Custom TaqMan® Assays

DESIGN AND ORDERING GUIDE

For SNP Genotyping and Gene Expression Assays

Publication Number 4367671

Revision G

ThermoFisher SCIENTIFIC

Manufacturer: Multiple Life Technologies Corporation manufacturing sites are responsible for manufacturing the products associated with the workflow covered in this guide.

Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

The information in this guide is subject to change without notice.

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Revision history: Pub. No. 4367671

Revision	Date	Description
G	12 May 2017	Updates to the assay products and formats, the assay search information, and the bioinformatics appendix.

Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

The Custom TaqMan® Assay Design Tool

The Custom TaqMan® Assay Design Tool is available for designing Custom TaqMan® Assays if a sequence is not available in our library of predesigned assays (**www.thermofisher.com/taqman**). Custom TaqMan® Assays use the sequence information that you supply, and all information is secure and confidential.

Use the Custom TaqMan® Assay Design Tool to order:

- **Custom TaqMan® SNP Genotyping Assays**— Custom assays for performing genotyping studies with singlenucleotide polymorphisms (SNPs), multiple nucleotide polymorphisms (MNPs), or insertions/deletions (indels). For more information, go to **thermofisher.com/taqmansnpdesign**.
- **Custom TaqMan® Gene Expression Assays**— Custom assays for quantitative gene expression analysis and DNA sequence detection. For more information, go to (**www.thermofisher.com/cadt**).
- **Custom Plus TaqMan® RNA Assays**— Custom assays, for quantitative analysis of coding and noncoding transcripts, that are designed using the bioinformatics feature of the Custom TaqMan® Assays Design Pipeline. For Custom Plus TaqMan® RNA Assays, the design pipeline performs bioinformatic analysis and quality control of the input sequences to ensure the best possible assay designs.

  IMPORTANT! We do not provide the assay sequences for Custom Plus TaqMan® RNA Assays. For more information, go to **www.thermofisher.com/cadt**.

After you select the sequences to study, access the Custom TaqMan® Assay Design Tool to enter target sequences, import sequence information from a file, or search our database for sequences. After selecting the target sequences, submit the sequences to us for custom designs.

From the Custom TaqMan® Assay Design Tool, you can:

- **Select an assay and submit your order**—We manufacture, package, and ship the Custom TaqMan® Assays to you.
- **Enter custom primer/probe pairs and submit your order**—Submit the sequences for the forward primer, reverse primer, and probe(s), then we synthesize the oligos and formulate a custom assay using your oligonucleotide sequences.

Custom TaqMan® Gene Expression Assays

The Custom TaqMan® Assay Design Tool can also:

- **Search for predesigned TaqMan® Gene Expression Assays**—Search the database for predesigned Inventoried or Made to Order Custom TaqMan® Gene Expression Assays.
- **Perform a bioinformatics analysis of input sequences**—Have the design tool perform bioinformatic analysis on your input sequences to design the best Custom TaqMan® Gene Expression Assays for your needs.

  The bioinformatics analysis of the design tool:
  - Generates custom assay designs based on input sequences.
  - Provides enhanced search that can restrict the assay design to include sequences that are unique to a specific organism (for transgenic experiments).
  - Displays candidate assay designs in context with genomic and transcript data.
  - Masks input sequences for SNPs and low complexity sequence.
  - Performs *in silico* quality control of candidate assay designs to ensure accuracy and uniqueness of potential assays.

  Note: We do not provide the sequences for Custom TaqMan® Gene Expression Assays designed using the bioinformatics analysis feature of the assay design pipeline.

Single tube products and formats

The products and formats for the Custom TaqMan® Assays are listed in Table 1 and Table 2 according to the type of assay and the number of reactions you want to order. The section, "Determine the sequence criteria" on page 9, explains instances when not to choose a human assay, even when the sequence is human.

Table 1 Single tube TaqMan® SNP Genotyping Assays

Product	Dye	Scale	Part number	Number of 5-µL reactions	Assay mix concentration
Custom TaqMan® SNP Genotyping Assays, Human	VIC™ and FAM™	Small	4331349	1,500	40X
		Medium	4332072	5,000	40X
		Large	4332073	12,000	80X
Custom TaqMan® SNP Genotyping Assays, Non Human	VIC™ and FAM™	Small	4332077	1,500	40X
		Medium	4332075	5,000	40X
		Large	4332076	12,000	80X

Table 2 Single tube TaqMan® Gene Expression Assays

Product	Dye	Scale	Part number	Number of 20-µL reactions	Assay mix concentration
Custom TaqMan® Gene Expression Assays	FAM™	Small	4331348	360	20X
		Medium	4332078	750	20X
		Large	4332079	2,900	60X
	VIC™	Small	4448508	360	20X
		Medium	4448509	750	20X
		Large	4448510	2,900	60X
Custom TaqMan® Gene Expression Assays, Primer-Limited (PL)	VIC™	Small	4448487	360	20X
		Medium	4448488	750	20X
		Large	4448492	2,900	60X
Custom Plus TaqMan® RNA Assays[1]	FAM™	Small	4441114	360	20X
		Medium	4441117	750	20X
		Large	4441118	2,900	60X
	VIC™	Small	4448514	360	20X
		Medium	4448515	750	20X
		Large	4448516	2,900	60X
Custom Plus TaqMan® RNA Assays, Primer-Limited (PL)	VIC™	Small	4448511	360	20X
		Medium	4448512	750	20X
		Large	4448513	2,900	60X

[1] Assay sequences are not provided for Custom Plus TaqMan® RNA Assay orders.

2 Design and order Custom TaqMan® SNP Genotyping Assays

Genotyping workflow

Note: There is a large database of predesigned TaqMan® SNP Genotyping Assays available. To search for predesigned TaqMan® SNP Genotyping Assays, use the keyword or chromosomal location search functions in the TaqMan® SNP Genotyping Assays section of our website: **www.thermofisher.com/taqmansnp**.
Predesigned TaqMan® SNP Genotyping Assays don't provide primer or probe sequences.

Determine the sequence criteria

▼

Mask target sequences

▼

Submit sequences

▼

Review the Design Details

▼

Select CustomTaqMan® SNP Assays

▼

Order assays

Note: For information on reordering Custom TaqMan® Assays, go to Chapter 4, "Reorder Custom TaqMan® Assays".

Determine the sequence criteria

The success of Custom TaqMan® SNP Genotyping Assay designs depends largely on the quality of the sequence data that you submit for the design process. After you select your target sequence, ensure that it meets criteria for:

- Allele frequency
- Sequence length
- Accuracy
- Uniqueness

Biological significance

Verify that:

- The SNP is confirmed by more than one line of experimental evidence, for example, that the SNP is a "double hit" or a validated SNP.
- Minor Allele Frequency (MAF) data are available for the SNP.
- The SNP occurs in the population (ethnic group) that you are examining.

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

Allele frequency criteria

The minor allele frequency (MAF) indicates the frequency of the uncommon allele in a population (Traditionally, only the minor allele frequency is reported. The major allele frequency is implied, and it is calculated as 1–MAF). With the MAF, you can estimate the size of the sample population required to detect a specified minor allele and to provide statistically significant results. If your sample is considerably smaller than the calculated value, consider using an assay for another SNP (one with a larger MAF) or increasing the sample size.

For a known SNP, try to find the allele frequency from resources such as the NCBI dbSNP, HapMap, or other project databases. Use the Hardy-Weinberg Equilibrium equation to determine the likelihood that a SNP with a known MAF in a specified population is detectable in a sample of a particular size in the same population.

The Hardy-Weinberg Equilibrium equation is:

$p^2 + 2pq + q^2 = 1$

where p and q represent the allele frequencies.

The values for p^2, 2pq, and q^2 correspond to the fraction of a known population that would be homozygous for the p allele (p:p), heterozygous (p:q), and homozygous for the q allele (q:q).

MAF example calculation

For a SNP with a MAF of 5% (0.05), the predicted spread of genotypes is 0.0025 q:q, 0.095 p:q, and 0.9025 p:p. In a test of 20 genomic DNA samples from this population, you might expect to see:

- Approximately 0 homozygotes for q, the minor allele
- 2 heterozygotes
- 18 homozygotes for p, the major allele

To detect a homozygote for the minor allele, the sample size would need to be approximately 400 individuals.

Sequence length criteria

A sequence length of approximately 600 bases is optimal, but the input sequence length can vary from 61 to 6,000 bases, with at least 30 bases on each side of the SNP.

Increasing the sequence length increases the assay design possibilities, although usually TaqMan® SNP Genotyping Assays produce amplicons of <200 base pairs. Provide a longer input sequence if your target sequence has low-complexity or contains N-masked nucleotides.

Select the sequence so that the target site is near the center of the submitted sequence.

Target site criteria

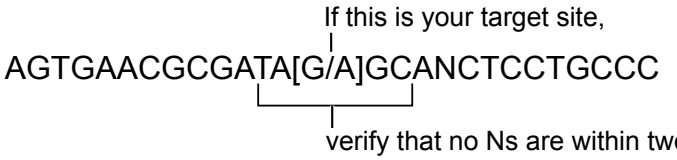
For TaqMan® SNP Assays, each target site identifies a SNP, an MNP, or an insertion/deletion (indel). Topics discussed in this chapter apply to SNPs, MNPs, or indels as the target site.

Note: Customers who have already prepared their sequences can go to the section "Submit sequences" on page 15.

Select:

- At least one target site.

  Note: No more than one assay is designed for a given sequence, regardless of the number of times an identical sequence is submitted. . Entering a longer sequence increases the assay design possibilities and can result in a different design than a shorter sequence. Contact Technical Support for assistance if required.
- Target sites that are more than 40 bases away from the 5′ and 3′ ends.
- Target sites that are more than two bases away from any Ns.



- Target sites that are more than two bases away from any other SNPs.
- At least one specific SNP for assay design (and mask all the remaining nontarget SNPs with Ns).

Target sequence requirements

Field	Requirement
Name	• Has 4 to 16 characters. • Uses only alphanumeric, underscore, hyphen, and period characters. • Has no spaces or tabs. • Does not start with a hyphen.

Field	Requirement
Sequence	• Enter the sequence in the 5′ to 3′ direction. • Enter ≤6,000 bases surrounding the SNP, with a minimum of 30 bases on each side of the SNP. • Use only A, C, G, T, and N, except where SNP or indel target sites are marked. • Convert the IUPAC codes R, Y, M, K, S, W, H, B, V, and D to N, except for marked SNP target sites where you translate the ambiguity code to the appropriate bases. The tool will accept codes for two bases but will prompt for selection of the desired order of alleles in brackets. • Enclose each target site with square brackets [ ]. • In the brackets that enclose: – **SNP targets**: Enter the base for the first allele followed by a forward slash (/), and then the base for the second allele. For example, convert R to [A/G]. – **MNP targets**: Enter from one to six bases for the first allele followed by a forward slash (/), and then one to six bases for the second allele. – **Insertion/deletion (indel) targets**: Enter from one to six bases for the insertion followed by a forward slash(/), then an asterisk (*) for the deletion. • Mask with Ns any SNPs, MNPs, or indels that are not targeted. **Note:** The probe for the first allele is labeled with a VIC™ dye. The probe for the second allele is labeled with a 6-FAM™ dye.
SNP, SNP#, SNP Name	• These fields are populated after the check format button is selected. If multiple polymorphisms appear in the sequence the tool will show them to the right of the sequence. Each SNP target site of interest: – Is marked with square brackets. – Has the bases for 2 alleles of the SNP, separated by a forward slash. • Each MNP target site of interest: – Is marked with square brackets. – Has 1 to 6 bases to the left of the forward slash and 1 to 6 bases to the right of the forward slash. (The number of bases on each side of the slash does not have to be the same.) • Each indel target site of interest: – Is marked with square brackets. – Has 1 to 6 bases to the left of a forward slash and an asterisk (indicating the deletion) to the right of the forward slash. – SNP# is the number of the SNP or polymorphism in the sequence in order from 5′ to 3′. – SNP Name: – Is an optional field used to name SNPs within a sequence. – Must be 1-4 characters long and consist solely of the characters A–Z, a–z, and 0–9.

Quality control

IMPORTANT! The quality control that is performed during manufacture of the primers and probes can ensure *only* that the yield and content of the primers and probes meet specifications. Although the manufacturer cannot guarantee the biological performance of the assays, reviewing your sequences as described in this document improves the possibility of success for your assay.

Each TaqMan® SNP Genotyping Assays undergoes a functional test the first time it is ordered. Genomic DNAs (gDNAs) from 20 unrelated individuals (from 4 populations and both sexes) are amplified under universal conditions with the SNP Genotyping Assay to test for amplification and clustering. Human SNP Genotyping Assays that fail this test are not shipped.

If you expect TaqMan® SNP Genotyping Assays for human targets to fail the functional test, order the nonhuman TaqMan® SNP Genotyping Assays. Failures can occur for the following reasons:

- Human cDNA sequences—Intronic sequences, or assay component binding sites, prevent primer or probe binding, which prevents efficient amplification because of longer amplicon size.
- Human Y-chromosome-specific sequences—More than 90% of the samples in the test must amplify to pass, and the female samples do not amplify.

Mask target sequences

Overview

The Custom TaqMan® Assays Design Pipeline does not design a primer or probe that spans an ambiguous base (an N). The presence of one or more Ns at a site forces the design pipeline to design the assay at another site in the sequence.

You can use Ns to mask (hide) sites where you do not want an assay to be designed, such as sites that contain ambiguous bases, known repeats, and/or polymorphisms. However, the more Ns used to mask undesired sites, the more restricted the assay design possibilities.

The next topic describes specific situations where you should consider masking your target sequence.

Evaluate sequence accuracy and uniqueness criteria

For more information about the tools that are used to evaluate target sequences, see Appendix B, "Bioinformatics tools for manually evaluating target sequences".

1. If you performed the sequencing yourself, perform multiple sequencing reactions to eliminate any ambiguities.

2. Except for the SNPs that you want to study, mask each ambiguous base in your sequence with an N.
   For example, the bases in bold text in the following sequence are ambiguous:
   ACGTGACGTGACGTGACGTGACGTGGAT**Y**GTG**RSRS**TCCT
   If you mask each ambiguous base with an N, the resulting sequence is:
   ACGTGACGTGACGTGACGTGACGTGGAT**N**GTG**NNNN**TCCT
   Too many Ns can restrict the possibilities for assay design. It may be better to resequence your gene to eliminate ambiguities than to mask the ambiguities with Ns.

3. Using other resources such as public databases, determine if your target sequence is unique in the organism of study or if similar sequences exist in the database.
   - Perform a BLAST-like Alignment Tool (BLAT) database search for genomic DNA alignments. See "Run a BLAT® database search" on page 43 for instructions.
   - Perform a Basic Local Alignment Search Tool (BLAST®) database search to find regions of your sequence that are similar to sequences in the database. See "Verify sequence uniqueness with a BLAST® database search" on page 43 for instructions.

   If other similar sequences exist, determine the degree of similarity. Multiple target sites in the genome interfere with the function of the assays.

4. If you find regions of your sequence that are similar to sequences in the database, mask those regions of your sequence with Ns.

Review sequences for repeats and polymorphisms

Assays that are designed in regions of a sequence that contain repeats and/or polymorphisms are likely to produce nonspecific amplification and probe binding. To reduce the likelihood of nonspecific amplification and probe binding, mask repeats and polymorphisms by performing the steps below.

1. Run the sequence through a program such as RepeatMasker to detect common repetitive elements. See "Mask sequence repeats" on page 51.

2. In the masked sequence that is generated by the program, determine if any of your target SNPs are:
   - In a masked repeat
   - Within 2 bases of a masked repeat

   If either condition exists, select another SNP because a primer or a probe cannot be designed within 2 bases of an N.

3. Run a BLAST® database search using the masked sequence from step 2 against the dbSNP, a database of SNPs from various species. See "Manually detect and mask non–target sequence polymorphisms" on page 53.

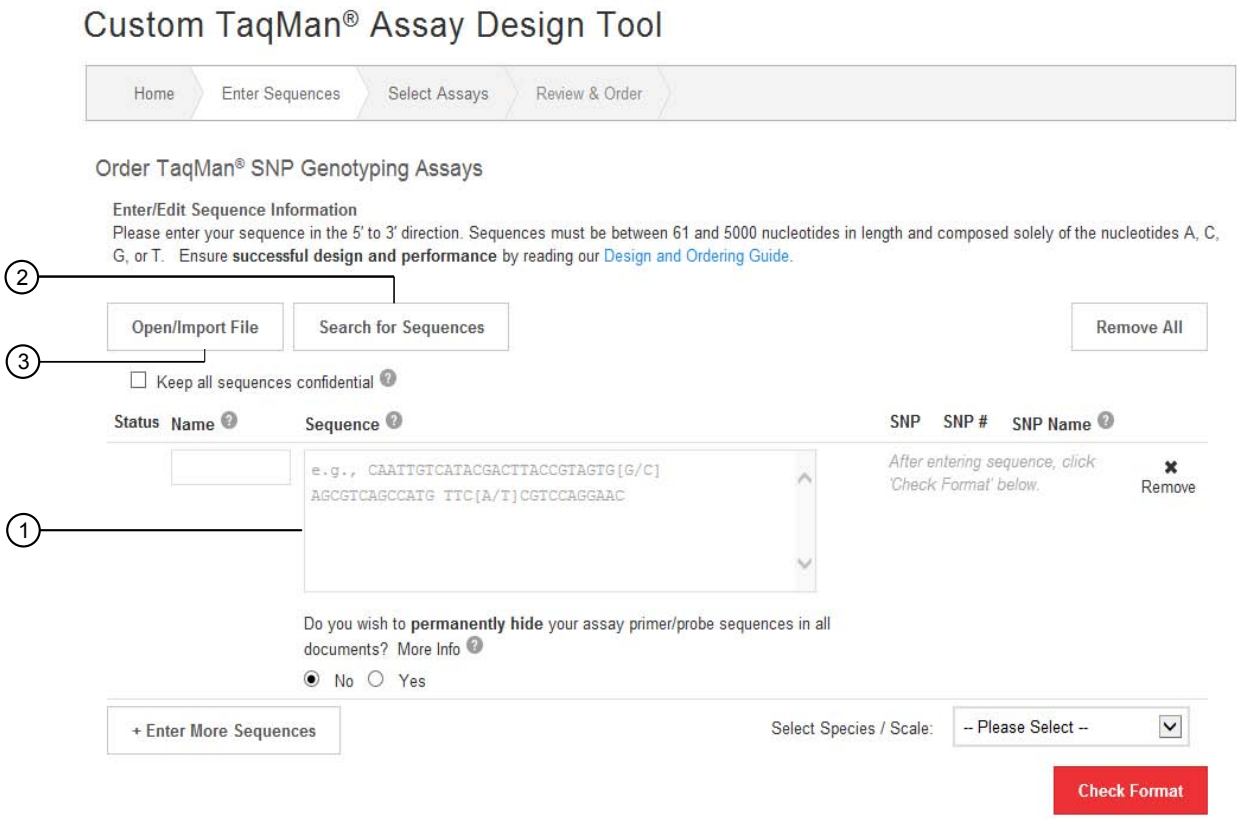
4. If you find nontarget SNPs, mask the nontarget SNPs with Ns. Select another SNP if any of your target SNPs is within 2 bases of a masked SNP.

