Designing TaqMan® MGB Probe and Primer Sets for Gene Expression Using Primer Express® Software Version 2.0

Overview

This tutorial details how a TaqMan® MGB Probe can be designed over a specific region of a template sequence such as an exon-exon junction (intron splice-site). Genomic DNA is often co-extracted with RNA and can therefore serve as a template in downstream processes such as PCR. Designing a TaqMan® MGB Probe over an exon-exon junction should enable the exclusion of genomic DNA as a template in a real-time PCR reaction.

This tutorial assumes basic working knowledge of the Primer Express® v2.0 Software. If you are unfamiliar with the software, please first review the following documents.

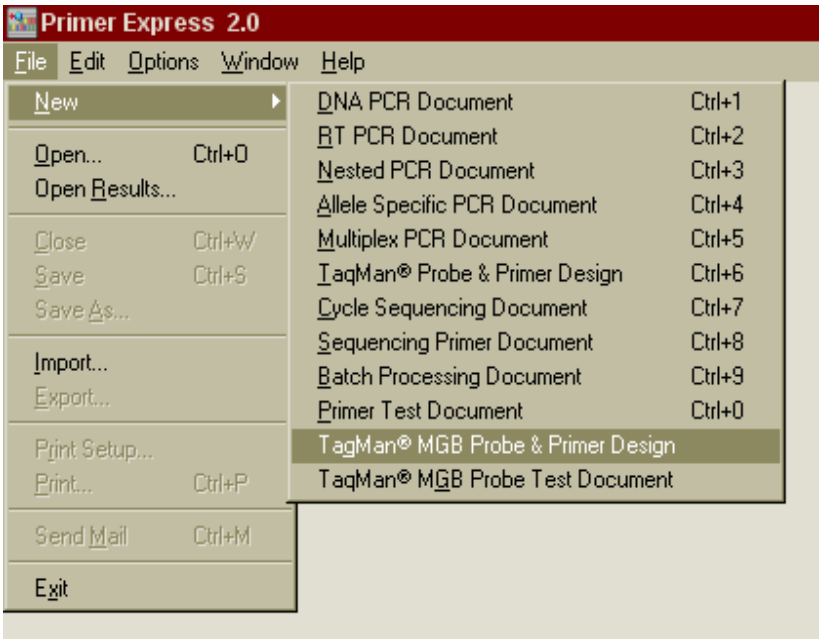
- Primer Express® Software v2.0 User's Manual, document part # 4329500
- Primer Express® Software v2.0 Applications Tutorials, document part # 4329501

Note: The documents above can be found electronically on your hard drive in Program Files/Applied Biosystems/Primer Express

