

Bioinformatic Evaluation of a Sequence for Custom TaqMan® SNP Genotyping Assays

Overview

The Custom TaqMan® SNP Genotyping Assays (formerly named TaqMan® Assays-by-Design® SNP Genotyping Service) are custom assays that are designed, synthesized, formulated, and delivered as analytically quality-controlled primer and probe sets for single nucleotide polymorphism (SNP) genotyping assays based on sequence information submitted by the customer. The goal of this tutorial is to help the researcher evaluate the quality of their sequence information before submitting an order for a Custom TaqMan® SNP Genotyping Assay. Specific information is given on how to assess a sequence using a variety of on-line tools.

The [Custom TaqMan® SNP Genotyping Assays](#) provide the researcher the opportunity to design an assay that is not currently available through the [TaqMan® SNP Genotyping Assays](#), TaqMan® Validated, Coding and Pre-Designed SNP Genotyping Assays offerings. Studies that involve new SNP discovery or SNPs in non-human species are examples of applications that would benefit from this custom design line of products. For human SNP assays, the TaqMan® SNP Genotyping Assays products should be used. If a particular SNP assay is currently not available then one should consider a custom design.

***Note:** Additional TaqMan® Assays are regularly added to the web site for ordering. Please visit the [Applied Biosystems web site](#) for regular updates.*

Process Overview

Ordering Custom TaqMan® SNP Genotyping Assays involves the following procedures:

1. Selecting a target sequence
- 2. Assessing the quality of the sequence**
3. Preparing the submission file using the [File Builder software](#).
4. Formatting the sequence for submission
5. Submitting the order via the File Builder software or e-mail.

Step two, **Assessing the quality of the sequence**, will be covered in this tutorial.

Step 1, and steps 3-5: Selecting a target sequence, Preparing the submission file, Formatting the sequence for submission, and Submitting the order are covered in:

- [Custom TaqMan® Genomic Assays: Protocol: Submission Guidelines](#)
- [Online Ordering Procedures Using the File Builder Software: Quick Reference Card](#)
- [Ordering TaqMan® SNP Genotyping Assays: Quick Reference Card](#).

Assessing the Quality of the Sequence

Overview

The most important factor in the success of the Custom TaqMan[®] Assays is the quality of the sequence data that you submit for the design process. Sequence analysis gives one a tool to eliminate poor sequence quality so it does not adversely impact the assay. Consider the following when selecting your target sequence:

- ❖ [Biological significance](#)
- ❖ [Sequence length](#)
- ❖ [Sequence quality](#)
- ❖ [Masking sequences](#)
- ❖ [Uniqueness of sequence](#)

Biological Significance

When choosing sequences to submit, first consider the biological significance of the desired assay. Points to take into consideration include:

- Has the SNP been substantiated by more than one line of experimental evidence, i.e., is the SNP a “double hit” SNP?
- Is there Minor Allele Frequency (MAF) data available for a SNP?
- Has this SNP been identified in the population, e.g., ethnic group, that you are examining?

Such biological qualifiers give confidence that a given SNP is well studied and may be useful as a marker in your particular study.

The quality assurance test of assays carried out during manufacture of the primers and probes ensure that the yield and content of the primers and probes meet specifications. For human SNP assays, an additional functional test is done with 20 genomes representing 3 populations (African American, Caucasian, and Japanese). This test ensures that a passed assay yields an amplified product and that at least one genotypable cluster forms in an allelic discrimination plot. However, Applied Biosystems is unable to guarantee the biological performance of the assays.

Example:

If you are studying a known SNP, try to get information on allele frequencies (in NCBI dbSNP, HapMap, or other project databases). By knowing the minor allele frequency, you can estimate the population size required to detect a given minor allele and to provide statistically significant results.

The Hardy-Weinberg Equilibrium (HWE) equation may be used to assess the likelihood that a SNP with a known MAF in a given population is detectable in the same population of a particular size:

$$q^2 + 2qp + p^2 = 1, \text{ where } q \text{ and } p \text{ represent the allele frequencies.}$$

The values obtained for q^2 , $2qp$, and p^2 correspond to the fraction of a given population that would be homozygous $q:q$, heterozygous $q:p$, and homozygous $p:p$. E.g., For a SNP with a MAF of 5% in a given population, the HWE predicted spread of genotypes is: 0.0025 $q:q$, 0.0095 $q:p$, and 0.9025 $p:p$. Thus, in a test of 20 gDNAs from this population, one might expect to see approximately 0 homozygotes for the minor allele, 2 heterozygotes, and 18 samples homozygous for the major allele. It would take a sample size of approximately 400 individuals to detect a homozygote for the minor allele.

