

FARG Tutorial 2003

Part 2

Single Base Extension: A Method
for Detecting Multiple SNPs Using
Automated DNA Sequencers

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Objective

- Discuss the the basics of Single Base Primer Extension
- Primer selection
- Examples using the ABI Prism[®] SNaPshot[™] ddNTP Primer Extension Kit
- Chemistry and cleanup methods
- Sample electrophoresis: Gels v. Caps

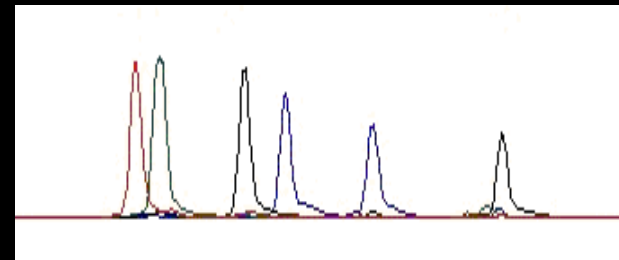
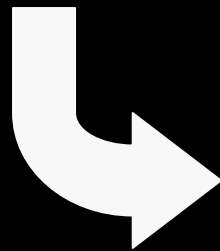
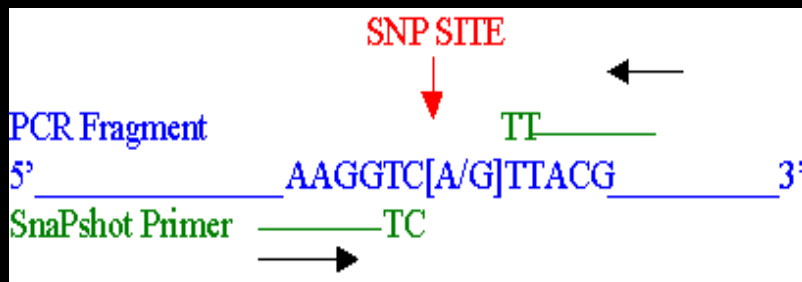
Single Base Extension: Advantages

- Base discrimination at the target SNP
 - Homozygous and Hetrozygous status
- Can be cheaper than conventional sequencing for multiple SNP sites
- Sense and anti-sense strands can be evaluated simultaneously for confirmation of SNP
- Can be run on any conventional DNA sequencer available in most labs.
 - No new equipment costs
 - Sequencing dyes used
- Allows verification of SNP before high throughput screening

SNaPshot[®] Overview

3 Step Process

- Amplify PCR fragment containing the SNP of interest
 - Unlabeled primers
- Perform primer extension
 - sense or anti-sense primers
 - Fluorescent ddNTP incorporated
- Visualization of SNP



Template Selection:

- Amplification of a PCR fragment containing the SNP site
 - Create any sized PCR fragment
 - 30 - >500 base pairs
 - Same primers can be used for both amplification steps as long as the primers are specific to the region of interest
 - Multiple SNPs can be contained in one large PCR template for primer extension
- Plasmid Templates
 - Do not require cleanup before primer extension

Primer Selection: SNaPshot™ Primers

- Primer is designed to terminate directly 5' of SNP site
- Primers can be designed in both the forward and reverse directions if conformation is needed
 - Some heterozygous peaks may be easier to see in a different direction depending on the bases
 - Check for normal pitfalls of self priming etc.
- Primers can be tailed 5' to allow for size multiplexing of the SNPs
 - Allow at least 4 base pairs between products
 - HPLC purify any oligo's larger than 30 base pairs to eliminate N-1 populations
 - Largest fragment we have done is 60 base pairs

Primer Example: Platelet-Activating Factor Acetylhydrolase (PAF-AH).