Starting the Design and Entering the Sequence

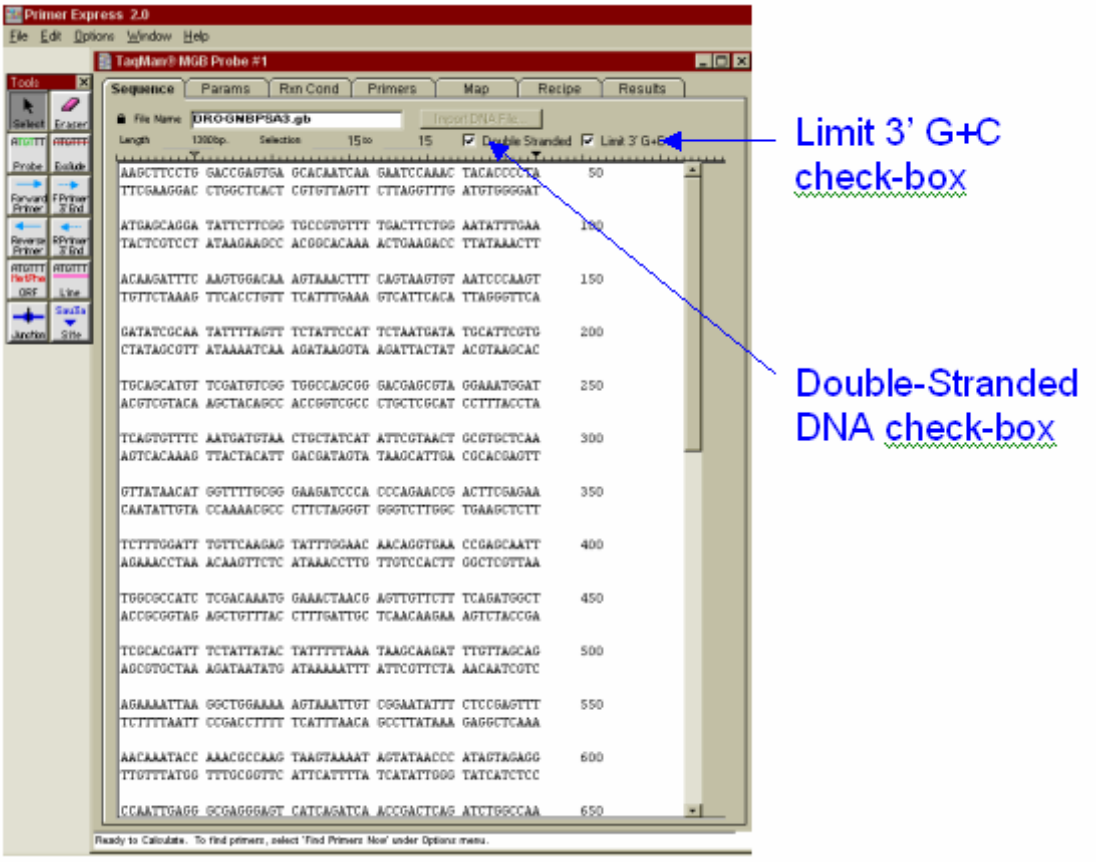
For gene expression assays, it is best to enter a cDNA sequence or an mRNA sequence into the Primer Express® Software. If entering an mRNA sequence and the sequence contains Uracils (U), one must first convert the Us to Thymidines (T) in a word processing document. To replace the Us with Ts, Copy and Paste the sequence into a word processing document such as Microsoft® Word. Go to the **Edit** menu and select **Replace**. In the **Find what** field, type the letter U. In the **Replace with** field, type the letter T. Click on the **Replace All** button. The sequence, which now contains Ts instead of Us, can be copied and pasted into the Primer Express® Software **Sequence** tab. Unless one is designing from a single-exon gene, it is not recommended to use genomic DNA sequences for the design of gene expression primer and probe sets.

For a gene expression assay, select the **TaqMan® MGB Probe and Primer Design** document from the **File/New** menu.

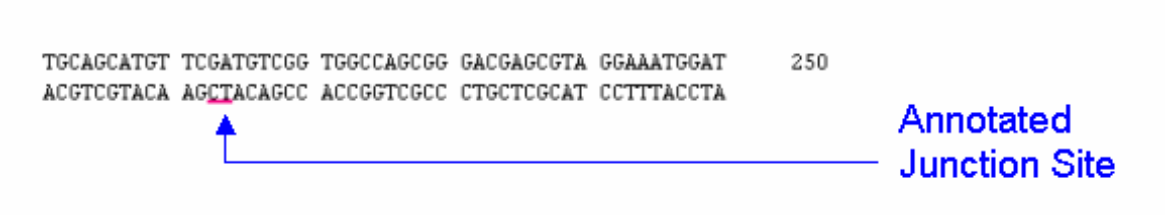


Enter the sequence either by going to **File/Import**, using the **Import DNA File** button in the **Sequence** Tab, or by copying and pasting the sequence through the **Edit** menu.