Sequence Length

To optimize your assay design, follow these guidelines:

- Submit a sequence length of approximately 600 bases. Increasing the sequence length increases the assay possibilities, albeit, most SNP assays produce amplicons of <300 bps.
- Select the sequence so that the target site is toward the center of the submitted sequence.

Note: Sequence length can range from 61 to 5000 bases. Short (fewer than 300 bases) sequences limit the potential number of assays that can be designed.

Sequence Quality

To Assess the Quality of the Sequence:

1. Obtain confidence in the sequence accuracy. You want to have the most accurate sequence of your desired target before you submit the sequence to have an assay designed. Inaccurate sequences can lead to failed assays due to poor binding, or no binding, of primers or probes.

Example: You have sequenced some clones, but there are some regions of the sequence that only have a single pass of sequence, or sequence from only one strand of the clone. You send the sequence in for a Custom TaqMan[®] Assay anyway. You may end up getting no amplification or nonspecific amplification with this assay because it may bind to other targets or to nothing at all, if your sequence contained incorrect sequences to which assay probes and primers were designed.

Note: If you performed the sequencing yourself, it is strongly recommended that you perform multiple sequencing reactions to remove any ambiguities.

2. Use other resources, such as public databases with curated sequences such as [NCBI](#) and [dbSNP](#) to determine the quality of your sequence.

Masking Sequences

The Custom TaqMan[®] Assays proprietary software for designing primers and probes will not design probes or primers to a region of sequence containing Ns. You can annotate your sequences with Ns to avoid specific regions of sequence in design (e.g., ambiguous sequences, repetitive sequences or other SNP sites), albeit the use of Ns may limit assay design choices.

To mask sequences:

1. You may substitute each ambiguous base with an N.

For example:

The **bolded** bases in this sequence are ambiguous:

ACGTGACGTGACGTGACGTGACGTGGATYGTG**RSR**STCCT

Where Y= C or T, R=A or G, and S= G or C; they would be substituted as:

ACGTGACGTGACGTGACGTGACGTGGAT**NGTGNNN**TCCT.

2. Minimize the substitution of Ns in the sequence.

Because the Custom TaqMan[®] Assays proprietary software does not include Ns in the probe or primer, having a sequence with Ns greatly reduces the number of available primers and probes from which to select an optimal assay.

3. Ensure that Ns are not too close to the target site.

Important! No probes can be designed if Ns are too close to the target site. When designing SNP assays, make sure that no Ns are within two bases of the target site

Uniqueness of Sequence

After you have selected a sequence, check whether unique primers and probes can be generated for the genomic DNA sequence by verifying that the target sequence is unique within the organism you are studying.

To Ensure Unique Primer and Probe Sequences for gDNA:

1. Follow one of two strategies:
 - Analyze the entire target sequence.
 - Limit analysis to the region around the target site (for example, 100 bp on either side of the SNP).
2. Run the sequence through a program such as [Repeat Masker*](#) to detect common repetitive elements found in genomic DNA.
3. If many regions with similar sequences are returned, try using a filter. For example, limit the search to Human genomic DNA for SNPs.
Note: The [BLAT server at the University of California Santa Cruz](#) carries out searches using the assembled genome, so that when conducting a BLAST search only one species is being queried.
4. Perform a [BLAST®](#) search against public databases to detect regions within your sequence that have similarity to other published sequences and repetitive elements.
5. Perform a [BLAST®](#) search against SNP databases, such as [dbSNP](#) and [dbSTS](#), to determine if there are any other polymorphisms in your target sequence.

TOOLS

I. Repeat Masker

While the use of Ns limits assay design (see [Masking Sequences](#)), it allows you to eliminate possible assay design in areas of similarity to other unrelated sequences or to regions of low complexity DNA. Neither repeat elements nor low complexity DNA should be used as potential PCR primer sites since they could produce non-specific amplification or probe binding.

