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GenomeLab™ SNPstream® Genotyping System

Autoprimer Tutorial

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Introduction

1.1 Autoprimer

Autoprimer (http://www.autoprimer.com) has guidelines and requirements for sequence content and format. The rule for minimum sequence length is mandatory, and it is highly recommended that other content guidelines be adhered to. The sequence format requirements are strict and must be followed exactly for successful primer design.

In addition to the sequence length, the content guidelines cover the sequence characteristics such as quality of the sequence data, and the GC content. There are also recommendations regarding BLASTing (Basic Local Alignment Sequence Tool) and masking of repeat/low complexity sequences.

If the user does not have the option of excluding a SNP with suboptimal characteristics, Autoprimer might still be able to design primers from the submitted sequence. However, certain deficiencies, such as insufficient number of bases surrounding the SNP locus, could result in failure to design the primers.

Once a sequence is qualified as a good candidate for the primer design, it must be formatted correctly for submission to Autoprimer.

This tutorial is a step-by-step guide to the primer design process. Italicized bullet points are from the SUPPORT section from *http://www.Autoprimer.com*. Some users may prefer a slightly different sequence of steps, i.e. performing repeat masking before BLASTing. It is important that all steps be performed, if possible.

Programming Tips

The Sections 6 and 7 provide tips on how to force Single Base Extension (SBE) primer design to one strand and how to use the *InSilico* PCR to check for PCR primer specificity.

URLs and Web References

The URLS and websites referenced in this tutorial are up-to-date at the time of print.

For updated information, send an email to autoprimersupport@beckmancoulter.com, or contact your local Beckman Coulter Technical Support Representative.

Autoprimer Design Process Tutorial

2.1 Sequence Content Guidelines

For this tutorial, panels will be designed from a test set of 70 human T/G SNPs obtained from the NCBI SNP database. The test sequences are shown in Appendix A.

It is helpful to have the sequences in FASTA format for direct submission to BLAST and RepeatMasker. A description of the FASTA format is found in www.ncbi.nlm.nih.gov/BLAST/fasta.shtml.

Briefly, the format requires:

- Single-line description preceded by ">"
- Lines of sequence data following the description line
- Standard IUPAC code
- Lower case letters
- A dash to indicate a gap of indeterminate length

NOTE Autoprimer does not accept dashes. Before submission to Autoprimer, dashes must be replaced by **N**s.

Determine the Sequence Length

Verify that the sequence meets the minimum sequence length requirement. (Mandatory)

- *The sequence should have at least 150 bases flanking each side of the SNP for a minimum sequence length of 301 bases.*

NOTE The SNP locus (represented by the IUPAC code "K") has been highlighted to make inspection easier. Since one line of sequence comprises ~100 bases, a SNP should have at least one-and-a-half lines of sequence on either side to qualify.

Determine the Acceptable Sequence

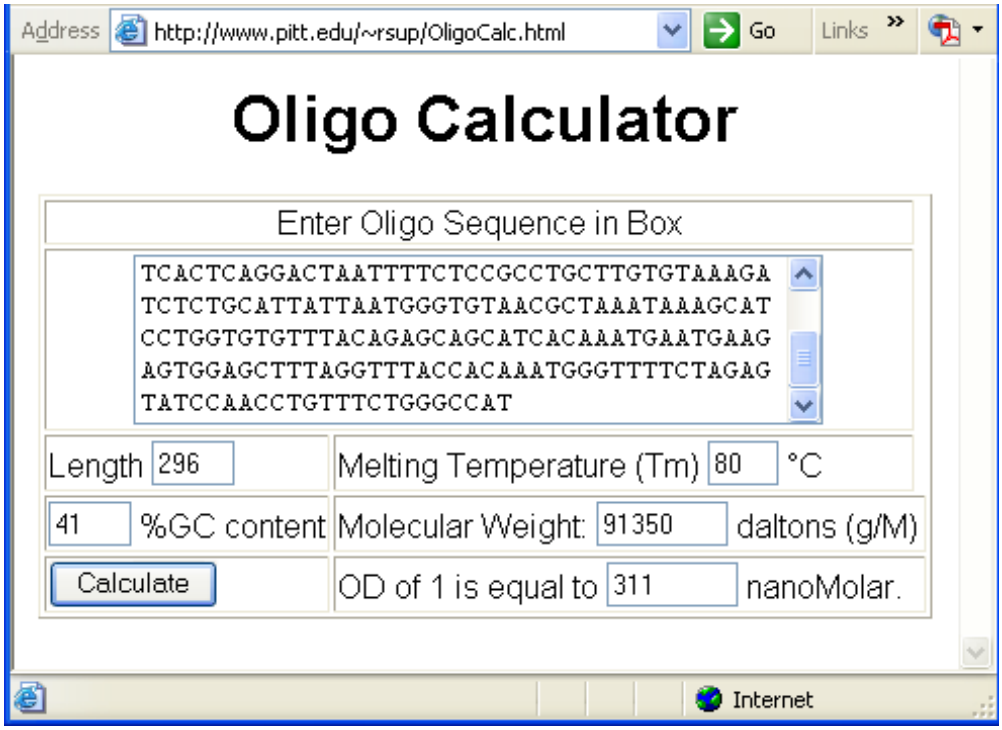
Determine if the sequence has acceptable GC-content (Recommended).

- *GC-content should be between 40 and 65%.*

  Check the region ±150 bases surrounding the SNP locus. Highly GC-rich regions could yield primers with "sticky" 3'-ends that bind non-specifically to other regions of the genome, or form heterodimers with other primers, or self-dimerize. Conversely, highly AT-rich primers do not bind efficiently to the target sequence.

GC-content calculators are usually available in the web as part of oligonucleotide analysis software. An exaxmple of a website that does not restrict sequence length is *http://www.pitt.edu/~rsup/OligoCalc.html*.The analysis of ~300 bases surrounding the SNP in the first test sequence, rs36950, is shown in Figure 2.1.

Figure 2.1 GC Content Calculator



Data Quality Score

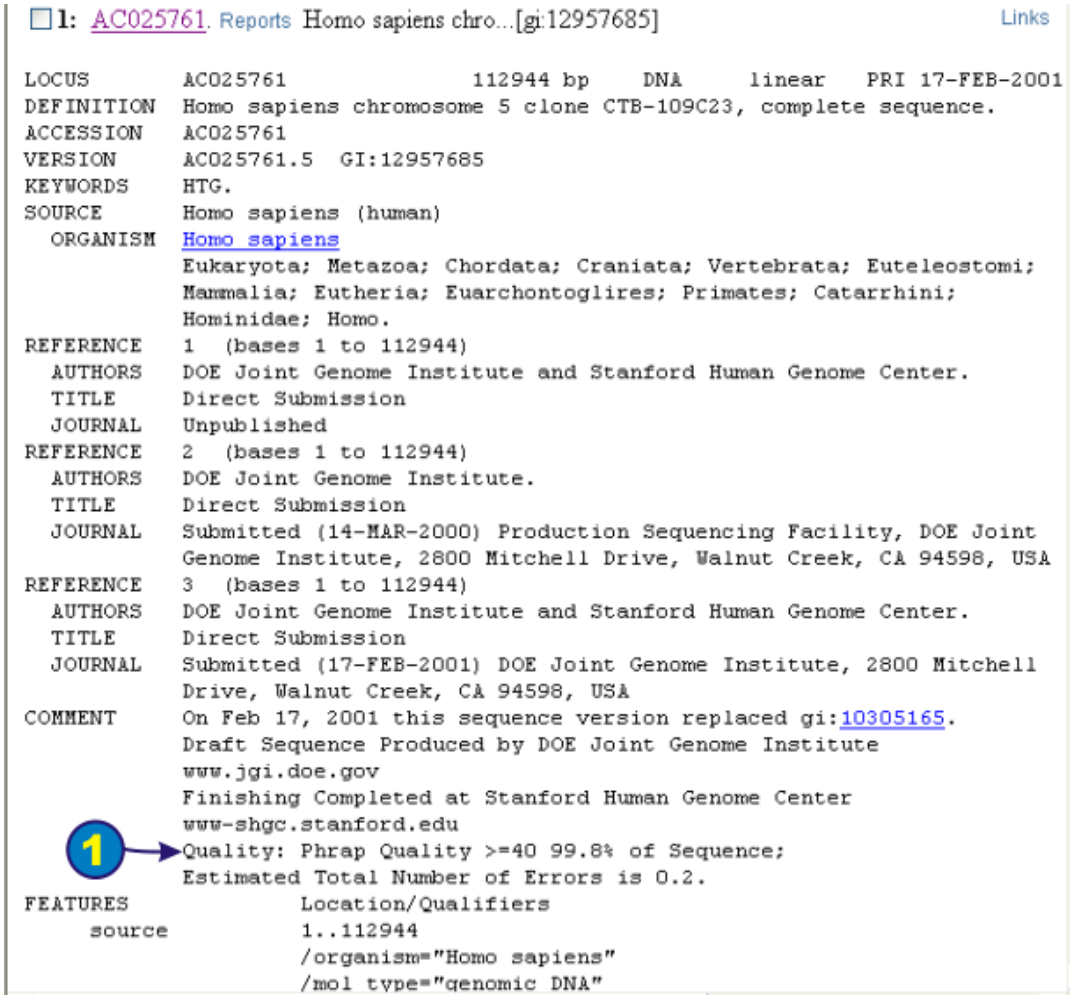
Obtain a Sequence Data Quality Score (Recommended).

- *Sequence quality should have a score of PHRED 20 or higher (alternative quality measurements to PHRED can also be used).*

NOTE PHRED is a common DNA quality measurement. For more information, please visit the following website: http://www.phrap.org

The PHRED software assigns a quality score to each called base in a sequence, while PHRAP assigns a quality score to each position on sequence contigs based on the PHRED scores. The error probability is approximately $10^{-Q/10}$, where Q is the quality score. For example, a PHRAP score of 20 corresponds to 99% accuracy for a base in the assembled sequence. The sequence quality scores, as shown below, may be included in the Genbank reports.

Figure 2.2 Sequence Quality Score in Genbank Report



Legend

1 = Quality Score

BLAST Search

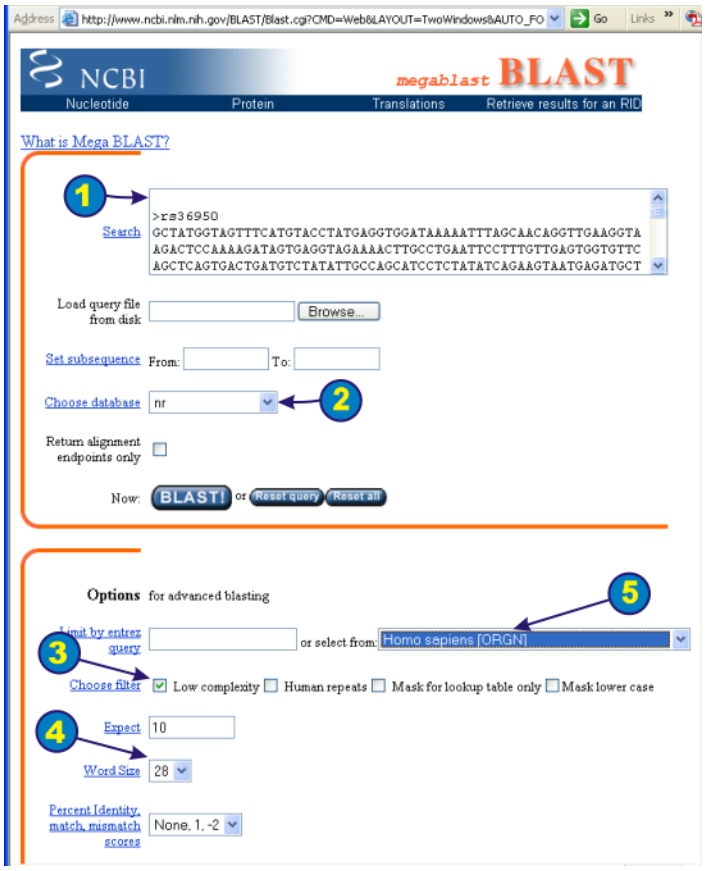
Perform a BLAST search. (Recommended)

- *You should BLAST your sequences to make sure they do not return High-Scoring Hits to more than one locus (e.g. pseudogenes).*

BLAST (Basic Local Alignment Sequence Tool; *http://www.ncbi.nlm.nih.gov/BLAST/*) compares nucleotide sequences to sequence databases and calculates the statistical significance of matches. There are several BLAST programs available. Megablast is suggested for performing quick searches for highly homologous sequences. Since the 70 test sequences are in a FASTA format, they may be submitted directly for BLASTing.

NOTE The Megablast accepts batch queries in a FASTA format.

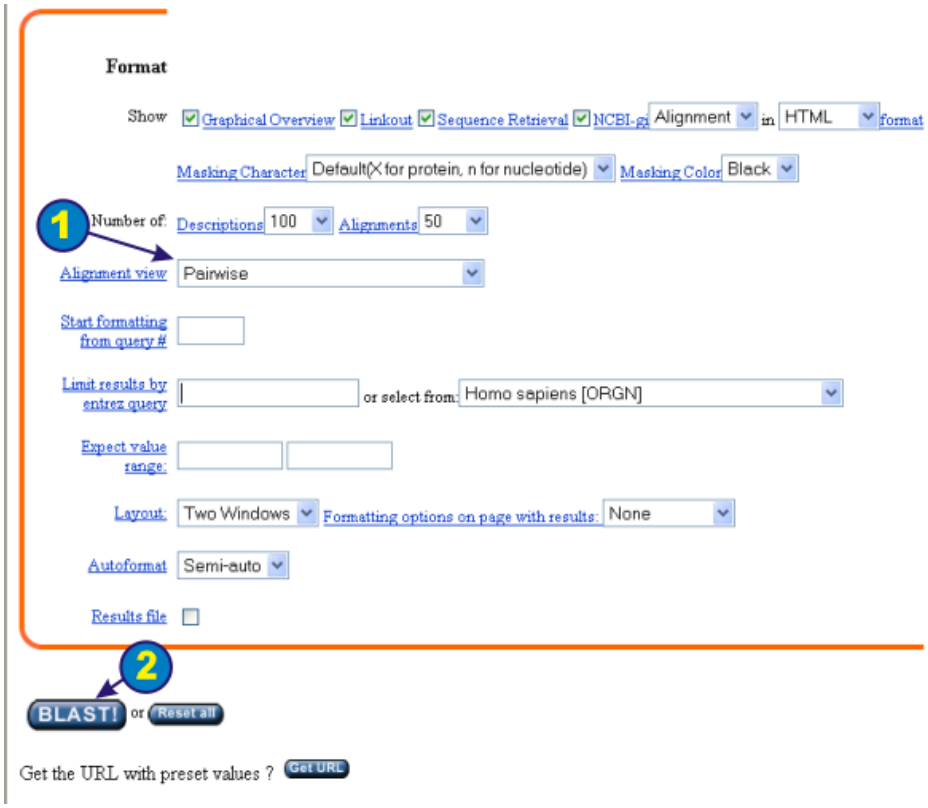
Figure 2.3 Sequence Submission to BLAST



Legend:

1. Paste sequences in FASTA format in the **Search** field or browse to the query file.
2. Select **nr** (non-redundant) database. This database includes GenBank + EMBL + DDBJ + PDB sequences (but no EST, STS, GSS, or phase 0, 1, or 2 HTGS sequences). According to the NCBI, is no “no longer ‘non-redundant’ due to computational cost.”
3. Select **Low complexity filter** (default) to remove the low-complexity sequences that may cause artifactual hits.
4. Use default **Expect** and **Word Size** values.
5. Limit the search by specifying the organism.

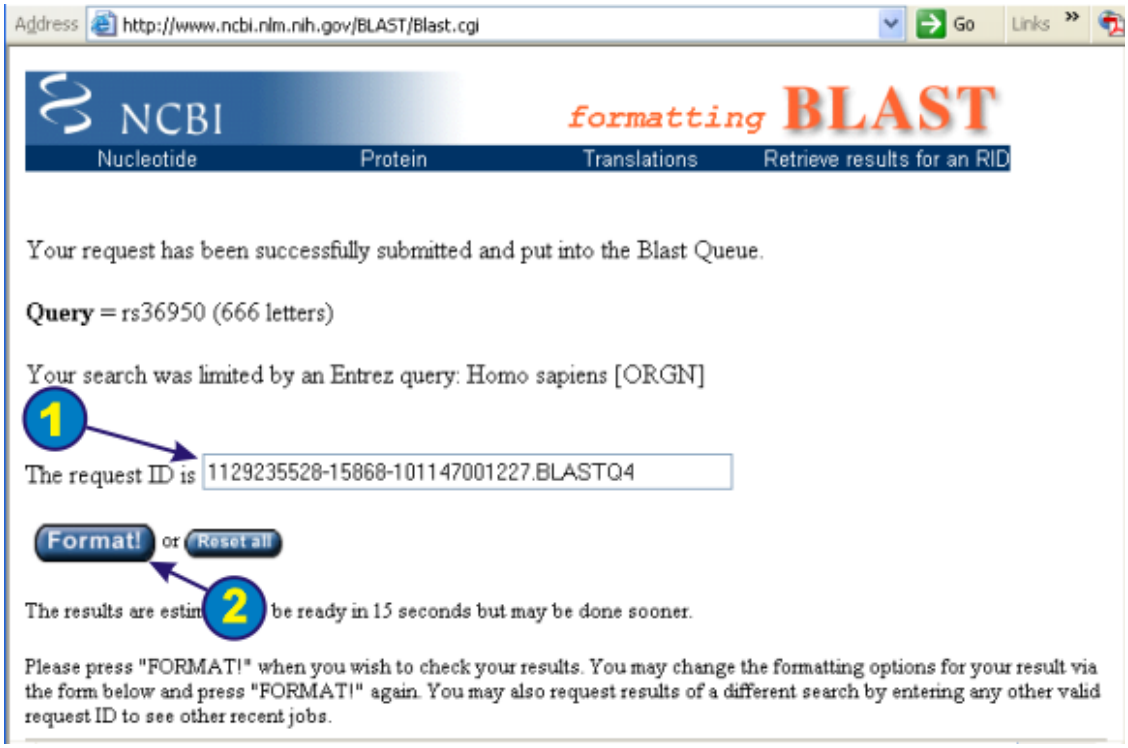
Figure 2.4 Sequence Submission to BLAST, cont'd.