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361  gtacagactt aatgtttgat cacactaata agggcacctt cttgcgttta tattatccat
421  cccaagataa tgatcgcctt gacacccttt ggatcccaa taagaatat ttttggggtc
481  ttagcaaatt tcttgaaca cactggctta tgggcaacat tttgaggtta ctctttgggt
541  caatgacaac tcttcaaac tgggaattccc ctctgaggcc tggtgaaaaa tatccacttg
601  ttgttttttc tcatgggtctt ggggcattca ggacacttta ttctgctatt ggcattgacc
661  tggcatctca tgggtttata gttgctgctg tagaacacag agatagatct gcatctgcaa
721  cttactatth caaggaccaa tctgctgcag aaatagggga caagtcttgg ctctacctta
781  gaaccctgaa acaagaggag gagacacata tagaaatga gcaggtagcg caaagagcaa
841  aagaatgttc ccaagctctc agtctgattc tctgacattga tcatggaaag ccagtgaaaga
901  atgcattaga tttaaagttt gatatggaac aactgaagga ctctattgat agggaaaaaa
961  tagcagtaat tggacattct tttgggtggag caacggttat tcagactctt agtgaagatc
1021 agagattcag atgtggtatt gccctggatg catggatggt tccactgggt gatgaagtat
1081 attccagaat tcctcagccc ctctttttta tcaactctga atattttcaa tatcctgcta
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1201 ggggttcagt ccaccagaat tttgctgact tcacttttgc aactggcaaa ataattggac
1261 acatgctcaa attaaagga gacatagatt caaatgtagc tattgatctt agcaacaag
1321 cttcattagc attcttacia aagcatttag gacttcataa agattttgat cagtgggact
```

3 codons of interest with the SNP sites highlighted in yellow

Primers Modified for Size Multiplexing

5'-3'	Primer Sequence	Size
SNE 4F	ccttcctt tattatccatccaagataatgac	32
SNE 4R	ccttccttc gggatccaaaggggtgtcaagg	30
SNE 7F	ccttc caaggaccaatctgctgcagaaa	28
