Keep all components on ice while preparing the reactions.

Introduction

The SNP-Primer Extension Kit is designed to provide robust single or multiplex genotyping of Single Nucleotide Polymorphisms (SNPs) in DNA templates using the Genetic Analysis System. SNP products are created by the hybridization of an unlabeled locus interrogation primer (or primers) to one or more complementary templates followed by single-base extension by fluorescent dye labeled terminators and DNA polymerase. The resulting labeled SNP fragments are combined with an aliquot of the DNA Size Standard 80 and loaded into the Genetic Analysis System for automated separation, detection, and genotyping.

Material Required

Materials provided by Beckman Coulter:

SNP-Primer Extension Kit (P/N 390280):
- DNA polymerase
- Dye Terminators (ddUTP, ddGTP, ddCTP, ddATP)
- Reaction Buffer
- Mineral Oil

Sample Loading Solution (P/N 608082)

Required materials not provided by Beckman Coulter:

- Deionized, distilled water (molecular biology grade)
- Sterile tubes - 0.5 mL microfuge, 0.2 mL thin wall thermal cycling tubes or microwell plates
- Thermal cycler with heated lid
- Shrimp Alkaline Phosphatase (SAP)
- Single Nucleotide Polymorphism (SNP) interrogation primer(s)
- Purified DNA Template(s)

Optional materials not provided by Beckman Coulter:

Exonuclease I (for PCR product purification)

Preparation and Storage of the SNP-Primer Extension Premix:

The following premix preparation protocol will provide optimal results for single and multiplex primer extension.

1. Prepare SNP-Primer Extension Premix in a sterile 1.5 mL microcentrifuge tube:

Component	Volume
10x Reaction Buffer	210 µL
ddUTP Dye Terminator	210 µL
ddGTP Dye Terminator	210 µL
ddCTP Dye Terminator	210 µL
ddATP Dye Terminator	210 µL
Polymerase Enzyme*	105 µL
Total	1155 µL

*__CAUTION__ The DNA polymerase enzyme is in a 50% glycerol solution. Pipette this solution slowly and carefully. The viscosity of the glycerol in the enzyme solution can lead to pipetting errors.

2. Mix and aliquot 190 µL into sterile 0.5 mL microfuge tubes. Each 190 µL aliquot is enough for 16 samples.
3. Store the aliquots in a -20°C non-frost free freezer. Minimize freezing and thawing of the SNP-Primer Extension Premix aliquots.

Preparation of the SNP-Primer Extension Reaction:

Refer to the Appendices for recommendations on DNA Template Preparation, SNP Interrogation Primer Design, and Troubleshooting.

Recommended starting points are 0.2 µM for each SNP primer and 100 fmol template. If performing a multiplex SNP reaction, create a primer premix such that each primer has a final concentration of 0.2 µM. Individual primer concentrations can be raised or lowered if allele signals are consistently low or high. Total primer per reaction should not exceed 4 pmol.

Prepare each 20 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.

Component	Volume
Sterile deionized water (to adjust total volume to 20 µL)	x.x µL
SNP-Primer Extension Premix	11.0 µL
dsDNA template [10 – 500 fmol]	x.x µL
SNP interrogation primer [0.1 – 1.0 µM]	1.0 µL
Total Volume	20.0 µL

Important: Mix reaction components thoroughly. Consolidate the liquid to the bottom of the tube or well by briefly centrifuging before thermal cycling.

Thermal Cycling Program:

Program the thermal cycler for 25 cycles of the temperature steps below followed by holding at 4°C. Use calculated temperature control, heated lid, and rapid thermal ramping between steps.

Temperature Step	Time
96°C	10 sec
50°C*	5 sec
72°C	30 sec

*Adjust annealing temperature to 5 degrees below the lowest T_m of the interrogation primer(s).

Post-reaction Sample Cleanup:

Unincorporated dye labeled terminators will co-migrate with small SNP products and interfere with analysis if left untreated. A simple digestion by shrimp alkaline phosphatase will hydrolyze 5' phosphate groups of unincorporated dye terminators to reduce their charge and thus their mobility during electrophoretic separation.