Legend:

1. Select **Pairwise** as the alignment view.
2. Click **BLAST!** to start the search.

Figure 2.5 Sequence Submission to BLAST, cont'd.

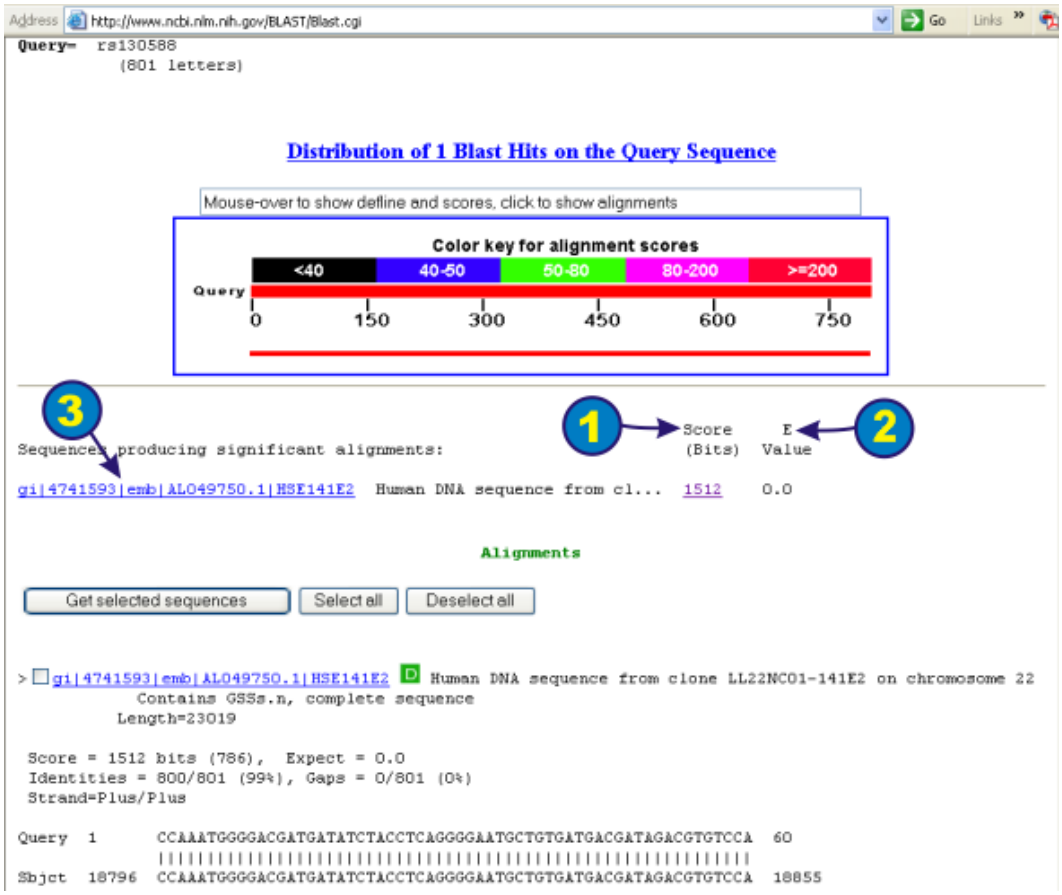


Legend:

1. A request ID is assigned to the query.
2. Click **Format!** to view the results.

Of the 70 test sequences submitted, 48 have single hits. The result for rs130588 is shown in the figure below.

Figure 2.6 BLAST Results: Single Hit

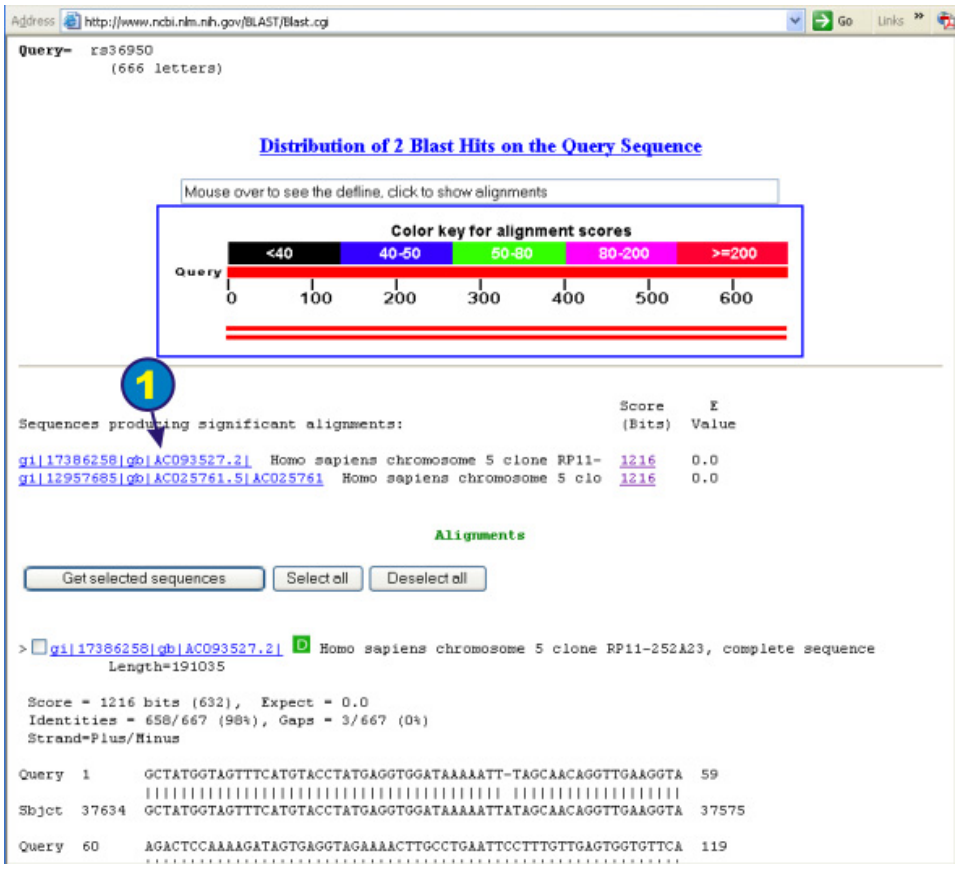


Legend:

1. The bit score gives an indication of how good the alignment is. The higher the score, the better the alignment.
2. The Expect Value (E-Value) gives an indication of the statistical significance of a given pairwise alignment and reflects the size of the database and the scoring system used. The lower the E-Value, the more significant the hit.
3. A link to the Genbank.

Sequence rs36950 has two hits (a single hit on each of two distinct clones), both on chromosome 5, with the same score, as shown in the figure below.

Figure 2.7 BLAST Results: Double Hit



Legend

1. The Genbank accession number

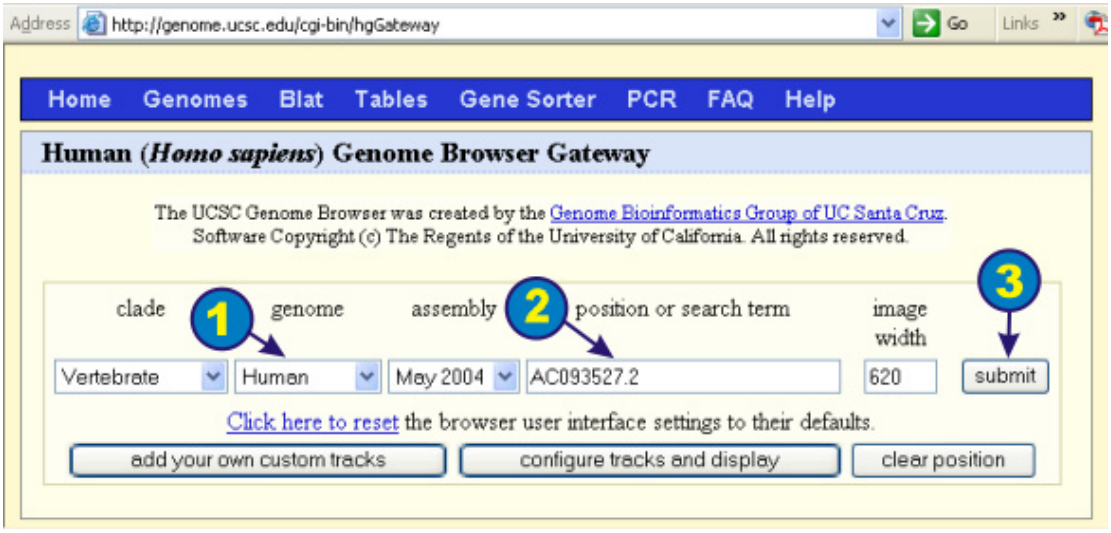
Are these hits overlapping and located in the same chromosomal position? In some cases, following the Genbank links and looking at the annotation for each clone does not give an unambiguous answer to this question.

It is possible to do a pairwise alignment of the two hits using Blast 2 Sequences (a program also available at the BLAST website) to determine how similar they are.

Another way to check the relationship between the two hits is to align them to the genome using the UCSC Genome Browser (*http://genome.ucsc.edu/cgi-bin/hgGateway*).

In the case of rs36950, the two hits have Genbank accession numbers of AC093527.2 and AC025761.5. See the figure below.

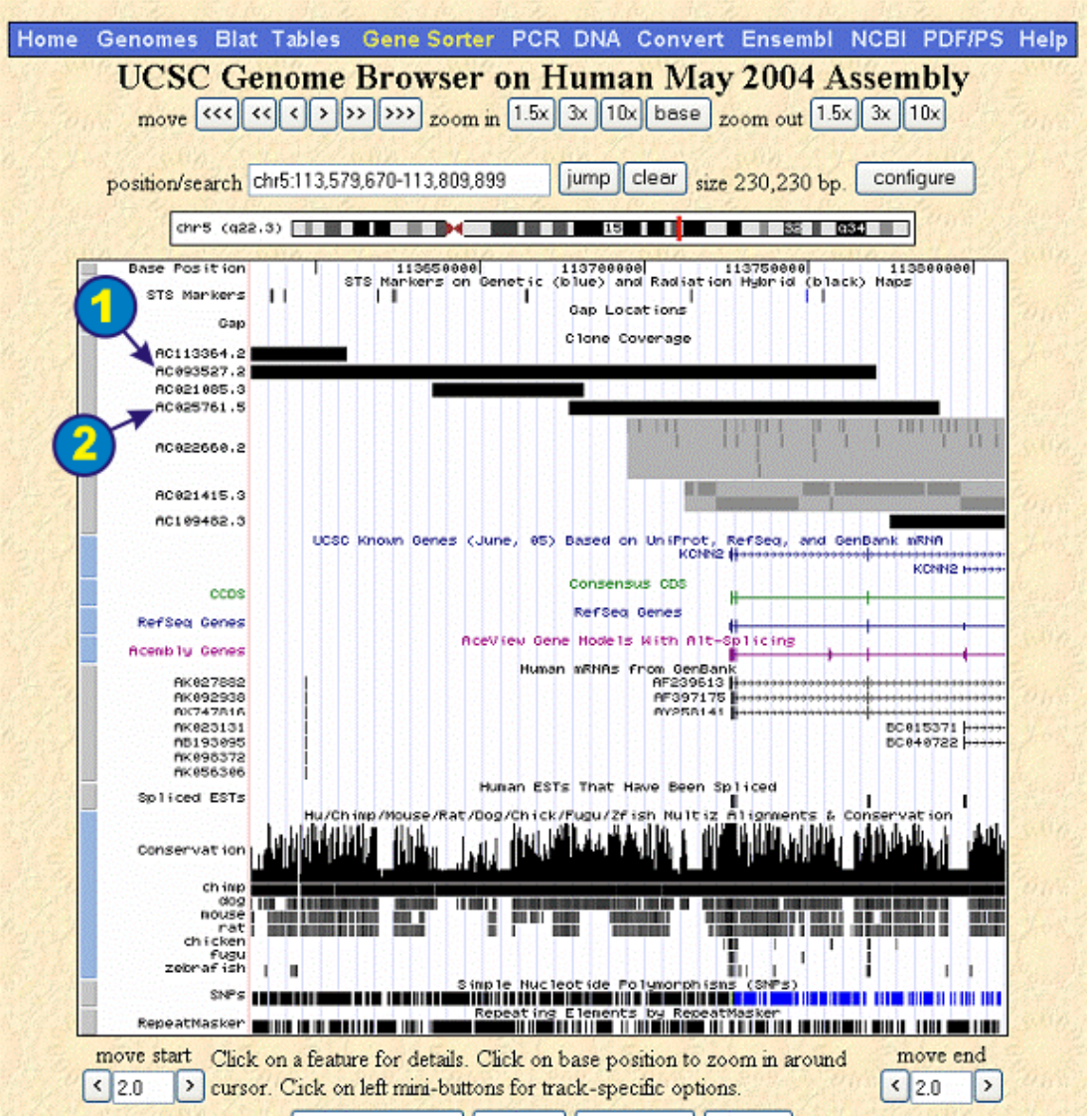
Figure 2.8 UCSC Genome Browser Search



Legend

1. Select the organism.
2. Enter the Genbank accession number of one of the two hits.
3. Click **Submit**.

Figure 2.9 Genome Browser Search Results



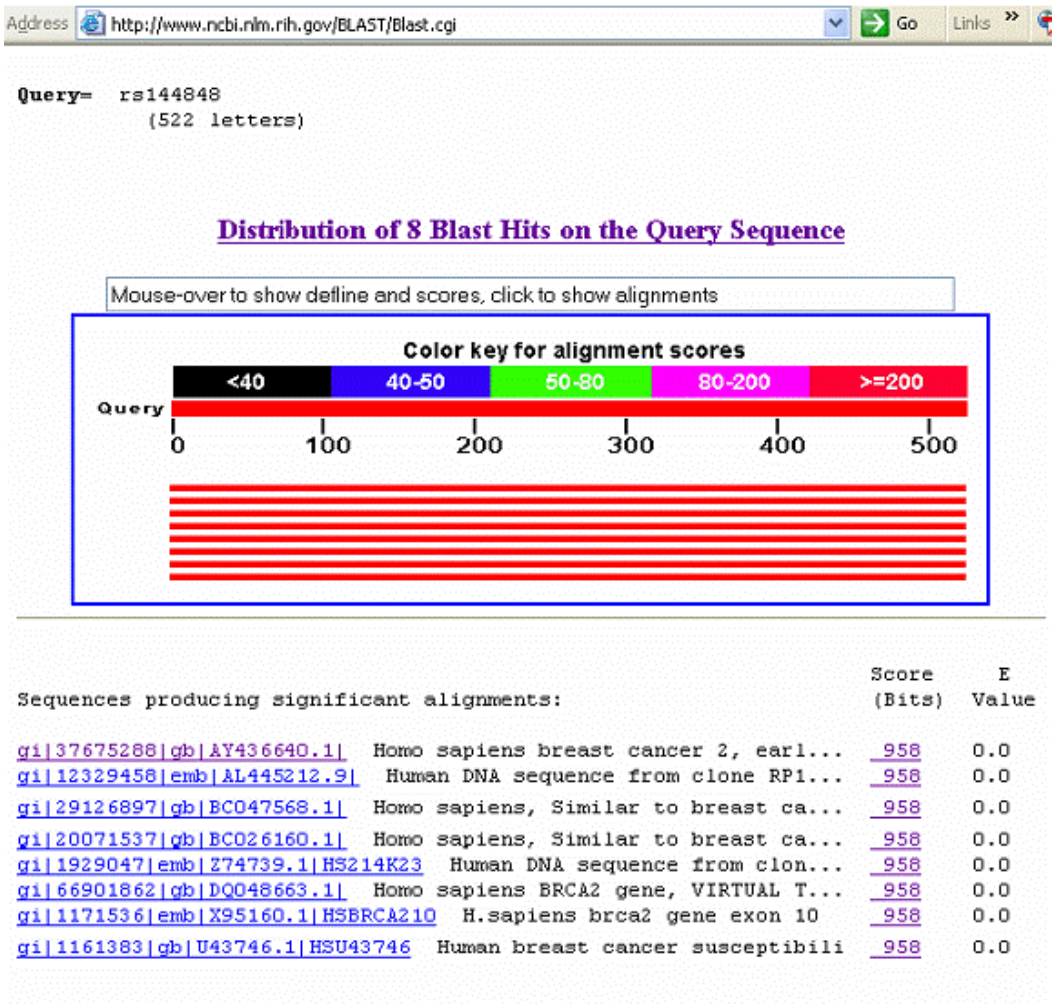
Legend

1. Hit #1, AC093527.2
2. Hit #2, AC025761.5

The results indicate that the two clones overlap, and suggest that there is in fact only one hit in chromosome 5.

NOTE This hypothesis may be checked by performing *InSilico* PCR with the Autoprimer-designed primers as shown in Section 7.