SNE 7R	cttc gtagagccaagacttgcccct	26
SNE 11F	tccttcctc cttaaaggagacatagattcaaatg	36
SNE 11R	ttccttcct tagctttgttgctaagatcaatagct	34

Cleanup of PCR Template:

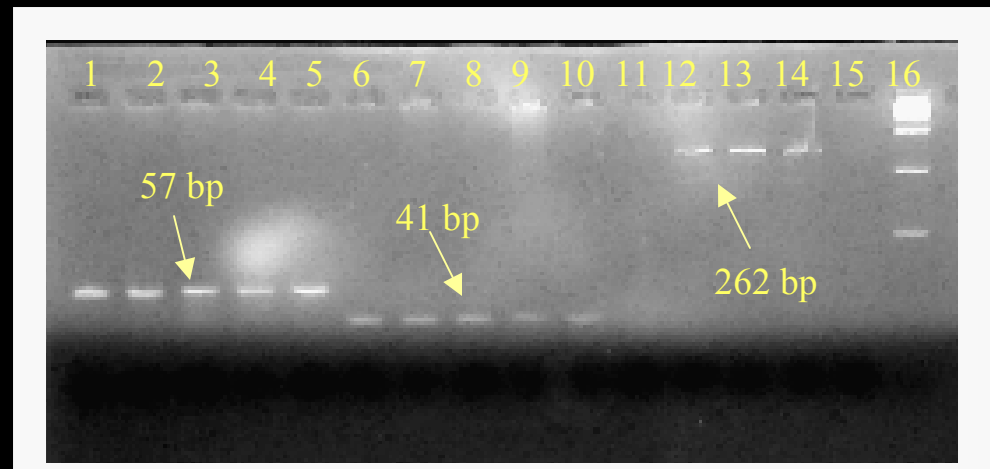
- Removal of unincorporated dNTPs
 - any excess dNTPs will be incorporated in the next reaction causing the primer extension to go past the SNP site and confuse results
- Removal of any excess primer
 - Any single stranded DNA will be extended in the SNaPshot[®] reaction and can cause confusion in data analysis

Clean-up Methods

- PCR Purification Kits: Column cleanup
 - Can be obtained from lots of different vendors
 - Multiple step process
 - Larger PCR volume may be needed
- Sap and Exo I Treatment
 - 45 min incubation time
 - 1 step process
 - Smaller PCR volumes can be used

Preparing PCR Template For Primer Extension

- Cleanup of template
- Quantification of template: PCR or Plasmid
 - 0.15pmole template needed
 - Multiple templates need to be balanced for multiplexing the SNaPshot[®] reaction
 - Spec analysis
 - Gel analysis
 - 3% agarose
 - Low DNA mass Ladder



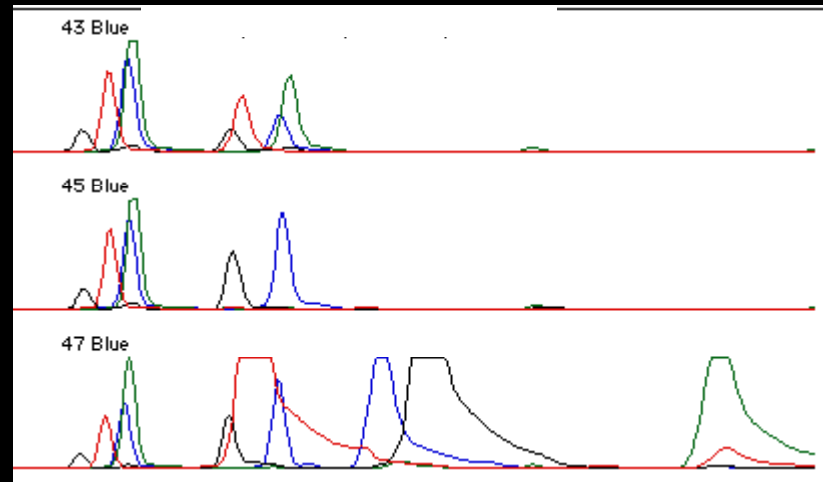
Primer Extension Reaction

- Cycling conditions: 3 step program
 - conditions from the protocol have worked on 18 of 18 SNPs tried
- Template concentration is critical
 - Overloading template causes reaction imbalance
- Primer concentration: 0.15pmole suggested
 - Can be titrated with other primers in multiplex for more product balance