Submit sequences

In the Enter Sequences tab, you can enter sequence information three different ways:

- Option 1: (Recommended) Enter the sequence information
- Option 2: Find target sequences in the sequence database
- Option 3: Import sequence information from a FASTA-formatted file

IMPORTANT! Log in to **thermofisher.com** to complete the order.



① Option 1
② Option 2
③ Option 3

Option 1: (Recommended) Enter the sequence information

You can enter or copy and paste the sequence information directly in the Enter Sequences tab.

1. Go to: **www.thermofisher.com/taqmansnpdesign**.
2. Click **Order Custom Genotyping Assays**.
3. Enter a SNP Name for each SNP in the entered sequence.

   Note: The combination of target name and sequence name must be unique.
4. In the Name field, enter the name for the sequence (for example, Sequence 1).
5. In the **Sequence** field, enter the entire sequence of interest in the 5′ to 3′ direction. Remove any spaces, tabs, and line breaks.
6. Select the species and the scale. See "Single tube products and formats" on page 7 for the number of reactions for each scale.

   Note: See "Determine the sequence criteria" on page 9 to determine whether to select human or nonhuman assays.
7. Click **Check Format** to validate the format of the sequence(s). If an error message is displayed, correct the format of the sequence information, then click **Check Format** again. Repeat until the format is validated.

   Note: For each IUPAC code that represents an ambiguous base, you are asked to provide your conversion option.
8. (*Optional*) To enter another sequence:
   a. Click **+ Enter More Sequences**.
   b. Repeat step 3 through step 7 in an empty row.
9. Click **Submit For Assay Design**.

   Note: The button is inactive until the format of the sequence information is free of errors.

(*Optional*) Permanently hide primer/probe sequence information

To hide primer and probe sequences in the AIF file (for example, to share a proprietary assay with someone without disclosing the oligo sequences):

For...	Do this...
Single sequence	Select the **Yes** button below the sequence next to the question, "**Do you want to permanently hide your assay primer/probe sequences in all documents?**
All sequences	Select the **Keep all sequences confidential** option at the top of the page.

The sequences will not be visible in the AIF shipped with the assay. Therefore, only select this option if you already have this information on file.

Option 2: Find target sequences in the sequence database

You can search the manufacturer database for target sequences to submit for custom assay designs.

1. In the Enter Sequences tab, click **Search for Sequences**.
2. Enter the chromosome location or search the database for the gene of interest.

Option	Description
To enter the chromosome location:	1. Select the Species, Chromosome (number), Chrom Start (chromosome start), and Chrom Stop (chromosome stop). 2. Click **Submit**.
To search the database for the transcript sequences:	1. Select the Field to Search (Keyword, Gene Name, Gene Symbol, Accession Number, Entrez Gene ID, rs Number, or Cytoband), Species, Criteria (Contains, Matches, Begins With, or Ends With), and the Search Term. 2. Click **Submit** to view a list of genes that meet your search criteria, which are sorted according to the gene symbol. 3. In the Search Results, click **Select** for the gene of interest.

3. In the Genome Map, select the sequence:
   a. View the genome map to find the SNPs of interest.
      - To zoom in: Click-drag the genome map left or right to center the desired location, then click [+] until the desired view is achieved.
      - To zoom out: Click [−] until the desired view is restored.
   b. To redefine the range for the genome map, enter the chromosome number and the chromosome start and stop locations, then click **Show Targets**.
   c. Click a SNP to add the sequence to the Possible Targets list.
4. Add the sequence(s) to the Target List. Enter a name for each sequence that you want to submit, then click **Add All** to add all the possible sequences, or click **Add** for each sequence for which you want a custom assay design.
5. In the Target List, click **Add to Design** when you are done adding sequences to add the sequence information for the selected sites to the Edit Sequences tab.
6. In the Enter or Search for Sequence Information, click **Check Format** to validate the format of the sequence(s). If an error message is displayed, correct the format of the sequence information, then click **Check Format** again. Repeat until the format is validated.

   Note: For each IUPAC code that represents an ambiguous base, you are asked to provide your conversion option.
7. Enter a SNP Name for each SNP in the entered sequence.

   Note: The combination of SNP name and sequence name must be unique.

8. Click **Submit For Assay Design**.

   Note: The button is inactive until the format of the sequence information is free of errors.

Option 3: Import sequence information from a FASTA-formatted file

1. In the Enter/Edit Sequence Information page, click **Open/Import File**.
2. For the File Type, select:
   - **FASTA** if your file was created in FASTA format.
3. Click **Browse** to find the file to import.
4. Select the text file (*.txt) that contains your sequences in FASTA format, then click **Open**.
5. Click **Import File**.

   The sequence information from the file is added to the Enter/Edit Sequence Information page.

   Note: For FASTA-formatted files, the Custom TaqMan® Assay Design Tool imports only the first 16 characters of the assay name into the Assay Name field. If your file contains multiple sequences with headers that are similar in the first 16 characters, edit the Assay Name field to distinguish between the sequences.

After you submit your sequences

After you submit your sequences for assay design, you can either wait for the Custom TaqMan® Assay Design Tool to complete your design, or you can close your browser and return to your order at a later time. You will receive an e-mail message to confirm that your sequences were submitted. The message contains a link to your design job in the Custom TaqMan® Assay Design Tool that you can use to view the status of your job.

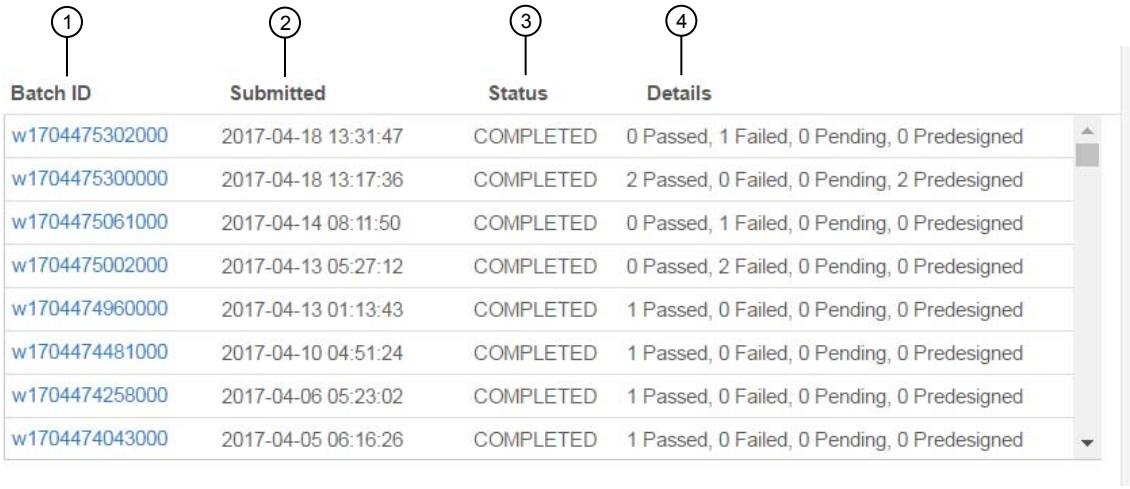
When the design job is complete, you will receive another e-mail to notify you that you can view the designs. You can use the link in the notification e-mail to view the design report in the Custom TaqMan® Assay Design Tool.

Review the Design Details

1. If you closed the Custom TaqMan® Assay Design Tool after submitting your sequence(s), follow the link in the notification email to the Custom TaqMan® Assay Design Tool.

2. In the Design Details section of the Select Assays tab, select the row that corresponds to the submission.

   For each design job, the Design Details table is displayed:



① **Batch ID**—The ID assigned to the submission by the manufacturer.
② **Submitted**—The date that you submitted the sequence information.
③ **Status**—The status of the design: Completed or Pending.
④ **Details**—The assays in each batch that passed, failed, are pending design, or are predesigned.

3. Review the Design Results in the Select Assays table.

Select Custom TaqMan® SNP Assays

1. For each assay that you want to order:
   a. In the **Size** column, select the assay size or scale in the dropdown list.
   b. In the **Quantity** column, enter the quantity of tubes to order.

2. Click **Add All** to add all assays to the order, or click **Add** next to each assay that you want to order. Selected assays are added to the **Shopping List**.

3. When you are done adding assays to your order, click **Order Now** to proceed to the checkout.

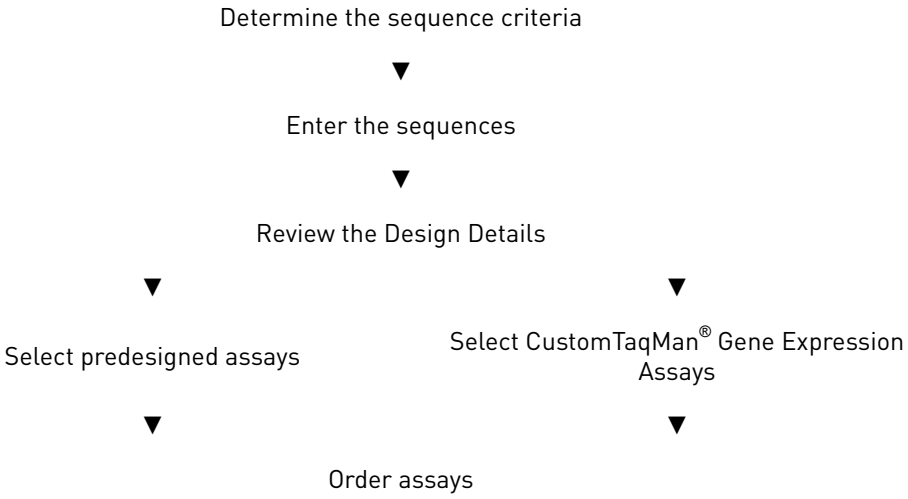
Order assays

1. Open your **Shopping List**.
2. Select the assay format:
   - **Single tubes**—assays are shipped in individual tubes.
   - **OpenArray™ plates**—assays are added to an open array plate.
3. (OpenArray™ plates) Select the assays to be plated.
4. Select the **Quantity** required.
5. Ensure that the checkbox under **Select** is checked for each assay you want to order.
6. Click **Add to cart**.
7. Ensure the assays to order are correct:
   - (*Optional*) Check the box next to the assay you want to delete and click **Remove**.
   - (*Optional*) Enter a different number in the **Quantity** field.
   - (*Optional*) Click the assay description and select a different size from the dropdown list.
8. Click **Begin checkout**.
9. Enter the Billing Address, Shipping Address, Shipping Method, Payment Method, Special Instructions, and Preferences. When you finish, click **Continue** to review.
10. Review the summary, then click **Place My Order**.

If you prefer to order by email, phone, or fax, reference the Catalog Number and assay ID.

3 Design and order Custom TaqMan® Gene Expression Assays

Gene expression workflow



Gene Expression assay types

The Custom TaqMan® Assay Design Tool (**thermofisher.com/cadt**) helps you in designing Custom TaqMan® Assays for quantitative gene expression analysis. The following table describes the TaqMan® assay products that you can order using the tool.

Product	Description
Custom TaqMan® Gene Expression Assays	Custom TaqMan® Assays manufactured to input sequences without the use of the Custom TaqMan® Assay Design Tool bioinformatics analysis feature. For more information, go to **www.thermofisher.com/cadt**.
Custom Plus TaqMan® RNA Assays	Custom TaqMan® Assays that are designed to input sequences using the Custom TaqMan® Assay Design Tool bioinformatics analysis feature. The bioinformatics analysis feature automates most aspects of the assay design process. The tool uses data from public molecular biology resources (NCBI Gene and GenBank) to help you in designing optimal TaqMan® assays. To use the Custom TaqMan® Assay Design Tool bioinformatics analysis feature to design Custom Plus assays, go to "Order assays" on page 32.
TaqMan® Gene Expression Assays	The Custom TaqMan® Assay Design Tool will suggest predesigned TaqMan® assays that match your sequence. To browse through our extensive library of inventoried and made-to-order predesigned assays , go to **www.thermofisher.com/allgenes** and enter your target gene name. All predesigned assays are designed using our validated bioinformatics pipeline, eliminating the need for primer design or PCR optimization.

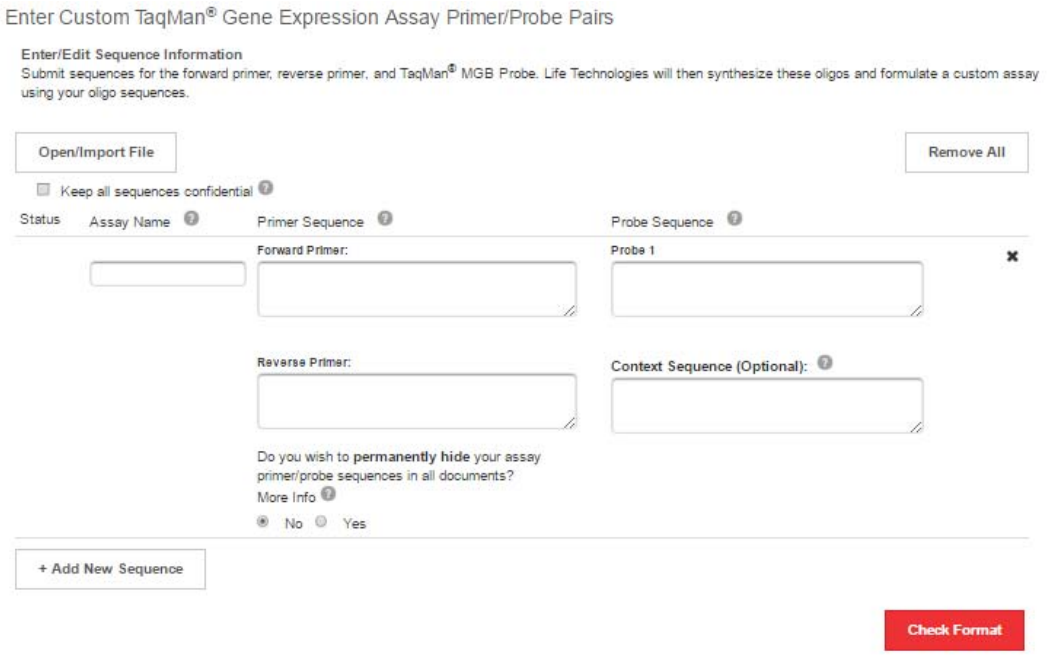
Target sequence requirements

Field	Requirement
Name	• Has 4 to 16 characters. • Uses only alphanumeric, underscore, hyphen, and period characters. • Has no spaces or tabs. • Does not begin with a hyphen.
Sequence	• Enter the sequence in the 5′ to 3′ direction. • Enter from 61 to 5000 bases. • Use only A, C, G, T, and N. • Convert the IUPAC codes R, Y, M, K, S, W, H, B, V, and D to N. • Convert U to T. • Remove any spaces, tabs, or line breaks.
Target Position	• The target position is the base position of the target site from the 5´ end. • The target position indicates where to design the TaqMan® probe.

Determine the sequence criteria

1. Go to the Custom TaqMan® Assay Design Tool website: **www.thermofisher.com/cadt**.

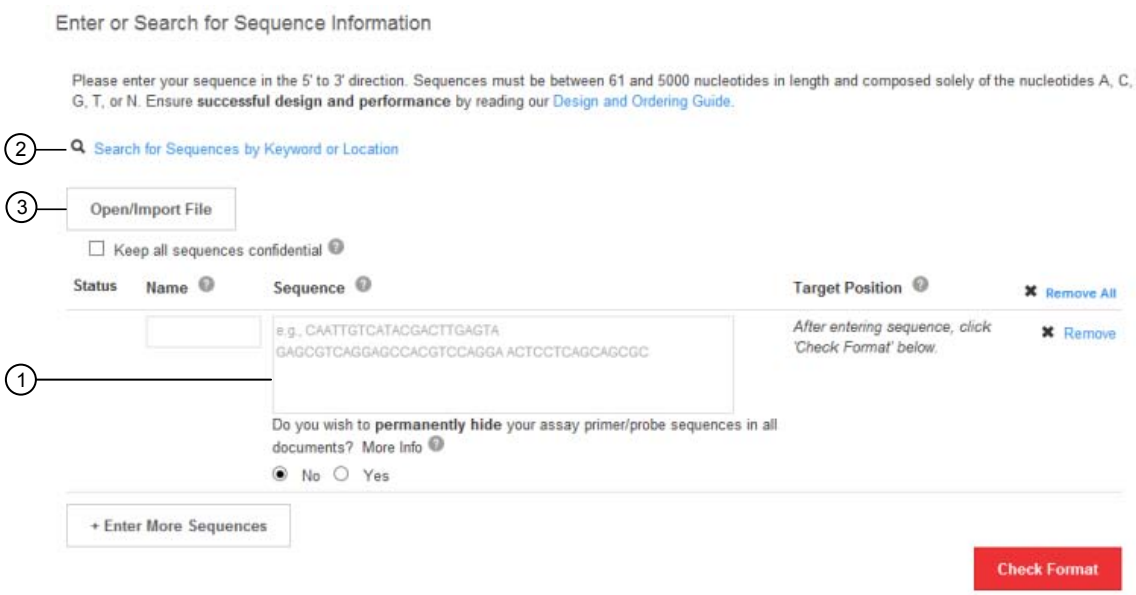
2. Click **Enter Custom Primer/Probe Pairs** if ordering specific primers and probes.