On average, close to 50% of the human genomic DNA sequence will be masked by RepeatMasker. It is a program that screens DNA sequences for interspersed repeats and low complexity DNA sequences. The output is a detailed annotation of the repeats that are present in the query sequence as well as a modified version of the query sequence in which all the annotated repeats have been masked (default: replaced by Ns). The masked sequence can be used for submission and can also be used in [BLAST®](#) searches.

*Examples of web sites that host RepeatMasker are:

<http://www.repeatmasker.org>

This website has a lot of useful information on the RepeatMasker program, including FAQs and documentation such as Interpreting Results, Sensitivity, and RepeatMasker uses. "RepeatMasker is most commonly used to avoid spurious matches in database searches. Generally this step is strongly recommended before doing BLASTN or BLASTX equivalent searches with mammalian DNA sequence."

<http://woody.embl-heidelberg.de/repeatmask>

This site is a mirror of the University of Washington site above. The [repeatmask help](#) on this site has similar information to that of the University of Washington.

How to use RepeatMasker

A. Submitting your sequence / Starting your query

- You may enter your sequence by either copying and pasting your sequence into the box provided, or uploading it from a file.
- Sequences can be submitted one at a time or in batch form.
- Sequence submissions must be in [FASTA format](#) (see input format)
- When selecting 'return format' and 'return method', if you choose "html" for both, your results will be displayed in your web browser window.
- Make sure you choose the appropriate source of your DNA. The default genome library is human. Because interspersed repeats are specific to a (group of) species, it is important to select the appropriate repeat library to search.
- Click on 'Submit Sequence'.

RepeatMasker Submission

Basic Options

[Large sequences](#) will be queued, and may take a while to process.

Enter the [file](#) to process:

Enter sequence(s) here

Or paste the sequence(s) in [FASTA format](#):

```
>gi|37552484:8878-11505 Homo sapiens chromosome 8 genomic
GAAATGAAAAATGACACTTTACTGTTTTAGTTTGCATTTCTCTGCTTACAAATGGATTACA
CGCATTTTTCATGTGCTGTTGGCTACTTATTCATTCAGAAAACATACTAAGTGCTGGCTCT
TTTTCATGTCCCTTTATCAAGTTTGGATCATGTCATTTGCTGTTTTCTTTCTGATGTA AAC
TCTCAAAGTTTGAAGGGTATTGTCTTTTCCTGACACATACATTGTAATAATTTCTGGC
TTACATTTTGACTTTTAAATTCATTCACGATGTTTTTAAATGAATAATTTAATTTTATG
```

Select [return format](#): html tar file links

Select [return method](#): html email

Advanced Options

[Speed/Sensitivity](#): rush quick default slow

[DNA source](#):

[Contamination](#):

[Repeat Options](#):

[Artifact Check](#):

[Alignment Options](#):

B. Viewing your Results

- RepeatMasker returns the submitted sequence(s) with all recognized interspersed or simple repeats masked. In the masked areas, each base is replaced with an N, so that the returned sequence is the same length as the original.
- A table annotating the masked sequences as well as a table summarizing the repeat content of the query sequence will be returned to your screen. In the "html" return format all output is returned to your screen in one file.
- The masked sequence can be copied directly from the web browser.
- We strongly recommend that when any sequence is submitted for a Custom TaqMan[®] Assay, the sequence be masked for repeat elements. This will reduce the possibility of poor sequence quality impacting assays.

II. **BLAST**[®] (Basic Local Alignment Search Tool)

After you have selected a target, there are other things that must be considered before submitting a sequence for a Custom TaqMan[®] Assay. Whether you have sequenced your target or taken the sequence from a sequence database, it is important to determine whether unique primers and probes can be generated for the sequence. It is also important to identify all polymorphisms in your sequence of interest. To do this, you can compare your target sequence to databases of sequences and search for regions of sequence similarities. In order to make your assay as specific as possible, regions of similarity can be masked out before submitting your sequence for design, so they are not considered in the assay design. The National Center for Biotechnology Information (NCBI) hosts a database of all published nucleotide and protein sequences. BLAST[®], a sequence comparison algorithm, is available to facilitate nucleotide and protein searching of the NCBI public databases.