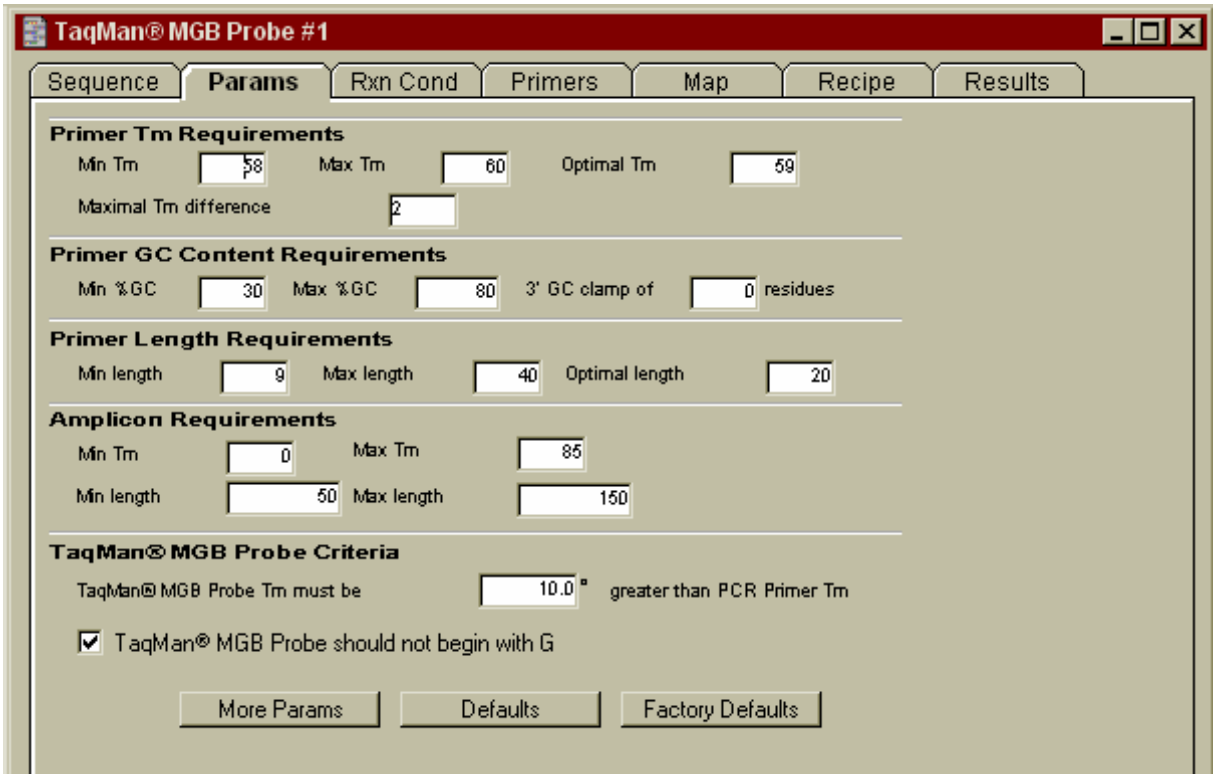
Next, if not checked, click in the **Double Stranded** and **Limit 3' G+C** boxes.



Use the **Line** tool to mark the junction site in the sequence. This only provides a visual aid and does not direct the software to design the primer/probe set over the site.



Click the **Params** tab. Click the **Defaults** button. Ideally, you will not have to alter the parameters, as the default parameters follow the TaqMan® Probe and Primer design guidelines as established by Applied Biosystems. For a list of the design guidelines, please refer to page 4-10 of the Primer Express® Software v2.0 User's Manual. One additional guideline specific to TaqMan® MGB probes is that the probe should be as short as possible, without being shorter than 13 nucleotides.



Return to the sequence page by clicking on the **Sequence** Tab.

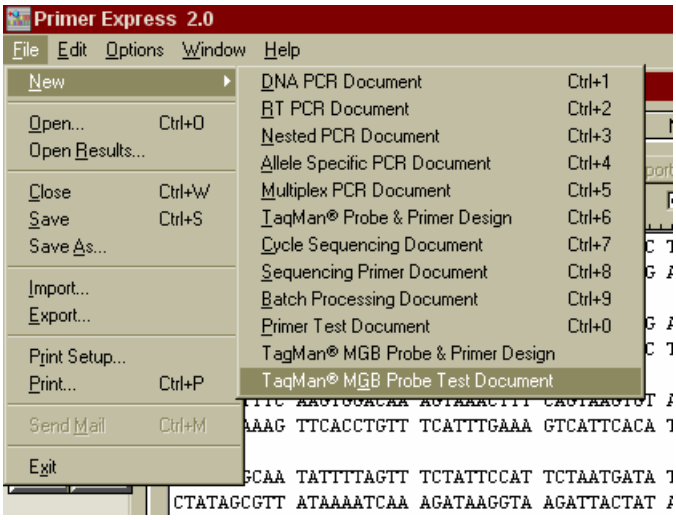
You are now ready to manually design the TaqMan® MGB probe.