Submit sequences

In the Enter Sequences tab, you can enter sequence information three different ways after you indicate your target species and bioinformatics analysis preference:

- Option 1: (Recommended) Enter the sequence information
- Option 2: Find target sequences in the sequence database
- Option 3: Import sequence information from a FASTA-formatted file



① Option 1
② Option 2
③ Option 3

Option 1: (Recommended) Enter the sequence information

You can enter or copy and paste the sequence information directly in the Enter Sequences tab.

1. In the Name field, enter a name for the sequence (for example, sequence1).
2. In the Sequence field, enter the entire sequence of interest in the 5′ to 3′ direction. Remove any spaces, tabs, and line breaks.
3. Click **Check Format** to validate the format of the sequences. If an error message is displayed, correct the format of the sequence information, then click **Check Format** again. Repeat until the format is validated.

   Note: The only characters that are allowed in a sequence for gene expression assays are: A, C, G, T, and N. The Custom TaqMan® Assay Design Tool converts all other characters to Ns.

4. In the Target Position column, select:
   - **Manual** to enter the target position. In the **Pos** field, enter the number of bases between the target site and the 5′ end. Click **+ Enter More Targets** to enter more target sites.
   - **Automatic** to have the Custom TaqMan® Assays design pipeline select a target from multiple unspecified sites.

     Note: If you select "Automatic" and you selected to perform a bioinformatic analysis on your input sequences (Appendix B, "Bioinformatics tools for manually evaluating target sequences"), then the design pipeline designs assays for all predicted exon junctions. If you did not select the bioinformatics option, the tool selects the best design, which could be anywhere in the submitted sequence.

5. (*Optional*) To enter another sequence:
   a. Click **+ Enter More Sequences**.
   b. Repeat step 1 through step 4 in an empty row.

6. Click **Continue**.

   Note: The button is inactive until the format of the sequence information is free of errors.

   Note: After you submit your sequences for design, the Custom TaqMan® Assay Design Tool automatically searches for predesigned assays that match your input sequences. If found, the tool displays all compatible predesigned TaqMan® Assays before presenting you with the custom assay designs.

7. Select a predesigned assay, then check out, or click **Proceed to Custom Assays**.

Option 2: Find target sequences in the sequence database

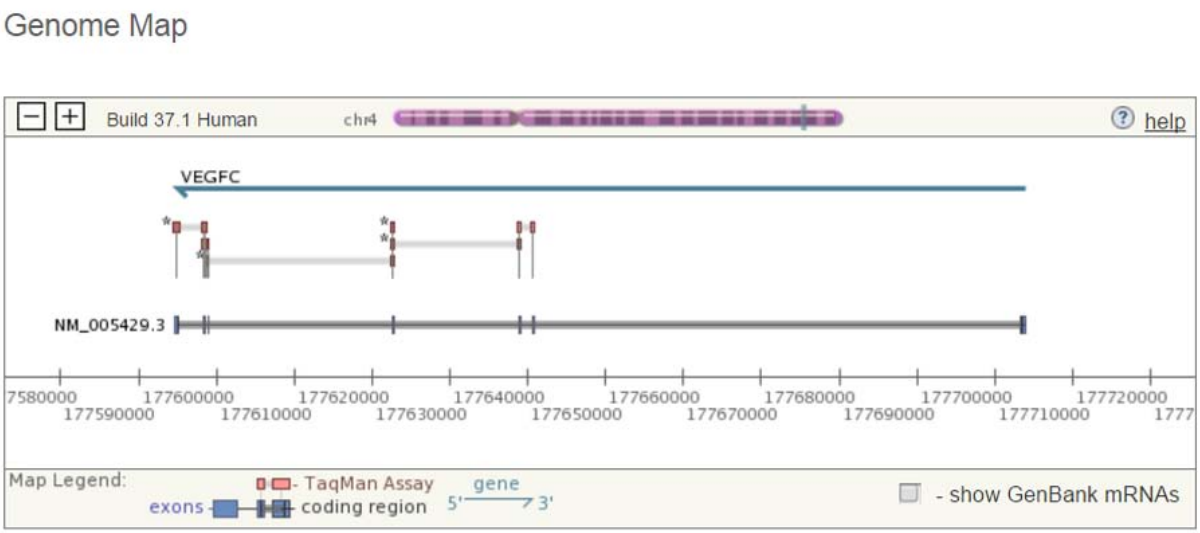
You can search the sequence database for target sequences to submit for custom assay designs.

1. In the **Enter Sequences** tab, click **Search for Sequences by Keyword or Location**.

2. Search the database for the gene of interest.

Search option	Description
Search the database for the gene	1. Select the Field to Search (Keyword, Gene Name, Gene Symbol, Accession Number, Entrez Gene ID), Species, Criteria (Contains, Matches, Begins With, or Ends With), and the Search Term. 2. Click **Submit** to view a list of genes that meet your search criteria, sorted according to the gene symbol. 3. In the Search Results, click **Select** for the gene of interest.
Enter the chromosome location	1. Select the Species, Chromosome (number), Chrom Start (chromosome start), and Chrom Stop (chromosome stop). 2. Click **Submit**.

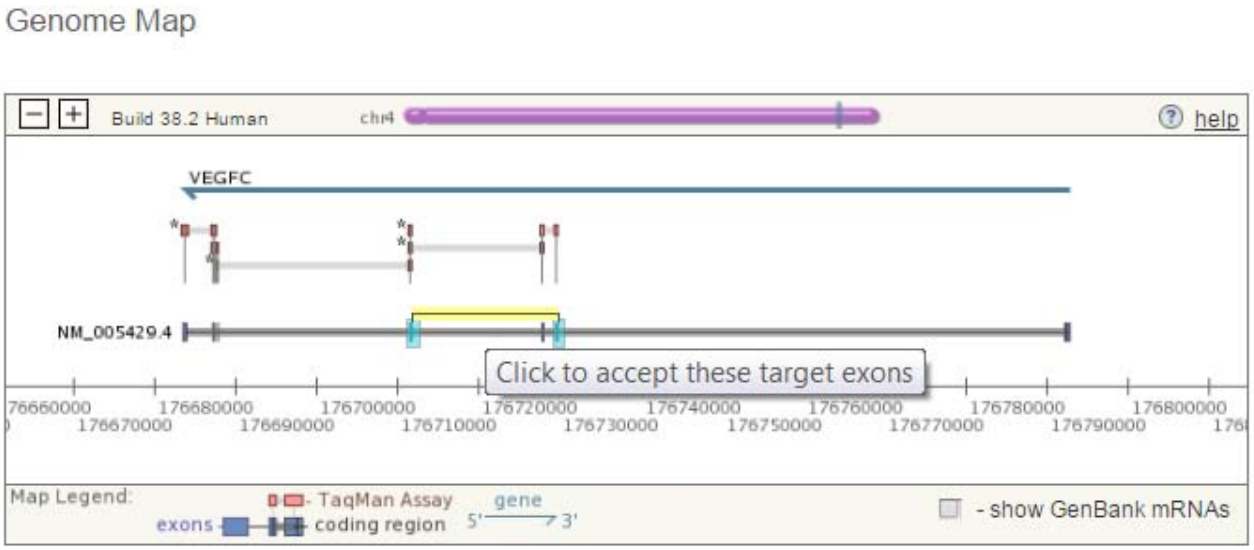
3. In the Genome Map, select the sequence:
   a. If available, view the genome map to find the transcripts of interest.
      - To zoom in: Click-drag the genome map left or right to center the desired location, then click [+] at the top left corner of the map until the desired view is achieved.
      - To zoom out: Click [−] until the desired view is restored.



   b. To view both GenBank and RefSeq mRNA transcripts in the displayed range of the genome map, select **Show GenBank mRNAs**.

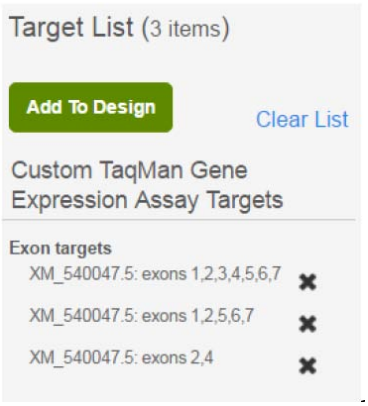
   c. To redefine the range for the genome map, enter the chromosome start and stop locations, then click **Submit**.

d. Select exons (blue boxes) from the transcript, then click the bracket that joins the exons to add the sequence to the Possible Sequences list. If the transcript contains only one exon, select the exon, then click the bracket that appears above the exon to add the sequence to the Possible Sequences list. To select the entire transcript, click the transcript between exons, then click the bracket joining all the exons to add the transcript to your possible targets.



4. Scroll down to enter a name. Enter a name for each sequence that you want to submit, then click **Add All** to add all the possible sequences, or click **Add** for each sequence for which you want a custom assay design.

5. In the Target List, click **Add to Design** when you are done adding sequences.



6. In the Enter or Search for Sequence Information, click **Check Format** to proceed.

7. Select **Automatic** to have the Custom TaqMan® Assays Design Pipeline select a target from the best site or across each exon junction if you selected to use the bioinformatics option.

   Note: If you searched for sequences and selected two or more exons, the target positions and names are automatically populated with the exon junction information.

8. Click **Continue**.

   Note: The button is inactive until the format of the sequence information is free of errors.

After you submit your sequences for design, the Custom TaqMan® Assay Design Tool automatically searches for predesigned assays that match your input sequences. If found, the tool displays all compatible predesigned TaqMan® assays before presenting you with the custom assay designs.

Option 3: Import sequence information from a FASTA-formatted file

You can import files that you created that contain sequences in FASTA format.

1. In the **Enter/Edit Sequence Information** page, click **Open/Import File**.
2. Click **Choose File** to find the file to import.
3. Select the text file (*.txt) that contains your sequences in FASTA format, then click **Open**.
4. Click **Import File**.

   The sequence information from the file is added to the **Enter/Edit Sequence Information** page.

   Note: For FASTA-formatted files, the Custom TaqMan® Assay Design Tool imports only the first 16 characters from the header line into the **Assay Name** field. If your sequence headers are similar in the first 16 characters, edit the **Assay Name** field to distinguish between the sequences.

After you submit your sequences for design, the Custom TaqMan® Assay Design Tool automatically searches for predesigned assays that match your input sequences. If found, the tool displays all compatible predesigned TaqMan® assays before presenting you with the custom assay designs.

After you submit your sequences

After you submit your sequences for assay design, you can either wait for the Custom TaqMan® Assay Design Tool to complete your design, or you can close your browser and return to your order at a later time. You will receive an e-mail message to confirm that your sequences were submitted. The message contains a link to your design job in the Custom TaqMan® Assay Design Tool that you can use to view the status of your job.

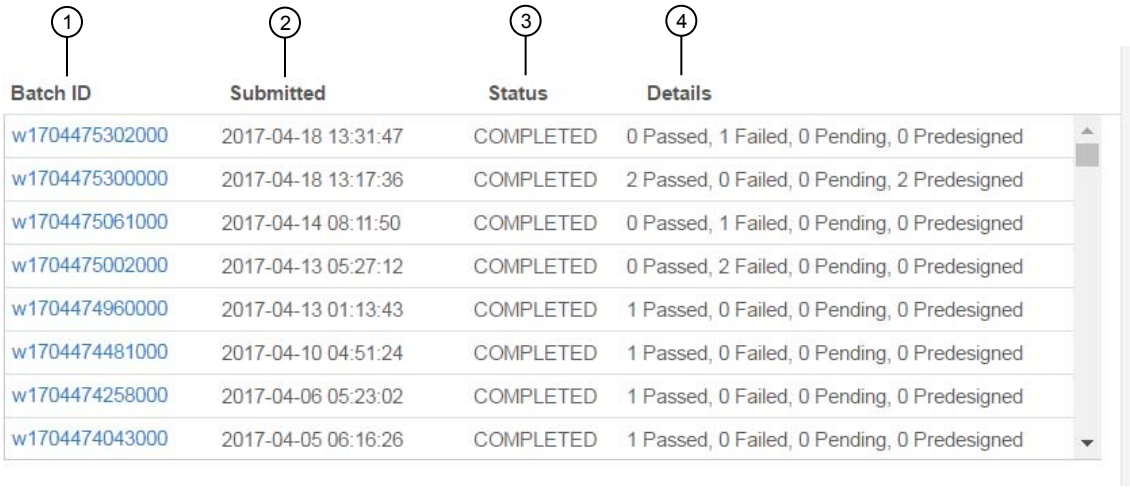
When the design job is complete, you will receive another e-mail to notify you that you can view the designs. You can use the link in the notification e-mail to view the design report in the Custom TaqMan® Assay Design Tool.

Review the Design Details

1. If you closed the Custom TaqMan® Assay Design Tool after submitting your sequence(s), follow the link in the notification email to the Custom TaqMan® Assay Design Tool.

2. In the Design Details section of the Select Assays tab, select the row that corresponds to the submission.

   For each design job, the Design Details table is displayed:

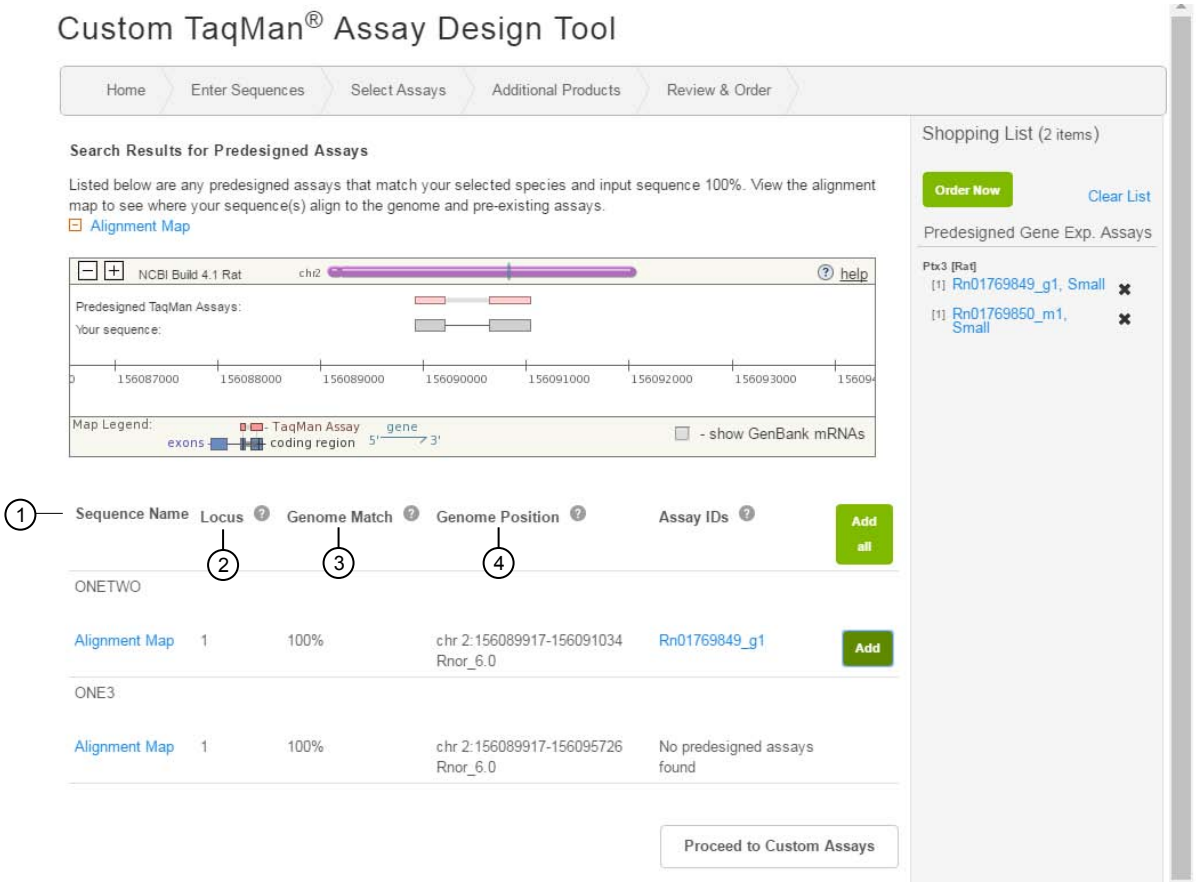


① **Batch ID**—The ID assigned to the submission by the manufacturer.
② **Submitted**—The date that you submitted the sequence information.
③ **Status**—The status of the design: Completed or Pending.
④ **Details**—The assays in each batch that passed, failed, are pending design, or are predesigned.

3. Review the Design Results in the Select Assays table.

Select predesigned assays

Before viewing the custom assay design results, review the alignment maps for your input sequences that have compatible predesigned TaqMan® assays. After receiving your sequences, the assay design tool searches for predesigned TaqMan® Gene Expression Assays that match your input sequences 100%. If you do not want to use the predesigned assays, you can proceed directly to the Custom Assay Design screen to review your assay designs.



① **Sequence Name**—The unique identifier that you gave to the sequence. The sequence name is referred to as the "Assay Name" after you order the assay.

② **Locus**—The unique loci to which the sequence aligns in the genome.
The assay design tool displays only the top three unique loci that have ≥90% alignment to the input sequence.

③ **Genome Match**—A measure of how closely the input sequence matches the selected genome, where the number is the percentage of nucleotides in the input sequence that match the selected genome. The assay design tool displays only matches greater than 90%.