A. **How to use BLAST**[®] **to search for Sequence Similarity**

1. **Submitting your sequence / Starting your query**

- Go to the [NCBI BLAST](#)[®] [site](#)
- Choose “Nucleotide-nucleotide BLAST (blastn)” under **Nucleotide**.
- Choose approximately 300 – 600 bases of sequence for your query
- Enter your sequence into the box provided. You may want to search with your masked sequence; the output from RepeatMasker. There are three sequence formats that may be entered into this box. (See pg. 9) For more information on this, click on the word [Search](#) next to the box.
- Choose the appropriate [database](#) to search.
Note: For SNP assay design, choose the “chromosome” or “nr” database. Sequences submitted to SNP assay design should not originate from mRNA sequences, as genomic DNA is the template for SNP assays, and assays made to mRNA sequences may be disrupted by intronic sequences.
- Under ‘Options for advanced blasting’ you can, among other things, [limit your search to a specific organism](#) using the drop down menu, and opt to [filter](#) your query for low complexity sequences (not necessary if searching with output from RepeatMasker).
- Click on ‘BLAST!’ to submit your search.

2. **For more information on how to use BLAST**[®]

NCBI has extensive help documentation on the NCBI BLAST[®] website. This includes:

- [FAQs](#)
- [Tutorials](#)
Included on the Tutorials page are also an [Introduction to Similarity Searches](#) and a [Glossary of Terms](#).

BLAST® Submission



Information on format of submission sequence. This sequence is in FASTA format

[Search](#)

```
>gi|37552484:8878-11505 Homo sapiens chromosome 8 genomic
AATGCAAGTTAAAAATAATTCTTTCATTGTGGTTTCTGACATGTCATGCCAATAAGGGTCT
TCCTCCTCCAAGAGCACAGAAAATTTTGCCAATACTGTCCTTAAAAATCGGTCACAGTTCA
TTTTTTATATATGCATTTTACTTCAATTGGGGCTTCATTTTACTGAATGCCCTATTTGAA
GCAAGTTTCTCAGTTAATTCTTTTCTCAAAGTGCTAAGTATGGTAGATTGCAAAACATAAG
```

[Set subsequence](#) From: To:

[Choose database](#)

Now: [Reset query](#) [Reset all](#)

- est_others
- gss
- htgs
- pat
- pdb
- month
- alu_repeats
- dbsts
- chromosome
- wgs
- env_nt

[Limit by entrez query](#) or select from:

[Choose filter](#) Low complexity Human repeats Mask for lookup table only Mask lower case

[Expect](#) **Options for Filtering for low complexity sequences if query sequence has not been masked**

[Word Size](#)

[Other advanced](#)

3. BLAST Results

There are three general parts to BLAST® results:

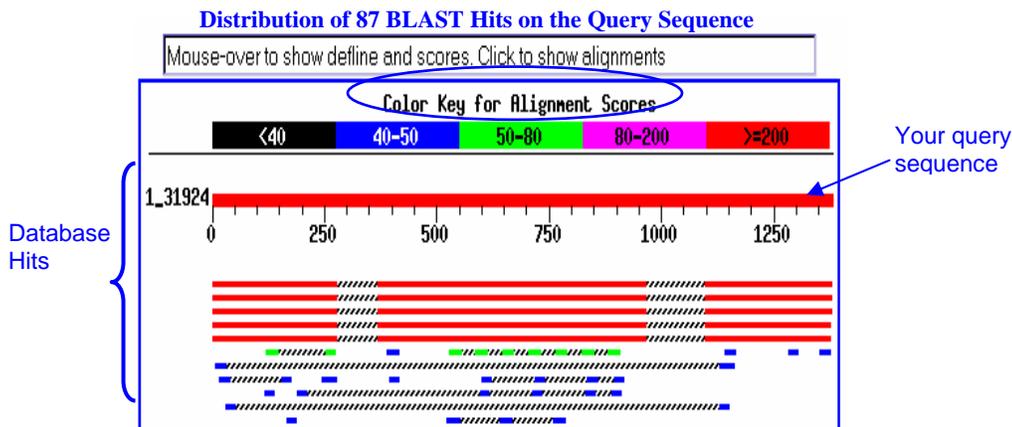
- Graphical overview
- List of Sequences producing significant alignments to your query
- Sequence alignments.