Designing the TaqMan® MGB Probe

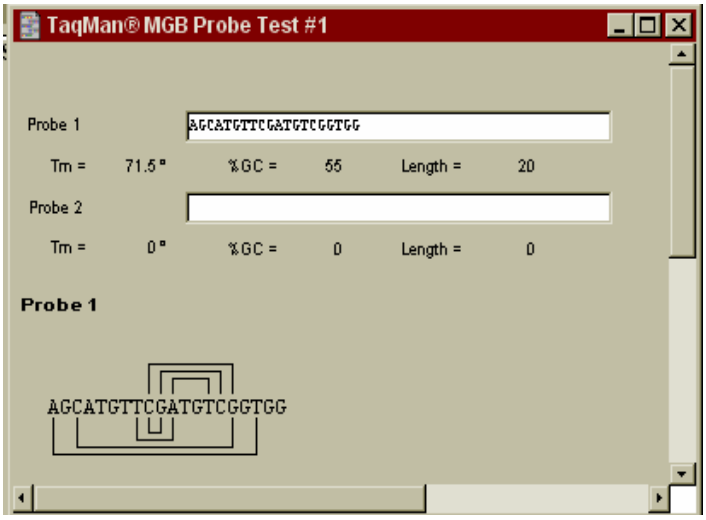
Make sure that the junction site is still labeled. If not, label the junction site using the **Line** tool.

Using the mouse, click and drag to highlight a portion of sequence approximately 20 bp long, keeping the junction site towards the middle third of the highlighted sequence. From the **Edit** menu, select **Copy**.

Under the **File/New** menu, select **TaqMan® MGB Probe Test Document**.



Click in the **Probe 1** text box, and then select **Paste** from the **Edit** menu.

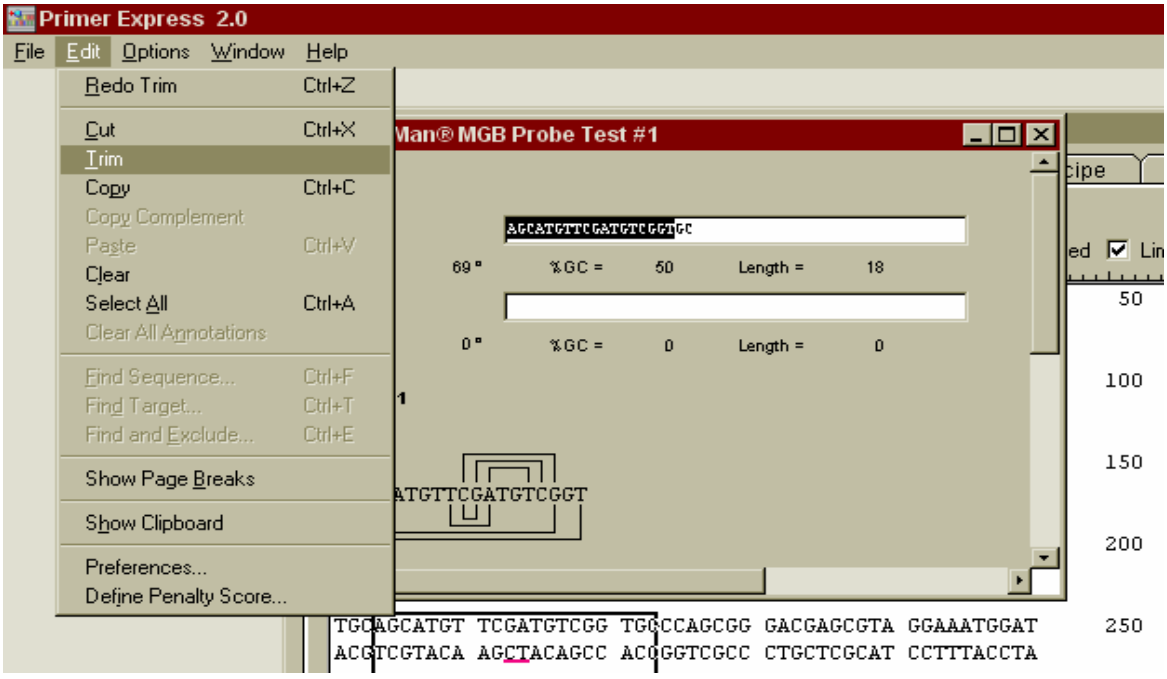


Check the melting temperature (Tm) of the sequence. TaqMan® MGB Probes for gene expression should have a Primer Express-estimated Tm of 68-70°C. TaqMan® MGB probes for gene expression should also be designed following the guidelines listed below.