Figure 2.10 Blast Results: Multiple Hits

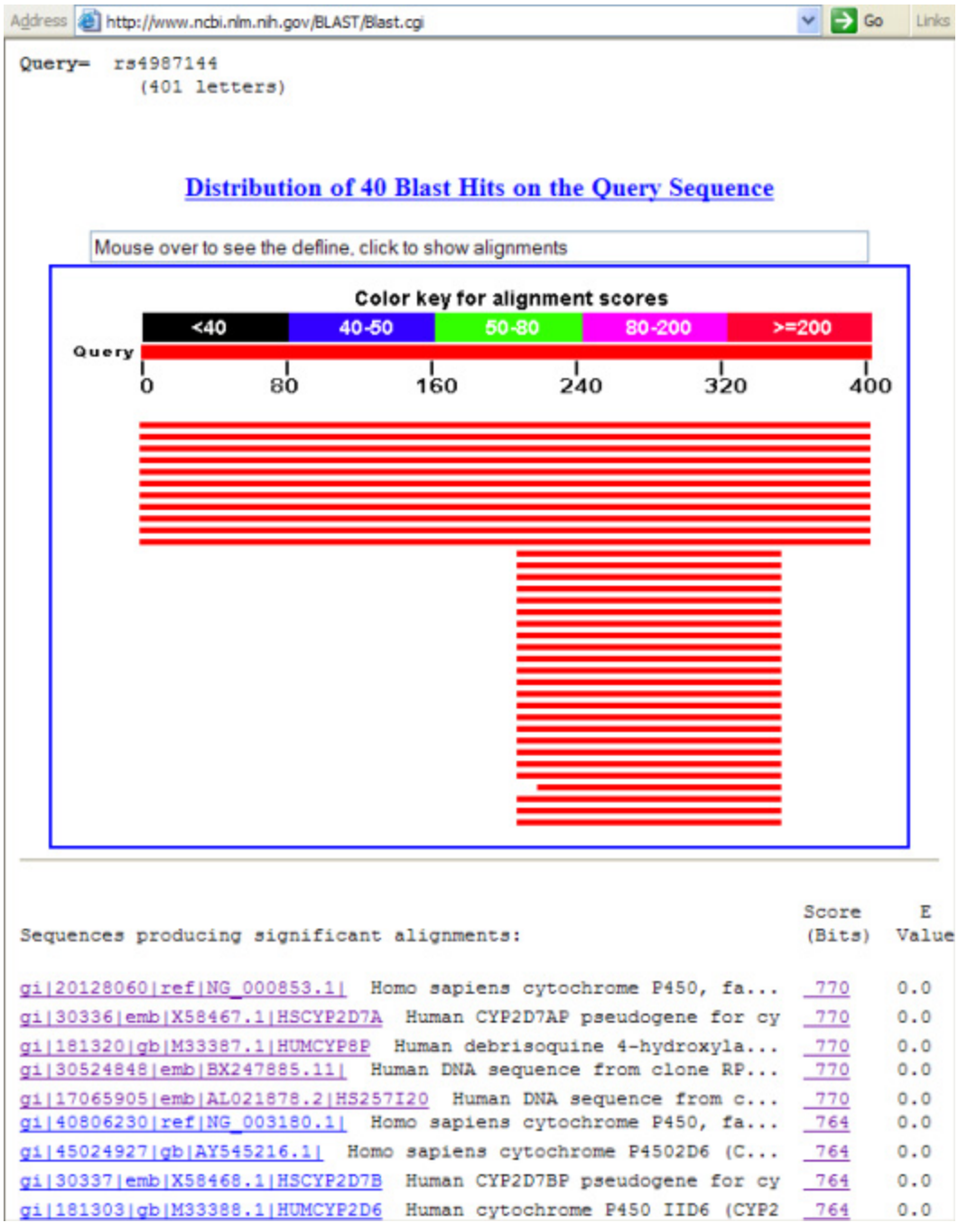


Most of the hits for rs144848 are clearly identified as the brca2 gene. Following the Genbank links on the other hits reveals that they are clones carrying the brca2 gene. Therefore, the multiple hits in BLAST are to the same locus. See Figure 2.10 above.

NOTE The alignments of the different hits may also be checked using the Gene Browser, as discussed in the previous example.

Other sequences in the test set with more than 2 high-scoring BLAST hits were analyzed using the Gene Browser. In most cases, the "multiple" hits turn out to be to the same locus. In a few cases, the accession numbers for one of the hits could not be found in the Gene Browser database. Again, primers designed for these latter sequences may be checked by *InSilico* PCR as shown in Section 7.

Figure 2.11 BLAST Results: True Multiple Hits



The first five hits have identical high scores. At least two hits map to distinct chromosomal regions, namely the CYP2D8P2 and CYP2D7AP genes. In this situation, additional sequence information is needed in order to design PCR primers that target less homologous regions, so that only the target SNP locus will be amplified. (The query sequence used in this example is not a member of the test set.)

Mask Repeat Sequences

(Recommended)

- *Repeat masking of the target sequence during primer design is highly recommended. If the repetitive elements are located outside 25 bases upstream or downstream of the SNP of interest, remove all repetitive elements by inserting N bases in their place. If the repetitive elements are located inside 25 bases upstream or downstream of the SNP of interest,* ***DO NOT*** *replace them with N bases.*
- *The Autoprimer needs both masked and unmasked sequences. The mask sequence indicates the positions to avoid when designing primers. The unmasked sequence gives the bases to use when calculating the energetics for primer design and when considering cross-hybridization.*

Repeat Masker

RepeatMasker screens DNA sequences for interspersed repeats and low complexity sequences. See Figure 2.12. The following information is from the RepeatMasker website (*http://www.RepeatMasker.org/*):

- Interspersed repeats:
  - Processed Pseudogenes, Retrotranscripts, SINES - Non-functional copies of RNA genes which have been reintegrated into the genome with the assistance of a reverse transcriptase
  - DNA Transposons
  - Retrovirus Retrotransposons
  - Non-Retrovirus Retrotransposons (LINES)
- Low complexity sequences:
  - Simple repeats (micro-satellites)
  - Poly-purine/poly-pyrimidine stretches
  - Regions of extremely high AT or GC

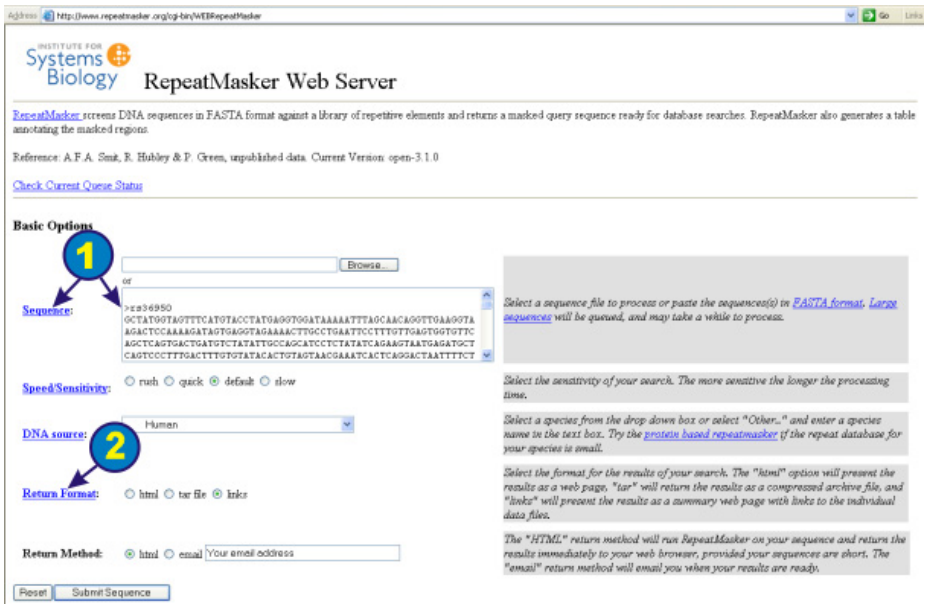
  "The settings are very stringent, and we think that few if any sequences informative in database searches are masked as low-complexity DNA."

  "A 100 bp stretch of DNA is masked when it is >87% AT or >89% GC, a 30 bp stretch has to contain 29 A/T (or GC) nucleotides."

The output is:

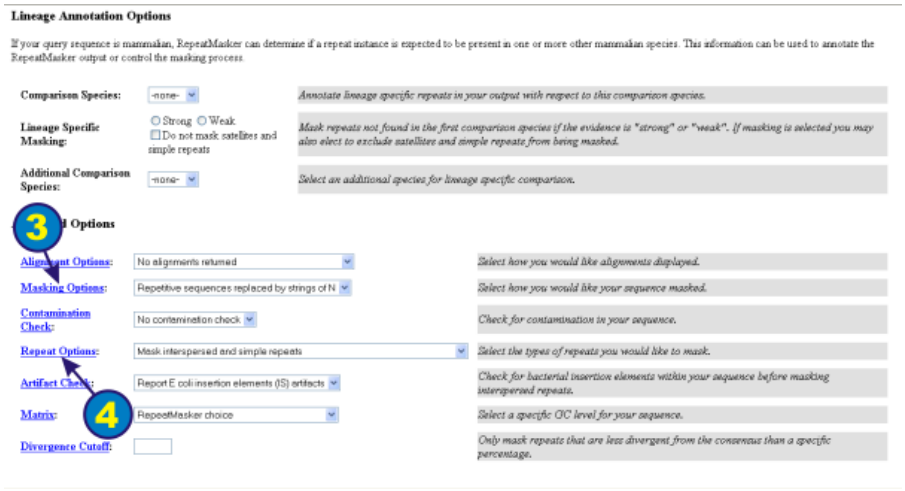
a. A detailed annotation of the repeats that are present in the query sequence, and
b. A modified version of the query sequence in which all the annotated repeats have been masked (default: replaced by Ns).

Figure 2.12 Sequence Submission to RepeatMasker



Legend

1. Paste the sequences in FASTA format; batch submissions are allowed.
2. Select **Links**, for the **Return Format**.



3. Select **Repetitive sequences replaced by strings of N,** for the **Masking Options**.
4. Select **Mask interspersed and simple repeats**, for the **Repeat Option**.

Figure 2.13 RepeatMasker Results

Legend:

1. Click on the link to open the results file.

Save the results as a .txt file. A portion of the file is shown below. The entire file is shown in Appendix B.

Figure 2.14 RepeatMasker Results (.txt)

```
>rs36950
GCTATGGTAGTTTCATGTACCTATGAGGTGGATAAAAATTTAGCAACAGG
TTGAAGGTAAGACTCCAAAAGATAGTGAGGTAGAAAACTTGCCTGAATTC
CTTTGTTGAGTGGTGTTCAGCTCAGTGACTGATGTCTATATTGCCAGCAT
CCTCTATATCAGAAGTAATGAGATGCTCAGTCCCTTTGACTTTGTGTATA
CACTGTAGTAACGAAATCACTCAGGACTAATTTTCTCCGCCTGCTTGTGT
AAAGATCTCTGCATTATTAATGGGTGTAACGCTAAATAAAGCKATCCTGG
TGTGTTTACAGAGCAGCATCACAAATGAATGAAGAGTGGAGCTTTAGGTT
TACCACAAATGGGTTTTCTAGAGTATCCAACCTGTTTCTGGGCCATGTTT
TGTACAGTTGAGAGATAAGGGCACTGTGGGCTGTCATCCCAAACCACGTT
TGGCAGGGAGCACCAAGGCAGCCTGCGTTGATTTAACTTCAGAGAAGTTA
TATTCAATTANGATAGGAAGTGGTTTTGGGGGGGTTGTTTTAAAGAGAAT
TTCTACTCATTAAGAAATAATATAATATTTGCTACTTTGGGAGTACATTT
TCAACCTGAAACTCCTTAATAATTCTATTTATAAAACTAGAAAACACATG
GNAGGGATCTTAATTA
>rs130588
CCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCTT
CAGCACCAGGGGAAATAAGAAAAAGCAAAGAAAGGGAAGTGCTGGTATAA
AGGGAAGGAAGGAAACCAAGGGCATCAGTGTGGATGGGGAAGGCAGAAAA
TGCTCTCCTGGGGAATCACAACCTTTGTATTATCAAGTGCTTTTTTTGAA
TTCAAGCCATGCTATTTCTTGCAGCTCTGACTATGTTTCAAGTTGGAATA
TCAAGCATCTCTGAGCCTTCACTACAGCAATTAAGGGCTCATAAAGCTAT
TCTTAGGAGTACAAAATAAACTGAGATATATTTGAGGTTGCCAGAAGGTT
KCCATGTGGAGAACTGAGTGCTAACCACTGGAAAGGTGCACATTAAAGAC
AGCAGGTGTGCAGAATGCTGTGGCCATAACTCTCTCCAGCTGAGGGCAGA
GGCCACAAAAAGGACCCTGCTGAGGGGAGGCTGCCAAGGAGCTCCACACT
CTGCCAAGAGGGGCCTGAGTTCTGGCGCCATCAGCAACTGTCTTGNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNATTCAACTAA
AGCCTGCAGATTCTTCCACTGCCCCCATGGTGGCCATCTGTTGAGGTGGG
AGGGAAACATGCTGGCCCTCCCCGTCTCCTACCCTAAAGAAGACACTGTG
C
>rs144848
GAATTCTCTGAAGTAAGAAAATCTTTCTTTCTTTTGTTCTCTGTGTCTAA
TAGGTCTTTTTCTGAAATATTTTGGTCACATGAAGAAATATGCAATAGGG
GTATTTTCTCCATCTGGGCTCCATTTAGACCTGAAAGGGTTAGTTGAGAC
CATTCACAGGCCAAAGACGGTACAACTTCCTTGGAGATTTTGTCACTTCC
ACTCTCAAAGGGCTTCTGATKTGCTACATTTGAATCTAATGGATCAGTAT
CATTTGGTTCCACTTCAGATACAAATGAGTATTTTTCTTTCACTTGGTTT
TTAGATTTTTCACATTCATCAGCGTTTGCTTCATGGAAAATTTTTTTCCT
AGTCTTGCTAGTTCTTACTTTTTGTAGATTTTTTGTTCTACATTTAGAAA
AACATAATGAAAAAACTATCTTCTTCAGAGGTATCTACAACTGTTTCATAG
ACTTCATCTTCTAGGACATTTGGCATTGACTTTCCCACGTGGTCTTTGCA
GCTATTTACTTTAAATGAATTC
>rs187031
```

Next, delete the sequences that were **not masked** by the RepeatMasker. The resulting file is shown in Appendix C.

Inspect the masked sequences. Make sure that there are at least 25 unmasked bases upstream **and** 25 unmasked bases downstream of the SNP. An easy way to do this is by **Edit | Find | K** (the SNP type for the test sequences). The following test sequences fail.

Figure 2.15 Example 1

```
>rs456798
CCCAAGGATGATCGGCTTAGCCTGTGATGCTGACAGGACTGAAACCTAGG
GCCTCTGCCTCTAAATCTCAGTTCATTTGACTCTGTCTGTGTCCCTGACA
GGCCTCAGCCTGGGACAGGGTGGGCAACGAAGAGGCCAGAGCAGCTGGGT
CCCAGGAAATGAGCACNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNGATCTCCCCACCAGGCATGAGAGTTGGGGGAGCGGG
GGGGCGGTTATTGCTGGGGATGCAGGTGAAATTATTCCAGTGATTCATTC
ATCCTAATTGACAAGGTTCTATTGAATTCCTTAAAAGCCCCAGGCTGTGC
ATTTTGGAAAACATGGCTGCAGTGCACTGGGAAATGTCCTCTGATATCAT
G
```

- Example 1: In Figure 2.15, the SNP locus is located inside a repeat region. If this is a must-have SNP, DO NOT submit the masked sequence to the Autoprimer.

Figure 2.16 Example 2

```
>rs717292
CCTGGTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNCACGTTCTAGA
```

- Example 2: In Figure 2.16, the entire sequence is a repeat region. If this is a must-have SNP, **DO NOT** submit the masked sequence to the Autoprimer.

Figure 2.17 Example 3

>rs727023
GGATCTCAAAAAATGTGGAGGTTGCATTCAATTACATTTGTGAATGTTAG
GAAACTGTCAACTGCCTTTGGAGTCCAAGGAGAGAGCCAGCAGACACCCT
TCTCACCACCCTGGGCTTTTCATGAGAGTGGCACTGCAATATCATCATCC
TAGCTTAGGGGAATTKGTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNCACACTGCTTCCCTTCTCTTCCGACAGGTTCCTGGAGA
CAAAGGAAAAGGGATATCTGGTGAGGCAGTAGAGGTCTAGTTCATCCACT
TAGAGATCATAGAGTCCTCCAGGTCGCAGGACTGGATGCTGAGATCC

- Example 3: In Figure 2.17, there are less than 25 unmasked bases downstream of the SNP. If this is a must-have SNP, modify the masked sequence before submission to the Autoprimer by restoring the original 25 bases downstream of the SNP. This is demonstrated on Section 3 on page 12.

Sequence Formatting Requirements

3.1 Transfer Requirements

The following are steps for transferring the sequence information, creating a .TXT file and the punctuation exceptions for use in the sequence.

- *Transfer the sequence information, one sequence per line, into the Notepad file. Be sure that no carriage returns are present within the sequence. Enter the SNP identifier/name at the beginning of the line and place one space between the identifier/name and the sequence. Sequence names should be restricted to the use of Alphanumeric characters with the following punctuation exceptions: Periods(.), dashes(-) and underscores(_). The sequence name should be 20 characters or less.*

NOTE Save the file with the .txt extension.