④ **Genome Position**—The chromosomal position to which the input sequence is aligned in the associated genome in the following format: `<chromosome number>:<chromosome start>-<chromosome stop>`

To order predesigned assays:

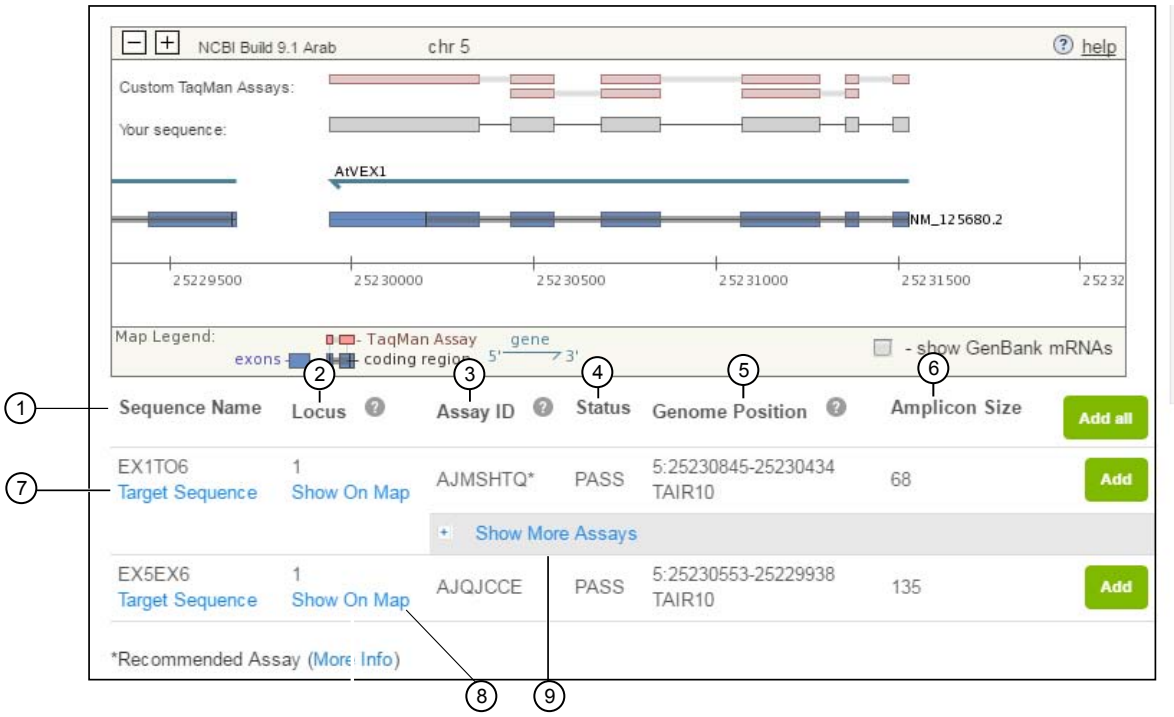
1. Select the desired predesigned assays.
2. Click **Add** next to the **assay ID**.

3. Click **Order Now** in the **Shopping List**.
4. Select the assay size or scale, and reporter dye.
5. Enter the quantity to order.
6. Click **Add to Cart**.

To see the new designs, click **Proceed to Custom Assays**.

Select Custom TaqMan® Gene Expression Assays

1. In the **Design Details** section of the **Select Assays** tab, click the **Batch ID** that corresponds to the submission (for example, w1703472531000).
2. Open Design Results. Only the recommended assays are displayed. Click **Show More** to see additional assays for your sequence.



① **Sequence Name**—The unique identifier that you gave the sequence.
② **Locus**—The unique loci that your input sequence aligns to in the genome.
③ **Assay ID**—A unique 7-character identifier assigned to each custom assay that contains the name entered during assay submission. You can use an Assay ID to reorder a Custom TaqMan® Assay.
④ **Status**—The status of the design: Passed, Failed, or Pending.
⑤ **Genome Position**—Position in the genome.
⑥ **Amplicon Size**—The size of the amplicon produced by your custom assay.
⑦ **Target Sequence**—Click to display the sequence that you entered.
⑧ **Show on Map**—Click to see your custom assays aligned to your input sequences and the gene it maps to.
⑨ **Show More Assays**—Click to view more assay designs.

① **Batch ID**—The ID assigned to the submission by manufacturer.
② **Submitted**—The date that you submitted the sequence information.
③ **Status**—The status of the design: Completed or Pending.
④ **Details**—The assays in each batch that passed, failed, are pending design, or are predesigned.

3. Click **Add** to add assays to the **Shopping List**.
4. Click **Order Now**, after you have added all required assays to your order.

Order assays

Place the order online

1. Open your **Shopping List**.
2. Select the assay format:
   - **Single tubes**—assays are shipped in individual tubes.
   - **OpenArray™ plates**—assays are added to an open array plate.
3. (OpenArray™ plates) Select the assays to be plated.
4. Select the **Quantity** required.
5. Ensure that the checkbox under **Select** is checked for each assay you want to order.
6. Click **Add To Cart**.
7. Ensure the assays to order are correct:
   - (*Optional*) Check the box next to the assay you want to delete and click **Remove**.
   - (*Optional*) Enter a different number in the **Quantity** field.
   - (*Optional*) Click the assay description and select a different size from the dropdown list.
8. Click **Begin checkout**.

9. Enter the Billing Address, Shipping Address, Shipping Method, Payment Method, Special Instructions, and Preferences. When you finish, click **Continue** to review.

10. Review the summary, then click **Place My Order**.

If you prefer to order by email, phone, or fax, reference the Catalog Number and assay ID.

4 Reorder Custom TaqMan® Assays

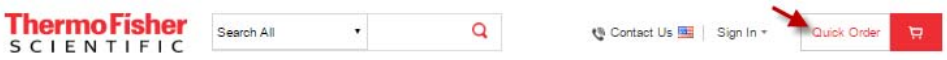
Reorder online

You can reorder assays through the company website in two ways: using the Quick Order service or the Custom TaqMan® Assay Design Tool.

Reorder using Quick Order

1. Go to **www.thermofisher.com**, then log in to the store:
   a. At the top of the home page, click **Sign In**.
   b. In the Store login page, enter your user name and password, then click **Sign In**.

   Note: Log in to the store to complete the order.
2. Click **Quick Order**.



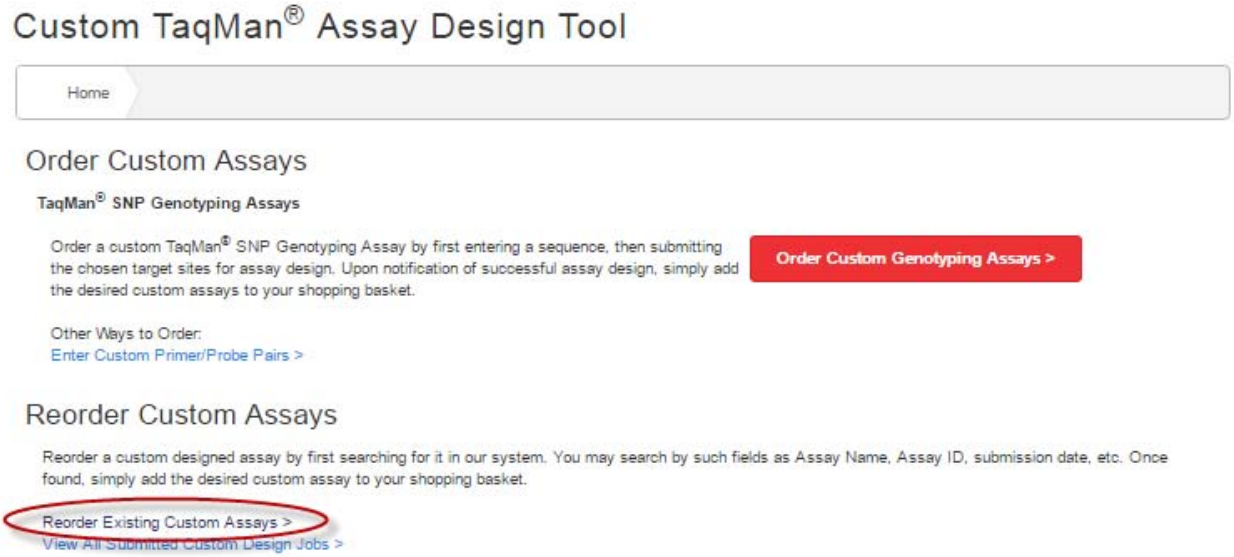
3. Enter or copy/paste the order data for the assays of interest:
   - **Manual entry**—Enter one or more catalog number-ID combinations, then enter a value in the Quantity field.
   - **Copy/paste entry**—Copy and paste the catalog numbers-ID combination data into the Catalog Numbers/IDs* field.
   - **Bulk upload**—Enter product information into spreadsheet template and upload.
4. Select a cart, then click **Add to Cart** to process the order.
5. Complete the order as directed.

Reorder using the standard ordering service

1. Go to **www.thermofisher.com** , then log in to the store:
   a. At the top of the home page, click **Sign In**.
   b. In the Store log in page, enter your user name and password, then click **Sign In**.

   Note: Log in to the store to complete the order.

2. Go directly to the Custom TaqMan® Assay Design Tool website:
   - Gene expression: **www.thermofisher.com/cadt**
   - SNP genotyping: **www.thermofisher.com/taqmansnpdesign**
3. Click **Reorder Existing Custom Assays**.



4. In the Search tab, search for existing custom assays:
   a. From the **Fields to be Searched** dropdown list, select: Assay ID, Legacy Assay-ID, Assay Name (Sequence Name), Date Range, Sales Order #.

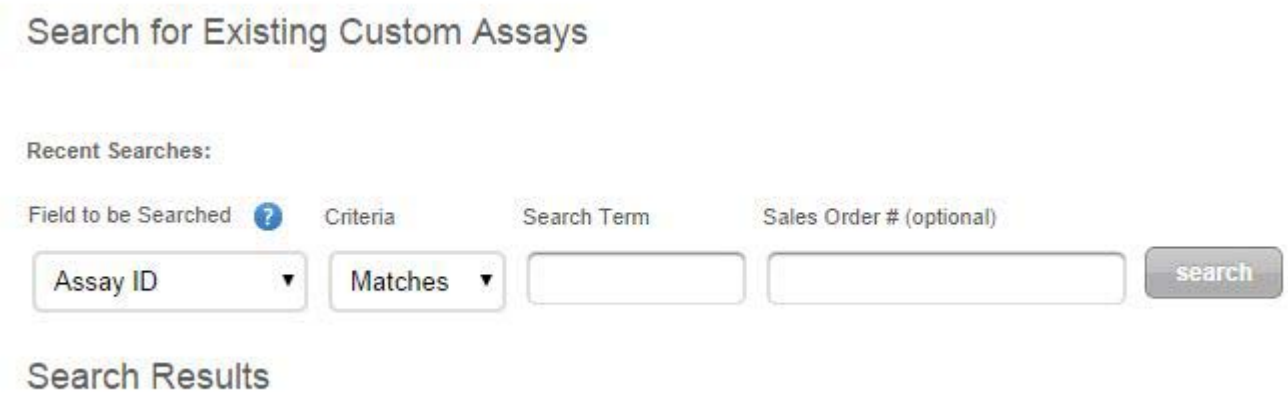
      Note: Legacy assays are Custom TaqMan® Assays that were designed before the release of the Custom TaqMan® Assay Design Tool. Legacy Assay IDs are IDs assigned to custom assays, ordered before February 2009, which consist of the sequence and target names that are separated by a hyphen.

   b. Select the criteria: Matches or Contains.

   c. Enter the Term to search.

   d. (Required for legacy IDs) Enter the Sales Order #.

e. Click **Search**.



5. For each assay that you want to order:
   a. (Gene expression only) Select the reporter dye in the **Dye** column.
   b. (Gene expression only) Select the assay size in the **Size** column.
   c. Enter the number of tubes in the **Quantity** column.
6. Click **Add** next to each assay to order. Selected assays are added to the **Shopping List**.

Reorder legacy assays

Legacy assays are Custom TaqMan® Assays that were designed before the release of the Custom TaqMan® Assay Design Tool. Legacy assay IDs were generated differently from Assay IDs. Use the procedures in this section to reorder legacy assays by email or by regular or express mail.

IMPORTANT! To reorder legacy Custom TaqMan® Assays, you need information from the Assay Information File (AIF) that was shipped to you with the previous assay.

Reorder online

To reorder legacy assays online using Quick Order, see "Reorder online" on page 34.

Reorder by email

1. Address the message:
   - For orders to North America: **mailto: genomicorders@thermofisher.com**
   - For other regions, visit **http://www.thermofisher.com/us/en/home/technical-resources/contact-us.html**
2. In the subject line, enter **Reorder for Custom TaqMan® Assay**.

3. In the message body enter the:
   - **Sales order** number—The number in the Assay Information File (AIF) that was shipped to you with your previous Custom TaqMan® Assays order.
   - **Assay ID**—The ID from the AIF.
   - **Part number**—The number that indicates the type of assay and the scale that you want to order.
   - **Quantity** —The number of tubes to order.
   - **Purchase order number**

     or

     Credit card information—The name as it appears on the card, the card number, and the expiration date.
   - **Contact information**—The name, email address, telephone number, and address of the person to contact if problems occur.
   - **Shipping information**—The name, address (including room number, building, department, and the ATTN line information), and telephone number of the person to receive shipment.
   - **Invoice information**—The name, email address, physical address, and telephone number of the purchasing agent or person to receive invoice details.
4. Send the message.

Reorder by regular or express mail (not recommended)

1. Prepare an order document containing the:
   - **Sales order number**—The number in the Assay Information File (AIF) that was shipped to you with your previous Custom TaqMan® Assays order.
   - **Assay ID**—The ID from the AIF.
   - **Part number**—The number that indicates the type of assay and the scale that you want to order.
   - **Quantity**—The number of tubes to order.
   - **Purchase order number**

     or

     Credit card information—The name as it appears on the card, the card number, and the expiration date.
   - **Contact information**—The name, email address, telephone number, and address of the person to contact if problems occur.
   - **Shipping information**—The name, address (including room number, building, and department), and telephone number of the person to receive shipment.
   - **Invoice information**—The name, email address, physical address, and telephone number of purchasing agent or person to receive invoice details.

   IMPORTANT! To prevent delays, include all information in the order document.
2. Print a copy of the order document.
   a. Click **Print for fax/mail order or hardcopy record** to display a printer-friendly order form.

b. Click **Print Window** to send the order form to your printer, then follow the instructions to configure the printed hardcopy.

3. Send the printed copy of your order document to the address on the printed form.

A Troubleshooting

This appendix gives detailed instructions for determining the reason that an assay failed preparation, design or manufacturing.

Failed sequence and assay troubleshooting

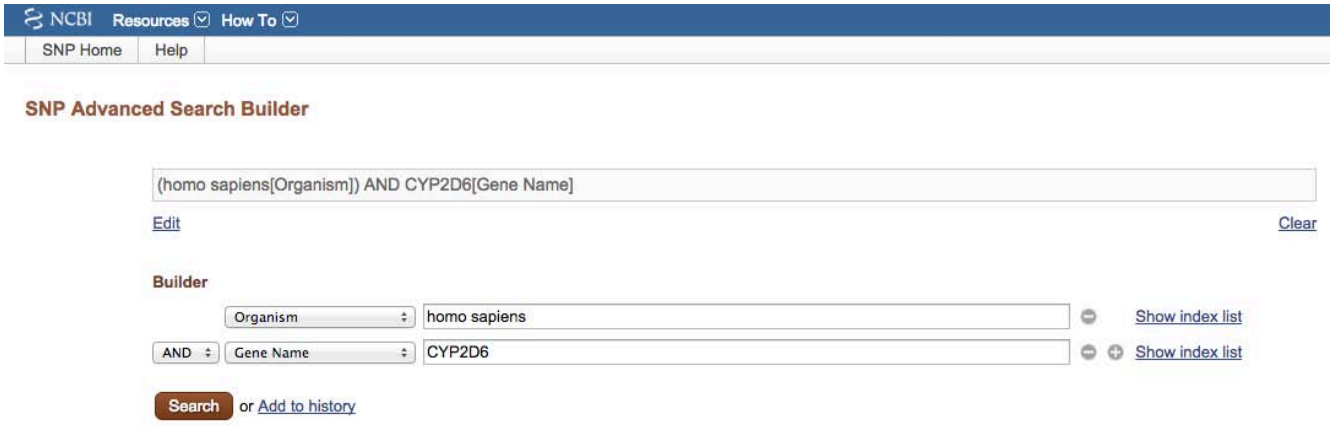
Observation	Possible cause	Recommended action
Sequence failed preparation	Incorrect format—sequence must contain at least 61 nucleotides.	Provide longer sequences.
	Invalid characters (for example, : or $)	Use "N" for masking.
Assay failed design	The input sequence is too short for the software to place primers and probes for a good assay design.	Extend the length of your input sequence to 150 bp both upstream and downstream of the target site if possible. The total recommended sequence length is 300 bp.
	Sequence complexity is too low. Low complexity sequences such as CCTCCACTCCTCTCCACCTCTCC can result in primers and probes that lack sufficient specificity for assay design.	Select another SNP sequence or try using a longer input sequence with greater complexity in the added sequence.
	The input sequence is overmasked and does not provide sufficient unmasked sequence for the software to place primers and probes for a good assay design.	If your input sequence has too many Ns because of masking with the RepeatMasker, extend the length of your input sequence or unmask those regions with less homology.
Assay failed manufacturing	If neighboring SNPs were not masked in the input sequence, the assay can yield no amplification for some samples or produce more than three clusters. If repeat sequences were not masked the assay can be nonspecific, producing a single cluster where the heterozygous cluster is typically found.	If a SNP Genotyping Assay failed manufacturing: • • Mask neighboring SNPs and repeats if masking was not done previously and submit the sequence again. • Select another nearby SNP. See "Select another nearby SNP" on page 40.

Select another nearby SNP

Search dbSNP using a gene name

You can search the National Center for Biotechnology Information (NCBI) dbSNP database by organism and gene name to find refSNP entries in a gene of interest. Other search criteria may also be used.

1. Go to: **http://www.ncbi.nlm.nih.gov/snp/advanced**.
2. For the first search field select **Organism** and select the organism from the dropdown menu.
3. For the second search field select **Gene Name** and enter the gene name.
4. Click **Search** and all known SNPs in the gene will be displayed on the next page.