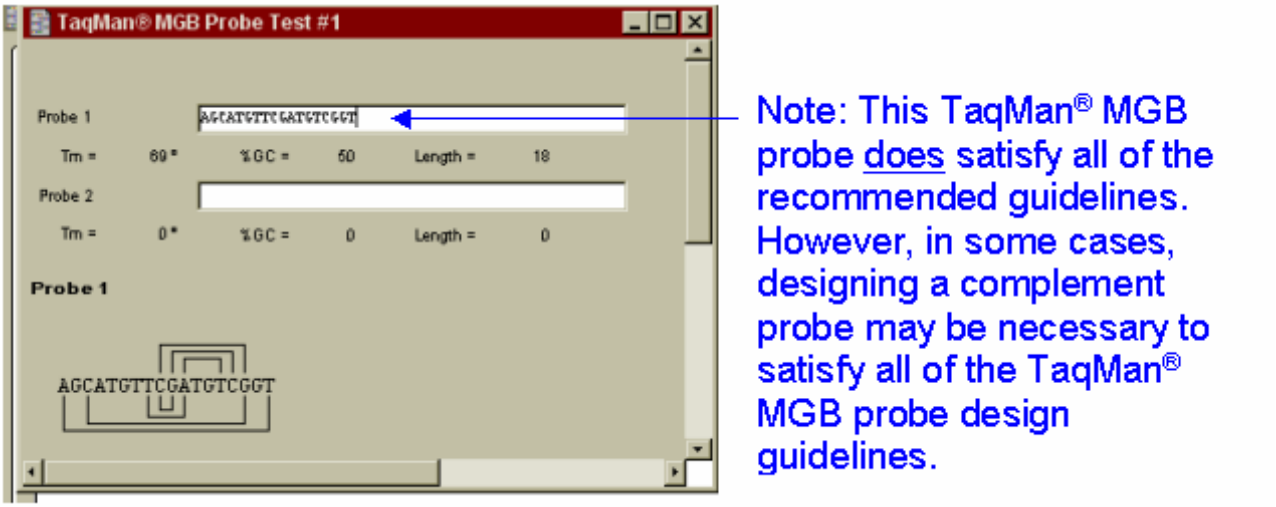
1) Keep G-C content in the 30-80% range.
2) Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
3) Do not put Gs on the 5' end.
4) Make TaqMan® MGB Probes as short as possible without being shorter than 13 nucleotides.

If the Tm of the potential probe sequence is too high, use the mouse to highlight a portion of the sequence in the **TaqMan® MGB Probe Test Document**. The **TaqMan® MGB Probe Test Document** will now report the Tm of the highlighted portion of the sequence.

When you have a sequence that has an appropriate Tm and also meets the guidelines above, select **Trim** from the **Edit** menu. This will remove the unhighlighted portion(s) of the sequence, and only a probe sequence that satisfies all of the guidelines will remain.



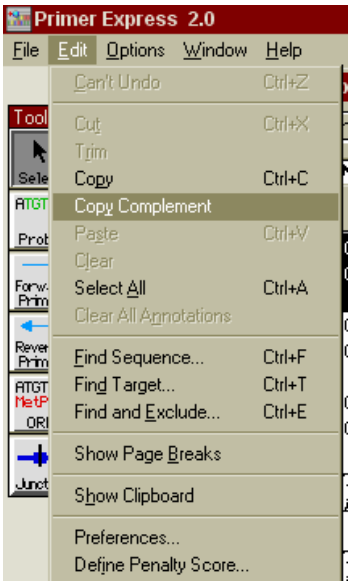
If all of the TaqMan® MGB probe design guidelines cannot be satisfied (ex. if it is not possible to select a probe without a guanine residue at the 5' end), you will need to design a probe on the complement (antisense) strand. If it is not necessary to design the TaqMan® MGB probe on the complement strand, go to the section entitled **Adding a Probe Annotation.**