- *Masking*
  - *The format for a sequence to be masked is:*

    [SNP Name] [whitespace] [SNP sequence] [whitespace] [Mask sequence]
  - *Entries that aren't to be masked only contain:*

    [SNP Name] [whitespace] [SNP sequence]
  - *Whitespace is any contiguous series of spaces and/or tab characters.*
  - *[Mask sequence] is the same length as the corresponding [SNP sequence].*
- *Sequence annotation*
  - *The SNP to be designed for should be represented by the two base types separated by a forward slash and encased in brackets (i.e. a T to C SNP would be represented as [T/C] in the sequence for ...ATGGATCTTTATT[T/C]TCTTATCTA...*
  - *Other SNPs located in the sequence are represented with the following single-letter annotation:*
    - *R = G or A*
    - *K = G or T*
    - *S = G or C*
    - *W = A or T*
    - *M = A or C*
    - *Y = T or C*
    - *D = G or A or T*
    - *V = G or A or C*
    - *B = G or T or C*
    - *H = A or T or C*
    - *N = G or A or T or C*
  - *Other sequence variations (e.g. insertion/deletions, repeat elements, etc.) should be annotated by an uppercase n: N.*

NOTE DO NOT use brackets to identify insertion/deletions or any other sequence elements other than the SNP to be interrogated.

RepeatMasker.txt Conversion

1. Convert the RepeatMasker .txt file into a "single line" format.

   This involves manipulating both masked and original sequence files in Microsoft Word, Excel and Notepad. Each application is optimal for performing specific tasks to achieve the required format.

Save the masked sequence .txt file. (Appendix C) as a .doc file. A portion of the file is shown below. The entire file is shown in Appendix D.

Figure 3.1 Masked Sequences (.doc)

```
>rs130588
CCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCTT
CAGCACCAGGGGAAATAAGAAAAAGCAAAGAAAGGGAAGTGCTGGTATAA
AGGGAAGGAAGGAAACCAAGGGCATCAGTGTGGATGGGGAAGGCAGAAAA
TGCTCTCCTGGGGAATCACAACCTTTGTATTATCAAGTGCTTTTTTTGAA
TTCAAGCCATGCTATTTCTTGCAGCTCTGACTATGTTTCAAGTTGGAATA
TCAAGCATCTCTGAGCCTTCACTACAGCAATTAAGGGCTCATAAAGCTAT
TCTTAGGAGTACAAAATAAACTGAGATATATTTGAGGTTGCCAGAAGGTT
KCCATGTGGAGAACTGAGTGCTAACCACTGGAAAGGTGCACATTAAAGAC
AGCAGGTGTGCAGAATGCTGTGGCCATAACTCTCTCCAGCTGAGGGCAGA
GGCCACAAAAAGGACCCTGCTGAGGGGAGGCTGCCAAGGAGCTCCACACT
CTGCCAAGAGGGGCCTGAGTTCTGGCGCCATCAGCAACTGTCTTGNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNATTCAACTAA
AGCCTGCAGATTCTTCCACTGCCCCCATGGTGGCCATCTGTTGAGGTGGG
AGGGAAACATGCTGGCCCTCCCCGTCTCCTACCCTAAAGAAGACACTGTG
C
>rs424293
AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACAC
TGTCATCTTATAACGCTTTCCAATTCCCAAATACCTTTAAGGTTTGGTAA
AAATAATATACCTTCCACAAGTCATTTGATTTTTGTGGGCCTTAGTTTAT
CGACCTATTTGGATAGATGGGATGAAAAACCTTAGAGCCATCTTTCCTTA
CTCATTCCCCCTGCTAGTGAGAGAGAATTCAGTGGAATCAGAAATGGAAT
GAAGCATATACTTGGAGAAGTAAAAGAAATTTCCCAKAACAAAAGACAGA
AAGGGCCCAGTGAGCAATATAAATATGTATTTTATGTCCATATTTAGGCA
TATTGCAATTAAAGTTCAAAATAACAAAGATGATTTGAAAATATTATGTA
TCAAACACGGTTTTACTTACTGGACAGTATGTAGTATAGGTTGTGNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNTTAGAAACATATGAGAGATGATTGGCATAGTCAGCTTTGG
CTGCCATAACAAGATGCT
```

NOTE Each sequence line ends in a line break (paragraph mark ¶).

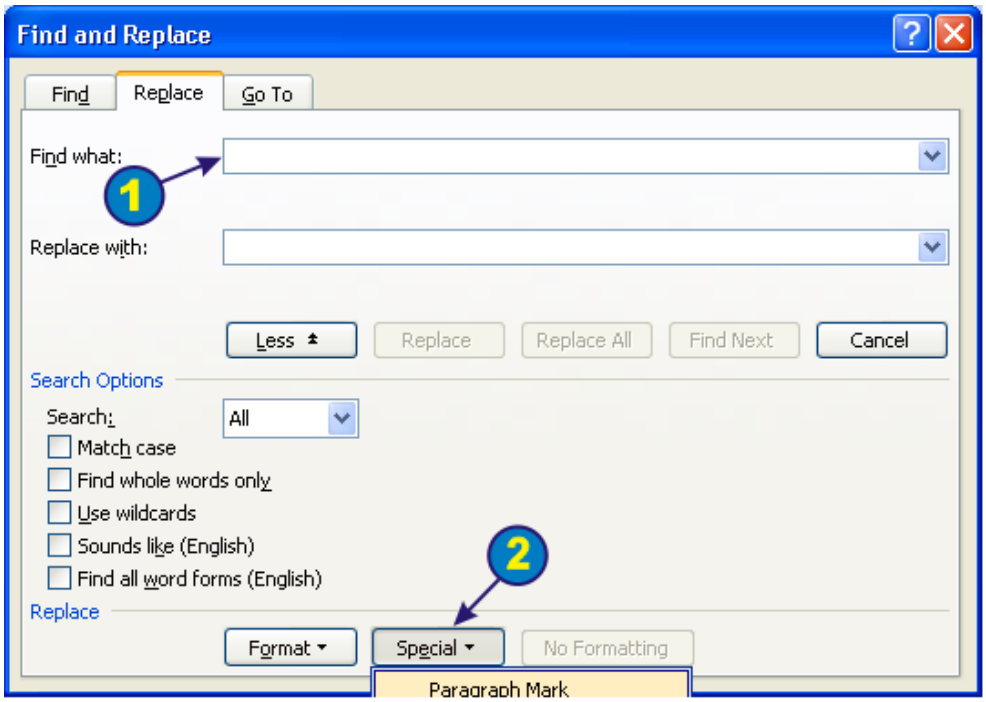
NOTE To display format marks click the ¶ icon in the Microsoft Word Standard toolbar.

2. Go to **Edit | Replace** and perform the following operations:

Function:	Find:	Replace with:	Effect:
Operation 1:	^p (paragraph mark)	(leave blank)	This removes all line breaks in the document.
Operation 2:	(initial character of the sequence name)	^p>	This will start each sequence, beginning with the sequence name, on a new line.

Use the **Find and Replace (CTL+F)** function box to perform operations 1 and 2 as shown below.

Figure 3.2 Find and Replace Dialog Box



Legend

1. Place the cursor on the **Find what** drop-down field.
2. Click **Special** and select **¶ Paragraph Mark** from the drop-down menu. The ¶ symbol displays in the **Find what** drop-down combo box.

NOTE For operation 2, substitute the input as described above.

A portion of the reformatted file is shown in Figure 3.3. The entire file is shown in Appendix E.

Figure 3.3 Masked Sequences, Single Line (.doc)

```
>rs130588CCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNCCTTCAGCACCAGGGGAAATAAGAAAAAGCAAAGAAAGGGAA
GTGCTGGTATAAAGGGAAGGAAGGAAACCAAGGGCATCAGTGTGGATGGG
GAAGGCAGAAAATGCTCTCCTGGGGAATCACAACCTTTGTATTATCAAGTGC
TTTTTTTGAATTCAAGCCATGCTATTTCTTGCAGCTCTGACTATGTTTCAAGT
TGGAATATCAAGCATCTCTGAGCCTTCACTACAGCAATTAAGGGCTCATAAA
GCTATTCTTAGGAGTACAAAATAAACTGAGATATATTTGAGGTTGCCAGAAG
GTTKCCATGTGGAGAACTGAGTGCTAACCACTGGAAAGGTGCACATTAAAG
ACAGCAGGTGTGCAGAATGCTGTGGCCATAACTCTCTCCAGCTGAGGGCAG
AGGCCACAAAAAGGACCCTGCTGAGGGGAGGCTGCCAAGGAGCTCCACACT
CTGCCAAGAGGGGCCTGAGTTCTGGCGCCATCAGCAACTGTCTTGNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNATTCAA
CTAAAGCCTGCAGATTCTTCCACTGCCCCCATGGTGGCCATCTGTTGAGGTG
GGAGGGAAACATGCTGGCCCTCCCCGTCTCCTACCCTAAAGAAGACACTGT
GC
>rs424293AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTA
ACACTGTCATCTTATAACGCTTTCCAATTCCCAAATACCTTTAAGGTTTGGTA
AAAATAATATACCTTCCACAAGTCATTTGATTTTTGTGGGCCTTAGTTTATCG
ACCTATTTGGATAGATGGGATGAAAAACCTTAGAGCCATCTTTCCTTACTCAT
TCCCCCTGCTAGTGAGAGAGAATTCAGTGGAATCAGAAATGGAATGAAGCA
TATACTTGGAGAAGTAAAAGAAATTTCCCAKAACAAAAGACAGAAAGGGCCC
AGTGAGCAATATAAATATGTATTTTATGTCCATATTTAGGCATATTGCAATTA
AAGTTCAAAATAACAAAGATGATTTGAAAATATTATGTATCAAACACGGTTTT
ACTTACTGGACAGTATGTAGTATAGGTTGTGNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNT
TAGAAACATATGAGAGATGATTGGCATAGTCAGCTTTGGCTGCCATAACAAG
ATGCT
>rs443523ATTACACTGTGAGCAGGANNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTGGACTGA
AGCCCACCTGCCTACTTCCCAAGCCTCATCTCTACCCACTCACCCCATTTCTC
TGTGCTCTGACCTGACTCAGTTCAAGGAAGCAATGAGACAGCCAAGAGAGA
AAATAGGAATTTCTTCCTGGTTTTCAATTTCAAAAAAGGTAGTCAAAACCTCA
GGTCATGGAGAAATTTTGGCGATTGTATKAAGAGTGAGAGCAAACTCCCCCA
CCCCTCAGATTATGTACTGATCCCTGGACTGATGTAAGTAGAAATGTATGCA
GTGGGCATACAGGGCTTGTGAGGAGTCGGAAGCAGTGGTGTGGTGTAGAG
ATTGTAGCTCCCTGGAAAGGTAGACCCTATGTTATATTTCCCCTAGACCAAG
TACTGGGGAAGACAATAGAATGCAGATAGTCTCGTGGGATCTGAAAATAATC
TAGAATTC
```

Save the above .doc file as a .txt file. From the main menu, select **Save As..** or copy and paste the text into Microsoft Notepad and from the main menu, select **File | Save**. A portion of the .txt file is shown in Figure 3.4 below. The entire file is shown in Appendix F.

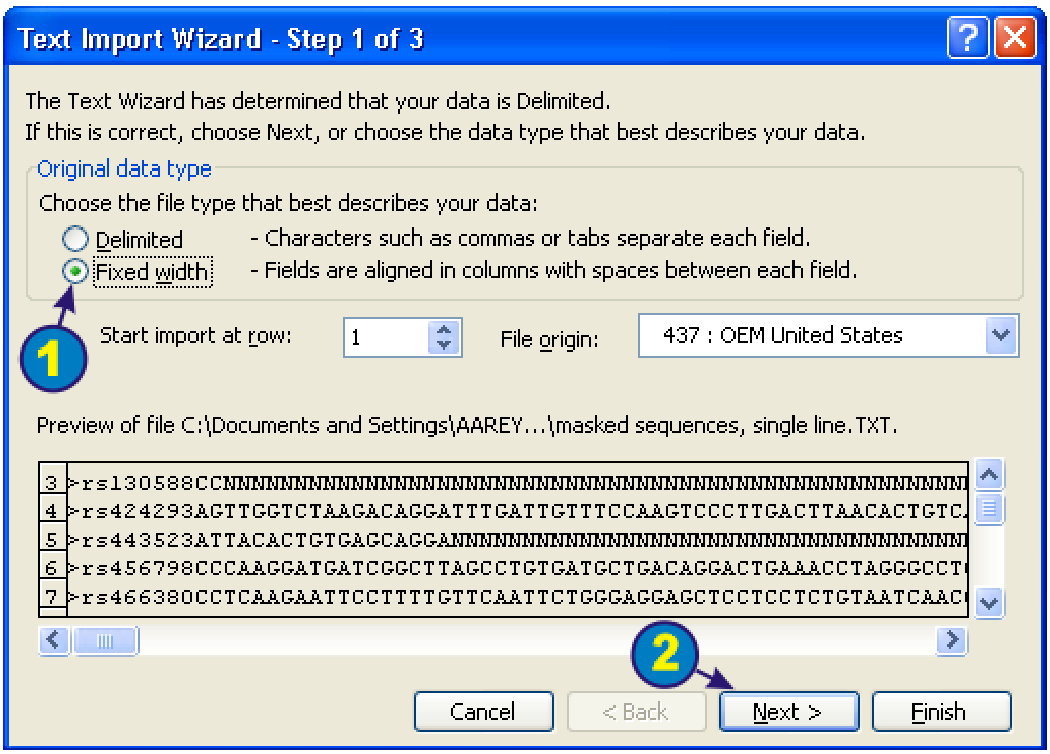
Figure 3.4 Masked Sequences, Single Line (.txt)

```
>rs130588CCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs424293AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACACTGTCATCTTATAACG
>rs443523ATTACACTGTGAGCAGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs456798CCCAAGGATGATCGGCTTAGCCTGTGATGCTGACAGGACTGAAACCTAGGGCCTCTGCCTCTAAA
>rs466380CCTCAAGAATTCCTTTTGTTCAATTCTGGGAGGAGCTCCTCCTCTGTAATCAACCAGATTAGCAT
>rs470490TCGCTTTGATATGTAAAGCTTGTTGATCCCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs717292CCTGGTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs718137TGCCCATACTGAGGATACATATAGAGTTCCAAGCATATGCTGGTGGTGCTAAATGATGAACACAC
>rs720460TCTANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs720513AANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs721580AACATTGGCAACATGTACAGAAGATTCCCTTACTAATTATAACTACGCTGTGAGCTGGAAAGAGC
>rs721992CATTTTTTCACATCAACTGTCCAGAGAGTGTTCAATCCATTTCCCCTTTGGTACAGGCATCCCTG
>rs723886TCTAGATTATTGCATTCCTGGGCTCAATCTACAGATGCTAGAGCTGGTCCCCCTGTTGGGATGGA
>rs724263NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs725046TCTAGAGCTGAGCAGCTTAGTTCCTGATAGACTAACATTGTTTTACACTCAGAACATGTCACCAC
>rs726207GAGAGACACAGAGAGAGAAAAAATCAAAGGCATACTCAATGAGAGAAGGAATTTCTAGAAGTTGG
>rs727023GGATCTCAAAAAATGTGGAGGTTGCATTCAATTACATTTGTGAATGTTAGGAAACTGTCAACTGC
>rs727678TCTAGATTGAGATATGATAGAATATACATTAATACCAGAATATTCTCTTGGTAAGTGAGGAGCTA
>rs729197TGTGGCTTCTCTCAACACCAGTATGTCCAAGGAGGATGCATTTTTATTGCTTTTGTGGTCTTAAC
>rs754257TAATGACGTTCGTTCTAAGAGTTTCAGACTTTAGAGCAGGAGTAAGAGTACCCATGATTTCCAAA
>rs763342CTATTAAATAGTTAGCAATCTTCAGCTAAGCACAAGTTGTAGCTGAGGTCAAAAGTAGAAACACT
>rs763405GGATCTGTTTGATAAATAGAACACAAGCTGGCAGAATTGGCCATCAGCCAACTTAAAAGAAGAAA
```

Next, insert a tab between the sequence name and the sequence. Do this manually for files containing a few sequences. For bigger files, open Microsoft Excel.

On the main menu, select **File | Open** and browse to the .txt file. The Text Import Wizard dialog box displays, as shown in the figures below.

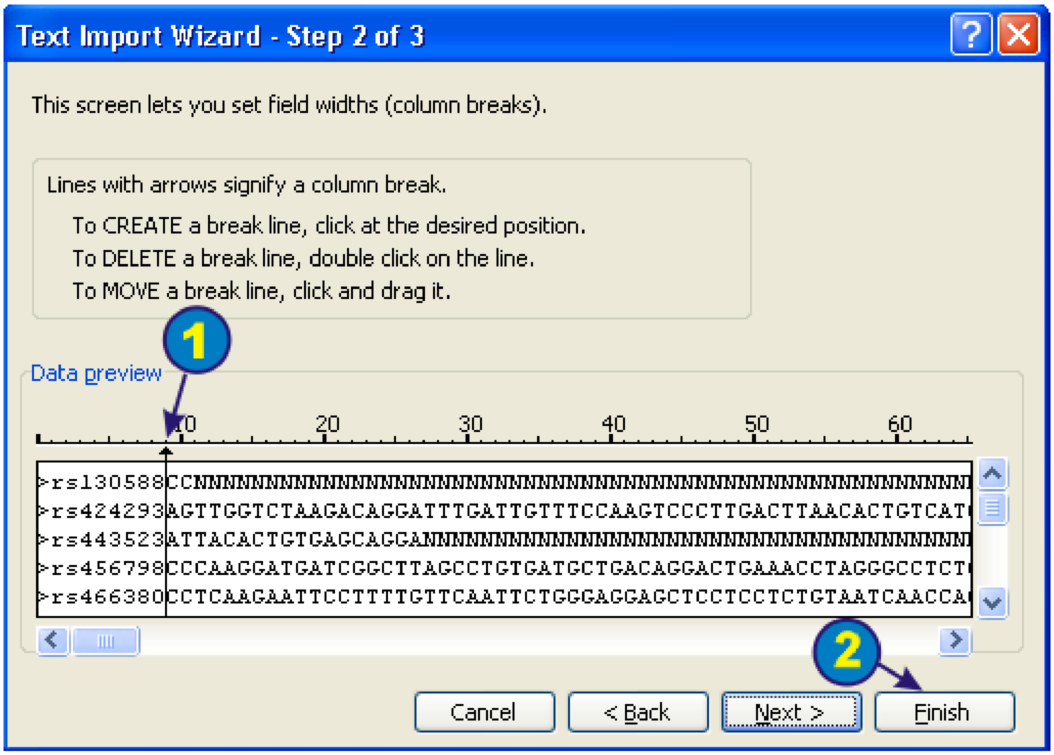
Figure 3.5 Text Import Wizard Box: Step 1



Legend:

1. Select **Fixed width**.
2. Click **Next**.

Figure 3.6 Text Import Wizard Box: Step 2



Legend:

1. Create a break line after the SNP name. If the SNP names vary in length, some sequence characters (bases) will end up in the name column, or vice-versa. Make corrections as necessary after saving the .xls file.
2. Click **Finish**.