These sections are described below to give you a better understanding of what information can be obtained from a BLAST search of the NCBI public nucleotide database.

a. Graphical overview

The graphical overview is a representation of the database sequences (hits) that align to your query sequence, with the query sequence represented by the thick red numbered line at the top of the graph. The color of the line represents the score of the alignment, and a striped line connects multiple alignments to the same database sequence.

BLAST Output



b. List of Sequences producing significant alignments to your query

The list of sequences is shown from best to worst alignment; the top hit being the best hit (and possibly the sequence with which you queried the database). Public ID information is available as hypertext to the GenBank records that align to your query sequence, as well as a sequence definition. Clicking on the Score hypertext will take you to the actual sequence alignment. The score reflects the degree of similarity between your sequence and the sequence to which it is being aligned. The higher the score is, the more similar the sequences. You should also be able to understand the [E value](#) in order to evaluate the significance of a particular result. The E value represents the number of hits one can "expect" to find by chance when searching a database of a particular size. In this case, the database is the NCBI database that you searched. The lower the E value is, the more significant the match. Hits with E values higher than around 0.1 are unlikely to be very significant.

Click on **Score** to go to sequence alignment

Sequences producing significant alignments:			Score (bits)	E Value
gi 42406292 ref NC_000008.8 NC_000008	Homo sapiens chromoso...	1181	0.0	
gi 42406303 ref NC_000016.7 NC_000016	Homo sapiens chromoso...	1031	0.0	
gi 42406218 ref NC_000001.7 NC_000001	Homo sapiens chromoso...	898	0.0	
gi 42406225 ref NC_000006.8 NC_000006	Homo sapiens chromoso...	515	e-143	
gi 42406224 ref NC_000005.7 NC_000005	Homo sapiens chromoso...	507	e-141	
gi 42406302 ref NC_000015.7 NC_000015	Homo sapiens chromoso...	52	0.001	
gi 42406301 ref NC_000014.6 NC_000014	Homo sapiens chromoso...	50	0.005	
gi 42406297 ref NC_000010.7 NC_000010	Homo sapiens chromoso...	46	0.079	
gi 42406298 ref NC_000011.7 NC_000011	Homo sapiens chromoso...	44	0.31	
gi 42406220 ref NC_000003.8 NC_000003	Homo sapiens chromoso...	44	0.31	
gi 42406221 ref NC_000004.8 NC_000004	Homo sapiens chromoso...	42	1.2	
gi 42406219 ref NC_000002.8 NC_000002	Homo sapiens chromoso...	42	1.2	
gi 50295403 ref NC_006030.1 	Candida glabrata strain CBS138...	40	4.9	
gi 23509224 ref NC_004317.1 	Plasmodium falciparum 3D7 chro...	40	4.9	
gi 31742517 ref NT_078267.2 	Anopheles gambiae str. PEST ch...	40	4.9	
gi 24762252 ref NT_037436.1 	Drosophila melanogaster chromo...	40	4.9	
gi 42406307 ref NC_000020.8 NC_000020	Homo sapiens chromoso...	40	4.9	
gi 42406305 ref NC_000018.7 NC_000018	Homo sapiens chromoso...	40	4.9	
gi 42406304 ref NC_000017.8 NC_000017	Homo sapiens chromoso...	40	4.9	
gi 42406293 ref NC_000009.8 NC_000009	Homo sapiens chromoso...	40	4.9	
gi 42406291 ref NC_000007.10 NC_000007	Homo sapiens chromos...	40	4.9	

By just browsing a list of hits one can get a good idea of the types of sequences that have been found to have some identity to your query. Notice that the first sequence in the list is the one that was used for the search in this example, NC_000008.8. The score is very high (1181), and the Expect value is 0. Remember that the closer an E-value is to "0" the more "significant" the match. For this particular query, most of the hits are to human chromosomes, which is the same gene as the query. Keep in mind that what you're looking for is the ability to design an assay that will uniquely detect your sequence of interest. If you find some regions of similarity between your sequence and another, those bases can be masked out, so that they will not be considered for assay design.

c. Sequence Alignments

This section is your query sequence aligned to every sequence on your list of hits. These alignments are to help assess the degree of similarity. The Score and Expect values are displayed underneath the sequence identifiers. The number of bases aligned and percent identity are shown, as well as the strand that was aligned of your query sequence and the database hit.