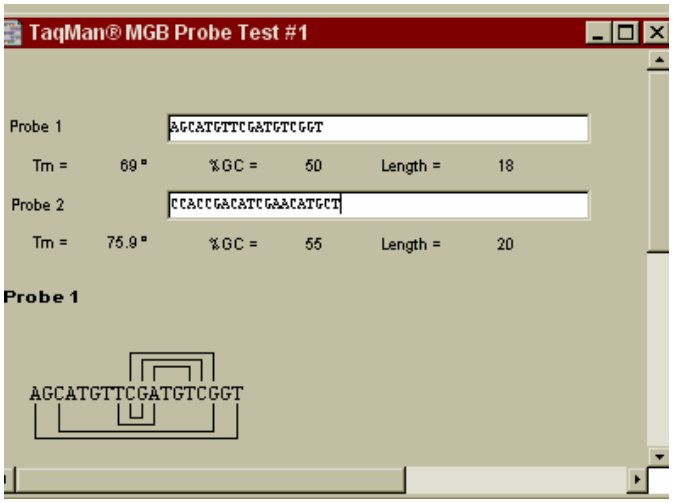
NOTE: Because of the asymmetric placement of the minor groove binder at the 3' end, complementary TaqMan® MGB probes do not necessarily have the same Tm as sense probe sequences. As shown in this tutorial, it is necessary to test the Tm of complement TaqMan® MGB probe sequences in a TaqMan® MGB Probe Test Document.

Designing the TaqMan® MGB Probe as a complement sequence

Return to the **Sequence** tab. Again, click and drag to highlight a portion of sequence approximately 20 bp long, keeping the junction site towards the middle third of the highlighted region. Select **Copy Complement** from the **Edit** menu.

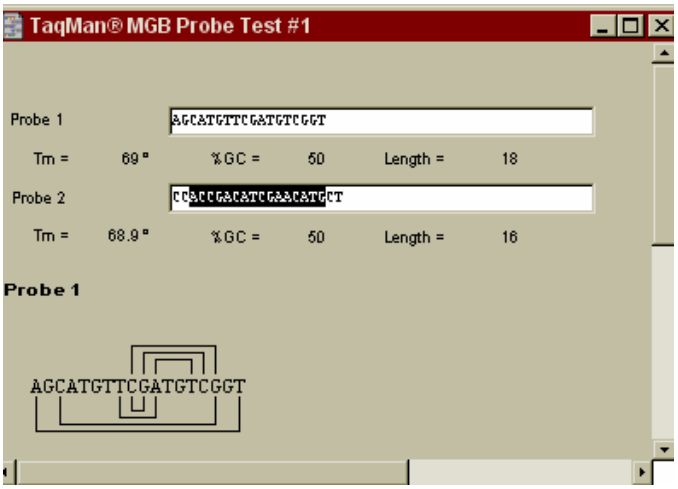


Click in the **Probe 2** text box, and then select **Paste** from the **Edit** menu. Check the Tm of the sequence. A TaqMan® MGB Probe for gene expression should have a T_m of 68-70°C.

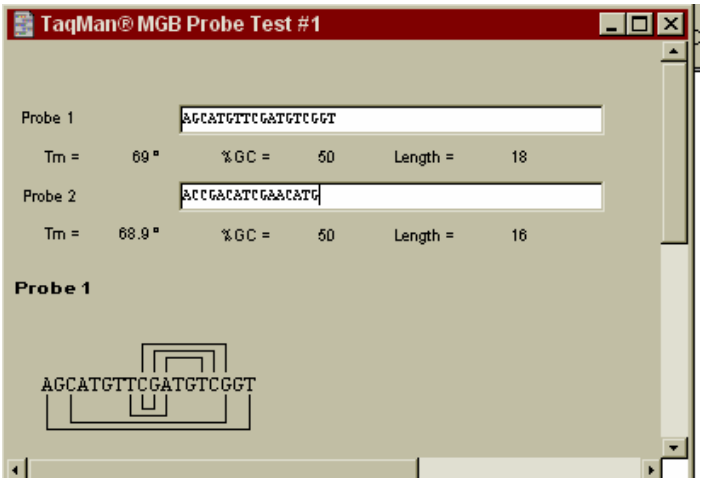


If the Tm of the potential probe sequence is too high, use the mouse to highlight a portion of the sequence in the **TaqMan® MGB Probe Test Document**.

The **TaqMan® MGB Probe Test Document** will now report the Tm of only the highlighted portion of the sequence.