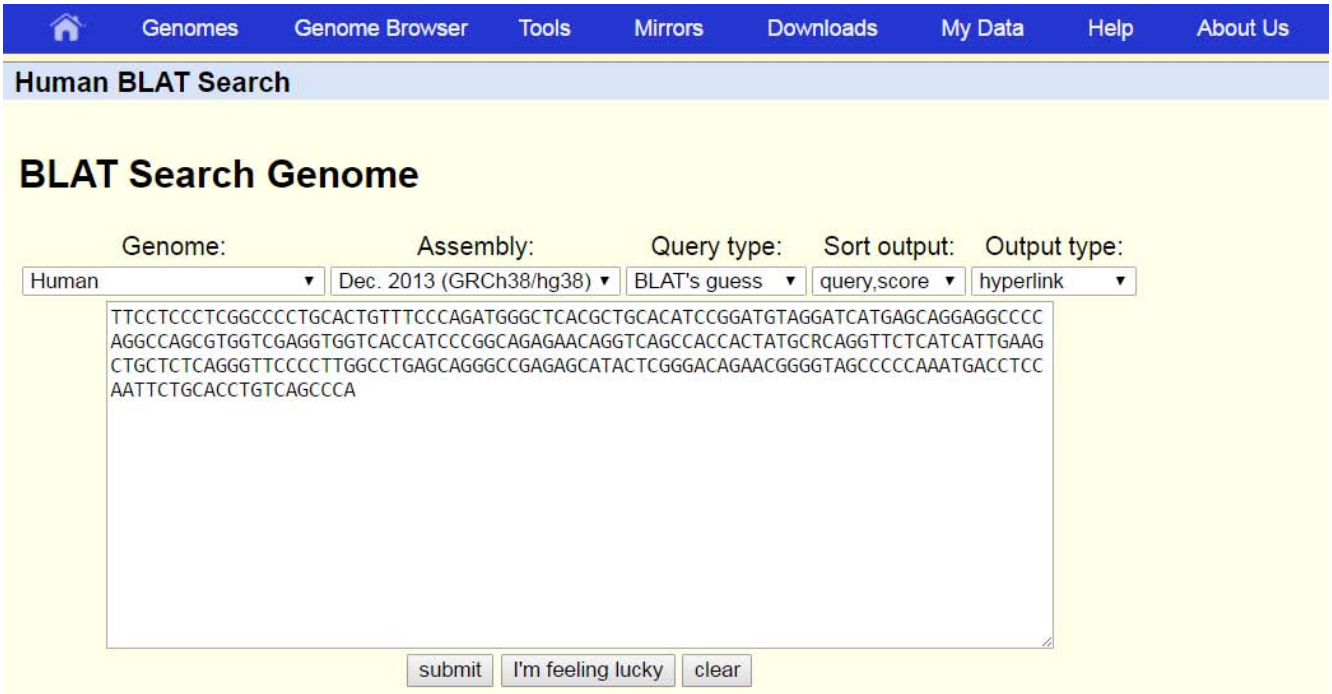


Select a new SNP from the search results.

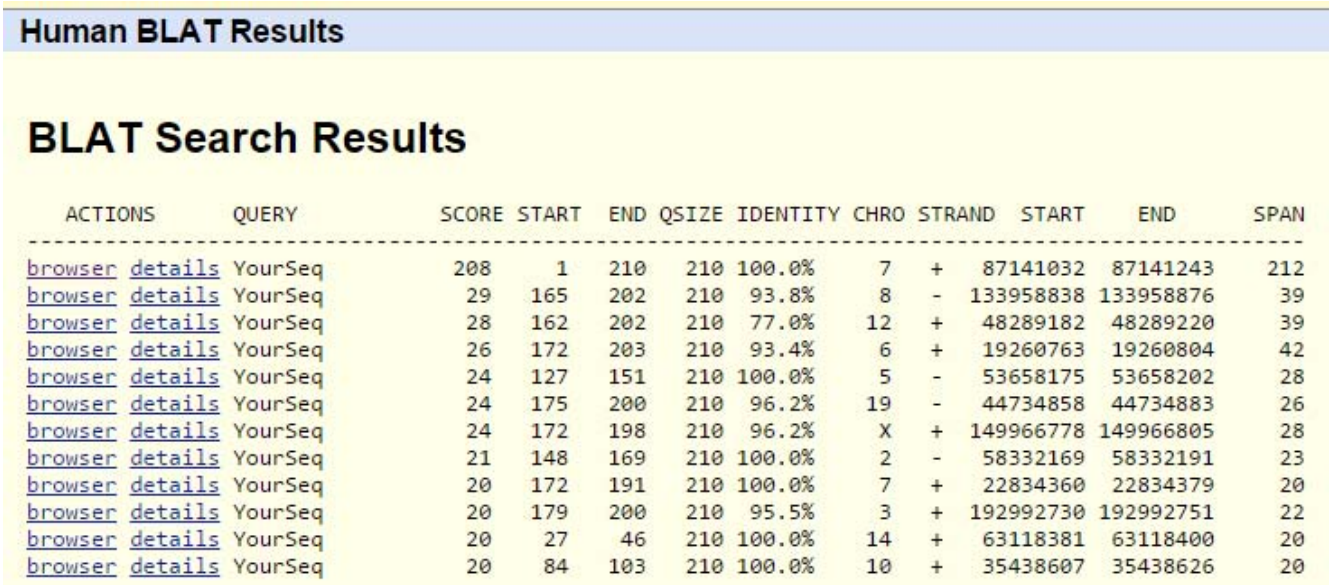
Search the UCSC Genome Browser using a sequence

The sequence can be used to search for nearby SNPs using the UCSC Genome Browser.

1. Go to:**http://genome.ucsc.edu/**, then click **BLAT** in the menu.
2. Select the organism and genome assembly from the dropdown menu.
3. Paste the sequence, then click **submit**.

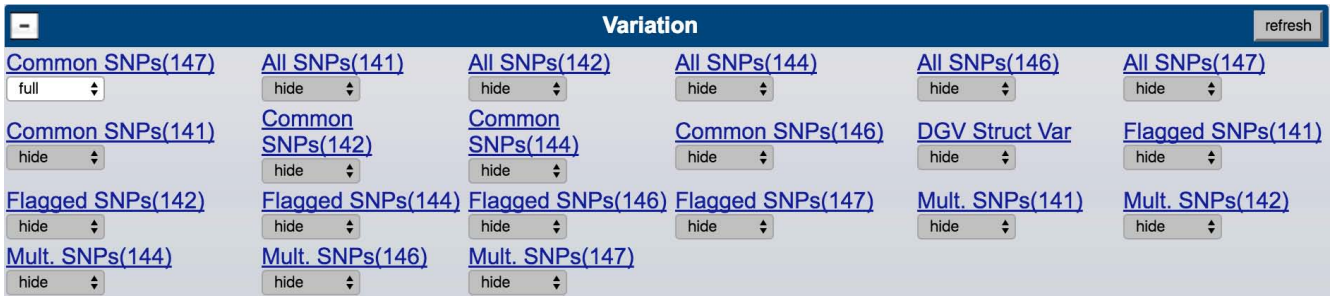


4. Select browser on the top line to see your sequence and the surrounding SNPs.

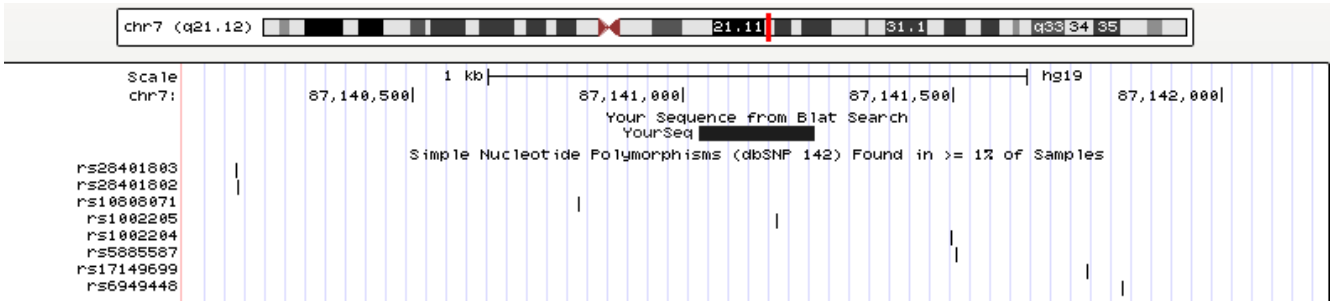


ACTIONS	QUERY	SCORE	START	END	QSIZE	IDENTITY	CHRO	STRAND	START	END	SPAN
browser details	YourSeq	208	1	210	210	100.0%	7	+	87141032	87141243	212
browser details	YourSeq	29	165	202	210	93.8%	8	-	133958838	133958876	39
browser details	YourSeq	28	162	202	210	77.0%	12	+	48289182	48289220	39
browser details	YourSeq	26	172	203	210	93.4%	6	+	19260763	19260804	42
browser details	YourSeq	24	127	151	210	100.0%	5	-	53658175	53658202	28
browser details	YourSeq	24	175	200	210	96.2%	19	-	44734858	44734883	26
browser details	YourSeq	24	172	198	210	96.2%	X	+	149966778	149966805	28
browser details	YourSeq	21	148	169	210	100.0%	2	-	58332169	58332191	23
browser details	YourSeq	20	172	191	210	100.0%	7	+	22834360	22834379	20
browser details	YourSeq	20	179	200	210	95.5%	3	+	192992730	192992751	22
browser details	YourSeq	20	27	46	210	100.0%	14	+	63118381	63118400	20
browser details	YourSeq	20	84	103	210	100.0%	10	+	35438607	35438626	20

5. Scroll down to the "Variations" section, then select the SNPs to be displayed. For example, a recommended selection is "Common SNPs (147)", which are SNPs with an allele frequency of 1% or greater in dbSNP build 147.



6. Scroll to the browser window to see nearby dbSNP entries. It may be necessary to zoom out to see the entries. This section can be moved upward so that rs numbers are displayed underneath "Your Sequence from Blat Search" to compare SNP locations relative to the original target SNP.



B Bioinformatics tools for manually evaluating target sequences

This appendix gives the user background information about the bioinformatics of manual sequence preparation. It provides instructions for bioinformatics tools available for manually evaluating target sequences. The following steps are covered:

- Ensuring sequence uniqueness with a BLAT® or BLAST® database search
- Finding exon information using the Entrez Gene database
- Masking sequence repeats
- Detecting and masking nontarget sequence polymorphisms that are found in dbSNP

Note: See *Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays* (Pub. No. 4371002) for a complete discussion of the bioinformatics analysis and techniques that are required for the design of Custom TaqMan® Gene Expression Assays.

Verify sequence uniqueness with a BLAST® database search

Goals for a BLAT® or BLAST® database search

Whether you have sequenced your target or obtained the sequence from a sequence database, you determine:

- If unique primers and probes can be generated for the sequence.
- If there are homologs. Homologs in gene families can present a problem, as can orthologous sequences when you work in a transgenic system.
- If there are polymorphisms in the sequence of interest.
- If the sequence is unique. Search for regions of sequence similarity between the target sequences and databases of known sequences. To make your assay as specific as possible, you can mask regions of similarity before submitting your sequence for design, so they are not considered in the assay design. You can use BLAT® or BLAST®, sequence comparison algorithms and database searching programs, to search the NCBI nucleic acid and protein public databases.

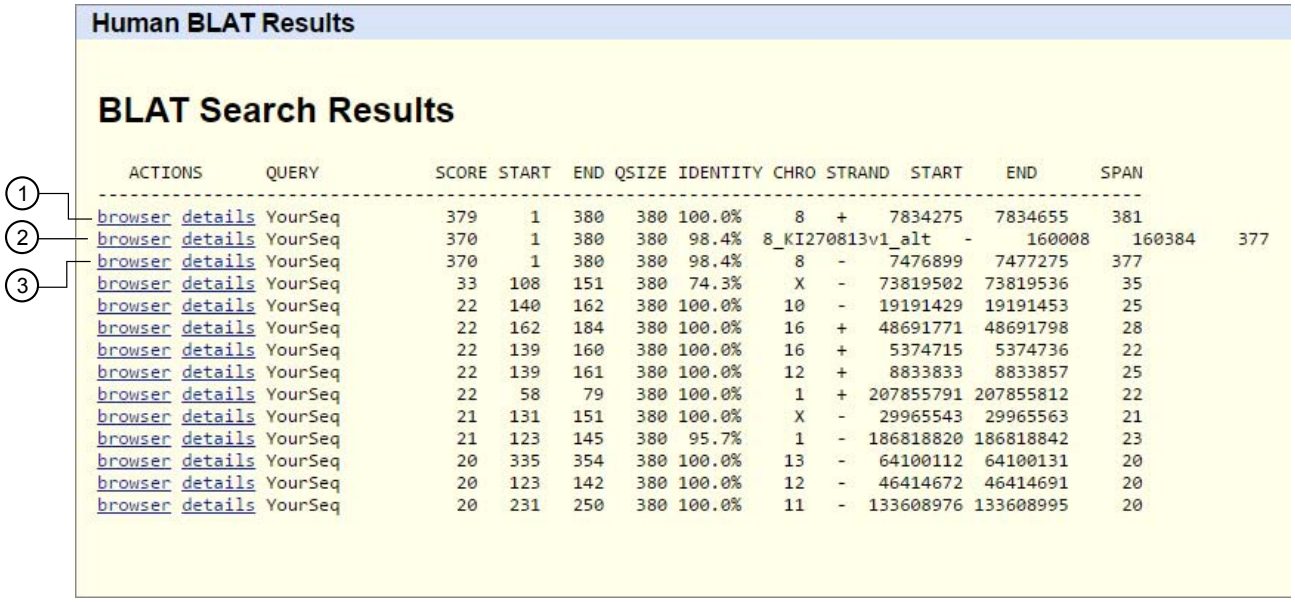
Run a BLAT® database search

1. Go to **https://genome.ucsc.edu/index**.
2. Click **BLAT** and paste the sequence in the window according to the online instructions.

   Note: Select the correct genome and the latest assembly.

3. Click **submit** to obtain the results.

4. In the **BLAT Search Results** window, make sure that your sequence produces a 100% match in the reference genome, and that there are no other sequences in the reference genome that have a strong match with the target sequence.



Human BLAT Results

BLAT Search Results

ACTIONS	QUERY	SCORE	START	END	QSIZE	IDENTITY	CHRO	STRAND	START	END	SPAN
browser details	YourSeq	379	1	380	380	100.0%	8	+	7834275	7834655	381
browser details	YourSeq	370	1	380	380	98.4%	8_KI270813v1_alt	-	160008	160384	377
browser details	YourSeq	370	1	380	380	98.4%	8	-	7476899	7477275	377
browser details	YourSeq	33	108	151	380	74.3%	X	-	73819502	73819536	35
browser details	YourSeq	22	140	162	380	100.0%	10	-	19191429	19191453	25
browser details	YourSeq	22	162	184	380	100.0%	16	+	48691771	48691798	28
browser details	YourSeq	22	139	160	380	100.0%	16	+	5374715	5374736	22
browser details	YourSeq	22	139	161	380	100.0%	12	+	8833833	8833857	25
browser details	YourSeq	22	58	79	380	100.0%	1	+	207855791	207855812	22
browser details	YourSeq	21	131	151	380	100.0%	X	-	29965543	29965563	21
browser details	YourSeq	21	123	145	380	95.7%	1	-	186818820	186818842	23
browser details	YourSeq	20	335	354	380	100.0%	13	-	64100112	64100131	20
browser details	YourSeq	20	123	142	380	100.0%	12	-	46414672	46414691	20
browser details	YourSeq	20	231	250	380	100.0%	11	-	133608976	133608995	20

① An exact match of the target sequence with the reference sequence.

② Also shows a close match, but the chromosome location **alt** indicates that this is a match with an alternate assembly, which can be ignored.

③ This match shows that another sequence is present in the reference genome with a 98.4% match to the target sequence. This close match indicates that an assay is likely to hybridize to both sequences, not just the target sequence.

5. To view non-target polymorphisms, click **details** and follow the instructions in "Search the UCSC Genome Browser using a sequence" on page 41.

Run a BLAST® database search

1. Go to **www.ncbi.nlm.nih.gov/blast**, then follow the instructions on the BLAST® home page.

   Instructions on how to use the software are posted on the website.

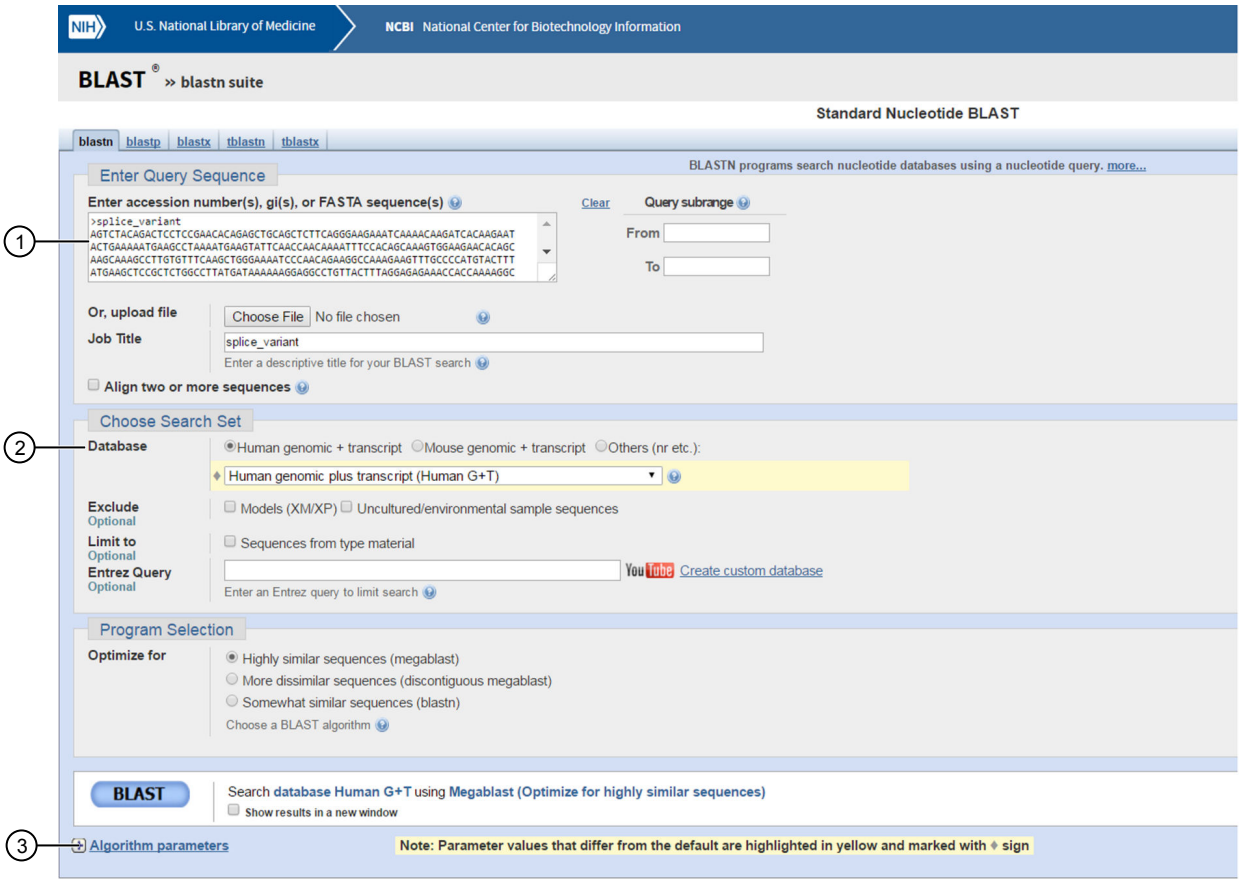
2. Select **nucleotide BLAST (blastn)** to access the BLAST® database search page.