Save the resulting file as an .xls file. A sample of the file is shown in Figure 3.7 below. The entire file is shown in Appendix G.

Figure 3.7 Masked Sequences, Single Line (.xls)

>rs130588	CCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs424293	AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACACTGTCATCTTATAACGCTT
>rs443523	ATTACACTGTGAGCAGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs456798	CCCAAGGATGATCGGCTTAGCCTGTGATGCTGACAGGACTGAAACCTAGGGCCTCTGCCTCTAAA
>rs466380	CCTCAAGAATTCCTTTTGTTCAATTCTGGGAGGAGCTCCTCCTCTGTAATCAACCAGATTAGCATGG
>rs470490	TCGCTTTGATATGTAAAGCTTGTTGATCCCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs717292	CCTGGTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs718137	TGCCCATACTGAGGATACATATAGAGTTCCAAGCATATGCTGGTGGTGCTAAATGATGAACACACC
>rs720460	TCTANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs720513	AANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs721580	AACATTGGCAACATGTACAGAAGATTCCCTTACTAATTATAACTACGCTGTGAGCTGGAAAGAGCT
>rs721992	CATTTTTTCACATCAACTGTCCAGAGAGTGTTCAATCCATTTCCCCTTTGGTACAGGCATCCCTGGTC
>rs723886	TCTAGATTATTGCATTCCTGGGCTCAATCTACAGATGCTAGAGCTGGTCCCCCTGTTGGGATGGAG
>rs724263	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs725046	TCTAGAGCTGAGCAGCTTAGTTCCTGATAGACTAACATTGTTTTACACTCAGAACATGTCACCACTC
>rs726207	GAGAGACACAGAGAGAGAAAAAATCAAAGGCATACTCAATGAGAGAAGGAATTTCTAGAAGTTG
>rs727023	GGATCTCAAAAAATGTGGAGGTTGCATTCAATTACATTTGTGAATGTTAGGAAACTGTCAACTGCCT
>rs727678	TCTAGATTGAGATATGATAGAATATACATTAATACCAGAATATTCTCTTGGTAAGTGAGGAGCTAGT
>rs729197	TGTGGCTTCTCTCAACACCAGTATGTCCAAGGAGGATGCATTTTTATTGCTTTTGTGGTCTTAACAAA
>rs754257	TAATGACGTTCGTTCTAAGAGTTTCAGACTTTAGAGCAGGAGTAAGAGTACCCATGATTTCCAAATA
>rs763342	CTATTAAATAGTTAGCAATCTTCAGCTAAGCACAAGTTGTAGCTGAGGTCAAAAGTAGAAACACTA
>rs763405	GGATCTGTTTGATAAATAGAACACAAGCTGGCAGAATTGGCCATCAGCCAACTTAAAAGAAGAAA

The masked sequences are now ready for alignment with the unmasked sequences.

3. Convert the original sequence file into a "single-line" format.

Convert the original, unmasked sequence file (Appendix A) to a Microsoft Excel file using steps similar to those used above for the masked file.

Briefly,

a. If necessary, format the .doc file so each sequence begins on a new line.

b. Save the file as a .txt.

c. Save the file as an .xls file.

A sample of the original sequence file saved as an .xls file, is shown in Figure 3.8 below. The entire file is shown in Appendix H.

Figure 3.8 Original Sequences, Single Line (.xls)

>rs36950	GCTATGGTAGTTTCATGTACCTATGAGGTGGATAAAAATTTAGCAACAGGTTGAAGGTAAGACTCC
>rs130588	CCAAATGGGGACGATGATATCTACCTCAGGGGAATGCTGTGATGACGATAGACGTGTCCAGCACA
>rs144848	GAATTCTCTGAAGTAAGAAAATCTTTCTTTCTTTTGTTCTCTGTGTCTAATAGGTCTTTTTCTGAAATAT
>rs187031	AGAAAGAAAGAAAAAAAACCCCTGCTGATTATGTATGCAAAAGTCGCAGCACGGTGCTAGAGTGCT
>rs277939	AGAGAAGTTGGCTCTTGCTTAAATTCCAACACTTATTCTTATGGGGAGAAAGCCATGGTTTACCTCT
>rs288422	GAATTCACCTTTTATTTTGATAGAATGGCAGANAGTCTAAGTAGAAGCAGGGCTTTGTGTATTCTGTA
>rs309133	CATAACATCCTGATGTCCCTGGCTTACTTCTATTTGGGAGAGGGGCCCCACTCGTTCTCATTTTATGA
>rs309773	TCTTCCTTCAGGCTAGGTGACTGCAAATCTCCCTAGAAACTGCTATAGCCACATGTTTATAATAAAC
>rs380607	AAGAGTGACACAGTCTCCATCAAGCTCTGGAGCCATGTAGTCATGGCAGAGCAAAGAGTATTTAG
>rs382515	AGCTAGAATACCCACAAGTGGCTTCACTCTGAGCTTCAGTTTATTCTACTGTTTATGGCCCTTAGAAC
>rs383885	AGAAGAAAACTGATTCTTTGCAGGACATTGTGGGTGAGTTTCTGCCAAAAATAAATTATCAATATTT
>rs424293	AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACACTGTCATCTTATAACGCTT
>rs443523	ATTACACTGTGAGCAGGACAAGATAAAGACCTTTACCTTCATTTTATAACTGGGGAAATTGAGGCTC
>rs456798	CCCAAGGATGATCGGCTTAGCCTGTGATGCTGACAGGACTGAAACCTAGGGCCTCTGCCTCTAAA
>rs466380	CCTCAAGAATTCCTTTTGTTCAATTCTGGGAGGAGCTCCTCCTCTGTAATCAACCAGATTAGCATGG
>rs470490	TCGCTTTGATATGTAAAGCTTGTTGATCCCCTTTTACTTTTTTTTTTTTTTTAGATGGAGTCTCGCTCTG
>rs554653	GGAATTTCCATTACAGCATATAGCATTCGTCCTATGAAGAAACCCCCTCGCTCAGTGACTTACACGA
>rs615474	CTGGAGAGCAAGCCACTCCAGCCCCACCCCAGCCATCCTGTGGTTCTGAGGGCCTCCTCAAGGCT
>rs638250	GGATCTCTTGTTTGCAATTGCTCTAATCTCTCAAGAATTATGAAGTGAGGTACTTCATTCTGCAGGGC
>rs713075	TCGTCGGAGGAATGCATAATATAAGAGTCGGTGGGCTAGTCTTTAGCCAGATGCCCTTCTGTAGAT
>rs713478	GGGGATCTCAAGATCACATCAAAATTAAAACAGCCTCCAACTATTGCCACAATCTTAGAAGAAATC
>rs713538	AAATATTTTGTGTAGTTAATGTAAGGATACTCAGACAGGAAAATAAACACAGTTATTTTTTAGAAAGA
>rs713718	CTTATAAAAATATTGTGCATTTAATACCTTTTTGGTTTCCTTTTATACTTGTCAAAAGTCATTTATACATA
>rs715755	TCTCCCACCAGGACCATCTTCCCCCGTTGTCCTCCTCTCTCCTTCACCAGGACTGCAGGTCACTGCT

4. Combine the original and masked sequences into one file.

   Copy the masked sequences from the single line .xls file (Figure 3.7 on page 8 and Appendix G), and append to the end of the original sequence .xls file. See Figure 3.8 on this page and Appendix H.

Sort the sequences by SNP name. Select **Data | Sort | Sort by SNP** name column.
The sequences are now arranged matching original and masked (if available) versions found in the adjacent rows, with the original sequence on the line above the masked sequence. A portion of the file is shown below. The entire file is shown in Appendix I.

Figure 3.9 Original and Masked Sequences, Each in Single Line (.xls)

>rs130588	CCAAATGGGGACGATGATATCTACCTCAGGGGAATGCTGTGATGACGATAGACGTGTCCAGCACA
>rs130588	CCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs144848	GAATTCTCTGAAGTAAGAAAATCTTTCTTTCTTTTGTTCTCTGTGTCTAATAGGTCTTTTTCTGAAATAT
>rs187031	AGAAAGAAAGAAAAAAACCCCTGCTGATTATGTATGCAAAAGTCGCAGCACGGTGCTAGAGTGCT
>rs277939	AGAGAAGTTGGCTCTTGCTTAAATTCCAACACTTATTCTTATGGGGAGAAAGCCATGGTTTACCTCT
>rs288422	GAATTCACCTTTTATTTTGATAGAATGGCAGANAGTCTAAGTAGAAGCAGGGCTTTGTGTATTCTGTA
>rs309133	CATAACATCCTGATGTCCCTGGCTTACTTCTATTTGGGAGAGGGGCCCCACTCGTTCTCATTTTATGA
>rs309773	TCTTCCTTCAGGCTAGGTGACTGCAAATCTCCCTAGAAACTGCTATAGCCACATGTTTATAATAAAC
>rs36950	GCTATGGTAGTTTCATGTACCTATGAGGTGGATAAAAATTTAGCAACAGGTTGAAGGTAAGACTCC
>rs380607	AAGAGTGACACAGTCTCCATCAAGCTCTGGAGCCATGTAGTCATGGCAGAGCAAAGAGTATTTAG
>rs382515	AGCTAGAATACCCACAAGTGGCTTCACTCTGAGCTTCAGTTTATTCTACTGTTTATGGCCCTTAGAAC
>rs383885	AGAAGAAAACTGATTCTTTGCAGGACATTGTGGGTGAGTTTCTGCCAAAAATAAATTATCAATATTT
>rs424293	AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACACTGTCATCTTATAACGCTT
>rs424293	AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACACTGTCATCTTATAACGCTT
>rs443523	ATTACACTGTGAGCAGGACAAGATAAAGACCTTTACCTTCATTTTATAACTGGGGAAATTGAGGCTC
>rs443523	ATTACACTGTGAGCAGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs456798	CCCAAGGATGATCGGCTTAGCCTGTGATGCTGACAGGACTGAAACCTAGGGCCTCTGCCTCTAAA
>rs456798	CCCAAGGATGATCGGCTTAGCCTGTGATGCTGACAGGACTGAAACCTAGGGCCTCTGCCTCTAAA
>rs466380	CCTCAAGAATTCCTTTTGTTCAATTCTGGGAGGAGCTCCTCCTCTGTAATCAACCAGATTAGCATGG
>rs466380	CCTCAAGAATTCCTTTTGTTCAATTCTGGGAGGAGCTCCTCCTCTGTAATCAACCAGATTAGCATGG
>rs470490	TCGCTTTGATATGTAAAGCTTGTTGATCCCCTTTTACTTTTTTTTTTTTTTTAGATGGAGTCTCGCTCTG
>rs470490	TCGCTTTGATATGTAAAGCTTGTTGATCCCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs554653	GGAATTTCCATTACAGCATATAGCATTCGTCCTATGAAGAAACCCCCTCGCTCAGTGACTTACACGA
>rs615474	CTGGAGAGCAAGCCACTCCAGCCCCACCCCAGCCATCCTGTGGTTCTGAGGGCCTCCTCAAGGCT
>rs638250	GGATCTCTTGTTTGCAATTGCTCTAATCTCTCAAGAATTATGAAGTGAGGTACTTCATTCTGCAGGGC

Next, convert the .xls file into a .txt file for better alignment viewing. In Notepad, select **Format** and uncheck **Wordwrap**, to remove the wordwrap formatting. A portion of the file is shown below. The entire file is shown in Appendix J.

Figure 3.10 Original and Masked Sequences, Each in Single Line (.txt)

```
>rs130588     CCAAATGGGGACGATGATATCTACCTCAGGGGAATGCTGTGATGACGATAGACGTGTCCAGCACACAGTAAC
>rs130588     CCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs144848     GAATTCTCTGAAGTAAGAAAATCTTTCTTTCTTTTGTTCTCTGTGTCTAATAGGTCTTTTTCTGAAATATTT
>rs187031     AGAAAGAAAGAAAAAAACCCCTGCTGATTATGTATGCAAAAGTCGCAGCACGGTGCTAGAGTGCTAGGCACA
>rs277939     AGAGAAGTTGGCTCTTGCTTAAATTCCAACACTTATTCTTATGGGGAGAAAGCCATGGTTTACCTCTTTGGT
>rs288422     GAATTCACCTTTTATTTTGATAGAATGGCAGANAGTCTAAGTAGAAGCAGGGCTTTGTGTATTCTGTAAAGT
>rs309133     CATAACATCCTGATGTCCCTGGCTTACTTCTATTTGGGAGAGGGGCCCCACTCGTTCTCATTTTATGAGACT
>rs309773     TCTTCCTTCAGGCTAGGTGACTGCAAATCTCCCTAGAAACTGCTATAGCCACATGTTTATAATAAACATTCA
>rs36950      GCTATGGTAGTTTCATGTACCTATGAGGTGGATAAAAATTTAGCAACAGGTTGAAGGTAAGACTCCAAAAGA
>rs380607     AAGAGTGACACAGTCTCCATCAAGCTCTGGAGCCATGTAGTCATGGCAGAGCAAAGAGTATTTAGCTCACTG
>rs382515     AGCTAGAATACCCACAAGTGGCTTCACTCTGAGCTTCAGTTTATTCTACTGTTTATGGCCCTTAGAACACTG
>rs383885     AGAAGAAAACTGATTCTTTGCAGGACATTGTGGGTGAGTTTCTGCCAAAAATAAATTATCAATATTTTCCTT
>rs424293     AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACACTGTCATCTTATAACGCTTTCCA
>rs424293     AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACACTGTCATCTTATAACGCTTTCCA
>rs443523     ATTACACTGTGAGCAGGACAAGATAAAGACCTTTACCTTCATTTTATAACTGGGGAAATTGAGGCTCTGAGG
>rs443523     ATTACACTGTGAGCAGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs456798     CCCAAGGATGATCGGCTTAGCCTGTGATGCTGACAGGACTGAAACCTAGGGCCTCTGCCTCTAAATCTCAGT
>rs456798     CCCAAGGATGATCGGCTTAGCCTGTGATGCTGACAGGACTGAAACCTAGGGCCTCTGCCTCTAAATCTCAGT
>rs466380     CCTCAAGAATTCCTTTTGTTCAATTCTGGGAGGAGCTCCTCCTCTGTAATCAACCAGATTAGCATGGACCTC
>rs466380     CCTCAAGAATTCCTTTTGTTCAATTCTGGGAGGAGCTCCTCCTCTGTAATCAACCAGATTAGCATGGACCTC
>rs470490     TCGCTTTGATATGTAAAGCTTGTTGATCCCCTTTTACTTTTTTTTTTTTTTTTTAGATGGAGTCTCGCTCTGT
>rs470490     TCGCTTTGATATGTAAAGCTTGTTGATCCCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs554653     GGAATTTCCATTACAGCATATAGCATTCGTCCTATGAAGAAACCCCCTCGCTCAGTGACTTACACGAAAAAC
>rs615474     CTGGAGAGCAAGCCACTCCAGCCCCACCCCAGCCATCCTGTGGTTCTGAGGGCCTCCTCAAGGCTATAGGGA
>rs638250     GGATCTCTTGTTTGCAATTGCTCTAATCTCTCAAGAATTATGAAGTGAGGTACTTCATTCTGCAGGGGGAAA
>rs713075     TCGTCGGAGGAATGCATAATATAAGAGTCGGTGGGCTAGTCTTTAGCCAGATGCCCTTCTGTAGATCCTGAG
>rs713478     GGGGATCTCAAGATCACATCAAAATTAAAACAGCCTCCAACTATTGCCACAATCTTAGAAGAAATCCCACTT
>rs713538     AAATATTTTGTGTAGTTAATGTAAGGATACTCAGACAGGAAAATAAACACAGTTATTTTTTAGAAAGATTTC
>rs713718     CTTATAAAAATATTGTGCATTTAATACCTTTTTGGTTTCCTTTTATACTTGTCAAAAGTCATTTATACATAA
>rs715755     TCTCCCACCAGGACCATCTTCCCCCGTTGTCCTCCTCTCTCCTTCACCAGGACTGCAGGTCACTGCTCAGAA
>rs717207     TCTAGATAGAAGTTAAAACCACCTCACTCTGGCTCGAGGAGGAGGGAGAAGGAGGCAGGGTTTCATTTCAAG
>rs717292     CCTGGTTGTTGATATTTATAGGTCTCTTTCCTTGGGCTGGTCAAATTCCCCAGAAAGATATCTTTCAATCTC
>rs717292     CCTGGTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
```

Make modifications to the masked sequences as necessary.

- Example 1. As shown in Figure 2.17 on page 19, rs727023 after repeat masking does not have the minimum 25 bases downstream of the SNP required for primer design. Assuming this is a must-have SNP, copy 25 bases from the unmasked sequence and replace the corresponding Ns in the masked sequence.