1. Add 1 unit of Shrimp Alkaline Phosphatase to each sample well or tube and mix thoroughly.
2. Incubate samples for 1 hour at 37°C to treat unincorporated dye terminators.

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3. Incubate samples 15 minutes at 75°C to deactivate the enzyme. SAP digested samples are now ready for dilution and addition of labeled size standards.

Store samples at 4°C or on ice if running the same day. Otherwise store samples at -20°C.

Sample Preparation for Loading into the instrument:

Add 0.5 µL purified SNP reaction to 0.5 µL Size Standard 80 (P/N 608395) and 39 µL of Sample Loading Solution for each sample in a fresh sample plate (P/N 609801) and overlay each of the diluted samples with one drop of light mineral oil. Load the sample plate into the instrument and select the desired method.

Important: 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.

Appendices

Appendix A - DNA Template Preparation:

Plasmid templates require no additional cleanup prior to their use in primer extension reactions.

PCR products contain unincorporated primers and dNTPs that will interfere with primer extension reactions. Exonuclease I (Exo I) digests residual PCR primers and Shrimp Alkaline Phosphatase (SAP) inactivates residual dNTPs. Follow the manufacturer's Exo/SAP protocol for preparing PCR templates for sequencing reactions. PCR products should be purified individually and combined if multiplexing.

Appendix B - Single and Multiplex SNP Interrogation Primer Design

The following recommendations should be considered when designing single and multiplex SNP primers.

- Primers should be purified via high-performance liquid chromatography to minimize the presence of n±1 primer products which may cause false allele signals.
- 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 step. Check primer sequences with suitable primer design software or run a primer extension reaction with the primer and no template to assay for self-priming interactions.
- The 3' terminal bases of the primers must not be complementary to any region of the primers. If locus interrogation is problematic for one strand, design primers to anneal to the complementary strand of DNA template.
- The minimum recommended spacing between SNP products and also between SNP products and the SNP Reference fragments is 4-8 bases (more spacing for smaller fragments and less for larger fragments). Lengths of primers can be adjusted by adding homopolymeric (poly dT, dC, or dA) tails to the 5' end of the primer.
- Due to effects of base composition and incorporated dyes, migration times of short fragments can differ significantly from predicted times based on size alone. Customers running multiplex primer sets are recommended to run individual reactions with probes of each allele size to determine if signal overlap will occur, and if necessary, design primers with a greater number of bases between adjacent SNP signals. Alternatively, it may be necessary to analyze products with overlapping signals in separate pools.

Appendix C - Troubleshooting

Low Allele Signals may be attributable to insufficient primer concentrations or inefficient hybridization of primers. Possible causes and remedies:

- *Insufficient annealed primer concentration.* Primer base composition and length significantly affect annealing efficiency. Increase the primer concentration to compensate for lower hybridization efficiency.
- *Suboptimal thermocycling conditions.* Decrease thermal cycling annealing temperature or increase annealing time from default values if shorter SNP signals are weak. Larger multiplexes may require longer extension times.
- *Poor template quality/quantity.* Run templates on an agarose slab gel to verify template quality and quantity.
  Note: Overall signal intensities can be raised by increasing the sample injection time of the SNP-1 method.

Extra Peaks may be attributable to insufficient cleanup of PCR products or extension reactions. Possible causes and remedies:

- *Incomplete Exo/SAP digestion of PCR product template.* Residual dNTPs will create allele peak patterns that resemble Sanger sequencing reactions. Residual PCR primers may cause extra allele signals. Ensure that fresh Exo/SAP is being used. Raising the enzyme concentrations or extending the time for the 37°C digestion step may yield cleaner PCR products.
- *Incomplete SAP digestion of unincorporated terminators after primer extension.* Dye terminators will co-migrate with short SNP products to create false allele signals. Ensure that fresh SAP is being used. Raising the enzyme concentrations or extending the time for the 37°C digestion step may yield cleaner SNP products.

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 an SNP product should remain the same in the analyzed data.

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