3. In the **Enter Query Sequence** field on the BLAST® database search page, enter the sequence in any of three formats:
   - **FASTA format**—">", followed by one description line, with the sequence on the following line
   - **Text-only sequence**—No numbers or non-sequence text
   - **Sequence identifiers**—Accession number, accession.version number, gi numbers, or others

   For more information on these formats, go to: **www.ncbi.nlm.nih.gov/blast/**

4. Select the appropriate database to search.
   - For **Gene Expression assays** , if you search with a cDNA sequence, search the default **Human genomic + transcript** database for human assays. For other species, click **Others** and specify the appropriate database.
   - For **SNP assays,** search the default **Human genomic + transcript** database for human assays. For other species, click **Others** and specify the appropriate database.

5. In the **Algorithm parameters** section, you can:
   - Filter species-specific repeats from a dropdown menu.
   - Filter and mask low-complexity regions.



① Enter Query Sequence
② Select the appropriate database
③ (*Optional*) Select the algorithm parameters

6. Click BLAST to submit your search.

7. Click **Formatting Options**. Wait for the results to be displayed.

Evaluating BLAST® database results

There are three parts to BLAST® database search results:

- Graphical overview
- List of matching sequences
- Sequence alignments

Graphical overview

The graphical overview is a graphical representation of the database sequences that align to your query sequence, with the query sequence represented by the thick blue numbered line at the top of the graph.

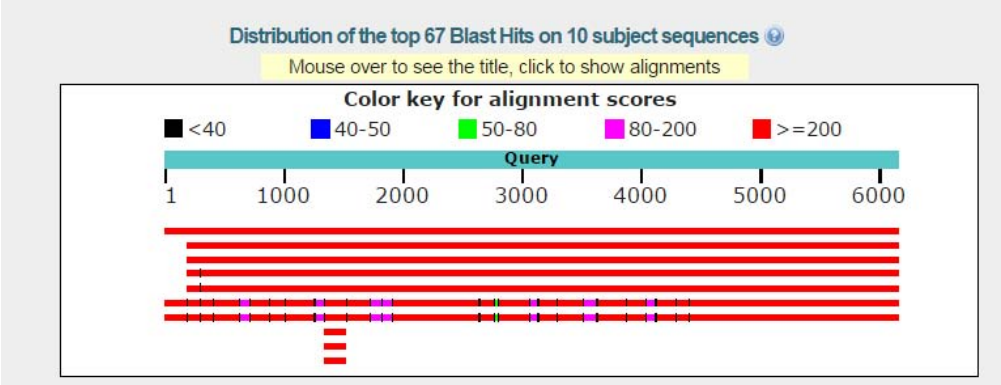


Figure 1 Graphical representation of BLAST® database search results

List of matching sequences

Matching sequences are listed from highest to lowest **Max score**. The top match is the best hit (which can be the sequence with which you queried the database). The link under **Accession** opens the GenBank record for the sequence. This record contains the complete sequence, species information, journal references, feature descriptions, and other information.

The Score indicates 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 are.

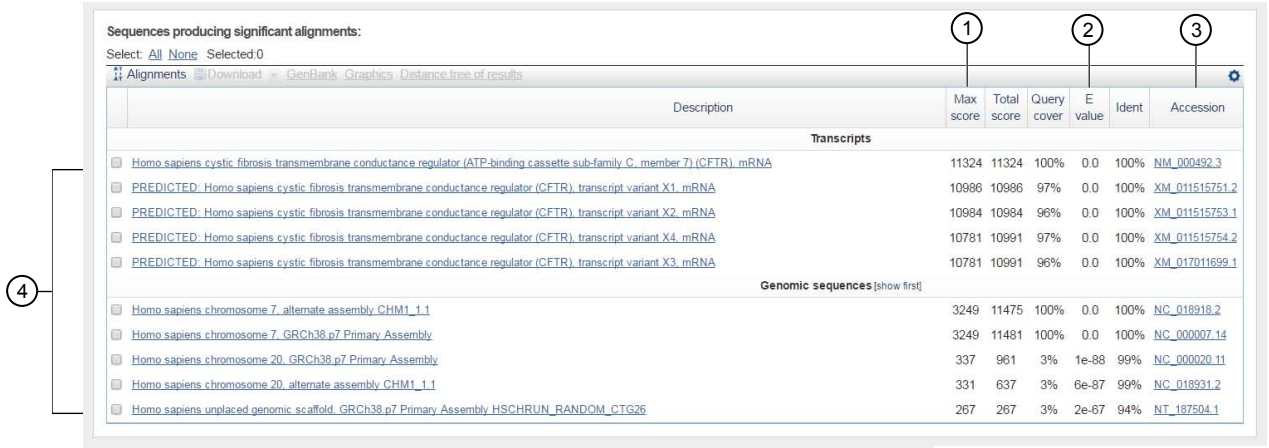


Figure 2 List of matching sequences in the results of a BLAST® database search (shortened for display purposes)

① Max score
② E value
③ Accession
④ Description

Each sequence alignment also has an E (Expect) value. The **E value** represents the number of hits you can "expect" to find by chance when searching a database of a particular size. The lower the E value, the more significant the match.

Look for matches with E values of less than ~0.1; matches with larger E values can be ignored.

You can go to the sequence alignment by clicking the **Description**.

Sequence alignments

This section shows the alignments between your query sequence (Query) and each sequence (Sbjct) in the list of hits. You can use these alignments to help evaluate the degree of similarity. The Score and Expect values are displayed underneath the sequence identifiers. The number of bases that are aligned, percent identity, and the strand that was aligned to your query sequence and the database hit are shown.

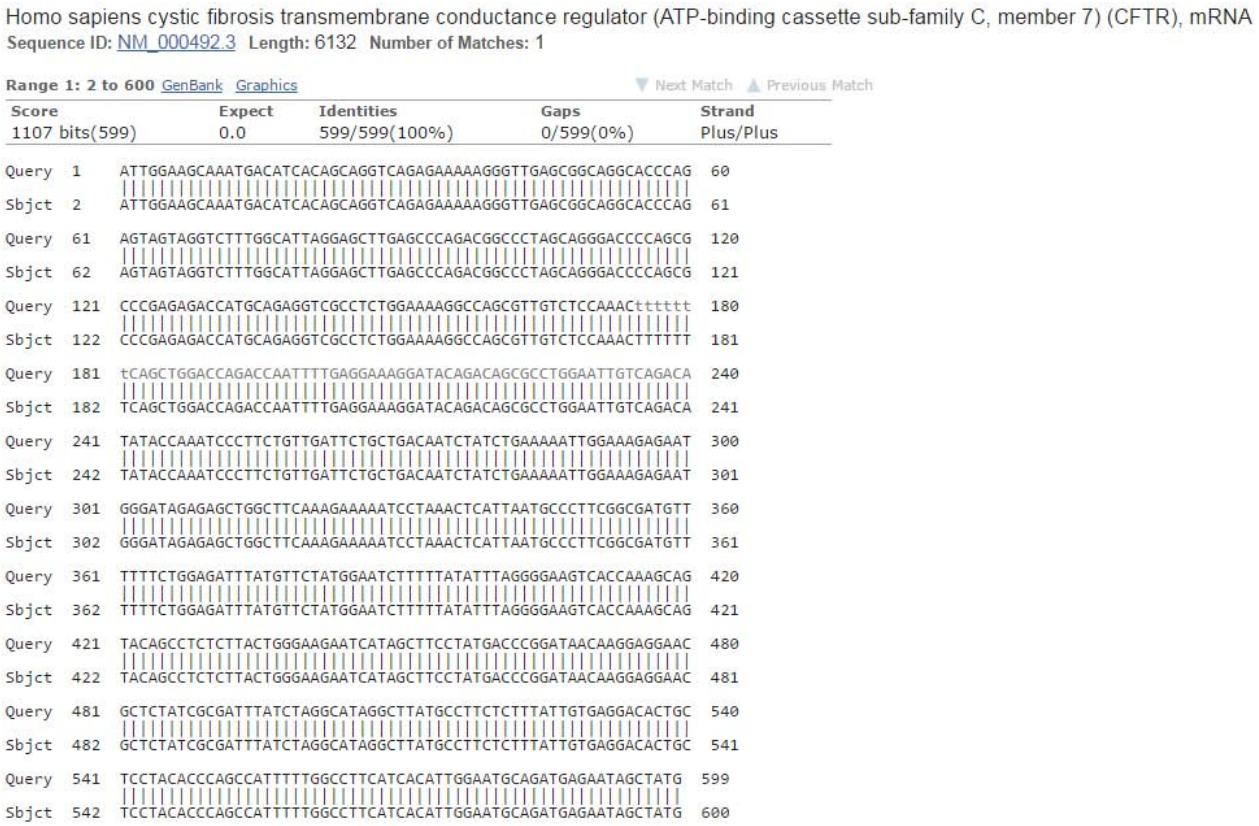


Figure 3 Individual sequence alignment from the results of a BLAST® database search

Find exon-exon boundaries (gene expression assays only)

For gene expression assays, you can use the exon-exon boundaries as the target positions in your submission file. Using the exon-exon boundaries as the target position excludes the detection of genomic DNA by the assay.

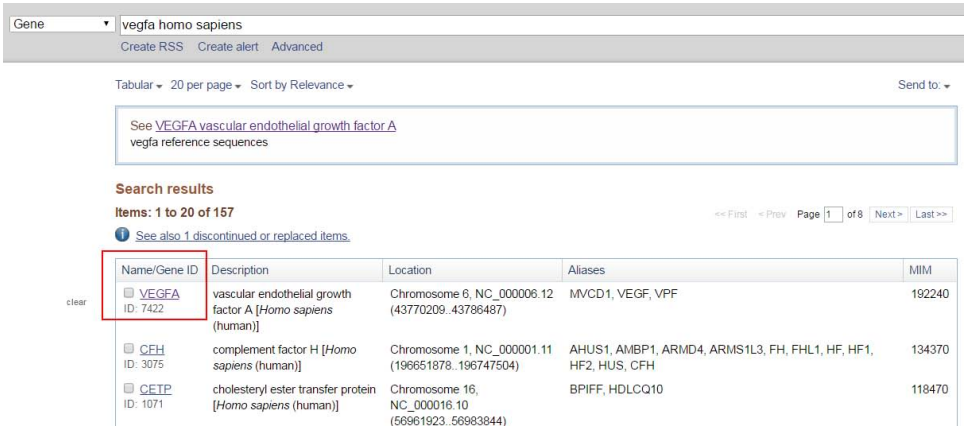
For instructions on searching for exon-exon boundaries in the Gene database, see the next section, "Find exon information using the Gene database" on page 48.

Find exon information using the Gene database

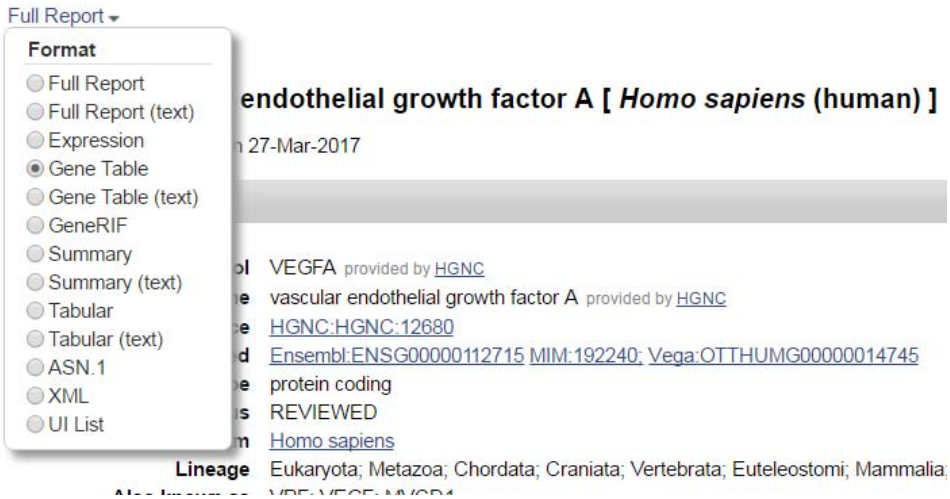
Gene is a searchable database of genes, from RefSeq genomes, which are defined by sequence and/or in the NCBI Map Viewer.

1. Go to **www.ncbi.nlm.nih.gov/gene**.
2. In the Gene page, enter a gene name, symbol, or keyword, then click **Search**.

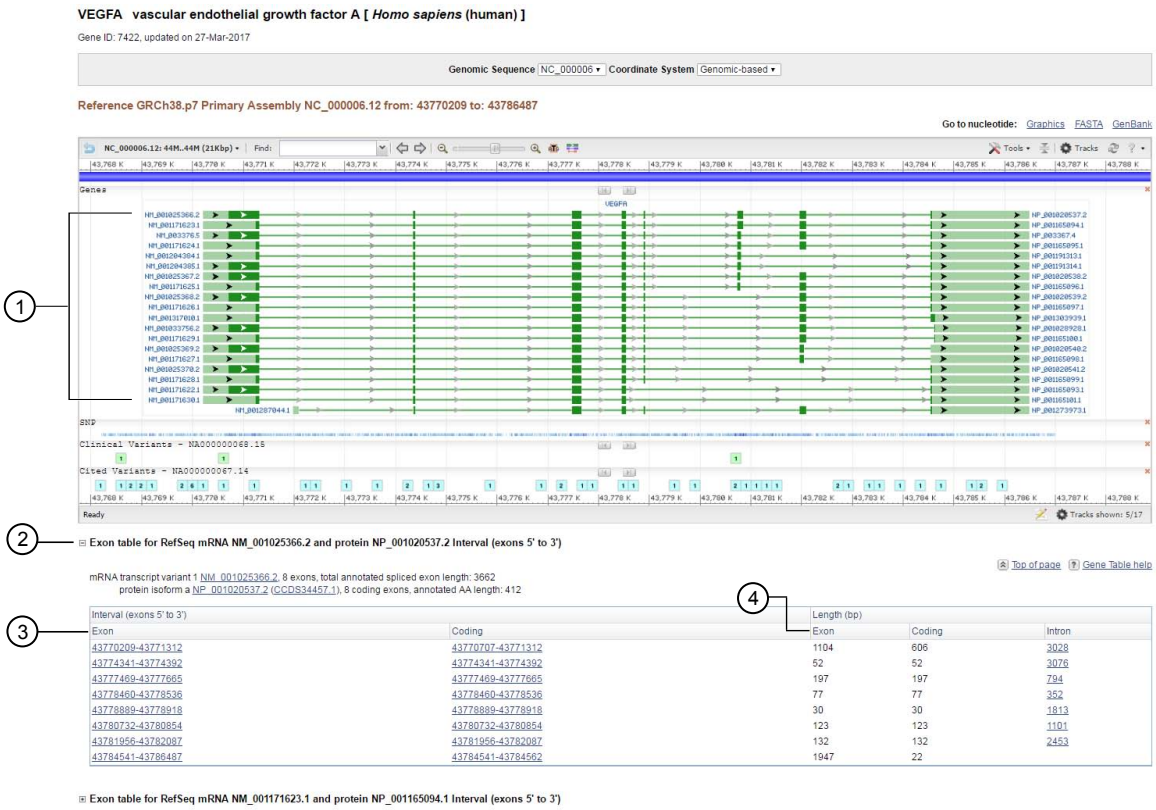
3. In the results list, click the gene name.



4. Under **Full Report**, select **Gene Table**.



5. Scroll to the transcript variant of interest, which includes the exon start and stop bases for the gene sequence.



① Transcript variants
② Exon table
③ Exon column displaying genome coordinates. Click the hyperlink to open the exon sequence of interest.
④ Exon length. The exon length helps determine the target position.

6. Download 2 exon sequences from the exon column, then identify the target position (the exon-exon boundary) using the exon lengths.

   After you select one or more target positions, identify and mask any repeats (“Mask sequence repeats“ on page 51) and polymorphisms (“Manually detect and mask non–target sequence polymorphisms“ on page 53).

Manually mask sequence repeats

Manually mask your sequences with RepeatMasker, a program that screens DNA sequences for interspersed repeats and low-complexity. The output is a detailed annotation of the repeats in the query sequence and a modified version of the query sequence in which all the annotated repeats have been masked.