In the figures below, note that the top sequence is changed to the bottom sequence.

```
ATCCTAGCTTAGGGGAATTKGTGTGCAATAAGCAAGAGGCAGGACTTGGGAGTCAGGAAGGTTTGAATCCTC
ATCCTAGCTTAGGGGAATTKGTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
```

```
ATCCTAGCTTAGGGGAATTKGTGTGCAATAAGCAAGAGGCAGGACTTGGGAGTCAGGAAGGTTTGAATCCTC
ATCCTAGCTTAGGGGAATTKGTGTGCAATAAGCAAGAGGCAGGACNNNNNNNNNNNNNNNNNNNNNNNNNN
```

- Example 2. In rs456798, the SNP is embedded in a repeat (Figure 2.15 on page 18). Assuming this is a necessary SNP, delete the entire line containing the SNP name and the masked sequence.

```
AATGAGCACTCCATTGGGGCTCGAACCCAGGACCACCTGGATAKGAAGCAGATGAGAGATCTCCCCACCAGC
AATGAGCACNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGATCTCCCCACCAGC
```

The "adjusted" file is shown in Appendix K.

For each SNP place the SNP name, the original sequence, and masked sequence on one line, separated by tabs.

Convert adjusted file to an .xls file. A sample of the resulting file is shown below. The entire file is shown in Appendix L.

Figure 3.11 Original and Masked Sequences, Single Line Each, Masking Adjusted (.xls)

	A	B
1	>rs130588	CCAAATGGGGACGATGATATCTACCTCAGGGGAATGCTGTGATGACGATAGACGTGTCCAGCACA
2	>rs130588	CCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
3	>rs144848	GAATTCTCTGAAGTAAGAAAATCTTTCTTTCTTTTGTTCTCTGTGTCTAATAGGTCTTTTTCTGAAATATT
4	>rs187031	AGAAAGAAAGAAAAAAAACCCCTGCTGATTATGTATGCAAAAGTCGCAGCACGGTGCTAGAGTGCTA
5	>rs277939	AGAGAAGTTGGCTCTTGCTTAAATTCCAACACTTATTCTTATGGGGAGAAAGCCATGGTTTACCTCTT
6	>rs288422	GAATTCACCTTTTATTTTGATAGAATGGCAGANAGTCTAAGTAGAAGCAGGGCTTTGTGTATTCTGTA
7	>rs309133	CATAACATCCTGATGTCCCTGGCTTACTTCTATTTGGGAGAGGGGCCCCACTCGTTCTCATTTTATGA
8	>rs309773	TCTTCCTTCAGGCTAGGTGACTGCAAATCTCCCTAGAAACTGCTATAGCCACATGTTTATAATAAACA
9	>rs36950	GCTATGGTAGTTTCATGTACCTATGAGGTGGATAAAAATTTAGCAACAGGTTGAAGGTAAGACTCCA
10	>rs380607	AAGAGTGACACAGTCTCCATCAAGCTCTGGAGCCATGTAGTCATGGCAGAGCAAAGAGTATTTAGC
11	>rs382515	AGCTAGAATACCCACAAGTGGCTTCACTCTGAGCTTCAGTTTATTCTACTGTTTATGGCCCTTAGAAC
12	>rs383885	AGAAGAAAACTGATTCTTTGCAGGACATTGTGGGTGAGTTTCTGCCAAAAATAAATTATCAATATTTT
13	>rs424293	AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACACTGTCATCTTATAACGCTTT
14	>rs424293	AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACACTGTCATCTTATAACGCTTT

For each original sequence that has a matching masked sequence, drag-and-drop the masked sequence into the empty cell to the right of the original sequence. Delete the sequence name of the masked sequence.

Legend:

1. Drag-and-drop the contents of cell B2 into cell C1.
2. Delete the sequence name in the masked sequence line.

Format the SNP symbol.

From the main menu select **Edit | Replace** and replace the SNP symbol ("K" in this example) with the required format: the two base types separated by a forward slash and enclosed in brackets, i.e. [T/G] or [G/T] (both are acceptable).

While a portion of the file is shown below, the correct format for the SNP loci is not visible. The entire file is shown in Appendix M.

Figure 3.12 Original and Masked Sequences, Single Line (.xls)

>rs130588	CCAAATG	CCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs144848	GAATTCTCTGAAGTAAGAAAATCTTTCTTTCTTTTGTTCTCTGTGTCTAATAGGTCTTTTTCTGAAATAT	
>rs187031	AGAAAGAAAGAAAAAAACCCCTGCTGATTATGTATGCAAAAGTCGCAGCACGGTGCTAGAGTGCT	
>rs277939	AGAGAAGTTGGCTCTTGCTTAAATTCCAACACTTATTCTTATGGGGAGAAAGCCATGGTTTACCTCT	
>rs288422	GAATTCACCTTTTATTTTGATAGAATGGCAGANAGTCTAAGTAGAAGCAGGGCTTTGTGTATTCTGT	
>rs309133	CATAACATCCTGATGTCCCTGGCTTACTTCTATTTGGGAGAGGGGCCCCACTCGTTCTCATTTTATG	
>rs309773	TCTTCCTTCAGGCTAGGTGACTGCAAATCTCCCTAGAAACTGCTATAGCCACATGTTTATAATAAAC	
>rs36950	GCTATGGTAGTTTCATGTACCTATGAGGTGGATAAAAATTTAGCAACAGGTTGAAGGTAAGACTCC	
>rs380607	AAGAGTGACACAGTCTCCATCAAGCTCTGGAGCCATGTAGTCATGGCAGAGCAAAGAGTATTTAG	
>rs382515	AGCTAGAATACCCACAAGTGGCTTCACTCTGAGCTTCAGTTTATTCTACTGTTTATGGCCCTTAGAA	
>rs383885	AGAAGAAAACTGATTCTTTGCAGGACATTGTGGGTGAGTTTCTGCCAAAAATAAATTATCAATATTT	
>rs424293	AGTTGGT	AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACACTGTCATCTTAT
>rs443523	ATTACAC	ATTACACTGTGAGCAGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs456798	CCCAAGGATGATCGGCTTAGCCTGTGATGCTGACAGGACTGAAACCTAGGGCCTCTGCCTCTAAA	
>rs466380	CCTCAAG	CCTCAAGAATTCCTTTTGTTCAATTCTGGGAGGAGCTCCTCCTCTGTAATCAACCAGATT
>rs470490	TCGCTTTG	TCGCTTTGATATGTAAAGCTTGTTGATCCCCNNNNNNNNNNNNNNNNNNNNNNNNNNN
>rs554653	GGAATTTCCATTACAGCATATAGCATTCGTCCTATGAAGAAACCCCCTCGCTCAGTGACTTACACG	
>rs615474	CTGGAGAGCAAGCCACTCCAGCCCCACCCCAGCCATCCTGTGGTTCTGAGGGCCTCCTCAAGGC	
>rs638250	GGATCTCTTGTTTGCAATTGCTCTAATCTCTCAAGAATTATGAAGTGAGGTACTTCATTCTGCAGGG	
>rs713075	TCGTCGGAGGAATGCATAATATAAGAGTCGGTGGGCTAGTCTTTAGCCAGATGCCCTTCTGTAGAT	

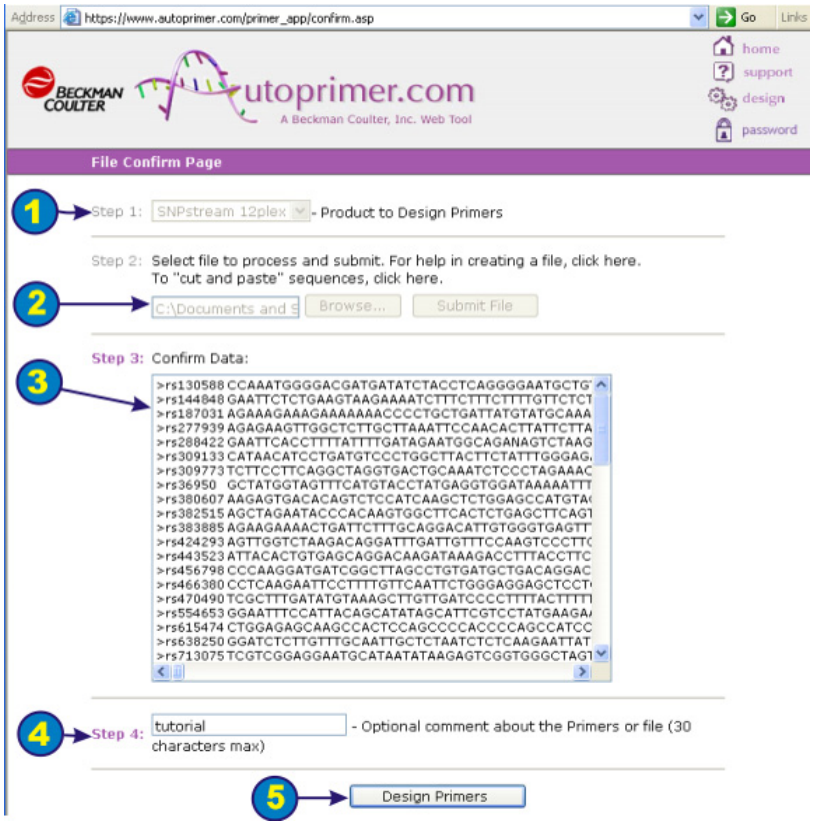
Finally, convert the .xls file into a .txt file for submission to the Autoprimer. A portion of the file is shown in Figure 3.13 below. The entire file is shown in Appendix N.

Figure 3.13 Original and Masked Sequences, Single Line (.txt)

Sequence Submission

4.1 Sequence Submission to the Autoprimer

Figure 4.1 Autoprimer File Confirm Page



Legend:

1. Select SNPstream 12plex or 48plex.
2. Browse to the sequence file then click **Submit File**. If no platform is selected in Step 1, the following error message displays: "You entered too many sequences. Only the first will be processed."
3. Confirm the sequence data.
4. Add any optional comments.
5. Click **Design Primers**.

Figure 4.2 Autoprimer Results

Legend:

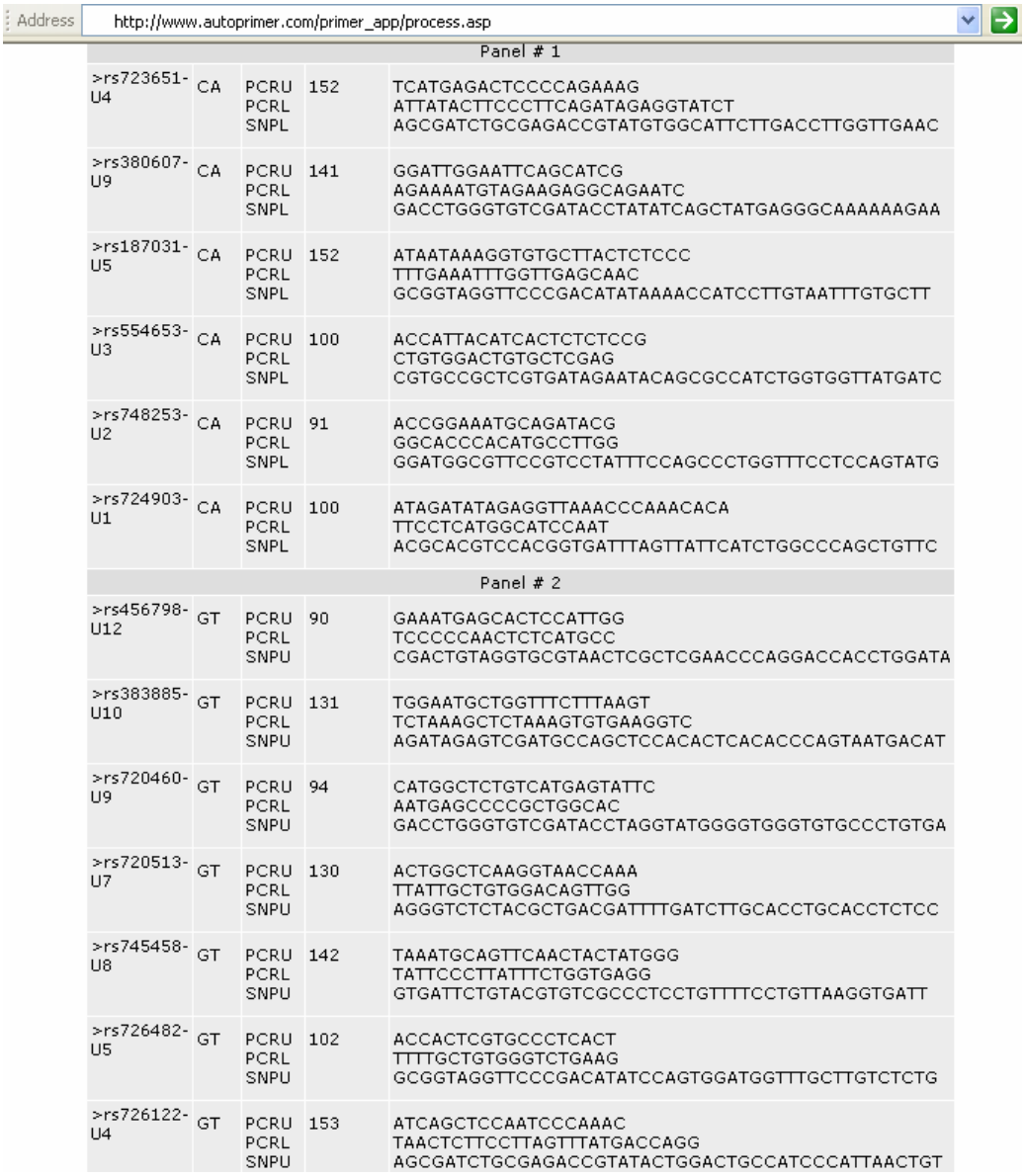
1. The sequences for which the Autoprimer could not design primers.

The Autoprimer designed a total of six panels:

- four 12plex panels with extension type G/T (Panels #2, 3, 4, 5),
- one 10plex G/T (Panel #6)
- one 6plex C/A (Panel #1)

A portion of the results file is shown below.

Figure 4.3 Autoprimer Results, cont'd.


Address http://www.autoprimer.com/primer_app/process.asp

Panel # 1				
>rs723651-U4	CA	PCRU PCRL SNPL	152	TCATGAGACTCCCCAGAAAG ATTATACTTCCCTTCAGATAGAGGTATCT AGCGATCTGCGAGACCGTATGTGGCATTCTTGACCTTGGTTGAAC
>rs380607-U9	CA	PCRU PCRL SNPL	141	GGATTGGAATTCAGCATCG AGAAAATGTAGAAGAGGCAGAATC GACCTGGGTGTCGATACCTATATCAGCTATGAGGGCAAAAAAGAA
>rs187031-U5	CA	PCRU PCRL SNPL	152	ATAATAAAGGTGTGCTTACTCTCCC TTTGAAATTTGGTTGAGCAAC GCGGTAGGTTCCCGACATATAAAACCATCCTTGTAATTTGTGCTT
>rs554653-U3	CA	PCRU PCRL SNPL	100	ACCATTACATCACTCTCTCCG CTGTGGACTGTGCTCGAG CGTGCCGCTCGTGATAGAATACAGCGCCATCTGGTGGTTATGATC
>rs748253-U2	CA	PCRU PCRL SNPL	91	ACCGGAAATGCAGATACG GGCACCCACATGCCTTGG GGATGGCGTTCCGTCCTATTTCCAGCCCTGGTTTCCTCCAGTATG
>rs724903-U1	CA	PCRU PCRL SNPL	100	ATAGATATAGAGGTTAAACCCAAACACA TTCCTCATGGCATCCAAT ACGCACGTCCACGGTGATTTAGTTATTCATCTGGCCCAGCTGTTC
Panel # 2				
>rs456798-U12	GT	PCRU PCRL SNPU	90	GAAATGAGCACTCCATTGG TCCCCCAACTCTCATGCC CGACTGTAGGTGCGTAACTCGCTCGAACCCAGGACCACCTGGATA
>rs383885-U10	GT	PCRU PCRL SNPU	131	TGGAATGCTGGTTTCTTTAAGT TCTAAAGCTCTAAAGTGTGAAGGTC AGATAGAGTCGATGCCAGCTCCACACTCACACCCAGTAATGACAT
>rs720460-U9	GT	PCRU PCRL SNPU	94	CATGGCTCTGTCATGAGTATTC AATGAGCCCCGCTGGCAC GACCTGGGTGTCGATACCTAGGTATGGGGTGGGTGTGCCCTGTGA
>rs720513-U7	GT	PCRU PCRL SNPU	130	ACTGGCTCAAGGTAACCAAA TTATTGCTGTGGACAGTTGG AGGGTCTCTACGCTGACGATTTTGATCTTGCACCTGCACCTCTCC
>rs745458-U8	GT	PCRU PCRL SNPU	142	TAAATGCAGTTCAACTACTATGGG TATTCCCTTATTTCTGGTGAGG GTGATTCTGTACGTGTCGCCCTCCTGTTTTCCTGTTAAGGTGATT
>rs726482-U5	GT	PCRU PCRL SNPU	102	ACCACTCGTGCCCTCACT TTTTGCTGTGGGTCTGAAG GCGGTAGGTTCCCGACATATCCAGTGGATGGTTTGCTTGTCTCTG
>rs726122-U4	GT	PCRU PCRL SNPU	153	ATCAGCTCCAATCCCAAAC TAACTCTTCCTTAGTTTATGACCAGG AGCGATCTGCGAGACCGTATACTGGACTGCCATCCCATTAACTGT

Download the matching .txt file as shown below. The entire file is shown in Appendix O.