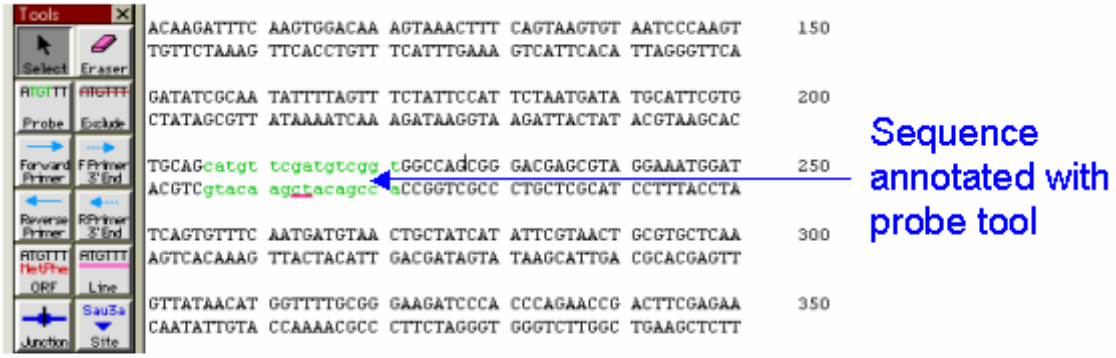


When you have a sequence that has an appropriate Tm and also meets the guidelines on page 6, select **Trim** from the **Edit** menu. This will remove the unhighlighted portion(s) of the sequence, and only a probe sequence that satisfies all of the guidelines will remain.



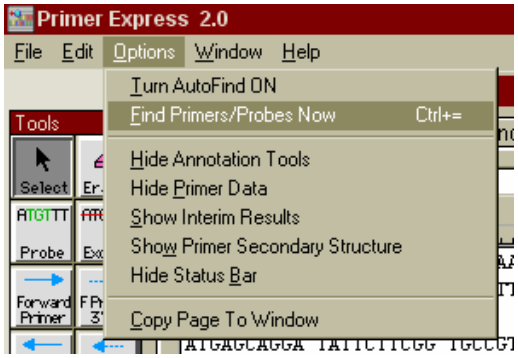
Adding a Probe Annotation

Return to the Primer Express® Software **Sequence** page. Select the **Probe** tool, and then highlight the portion of sequence matching the TaqMan® MGB probe.

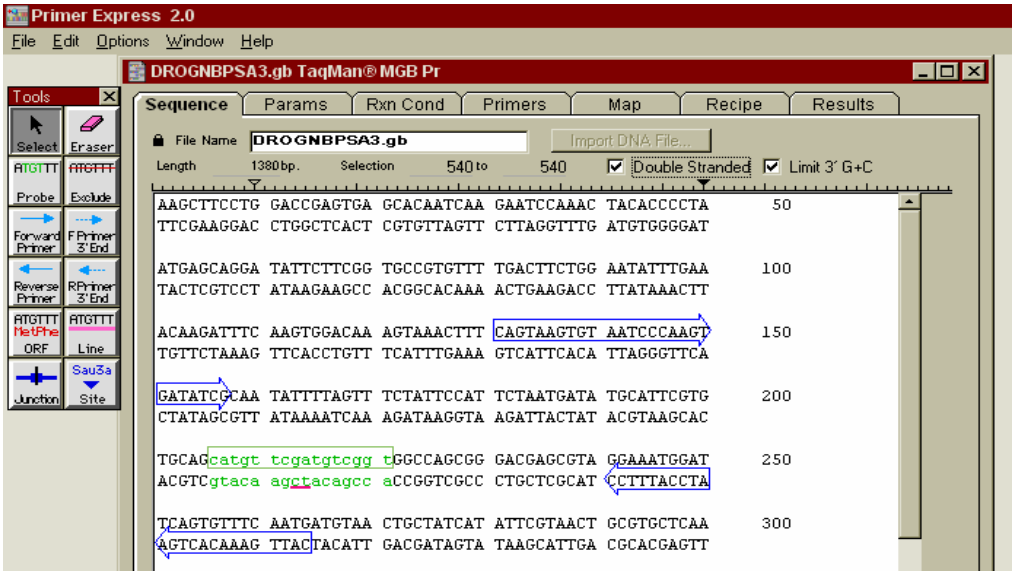


Designing the Primers

Ensure that the **Limit 3' G+C** checkbox is still checked. Select **Find Primers/Probes Now** from the **Options** menu.