If you'll notice, the first hit in this list is the query sequences aligned to itself. This will be the first alignment shown, and will be a 100% match to itself.

```
Score = 1181 bits (596), Expect = 0.0
Identities = 596/596 (100%)
Strand = Plus / Plus
```

The alignments shown below are from the following genomic sequence from the database.

[>gj|42406224|ref|NC_000005.7|NC_000005](#) Homo sapiens chromosome 5, complete sequence Length = 181034922

Expect = e-141
Identities = 271/276 (98%)
Expect = e-135
Identities = 271/279 (97%)
Expect = e-113
Identities = 230/237 (97%)
Expect = 8e-79
Identities = 158/160 (98%)
Expect = 8e-76
Identities = 153/155 (98%)

Expect = 8e-76
Identities = 153/155 (98%)
Expect = 5e-71
Identities = 151/155 (97%)
Expect = 1e-68
Identities = 144/147 (97%)
Expect = 1e-68
Identities = 144/147 (97%)

Shown above is a list of the Expect values and percent identities for each of the 9 alignments (high-scoring segment pairs; see *HSP* in [Glossary](#)) for this database hit, NC_000005. The alignments shown on page 12 are the first and the third alignments listed above from NC_000005, with E values of e-141 and e-113.

the likely reason for the first two HSPs (shown in the above figure). If a segment of your query sequences came up with a significant match to part of a sequence from another gene, you should mask out that region of the sequence in your sequence for submission or simply not include that region in your submission.

B. How to use BLAST® dbSNP to search for Sequence Polymorphisms

1. Submitting your sequence / Starting your query

- Go to the [NCBI BLAST® SNP site](#). The default Program is blastn. This is the program you should use.
- Choose approximately 300 – 600 bases of sequence for your query
- Enter your sequence into the box provided. The sequence format should be [FASTA](#). You may either search with your masked sequence (output from RepeatMasker) or have the sequence filtered for you by the program. To have the sequence filtered for you, simply check the appropriate boxes next to the word [FILTER](#), as shown below.
- If you are searching with a gDNA sequence containing a SNP of interest, use an IUPAC code or 'N' for your SNP of interest so that it is readily identifiable. For example, if your SNP is [A/G], you may want to annotate it is as 'R'. Do not leave it as [A/G] because this will be interpreted as two bases.
- Click on 'Submit Query' to submit your search.

NCBI dbSNP homepage

BLAST Home Page

BLAST overview

BLAST FAQs

BLAST news

BLAST manual

Single Nucleotide Polymorphism

Select the BLAST program to use and enter your sequence in the text area below.

Program

Query Sequence

Enter your sequence as:

```
>gnl|dbSNP|rs25|alle1ePos=201
AGTAAGAGGAATCAATGTCATAGGCTTTAGATAGCATTATGACTGTGTG
CTCGTGTGTGTGAAAACCTTATAGGATGTAAGAGTGCCTTACAAATTGTCTT
CAAGTTTAAATTACAAACAGACATAGTACTTTCATTTAAAAGTTAGGAAA
ATGTAAGTTTAAATTTTTTAAATTTCTCTGTGAGCTTCTGCATGCAATCCT
RTGCAATTGGAATTTGATAGTCCTTTCACACAGGAGAATGAGAAATAGCT
AAGCATCCATTATTTAAGTCATTTTTCTGCAAGTGTGGGCTCACCCAAAT
CATGAGAGTGATAAAGGAACCTGGAACCTAGCTATTATTCAGGAAATGTGTG
```

Snp Blast Databases(Human)