Figure 4.4 Autoprimer Results (.txt)

```
Primers Designed For:
SNPstream Customer
Senior Scientist
BCI
D25B
4300 N. Harbor Blvd
Fullerton, CA 92834

Telephone: 7149923669
Facsimile: 7149923669

General Info:
Submission Date: 10/13/2005
Primers Designed for SNPstream 12plex Kits
Comments: tutorial

Powered By:   Autoprimer v2.3 (Build 48)


                        SNP      Oligo      Amplicon
SNP Name                Type    Type-Mod.    Length       Sequence
>rs763342                    No Primers could be found for this sequence...

>rs754257                    No Primers could be found for this sequence...

>rs727678                    No Primers could be found for this sequence...

>rs727023                    No Primers could be found for this sequence...

>rs717292                    No Primers could be found for this sequence...

>rs721580                    No Primers could be found for this sequence...

Panel # 1
>rs723651-U4             CA      PCRU          152    TCATGAGACTCCCCAGAAAG
                                 PCRL                 ATTATACTTCCCTTCAGATAGAGGTATCT
                                 SNPL                 AGCGATCTGCGAGACCGTATGTGGCATTCTTGACCTTGGTTGAAC

>rs380607-U9             CA      PCRU          141    GGATTGGAATTCAGCATCG
                                 PCRL                 AGAAAATGTAGAAGAGGCAGAATC
                                 SNPL                 GACCTGGGTGTCGATACCTATATCAGCTATGAGGGCAAAAAAGAA

>rs187031-U5             CA      PCRU          152    ATAATAAAGGTGTGCTTACTCTCCC
                                 PCRL                 TTTGAAATTTGGTTGAGCAAC
                                 SNPL                 GCGGTAGGTTCCCGACATATAAAACCATCCTTGTAATTTGTGCTT

>rs554653-U3             CA      PCRU          100    ACCATTACATCACTCTCTCCG
                                 PCRL                 CTGTGGACTGTGCTCGAG
                                 SNPL                 CGTGCCGCTCGTGATAGAATACAGCGCCATCTGGTGGTTATGATC

>rs748253-U2             CA      PCRU          91     ACCGGAAATGCAGATACG
                                 PCRL                 GGCACCCACATGCCTTGG
                                 SNPL                 GGATGGCGTTCCGTCCTATTTCCAGCCCTGGTTTCCTCCAGTATG

>rs724903-U1             CA      PCRU          100    ATAGATATAGAGGTTAAACCCAAACACA
                                 PCRL                 TTCCTCATGGCATCCAAT
                                 SNPL                 ACGCACGTCCACGGTGATTTAGTTATTCATCTGGCCCAGCTGTTC

Panel # 2
>rs456798-U12            GT      PCRU          90     GAAATGAGCACTCCATTGG
                                 PCRL                 TCCCCCAACTCTCATGCC
                                 SNPU                 CGACTGTAGGTGCGTAACTCGCTCGAACCCAGGACCACCTGGATA
```

Completing Partial Panels

5.1 Reverse Complementing G/A:T/C or C/A:T/G SNPs

Partial panels are formed when not enough SNPs of one extension type are submitted to the Autoprimer. Submission size for a given extension type may be increased by taking the reverse complement of sequences that have the complementary SNP type. For example, the following panel, as shown below, was submitted for 12plex design as 8 C/T and 5 G/A SNPs. With one exception, the extension type selected by Autoprimer was the same as the input extension type; thus, the panel consists of 7 C/T and 6 G/A extension types.

Figure 5.1 Partial SNP Panels

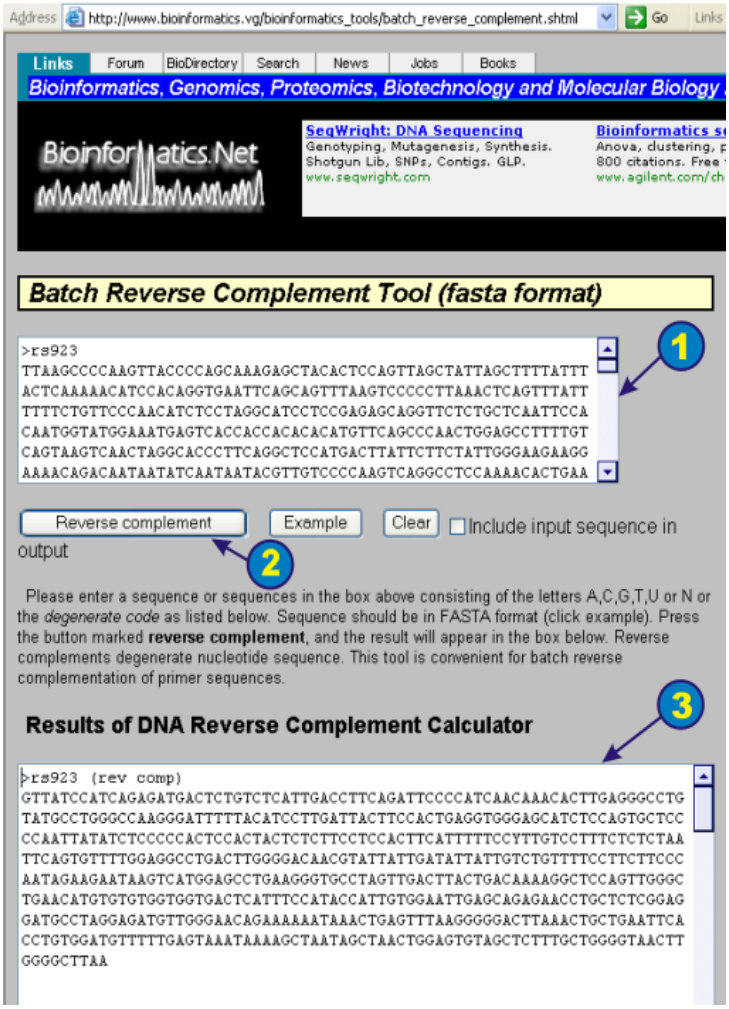
SNP Name-Probe Name	SNP Type	Primer Type-Mod.	Amplicon Length	Sequence
				Panel # 1
>rs22124-U6	CT	PCRU PCRL SNPU	146	TTCCTCATGTGGGGTTCTT TTATGGTGCTAAACACTGTACCAC GGCTATGATTCGCAATGCTTTTCTTCACTGCAAACCAGTTTAGAG
>rs52911-U5	CT	PCRU PCRL SNPU	152	TATACTCTACTCAACAACATTCCCATAC ATTTTAGCAGCTCTGCTTGATG GCGGTAGGTTCCCGACATATTTCTCTTTGCCTCCTCTTAATCAAA
>rs2624-U3	CT	PCRU PCRL SNPU	112	AATGTCCAGAAATCACATTATTGC AATGTCCCAATATCAAGGACAA CGTGCCGCTCGTGATAGAATCATAGACCGTGTAGTCTTGATCTAA
>rs55778-U4	CT	PCRU PCRL SNPU	131	AGAGGACATATGCTCTTCTATCAAAT AAGAATACATTAAATTTTCTTTGGAGG AGCGATCTGCGAGACCGTATCCACTGGGGAAAGTATTTATTTCAT
>rs49698-U1	CT	PCRU PCRL SNPU	96	TAAAACACTATATTACCCAGCACCT AGGGCCAATTAGGATAGGAG ACGCACGTCCACGGTGATTTTGTTGGGTTCCTCCTAGCTCACACA
>rs31226-U10	CT	PCRU PCRL SNPU	153	ATTCTACCAAAAAACAAATTCCAA ATGATCTGTGTCATGGGGAA AGATAGAGTCGATGCCAGCTTTGTCTTTGTTGTCTAAAAAATAAT
>rs13180-U2	CT	PCRU PCRL SNPU	99	TGATTAGAGGGAATAAAATGCAC CCAATTCCAATCAAATGATCTT GGATGGCGTTCCGTCCTATTTACAGGGTGTGAAAGCTGTTTTGGC
				Panel # 2
>rs2246-U7	GA	PCRU PCRL SNPU	152	AGTTGAACTGATGGCCAGC TTTCTTGTGGATTTGCATGC AGGGTCTCTACGCTGACGATACAAACCCTAAGAAACACAGAAATG
>rs19417-U5	GA	PCRU PCRL SNPU	152	TATATTGGTCAGAGACTGAGTATGTCC AAGTCAATCAGATGAAATAAAAATTCC GCGGTAGGTTCCCGACATATGAACTAAAGGAAGATTCATAAACTT
>rs923-U4	GA	PCRU PCRL SNPU	90	TCCCCAAGTCAGGCCTCC ATCTCCCCCACTCCACTACT AGCGATCTGCGAGACCGTATACACTGAATTAGAGAGAAAGGACAA
>rs17422-U3	GA	PCRU PCRL SNPL	116	ACAAGCTGAGTCTGAGACC TTTAGGACCTGGACATCCAC CGTGCCGCTCGTGATAGAATGGACCTAGAACACTCTGTACCTGAT
>rs17504-U1	GA	PCRU PCRL SNPU	89	CTTCCAGTGCTTCCAGAAA AGGAATTTATGTTTACCTTTCCC ACGCACGTCCACGGTGATTTTGACCCTTCTAGTGACGTCTGCTAC
>rs9239-U2	GA	PCRU PCRL SNPU	107	ATTTGAAGTCTCAGAGCTTGC CTGTAAAGAGGATTATCTTAGTTTATACTTTG GGATGGCGTTCCGTCCTATTAGTTAAACACCGCCTTCAGTACCGA

The two partial panels may be combined into one full panel, for example, C/T.

1. Reverse complement all the G/A SNP sequences.

   Use the website below to reverse complements degenerate positions (e.g. SNPs) and accepts batch files, as shown in the figure below.
   http://www.bioinformatics.vg/bioinformatics_tools/batch_reverse_complement.shtml.

Figure 5.2 Batch Reverse Complement



Legend:

1. Paste the sequence in FASTA format.
2. Click **Reverse complement**
3. View the results.

2. Assemble a new Autoprimer submission file.

   Retain the original C/T SNPs. Replace the G/A SNP sequences with the corresponding C/T sequences.

3. Submit to the Autoprimer.

   The resulting Autoprimer design, as shown below, shows a full 12plex C/T panel.

NOTE The "refractory" G/A SNP, rs17422, may be switched to C/T using the method described in Section 6.

Figure 5.3 Full SNP Panel After Reverse Complementation

SNP Name-Probe Name	SNP Type	Primer Type-Mod.	Amplicon Length	Sequence
				Panel # 1
>rs2246rev-U12	CT	PCRU PCRL SNPU	142	ATTTGCATGCTATGGGGA AGTTGAACTGATGGCCAGC CGACTGTAGGTGCGTAACTCGAAAGCATCTTCATGGGCAGGAATT
>rs22124-U11	CT	PCRU PCRL SNPU	146	TTCCTCATGTGGGGTTCTT TTATGGTGCTAAACACTGTACCAC AGAGCGAGTGACGCATACTATTCTTCACTGCAAACCAGTTTAGAG
>rs19417rev-U9	CT	PCRU PCRL SNPU	152	AAGTCAATCAGATGAAATAAAAATTCC TATATTGGTCAGAGACTGAGTATGTCC GACCTGGGTGTCGATACCTAGTGTCCACTCAATCTTTAAGCAAAA
>rs2624-U7	CT	PCRU PCRL SNPU	112	AATGTCCAGAAATCACATTATTGC AATGTCCCAATATCAAGGACAA AGGGTCTCTACGCTGACGATCATAGACCGTGTAGTCTTGATCTAA
>rs17504rev-U6	CT	PCRU PCRL SNPU	113	GAGGTCTTAGATATATCCTGAGTTCAG TTCCAGTGCTTCCAGAAACT GGCTATGATTCGCAATGCTTTTCCCATTTAGGTTTCCAAAACAAC
>rs923rev-U8	CT	PCRU PCRL SNPU	93	AATTATATCTCCCCCACTCCAC CCAAGTCAGGCCTCCAAA GTGATTCTGTACGTGTCGCCCTCTCTTCCTCCACTTCATTTTTCC Possible PCR Primer Cross Hybridization... Possible SBE Primer vs Amplicon Cross Hybridization...
>rs52911-U5	CT	PCRU PCRL SNPU	152	TATACTCTACTCAACAACATTCCCATAC ATTTTAGCAGCTCTGCTTGATG GCGGTAGGTTCCCGACATATTTCTCTTTGCCTCCTCTTAATCAAA
>rs49698-U3	CT	PCRU PCRL SNPU	96	TAAAACACTATATTACCCAGCACCT AGGGCCAATTAGGATAGGAG CGTGCCGCTCGTGATAGAATTGTTGGGTTCCTCCTAGCTCACACA
>rs31226-U10	CT	PCRU PCRL SNPU	153	ATTCTACCAAAAAACAAATTCCAA ATGATCTGTGTCATGGGGAA AGATAGAGTCGATGCCAGCTTTGTCTTTGTTGTCTAAAAAATAAT Possible SBE Primer Cross Hybridization...
>rs55778-U4	CT	PCRU PCRL SNPU	131	AGAGGACATATGCTCTTCTATCAAAT AAGAATACATTAAATTTTCTTTGGAGG AGCGATCTGCGAGACCGTATCCACTGGGGAAAGTATTTATTTCAT Possible PCR Primer Cross Hybridization... Possible SBE Primer vs Amplicon Cross Hybridization...
>rs9239rev-U1	CT	PCRU PCRL SNPU	124	TTTTAATGCACTAATCTGAATTCTGTA AAGTCTCAGAGCTTGCCAAGT ACGCACGTCCACGGTGATTTATTTTATAATGTTCTTGTATAGCAG
>rs13180-U2	CT	PCRU PCRL SNPU	99	TGATTAGAGGGAATAAAATGCAC CCAATTCCAATCAAATGATCTT GGATGGCGTTCCGTCCTATTTACAGGGTGTGAAAGCTGTTTTGGC
				Panel # 2
>rs17422-U1	GA	PCRU PCRL SNPL	116	ACAAGCTGAGTCTGAGACC TTTAGGACCTGGACATCCAC ACGCACGTCCACGGTGATTTGGACCTAGAACACTCTGTACCTGAT

Completing Partial Panels

6.1 Forcing SBE Primer Design to One Strand

As seen in the panels designed for the 70 G/T SNPs (Section 4 and Appendix Q), the Autoprimer switches the extension type of a submitted SNP if it calculates more favorable energetics for the opposite strand. This could result in partial panels. For example, Panel #6 comprises 10 G/T SNPs. If a full 12plex G/T panel is preferred, two SNPs that were designed as C/A (Panel #1) may be "forced" to the G/T extension type. To do this, follow the steps below:

1. Obtain the sequence of the strand that, as written, shows the desired SNP extension type.

   This could be the sequence that was originally submitted. If not, get the reverse complement of the original sequence.

   Suppose that C/A SNPs rs723651and rs380607 from Panel #1 are selected for inclusion in the new G/T panel. Locate the sequences in the Autoprimer submission file. Only the regions surrounding the SNP loci are shown below:

```
rs723651    GGGTCTAGGCCCCTCTTGCTGTTTGGGT[G/T]GTTCAACCAAGGTCAAGAATGCCACATTC
rs380607    TCTCCTTGGATGTCTGGATAATCTCCAC[G/T]TTCTTTTTTGCCCTCATAGCTGATATGGT
```

2. Replace the base immediately upstream of the SNP locus with **N**.

   Repeat with the corresponding masked sequence, if available.

```
rs723651    GGGTCTAGGCCCCTCTTGCTGTTTGGGN[G/T]GTTCAACCAAGGTCAAGAATGCCACATTC
rs380607    TCTCCTTGGATGTCTGGATAATCTCCAN[G/T]TTCTTTTTTGCCCTCATAGCTGATATGGT
```

3. Create a new submission file.

   Create a new .txt file for the new panel. It consists of the original 10 sequences from Panel #6, plus the two modified sequences from Panel #1. A portion of the file is shown below. The entire file is shown in Appendix P.

Figure 6.1 Forced panel (.txt)

```
>rs722979       GGATCTCTGCTGAGGTGTTGAAGCTTTGAGGAAGAGATAGCATTTTATACATGCATTGGAGATCAAGTTGTAACAAATGGAATTTTAGTACA
>rs309773       TCTTCCTTCAGGCTAGGTGACTGCAAATCTCCCTAGAAACTGCTATAGCCACATGTTTATAATAAACATTCAAGGCCAGTAGAATACTGAGA
>rs721757       TCTAACCATGTCATATTCAAAGTTTCTGTCTTTGTGCCTTTTTTGGCGATCTTTATCATCATCCCAAGCAATTAATGCATTCCATAAATACA
>rs424293       AGTTGGTCTAAGACAGGATTTGATTGTTTCCAAGTCCCTTGACTTAACACTGTCATCTTATAACGCTTTCCAATTCCCAAATACCTTTAAGG
AGGTTGTGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTTAGAAACATATGAGAGATGATTGGCATAGTCAGC
>rs727432       TTATTCTCTTACCCCCAAAGCCACCGCGGTTTCCTCCATCATCCTTAGCCTCACTTACCTGGATGCTGTTGCACACGGACCACCCTATCCTG
>rs36950        GCTATGGTAGTTTCATGTACCTATGAGGTGGATAAAAATTTAGCAACAGGTTGAAGGTAAGACTCCAAAAGATAGTGAGGTAGAAAACTTGC
>rs720596       TCTAGACATCTTCAGTGCTGCCACACTGCGCAGACCCAGGACGTCGTTACGTTGAAGTCCAGCATTCAGGGGCTGCTGTTCACGTGGAATAT
>rs721623       TTTGGTTTCATGTAGAAGTCAAAATTCGTCTACAAATGAAAGTAGTTCCCAATCCAGTTGTGAAATAAAAGCAGGGCCTGTCACAGTCATTA
>rs470490       TCGCTTTGATATGTAAAGCTTGTTGATCCCCTTTTACTTTTTTTTTTTTTTTTTAGATGGAGTCTCGCTCTGTTGCCCGGGCTAGAGTGCAGT
GTTAAGAATATTTTCTAGAAGAAAACACAGTAAGGAAATGGGGAAAGTTGAAGATGCCAAACAAAGGTAAATCTTCAGGTAAATGTGTAGCCTCTGCATAATCTCATA
>rs756384       TCCTGCTTTAGCAAACTTGCAAGTACCTACTGAGATGTTAGAGAGTCTGTAAGTCTGGGTACCTAGAGTGACTACATTGAGTACCCTGTGGA
>rs723651       GGTGGCAAATGGGGTTCGCTGAATTATTCTTGTTTGTCAATCTTGAGGCCCTTACTATCTGGAGAGCAAAAAGAGGTGATGAACCCTGCTGT
```

4. Submit the file to the Autoprimer.

   The resulting design of 12-SNP T/G panel is shown in Figure 6.2 below. The entire results file is shown in Appendix Q.