Sequence format requirements

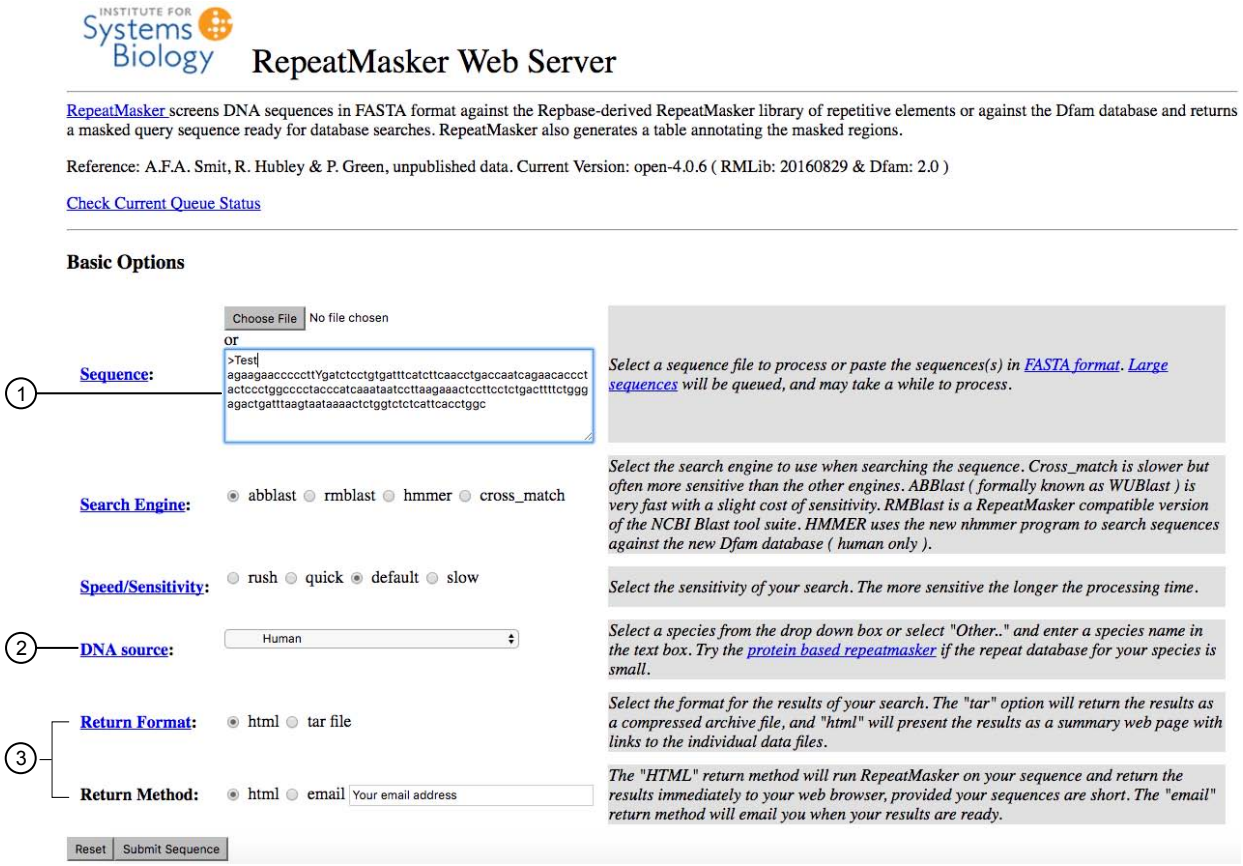
You can enter one sequence at a time or multiple sequences (in a batch).

The sequence should be in FASTA format – “>”, followed by one description line, with the sequence on the following line. RepeatMasker does not allow SNPs marked with brackets. If your sequence has SNPs, you must remove the brackets and convert the

bases to the corresponding IUPAC ambiguity code before submitting the sequence to search.

Mask sequence repeats

1. Go to **www.repeatmasker.org**, then click **RepeatMasking**.
2. In the web page that appears, enter your sequence into the form on the web page by:
   - Copying and pasting your sequence.
     or
   - Uploading it from a file.



① Enter your sequence.
② Select your DNA source.
③ Select the Return Format and Return Method.

3. Select the appropriate source of DNA from the DNA source list.

   Note: The default genome library is Human. Because interspersed repeats are species-specific, be sure to select the appropriate repeat library to search.

4. To display the results of the search in your web browser, select **html** for Return Format and Return Method.
5. Click **Submit Sequence**.

Evaluate RepeatMasker results

The RepeatMasker results are displayed as:

- A summary of the types of repeats found
- A table annotating the masked sequences
- The masked sequence

Note: The results in "Examples of summary of the types of repeats found" are from the Web service RepeatMasker.

Examples of summary of the types of repeats found

Summary:

```
==================================================
file name: RM2sequpload_1491405698
sequences:             1
total length:        165 bp  (165 bp excl N/X-runs)
GC level:         44.51 %
bases masked:        154 bp ( 93.33 %)
==================================================
                number of      length   percentage
                elements*    occupied  of sequence
--------------------------------------------------
SINEs:                 0            0 bp    0.00 %
      ALUs             0            0 bp    0.00 %
      MIRs             0            0 bp    0.00 %

LINEs:                 0            0 bp    0.00 %
      LINE1            0            0 bp    0.00 %
      LINE2            0            0 bp    0.00 %
      L3/CR1           0            0 bp    0.00 %

LTR elements:          1          154 bp   93.33 %
      ERVL             0            0 bp    0.00 %
      ERVL-MaLRs       0            0 bp    0.00 %
      ERV_classI       1          154 bp   93.33 %
      ERV_classII      0            0 bp    0.00 %

DNA elements:          0            0 bp    0.00 %
     hAT-Charlie       0            0 bp    0.00 %
     TcMar-Tigger      0            0 bp    0.00 %

Unclassified:          0            0 bp    0.00 %

Total interspersed repeats:      154 bp   93.33 %


Small RNA:             0            0 bp    0.00 %

Satellites:            0            0 bp    0.00 %
Simple repeats:        0            0 bp    0.00 %
Low complexity:        0            0 bp    0.00 %
==================================================

* most repeats fragmented by insertions or deletions
  have been counted as one element


The query species was assumed to be homo sapiens
RepeatMasker version open-4.0.6 , default mode

run with blastp version 3.0SE-AB [2009-10-30] [linux26-x64-I32LPF64 2009-10-30T17:06:09]
RepBase Update 20160829, RM database version 20160829
```

Example of a table annotating the masked sequences

SW score	perc div.	perc del.	perc ins.	query sequence	position in query begin	position in query end	(left)	matching repeat	repeat class/family	position in repeat begin	position in repeat end	position in repeat (left)	ID
575	23.6	4.5	3.9	Test	12	165	(0)	+ LTR8	LTR/ERV1	441	595	(96)	1

The masked sequence

The search returns the submitted sequence with all low-complexity sequences replaced with Ns, so that the returned sequence is the same length as the original.

```
>Test
AGAAGAACCCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNN
```

Figure 4 Masked sequence from the RepeatMasker "Masked File"

You can copy the masked sequence directly from the results and use it for other searches, or paste the masked sequence into the Custom TaqMan® Assay Design Tool.

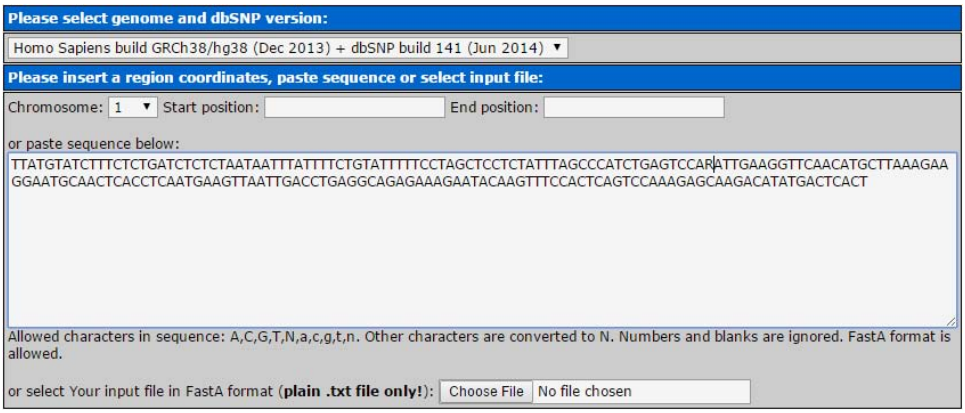
Manually detect and mask non–target sequence polymorphisms

SNPmasker is one of many programs with the ability to mask SNPs in a given sequence using information from dbSNP. You can use this program to find and mask nontarget SNPs in your sequence.

To search for sequence polymorphisms in your sequence:

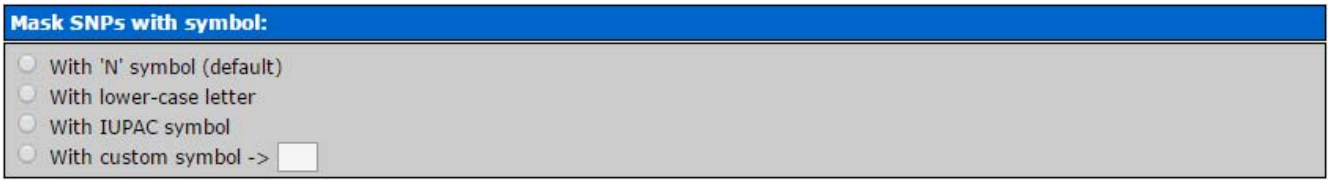
1. Go to: **http://bioinfo.ebc.ee/snpmasker/**
2. Under "**Please select genome and dbSNP version:**" in the dropdown list, make the appropriate selection.

3. Under "**Please insert a region coordinates, paste sequence or select input file:**", do one of the following:
   - Enter the sequence coordinates.
   - Paste the sequence in the window provided.
   - Upload the input sequence in FASTA format (as a plain .txt file).

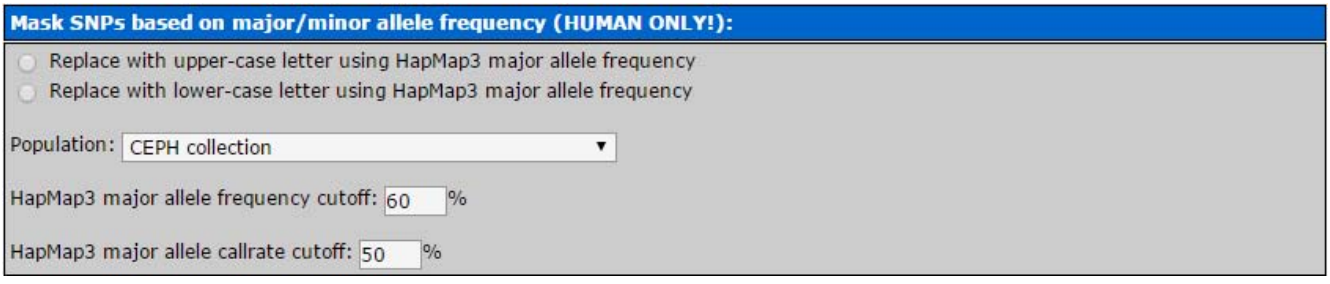


4. Mask SNPs in one of the following ways:
   a. To mask all SNPs with N, select "With 'N' symbol (default)".

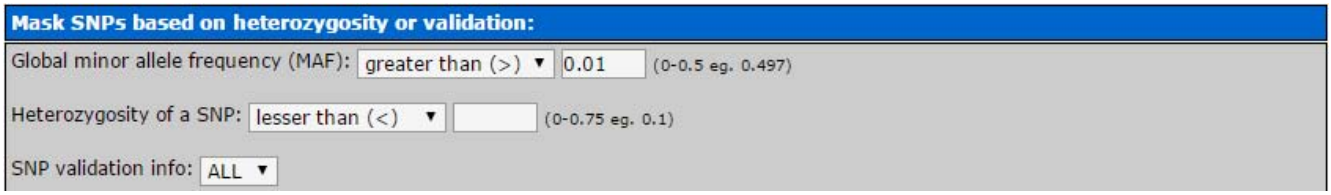
      Note: If the target SNP is in the database, it is also masked with N. Otherwise, it is replaced by the allele in the reference sequence. It can be preferable to screen the sequence on the 5′ side of the SNP and the sequence on the 3′ side of the SNP separately to avoid searching for the target SNP in the output sequence.



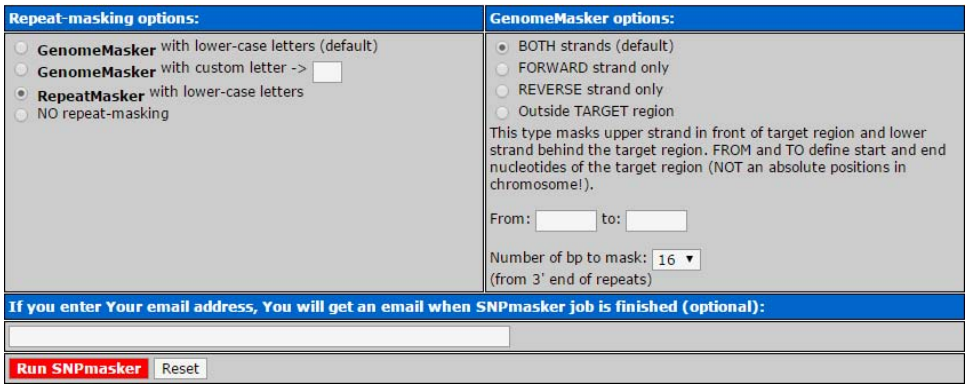
   b. To mask human SNPs selectively based on known allele frequency information, use "SNPs selectively based on known allele frequency information". Select the option to display SNPs in upper- or lower-case letters. Select the population from the dropdown list. Select the major allele frequency cutoff and call rate cutoff.



c. To mask SNPs based on global minor allele frequency, make a selection from the dropdown menu for the type of cutoff to apply and the cutoff value. A suggested cutoff of greater than 0.01 global minor allele frequency is shown. This cutoff level decreases the total number of masked SNPs to only those with a minor allele frequency of greater than 1%. It also increases the likelihood of obtaining an assay design from CADT by increasing the selection of potential primers and probes.



5. This tool can also be used to mask repeats using the RepeatMasker algorithm described earlier in this section. Select **RepeatMasker** then click **Run SNPmasker** after entering an email address if desired.



6. The SNPmasker output shows masked SNPs in red with links to dbSNP entries with more details. Replace the SNP base with the alleles in brackets (for example [A/G] or [*/G] for indels). The sequence can then be named and entered or uploaded into the Custom TaqMan® Assay Design Tool.

C

Order other TaqMan® assays

Order other TaqMan® SNP Genotyping Assays

Enter custom primer- probe pairs

Submit the sequences for the forward primer, reverse primer, and TaqMan® MGB probes, then we synthesize the oligos and formulate a custom assay using your oligonucleotide sequences.

Format requirements

Field	Requirement
Assay Name	Enter 4 to 16 characters.
Forward Primer	• Enter the sequence in the 5′ to 3′ direction. • Enter 9 to 50 nucleotides. • Use only A, C, G, and T. • Remove any spaces, tabs, or line breaks.
Reverse Primer	• Enter the sequence in the 5′ to 3′ direction. • Enter 9 to 50 nucleotides. • Use only A, C, G, and T. • Remove any spaces, tabs, or line breaks.
Probe 1	• Enter the sequence in the 5′ to 3′ direction. • Enter 9 to 50 nucleotides. **Note:** We recommend ≤26 nucleotides. • Use only A, C, G, and T. • Remove any spaces, tabs, or line breaks. **Note:** The probe for the first allele is labeled with VIC dye.
Probe 2	• Enter the sequence in the 5′ to 3′ orientation. • Enter 9 to 50 nucleotides. **Note:** We recommend ≤26 nucleotides. • Use only A, C, G, and T. • Remove any spaces, tabs, or line breaks. **Note:** The probe for the second allele is labeled with 6-FAM dye.

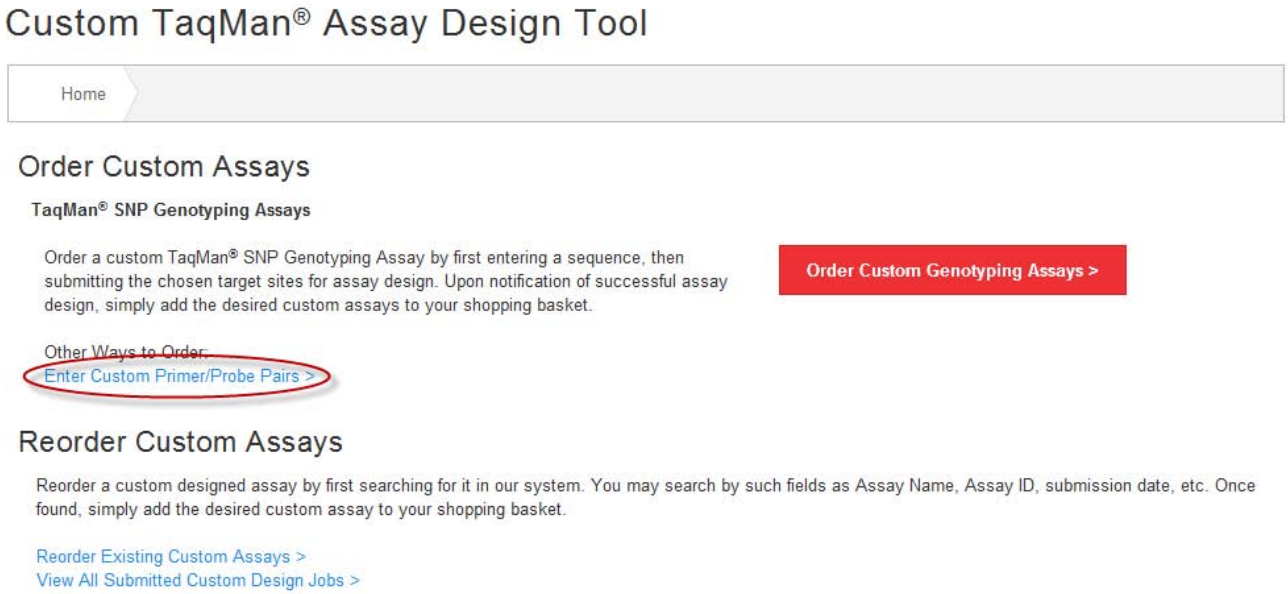
Log in to the Custom TaqMan® Assay Design Tool

1. Go to **thermofisher.com**, then log in to the store:
2. At the top of the home page, click **Sign In**.
3. In the Store log in page, log in to the website as instructed.
   - Existing customer, enter your user name and password, then click **Sign In**.
   - New customer, click **Register Now**, then complete the registration process as instructed.