<input checked="" type="checkbox"/> Chr. 1	<input checked="" type="checkbox"/> Chr. 7	<input checked="" type="checkbox"/> Chr. 13	<input checked="" type="checkbox"/> Chr. 19
<input checked="" type="checkbox"/> Chr. 2	<input checked="" type="checkbox"/> Chr. 8	<input checked="" type="checkbox"/> Chr. 14	<input checked="" type="checkbox"/> Chr. 20
<input checked="" type="checkbox"/> Chr. 3	<input checked="" type="checkbox"/> Chr. 9	<input checked="" type="checkbox"/> Chr. 15	<input checked="" type="checkbox"/> Chr. 21
<input checked="" type="checkbox"/> Chr. 4	<input checked="" type="checkbox"/> Chr. 10	<input checked="" type="checkbox"/> Chr. 16	<input checked="" type="checkbox"/> Chr. 22
<input checked="" type="checkbox"/> Chr. 5	<input checked="" type="checkbox"/> Chr. 11	<input checked="" type="checkbox"/> Chr. 17	<input checked="" type="checkbox"/> Chr. X
<input checked="" type="checkbox"/> Chr. 6	<input checked="" type="checkbox"/> Chr. 12	<input checked="" type="checkbox"/> Chr. 18	<input checked="" type="checkbox"/> Chr. Y
<input checked="" type="checkbox"/> MultiChr.	<input checked="" type="checkbox"/> NotOnChr.	<input checked="" type="checkbox"/> All of the Above	

BLAST Search Options

Expect **Descriptions** **Alignments**

Filter Low complexity Human repeats Mask for lookup table only

Other advanced options:

2. dbSNP BLAST® Results

The output is typical of BLAST® results, a list of sequences producing significant alignments to your query and the sequence alignments. Notice the Scores and Expect values, as well as the public identifiers. These are all discussed in the section entitled "[List of Sequences producing significant alignments to your query](#)".

<u>Sequences producing significant alignments:</u>	Score (bits)	E Value
gnl dbSNP rs25_allelePos=20 totalLen=691	1206	0.0
gnl dbSNP rs2883564_allelePos=500 totalLen=1000	1202	0.0
gnl dbSNP rs2354963_allelePos=499 totalLen=999	1202	0.0
gnl dbSNP rs2040818_allelePos=381 totalLen=730	909	0.0
gnl dbSNP rs57_allelePos=444 totalLen=730	909	0.0
gnl dbSNP rs2040819_allelePos=491 totalLen=731	887	0.0
gnl dbSNP rs58_allelePos=491 totalLen=731	843	0.0
gnl dbSNP rs12531657_allelePos=201 totalLen=401	675	0.0
gnl dbSNP rs26_allelePos=531 totalLen=731	575	e-161
gnl dbSNP rs9937771_allelePos=320 totalLen=820	371	e-100
gnl dbSNP rs9939845_allelePos=388 totalLen=888	367	1e-98
gnl dbSNP rs9925030_allelePos=306 totalLen=806	367	1e-98
gnl dbSNP rs9924427_allelePos=276 totalLen=776	367	1e-98
gnl dbSNP rs4591651_allelePos=401 totalLen=801	349	4e-93

Sequence Alignments

In the alignment on page 15 there are a few things of which to take note:

1. There is a stretch of 'N's in your query sequence. This is where BLAST® has filtered out a region of low complexity sequence. This region should be masked in your submission sequence.
2. You may or may not be able to see your SNP in the alignment, depending on what part of your sequence is aligned to the database hit.
3. By looking for mismatches in the alignment (no hash marks) you will be able to identify other known, documented SNPs. These SNPs should also be masked out in your submission sequence so that no primer or probe is designed over this area.

```
>gnl|dbSNP|rs2354963_allelePos=499totalLen=999
Length = 999
```

```
Score = 1203 bits (606), Expect = 0.0
Identities = 644/664 (96%)
Strand = Plus / Plus
```

```
Query: 1 agtaagaggaatcaatgtcataggccttagatagcatttatgactgtgtgctcgtgtgtg 60
|||||
Sbjct: 220 agtaagaggaatcaatgtcataggccttagatagcatttatgactgtgtgctcgtgtgtg 279
```

```
Query: 61 tgaaaacttataggatgtaaaagtgccttacaatttgtcttcaagtttaaattacaaacag 120
|||||
Sbjct: 280 tgaaaacttataggatgtaaaagtgccttacaatttgtcttcaagtttaaattacaaacag 339
```

```
Query: 121 acatagtactttcatttaaaagttaggaaaatgtagtttaaaannnnnnaatttctctgt 180
|||||
Sbjct: 340 acatagtactttcatttaaaagttaggaaaatgtagtttaaaatTTTTTaaatttctctgt 399
```