Figure 6.2 Full SNP Panel After Strand Forcing

SNP Name-Probe Name	SNP Type	Primer Type-Mod.	Amplicon Length	Sequence
Panel # 1				
>rs380607-U11	GT	PCRU PCRL SNPU	141	GGATTGGAATTCAGCATCG AGAAAATGTAGAAGAGGCAGAATC AGAGCGAGTGACGCATACTACCTTGGATGTCTGGATAATCTCCAN
>rs722979-U10	GT	PCRU PCRL SNPU	90	TGAAGGCAAAAAAAATCCAA AGGAGGTAAAAAGGTATCTACAGACTTAT AGATAGAGTCGATGCCAGCTGTCCAAAGCGTGCGTAAGAGTCGTT
>rs309773-U12	GT	PCRU PCRL SNPU	141	AGTCAAATGTTGCATCCTAAAAG ATCTAATAGCATATATACTAGGTCTTCTGAGA CGACTGTAGGTGCGTAACTCGGCCACAGATGATCATACACTTGCT
>rs727432-U9	GT	PCRU PCRL SNPU	108	TTTACCCAAACCATCGTCTG TCAATTGCTAATTCAAAATGAATG GACCTGGGTGTCGATACCTAGGTATGAGGTCTTCACCCCATGCCT Possible PCR Primer Cross Hybridization...
>rs36950-U7	GT	PCRU PCRL SNPU	97	CTGCTTGTGTAAAGATCTCTGC ACTCTTCATTCATTTGTGATGCT AGGGTCTCTACGCTGACGATTAATGGGTGTAACGCTAAATAAAGC Possible PCR Primer Cross Hybridization...
>rs424293-U8	GT	PCRU PCRL SNPU	101	AATCAGAAATGGAATGAAGCATAT CATAAAATACATATTTATATTGCTCACTGG GTGATTCTGTACGTGTCGCCGAGAAGTAAAAGAAATTTCCCA Possible SBE Primer vs Amplicon Cross Hybridization...
>rs721757-U5	GT	PCRU PCRL SNPU	132	AACATGGAAAAATTCATCCTAACT AACAATATTGGGGATGTTATTAGG GCGGTAGGTTCCCGACATATTCTATCAACACTTGGAATAACAGAT
>rs720596-U6	GT	PCRU PCRL SNPU	151	AGGGTTGGGATAAAACAACAG ATTATATTTCAGTACAACATCTACTCTTCAA GGCTATGATTCGCAATGCTTATGAATAGAAAAGGATTAGAGCAGG
>rs756384-U4	GT	PCRU PCRL SNPU	153	AAAGGTAAATATCTGACCAATGTGA ATTTCATAGCTCCCAGGATTACA AGCGATCTGCGAGACCGTATCTGCTCAGATTTTATATCCCAGGAA
>rs721623-U2	GT	PCRU PCRL SNPU	107	AGTATTTGATATGAAAATAATTGTGCTG AATTCTATTTGGCACAGGTGG GGATGGCGTTCCGTCCTATTCTTCATAAATGTAACTATCAAGCAC
>rs470490-U3	GT	PCRU PCRL SNPU	102	AATTCAAGGCGAGATGACAA AAATGTATGTCTGCTTTCTTCCC CGTGCCGCTCGTGATAGAATCTCACAGCAGTTATTGGCCAAGGAC Possible SBE Primer vs Amplicon Cross Hybridization...
>rs723651-U1	GT	PCRU PCRL SNPU	90	ATGAGACTCCCCAGAAAGG TTTAAGTGATGATTTGAATGTGG ACGCACGTCCACGGTGATTTTCTAGGCCCCTCTTGCTGTTTGGGN

NOTE For the forced SNPs, the SBE primer has an **N** at the 3'-end.

5. Replace the 3' N in the SBE primer with the original base prior to ordering the oligonucleotide.

   Although this procedure has been shown to be a reliable method for forcing AutoPrimer to pick a specific strand, there exist conditions where the AutoPrimer algorithm will select only one primer orientation no matter what is done to the opposite strand sequence. In these cases, replacing the SNP may be necessary to complete the panel.

InSilico PCR

7.1 *InSilico* PCR

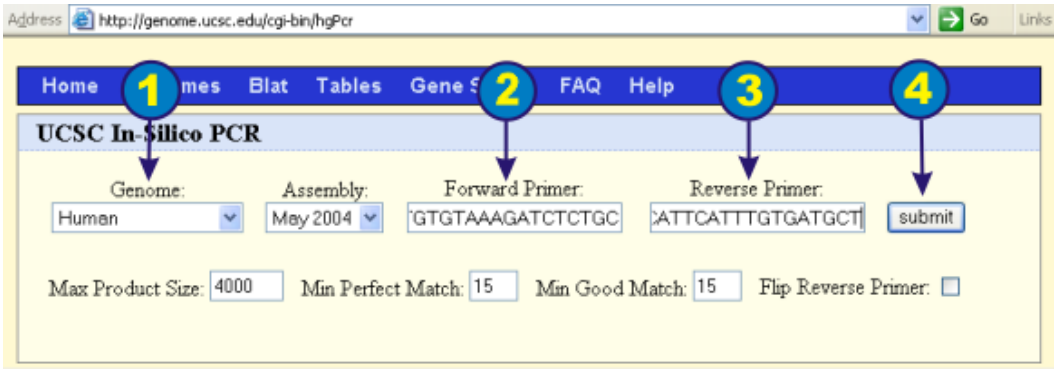
InSilico PCR, available at *http://genome.ucsc.edu/cgi-bin/hgPcr*, predicts the number of distinct amplicons that may be generated from a given primer pair. This method is useful for checking a sequence with multiple BLAST hits that can not be unambiguously determined to overlap and align to the same chromosomal position.

For example, rs36950 has two BLAST hits (Figure 2.3 on page 5). PCR primer sequences are given in the Autoprimer results file, as shown below.

Figure 7.1 Autoprimer Results

SNP Name Panel # 1	SNP Type	Oligo Type-Mod.	Amplicon Length	Sequence
>rs380607-U11	GT	PCRU	141	GGATTGGAATTCAGCATCG
		PCRL		AGAAAATGTAGAAGAGGCAGAATC
		SNPU		AGAGCGAGTGACGCATACTACCTTGGATGTCTGGATAATCTCCAN
>rs722979-U10	GT	PCRU	90	TGAAGGCAAAAAAAATCCAA
		PCRL		AGGAGGTAAAAAGGTATCTACAGACTTAT
		SNPU		AGATAGAGTCGATGCCAGCTGTCCAAAGCGTGCGTAAGAGTCGTT
>rs309773-U12	GT	PCRU	141	AGTCAAATGTTGCATCCTAAAAG
		PCRL		ATCTAATAGCATATATACTAGGTCTTCTGAGA
		SNPU		CGACTGTAGGTGCGTAACTCGGCCACAGATGATCATACACTTGCT
>rs727432-U9	GT	PCRU	108	TTTACCCAAACCATCGTCTG
		PCRL		TCAATTGCTAATTCAAAATGAATG
		SNPU		GACCTGGGTGTCGATACCTAGGTATGAGGTCTTCACCCCATGCCT
				Possible PCR Primer Cross Hybridization...
>rs36950-U7	GT	PCRU	97	CTGCTTGTGTAAAGATCTCTGC
		PCRL		ACTCTTCATTCATTTGTGATGCT
		SNPU		AGGGTCTCTACGCTGACGATTAATGGGTGTAACGCTAAATAAAGC
				Possible PCR Primer Cross Hybridization...
>rs424293-U8	GT	PCRU	101	AATCAGAAATGGAATGAAGCATAT
		PCRL		CATAAAATACATATTTATATTGCTCACTGG
		SNPU		GTGATTCTGTACGTGTCGCCGAGAACTAAAAGAAATTTCCCA
				Possible SBE Primer vs Amplicon Cross Hybridization...
>rs721757-U5	GT	PCRU	132	AACATGGAAAAATTCATCCTAACT
		PCRL		AACAATATTGGGGATGTTATTAGG
		SNPU		GCGGTAGGTTCCCGACATATTCTATCAACACTTGGAATAACAGAT
>rs720596-U6	GT	PCRU	151	AGGGTTGGGATAAAACAACAG
		PCRL		ATTATATTTCAGTACAACATCTACTCTTCAA
		SNPU		GGCTATGATTCGCAATGCTTATGAATAGAAAAGGATTAGAGCAGG
>rs756384-U4	GT	PCRU	153	AAAGGTAAATATCTGACCAATGTGA
		PCRL		ATTTCATAGCTCCCAGGATTACA
		SNPU		AGCGATCTGCGAGACCGTATCTGCTCAGATTTTATATCCCAGGAA
>rs721623-U2	GT	PCRU	107	AGTATTTGATATGAAAATAATTGTGCTG
		PCRL		AATTCTATTTGGCACAGGTGG
		SNPU		GGATGGCGTTCCGTCCTATTCTTCATAAATGTAACTATCAAGCAC
>rs470490-U3	GT	PCRU	102	AATTCAAGGCGAGATGACAA
		PCRL		AAATGTATGTCTGCTTTCTTCCC
		SNPU		CGTGCCGCTCGTGATAGAATCTCACAGCAGTTATTGGCCAAGGAC
				Possible SBE Primer vs Amplicon Cross Hybridization...
>rs723651-U1	GT	PCRU	90	ATGAGACTCCCCAGAAAGG
		PCRL		TTTAAGTGATGATTTGAATGTGG
		SNPU		ACGCACGTCCACGGTGATTTTCTAGGCCCCTCTTGCTGTTTGGGN

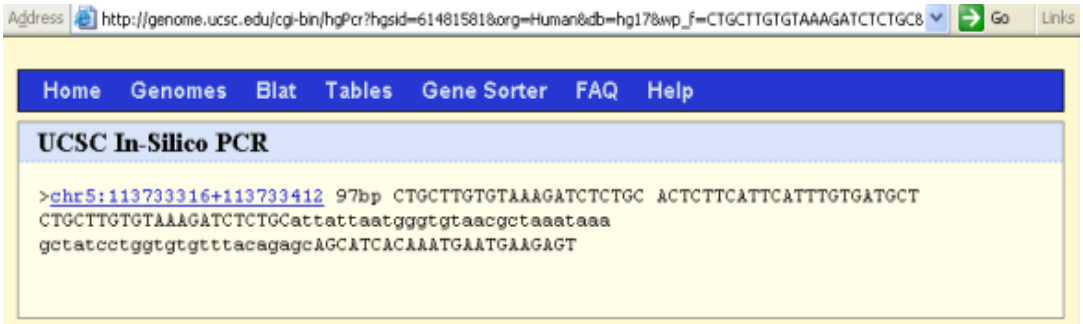
Figure 7.2 Primer Submission for *InSilico* PCR



Legend

1. Select **Genome**.
2. Click in the field and paste the forward primer.
3. Click in the field and paste the reverse primer.
4. Click **Submit**.

Figure 7.3 *InSilico* PCR Results



The results show the chromosomal location and size of a single amplicon generated from the submitted primers. See Figure 7.3 above.

NOTE The predicted size agrees with that calculated by the Autoprimer from the submitted sequence. The web-based tool does not accept batch queries.

Original Sequences

A.1 Original Sequences (.doc)

>rs36950

GCTATGGTAGTTTCATGTACCTATGAGGTGGATAAAAATTTAGCAACAGGTTGAAGGTAA
GACTCCAAAAGATAGTGAGGTAGAAAACTTGCCTGAATTCCTTTGTTGAGTGGTGTTCA
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GTCCCTTTGACTTTGTGTATACACTGTAGTAACGAAATCACTCAGGACTAATTTTCTCCG
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GATCTTAATTA

>rs130588

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>rs144848

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>rs187031

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GCAACCTCC

>rs277939

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>rs288422

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>rs309133

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>rs380607

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>rs382515

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>rs383885

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>rs424293

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>rs443523

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>rs456798

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>rs466380

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>rs470490

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>rs554653

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>rs615474

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>rs638250

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>rs713075

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>rs713478

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Original Sequences (.doc)

RepeatMasker Results

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C.1 Masked Sequences (.txt)

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Masked Sequences

D.1 Masked Sequences (.doc)

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Masked Sequences
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Masked Sequences, Single Line

E.1 Masked Sequences, Single Line (.doc)

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F.1 Masked Sequences, Single Line (.txt)

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Original Sequences

H.1 Original Sequences, Single Line (.xls)

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Original and Masked Sequences

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Original and Masked Sequences

J.1 Original and Masked Sequences, Single Line Each (.txt)

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Original and Masked Sequences

K.1 Original and Masked Sequences, Single Line Each, Masking Adjusted (.txt)

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Original and Masked Sequences

L.1 Original and Masked Sequences, Single Line Each, Masking Adjusted (.xls)

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Original and Masked Sequences

Original and Masked Sequences, Single Line Each, Masking Adjusted (.xls)

Original and Masked Sequences

M.1 Original and Masked Sequences, Single Line (.xls)

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Autoprimer Results

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Forced Panel

P.1 Forced Panel (.txt)

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Autoprimer Results

Q.1 Autoprimer Results, Forced Panel (.txt)

Primers Designed For:
SNPstream Customer
Senior Scientist
BCI
D25B
4300 N. Harbor Blvd
Fullerton, CA 92834

Telephone: 7149923669
Facsimile: 7149923669

General Info:
Submission Date: 9/7/2005
Primers Designed for SNPstream 12plex Kits
Comments: tutorial new panel 12plex

Powered By: Autoprimer v2.3 (Build 48)

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		SNPU		
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>rs722979-U10	GT	PCRU	90	TGAAGGCAAAAAAAAATCCAA
		PCRL		AGGAGGTAAAAAGGTATCTACAGACTTAT
		SNPU		
AGATAGAGTCGATGCCAGCTGTCCAAAGCGTGCGTAAGAGTCGTT				
>rs309773-U12	GT	PCRU	141	AGTCAAATGTTGCATCCTAAAAG
		PCRL		ATCTAATAGCATATATACTAGGTCTTCTGAGA
		SNPU		
CGACTGTAGGTGCGTAACTCGGCCACAGATGATCATACACTTGCT				
>rs727432-U9	GT	PCRU	108	TTTACCCAAACCATCGTCTG
		PCRL		TCAATTGCTAATTCAAAATGAATG
		SNPU		
GACCTGGGTGTCGATACCTAGGTATGAGGTCTTCACCCCATGCCT				

Possible PCR Primer Cross Hybridization...

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>rs36950-U7          GT    PCRU       97    CTGCTTGTGTAAAGATCTCTGC
                     PCRL             ACTCTTCATTCATTTGTGATGCT
                     SNPU
AGGGTCTCTACGCTGACGATTAATGGGTGTAACGCTAAATAAAGC
                                 Possible PCR Primer Cross Hybridization...

>rs424293-U8         GT    PCRU       101   AATCAGAAATGGAATGAAGCATAT
                     PCRL             CATAAAATACATATTTATATTGCTCACTGG
                     SNPU
GTGATTCTGTACGTGTCGCCGAGAAGTAAAAGAAATTTCCCA
                                 Possible SBE Primer vs Amplicon Cross Hybridization...

>rs721757-U5         GT    PCRU       132   AACATGGAAAAATTCATCCTAACT
                     PCRL             AACAATATTGGGGATGTTATTAGG
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>rs720596-U6         GT    PCRU       151   AGGGTTGGGATAAAACAACAG
                     PCRL             ATTATATTTCAGTACAACATCTACTCTTCAA
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GGCTATGATTCGCAATGCTTATGAATAGAAAAGGATTAGAGCAGG

>rs756384-U4         GT    PCRU       153   AAAGGTAAATATCTGACCAATGTGA
                     PCRL             ATTTCATAGCTCCCAGGATTACA
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>rs721623-U2         GT    PCRU       107   AGTATTTGATATGAAAATAATTGTGCTG
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GGATGGCGTTCCGTCCTATTCTTCATAAATGTAACTATCAAGCAC

>rs470490-U3         GT    PCRU       102   AATTCAAGGCGAGATGACAA
                     PCRL             AAATGTATGTCTGCTTTCTTCCC
                     SNPU
CGTGCCGCTCGTGATAGAATCTCACAGCAGTTATTGGCCAAGGAC
                                 Possible SBE Primer vs Amplicon Cross Hybridization...

>rs723651-U1         GT    PCRU       90    ATGAGACTCCCCAGAAAGG
                     PCRL             TTTAAGTGATGATTTGAATGTGG
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ACGCACGTCCACGGTGATTTTCTAGGCCCCTCTTGCTGTTTGGGN


>========== UHT Probe Panel Info ==========<
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