Post-Extension Cleanup

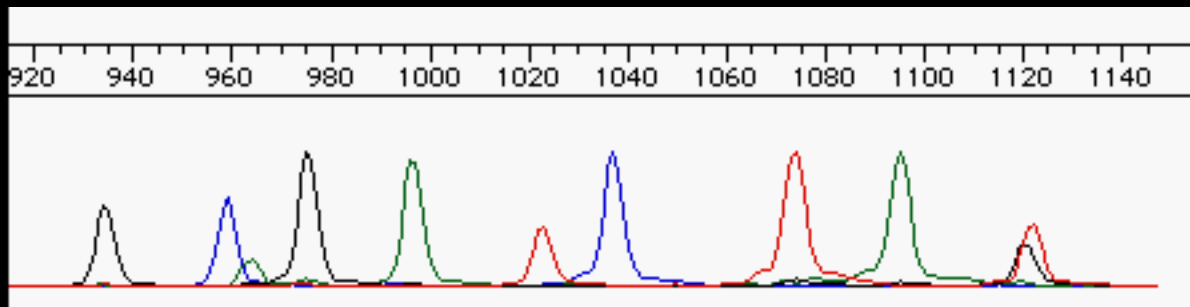
- Removal of excess labeled ddNTPs: SAP or CIP
 - 1 hour incubation
 - Hard to aliquot accurately
 - Dye blobs can confuse the picture

SAP was not
added to sample



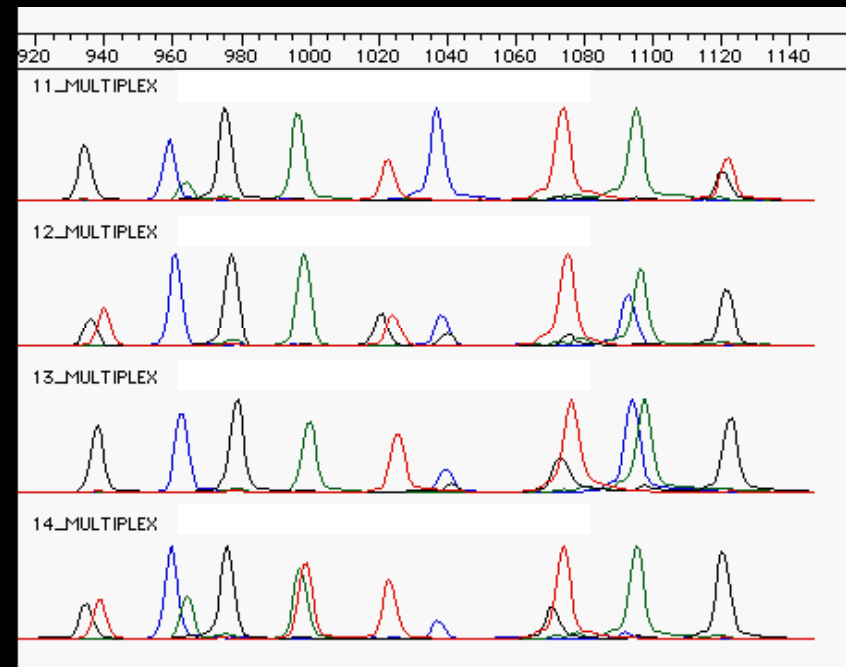
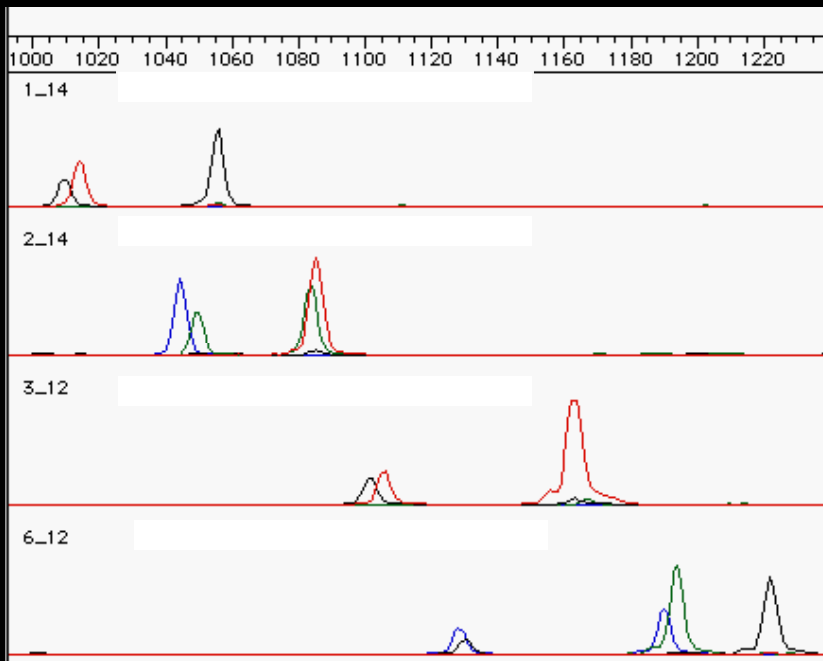
Running your Samples

- Which platform?
 - Capillary
 - ABI310, ABI3100, ABI3700
 - 120 Liz size standard
 - Automated calls
 - Gels
 - ABI377 – Filter set E
 - No sizing, view by scan lines
 - Manual calling of peaks



Multiplexing

- Smaller multiplexes are easier to balance
 - More expensive
- Mobility will be different than expected
 - Test sizes by running them separated



Problems

- Template contamination:
 - Use of same primer set can cause primer contamination in pcr templates
- SAP cleanup failing
 - Viscous solution, hard to aliquot 0.5uL
 - Dilution of SAP still can yield imperfect cleanup
 - Spinning samples after addition of SAP seems to help some

Problems Continued

- Template types
 - Whole genome amplification
 - Did not give consistent results when repeated, not sure of the reason
 - Try different amplification techniques, Genomiphi
- Verification of Target
 - Are we seeing the right thing?
 - Need to sequence the target template if uncertain
- Constant bands in PCR template
 - Can cause multiple peaks in SNP reaction
- Primer dimers forming from multiplexed primers in snapshot reaction
 - Check for primer interactions before setting up multiplexes