Filtered sequence

```
Query: 181 gagcttctgcatgcaatcctrtgcaattggaatttgatagtcctttcacacaggagaatg 240
|||||
Sbjct: 400 gagcttctgcatgcaatcctatgcaattggaatttgatagtcctttcacacaggagaatg 459
```

SNP of interest

```
Query: 241 agaaatagctaagcatccattatttaagtcatttttctgcaagtgtgggctcacccaat 300
|||||
Sbjct: 460 agaaatagctaagcatccattatttaagtcatttttctgcaagtgtgggctcacccaat 519*
```

Documented SNP in dbSNP
It is important to mask this base before submission.

*Alignment shortened for display purposes

Having evaluated the quality of your sequence information, you are now ready to move on to preparing your submission file using the [File Builder software](#).

Appendix

```
>gij|37552484:8878-11505 Homo sapiens chromosome 8 genomic
AATGCAAGTTAAAATAATTCTTTTCATTGTGGTTTCTGACATGTCATGCCAATAAGGGTCTTCTCCTCCAAGAGCACAGA
AATATTTGCCAATACTGTCCCTTAAAATCGGTCACAGTTTCATTTTTATATATGCATTTTACTTCAATTGGGGCTTCATTT
TACTGAATGCCCTATTTGAAGCAAGTTTCTCAGTTAATTCTTTTCTCAAAGTGCTAAGTATGGTAGATTGCAAACATAA
GTGGCCACATAAATACTCCACCTCCTTGGCCTCCTCTCCCAGGAGGAGATAGCCTCCATCTTCCACTCCTTAATCTG
GGCTTGGCCATGTGACTTACACTGGCCAATGGGATATTAACAAGTCTGATGTGCACAGAGGCTGTAGAATGTGCACT
GGGGCTTGGTCTCTCTTGGCTGCCCTGGAGACCAGCTGCCCCACGAAGGAAACAGAGCCAACCTGCTGCTTCCTGGG
GGGAGACAGTCCCTCAGTCCCTCTGTCTCTGCCAACCAGTTAACCTGCTGCTTCTGGAGGAAGACAGTCCCTCAGT
CCCTCTGTCTCTGCCAACCAGTTAACCTGCTGCTTCTGGAGGAAGACAGTCCCTCTGTCCCTCTGTCTCTGCCAAC
CAGTTAACCTGCTGCTTCTGGAGGAAGACAGTCCCTCAGTCCCTCTGTCTCTGCCAACCAGTTAACCTGCTGCTTC
CTGGAGGAAGACAGTCCCTCTGTCCCTCTGTCTCTGCCAACCAGTTAACCTGCTGCTTCTGGAGGAAGACAGTCCC
TCAGTCCCTCTGTCTCTGCCAACCAGTTAACCTGCTGCTTCTGGAGGAAGACAGTCCCTCTGTCCCTCTGTCTCTGC
CAACCAGTTAACCTGCTGCTTCTGGAGGAAGACAGTCCCTCTGTCCCTCTGTCTCTGCCAACCAGTTAACCTGCTG
CTTCTGGAGGAAGACAGTCACTCTGTCTCTGCCAACCAGTTGACCGCAGACATGCAGGTCTGCTCAGGTAAGACC
AGCACAGTCCCTGCCCTGTGAGCCAAACCAATGGTCCAGCCACAGAATCGTGAGCAAATAAGTATGCTTAAGTCA
CTAAGATTTGGGCAAAAGCTGAGCATTTATCCCAATCCCAATACTGTTTGTCTTCTGTTTATCTGTCTGTCTTCTCT
GCTCATTTAAAATGCCCCACTGCATCTAGTACATTTTTATAGGATCAGGGATCTGCTCTTGGATTTATGTCATGTTCC
CACCTCGAGGCAGCTTTGTAAGCTTCTGAGCACTTCCCAATTCCGGGTGACTTCAGGCGCTGGGAGCCCTGTGCATC
AGCTGCTGCTGTCTGTAGCTGAGTTCCTTACCCTCTGCTGTCTCAGCTCCTTCGC
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This is the sequence used for the RepeatMasker and nucleotide BLAST sections

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