     Note: Log in to the store to complete orders.

Order TaqMan® Assays

1. Access the Custom TaqMan® Assay Design Tool at **www.thermofisher.com/taqmansnpdesign**.
2. Click **Enter Custom Primer/Probe Pairs**.



Enter the sequences

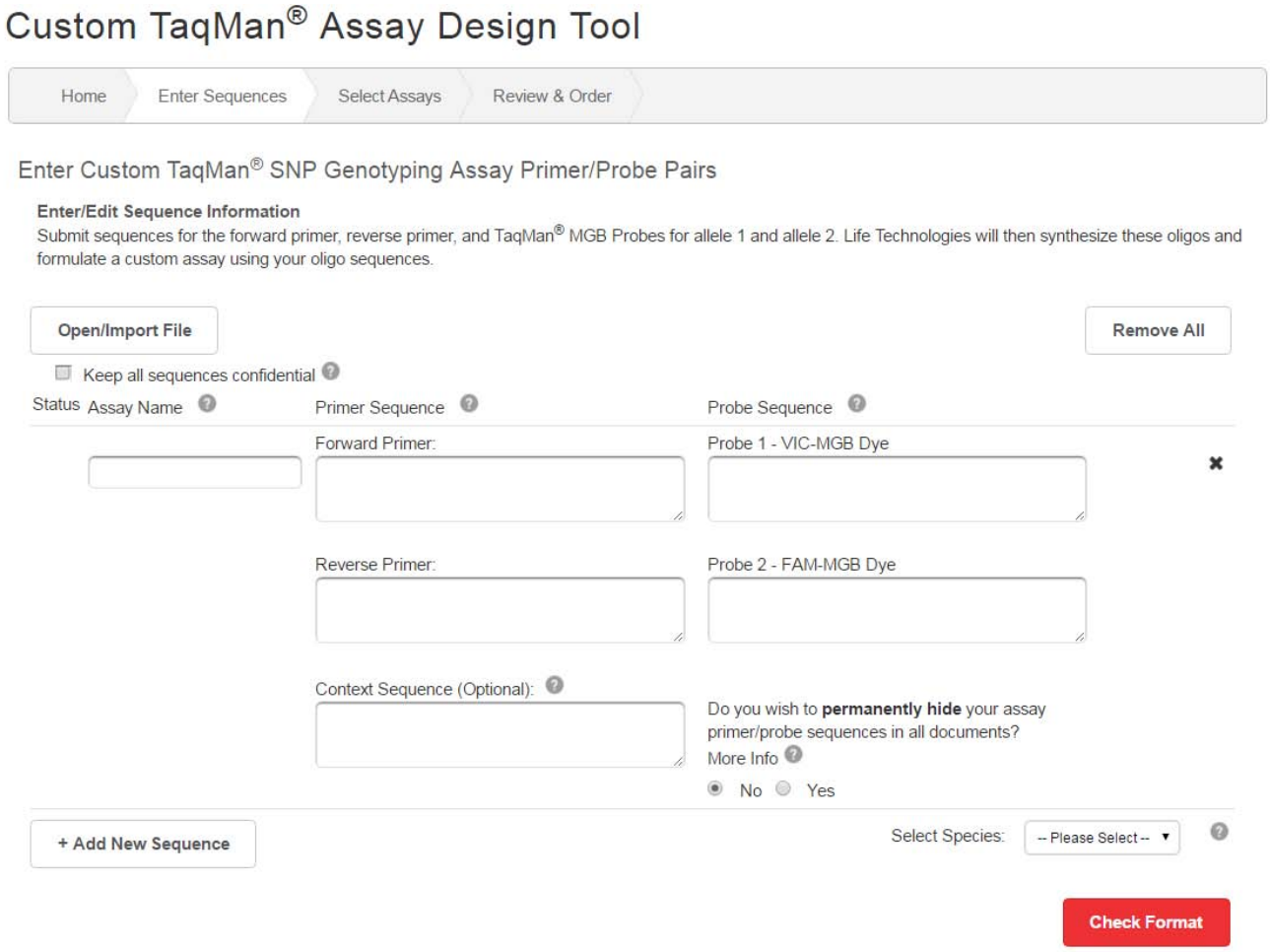
1. Enter 4 to 16 characters for the Assay Name.
2. Enter 9 to 50 nucleotides (A, C, G, or T) for the Forward Primer sequence.
3. Enter 9 to 50 nucleotides (A, C, G, or T) for the Reverse Primer sequence.
4. Enter 9 to 50 nucleotides (A, C, G, or T) for the Probe 1 sequence.

   Note: We recommend ≤26 nucleotides for MGB probes.

5. Enter 9 to 50 nucleotides (A, C, G, or T) for the Probe 2 sequence.

   Note: We recommend ≤26 nucleotides for MGB probes.

6. (*Optional*) To enter more than one custom primer/probe pair, click **+ Add New Sequence**, then repeat step 1 through step 5.



(*Optional*) Upload sequence from a file

The uploaded file must be a tab-delimited text file (.txt) formatted with all the following information, including the column headers.

- **Assay Name** (first column)—Enter a name for the assay (followed by a Tab).
- **VIC™ probe sequence** (second column)—Enter the sequence of the VIC™/MGB probe in the 5' to 3' direction (followed by a Tab).
- **FAM™ probe sequence** (third column)—Enter the sequence of the probe in the 5' to 3' direction (followed by a Tab).
- **Forward primer sequence** (fourth column)—Enter the forward primer sequence in the 5′ to 3′ direction (followed by a Tab).
- **Reverse primer sequence** (fifth column)—Enter the reverse primer sequence in the 5′ to 3′ direction.

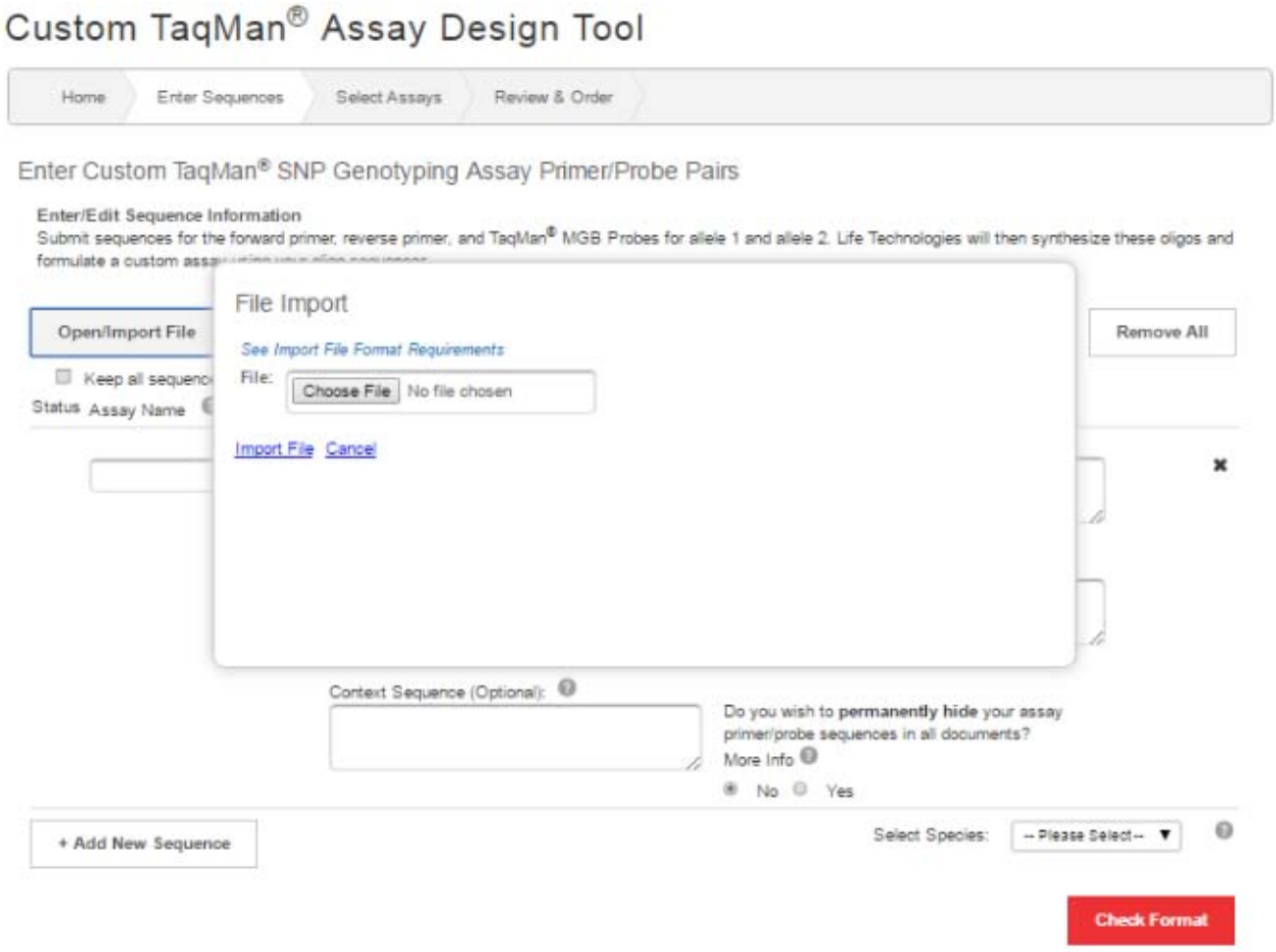
- **(*Optional*) Context sequence** (sixth column)—Enter the context sequence of the assay in the 5' to 3' direction (followed by a Tab).
- **(*Optional*) Private** (seventh column)—Indicate whether the sequence information is to be kept confidential.

The following is an example of properly formatted file:

Assay Name	VIC Probe Sequence	FAM Probe Sequence	Forward Primer Sequence	Reverse Primer Sequence	Context sequence	Private
mysequence	ATGGGCACTAATTTTCT	ATGGGCACAAATTTTCT	TGGAGTTGTCCCAATTCTTGTTGAA	ACCTTCACCCTCTCCACTGA	ATGGGCAC[T/A]AATTTTCTCGTAGTCG	FALSE

Note: For more information on how to format the file, click **Import File Format Requirements**.
To include multiple sequences in the file, enter the assay information in the following lines of the file in the same format. Do not repeat the header information.
The uploaded file must be a tab-delimited text file (.txt) that includes all the following information, including the column headers:

1. Click **Open/Import File**.
2. Click **Browse**, select the file, then click **Open**.
3. Click **Import File**.



Check the format and submit your sequences

After you finish entering custom primer/probe pairs, check the format of the sequences.

1. Click **Check Format**, then correct the sequence information if needed.
2. (*Optional*) To hide your assay sequences, answer **Yes** to the question, "**Do you wish to permanently hide your assay primer/probe sequences in all documents?**".
3. Click **Submit forAssay Design**.
   A confirmation message is displayed with a **Batch ID**. If the **Batch ID** is not shown, click **Refresh Batch List**.
4. (*Optional*) Save the sequence information in a *.txt file.
   a. Click **Save File**.
   b. In the Save File dialog box, select a location for the file, enter a file name, then click **Save**.
5. After the submitted batch status changes to **COMPLETED**, the passing **Assay IDs** are listed in a table at the bottom of the page. Select the **Size** and **Quantity** of assays, then click **Add** or **Add all** to add the assays to the **Shopping List** on the right of the page.
6. To order, click **Order Now** in the **Shopping List**.

Order other TaqMan® Gene Expression Assays

Enter custom primer probe pairs

Submit the sequences for the forward primer, reverse primer, and TaqMan® MGB probe, then we synthesize the oligos and formulate a custom assay using your oligonucleotide sequences.

Format requirements

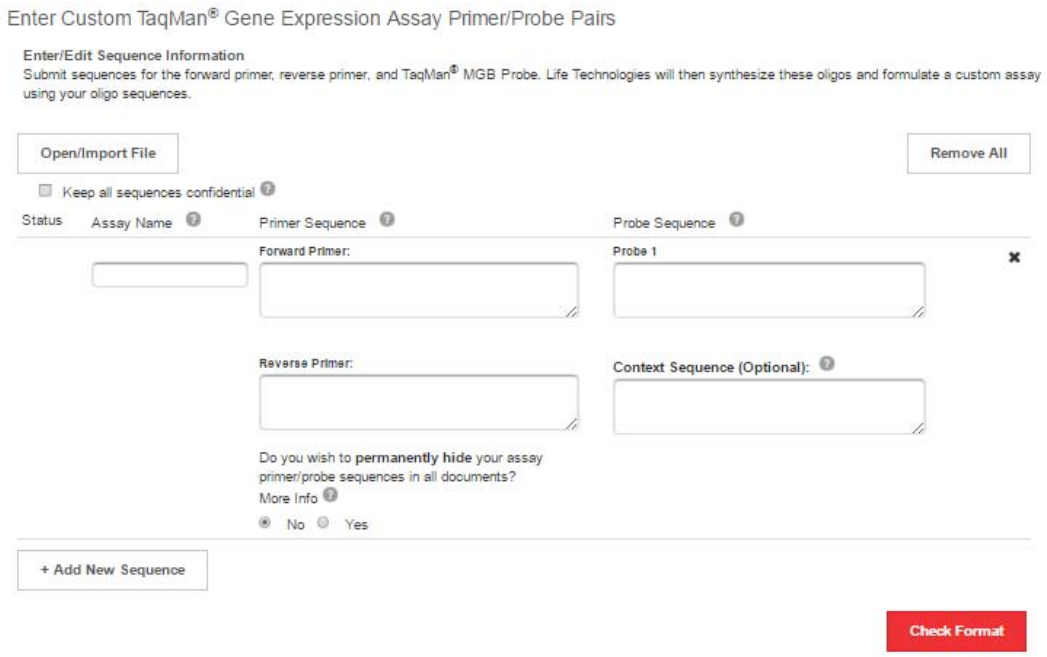
Field	Requirement
Assay Name	Enter 4 to 16 characters.
Forward Primer	• Enter the sequence in the 5′ to 3′ direction. • Enter 9 to 45 nucleotides. • Use only A, C, G, and T. • Convert U to T. • Remove any spaces, tabs, or line breaks.

Field	Requirement
Reverse Primer	• Enter the sequence in the 5′ to 3′ direction. • Enter 9 to 45 nucleotides. • Use only A, C, G, and T. • Convert U to T. • Remove any spaces, tabs, or line breaks.
Probe	• Enter the sequence in the 5′ to 3′ direction. • Enter 9 to 45 nucleotides. **Note:** We recommend ≤26 nucleotides. • Use only A, C, G, and T. • Convert U to T. • Remove any spaces, tabs, or line breaks. **Note:** The probe sequence must be for a TaqMan® MGB probe.

Determine the sequence criteria

1. Go to the Custom TaqMan® Assay Design Tool website: **www.thermofisher.com/cadt**.

2. Click **Enter Custom Primer/Probe Pairs** if ordering specific primers and probes.



Enter the sequences

1. Enter 4 to 16 characters for the Assay Name.
2. Enter 9 to 45 nucleotides (A, C, G, or T) for the Forward Primer sequence.
3. Enter 9 to 45 nucleotides (A, C, G, or T) for the Reverse Primer sequence.
4. Enter 9 to 45 nucleotides (A, C, G, or T) for the probe sequence.
   We recommend probes ≤26 nucleotides.
5. (*Optional*) To enter more than one custom primer/probe pair, click **+ Add New Sequence**, then repeat step 1 through step 4.

Import sequence information from a file

The uploaded file must be a tab-delimited text file (.txt) formatted with all the following information, including the column headers.

- **Assay Name** (first column)—Enter a name for the assay (followed by a Tab).

  Note: To include multiple sequences in the file, enter the assay information in the next row of the text file.
- **Probe sequence** (second column)—Enter the sequence of the probe in the 5' to 3' direction (followed by a Tab).
- **Forward primer sequence** (third column)—Enter the forward primer sequence in the 5′ to 3′ direction (followed by a Tab).
- **Reverse primer sequence** (fourth column)—Enter the reverse primer sequence in the 5′ to 3′ direction (followed by a Tabr).
- **(*Optional*) Context sequence** (fifth column)—Enter the context sequence of the assay in the 5' to 3' direction (followed by a Tab).
- **(*Optional*) Private** (sixth column)—Indicate whether the sequence information is to be kept confidential.

The following is an example of properly formatted file:

Assay Name	Probe Sequence	Forward Primer Sequence	Reverse Primer Sequence	Context sequence	Private
mysequence	ATGGGCACAAATTTTCT	TGGAGTTGTCCCAATTCTTGTTGAA	ACCTTCACCCTCTCCACTGA	ATGGGCACAAATTTTCTCGTAGTCG	FALSE

Note: For more information on how to format the file, click **Import File Format Requirements**.
To include multiple sequences in the file, enter the assay information in the following lines of the file in the same format. Do not repeat the header information.
The uploaded file must be a tab-delimited text file (.txt) that includes all the following information, including the column headers:

1. Click **Open/Import File**.
2. Click **Browse**, select the file, then click **Open**.
3. Click **Import File**.

Check the format, then submit your sequences

After you finish entering custom primer/probe pairs, check the format of the sequences.

1. Click **Check Format**, then correct the sequence information if needed.
2. (*Optional*) To hide your assay sequences, answer **Yes** to the question, "**Do you wish to permanently hide your assay primer/probe sequences in all documents?**".
3. Click **Continue**.

   A confirmation message is displayed with a **Batch ID**. If the **Batch ID** is not shown, click **Refresh Batch List**.
4. After the submitted batch status changes to **COMPLETED**, click the line in the **Design Job Details** for that job. The passing **Assay ID**s are listed in a table at the bottom of the page. Select the **Size** and **Quantity** of assays, then click **Add** or **Add all** to add the assays to the **Shopping List** on the right of the page.
5. To order, click **Order Now** in the **Shopping List**.

Documentation and support

Related documentation

Document	Pub. no.
TaqMan® Gene Expression Assays Protocol	4333458
TaqMan® Gene Expression Assays Quick Reference Card	4401212
Introduction to Gene Expression Getting Started Guide	4454239
TaqMan® SNP Genotyping Assays User Guide	MAN0009593

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- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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