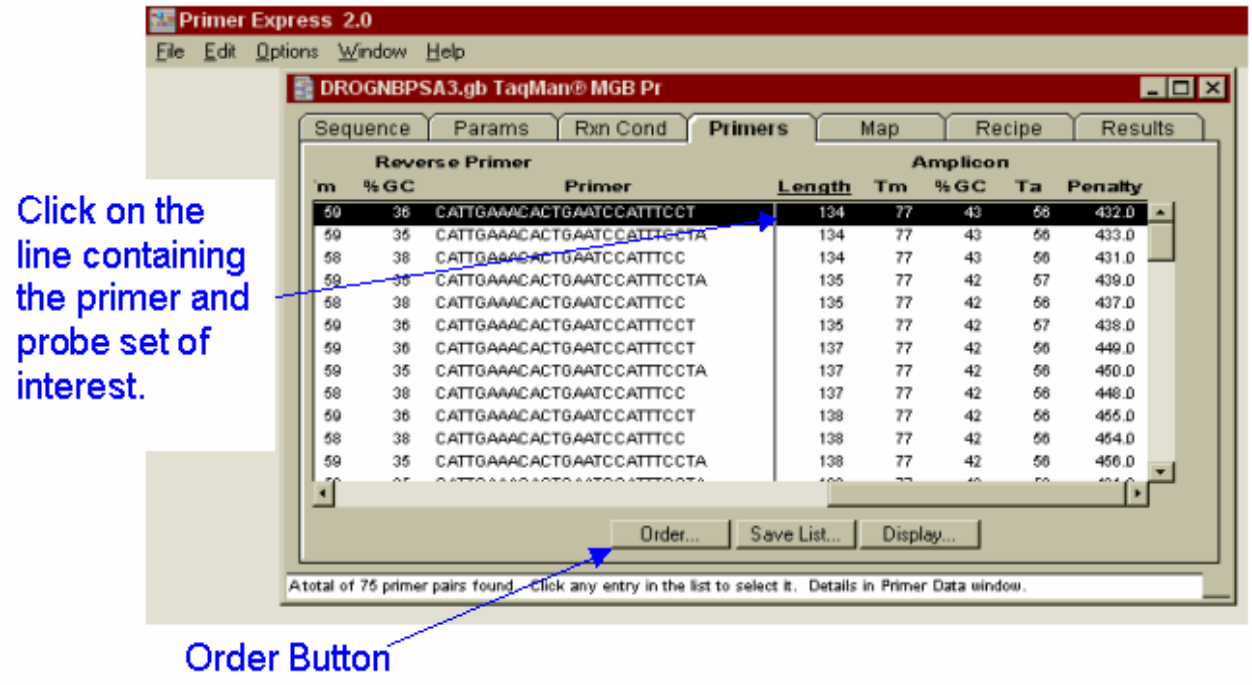


If the software finds acceptable primers, click the **Primers** tab. If the software cannot find acceptable primers, skip the following and proceed directly to the next section entitled **Manually designing primers through the Primer Express® Software.**

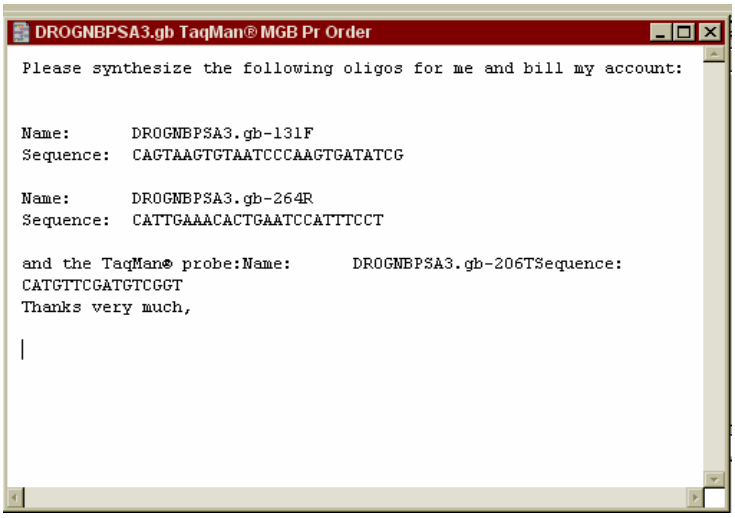


Select a primer pair from the list that will produce the shortest amplicon while satisfying all design guidelines.

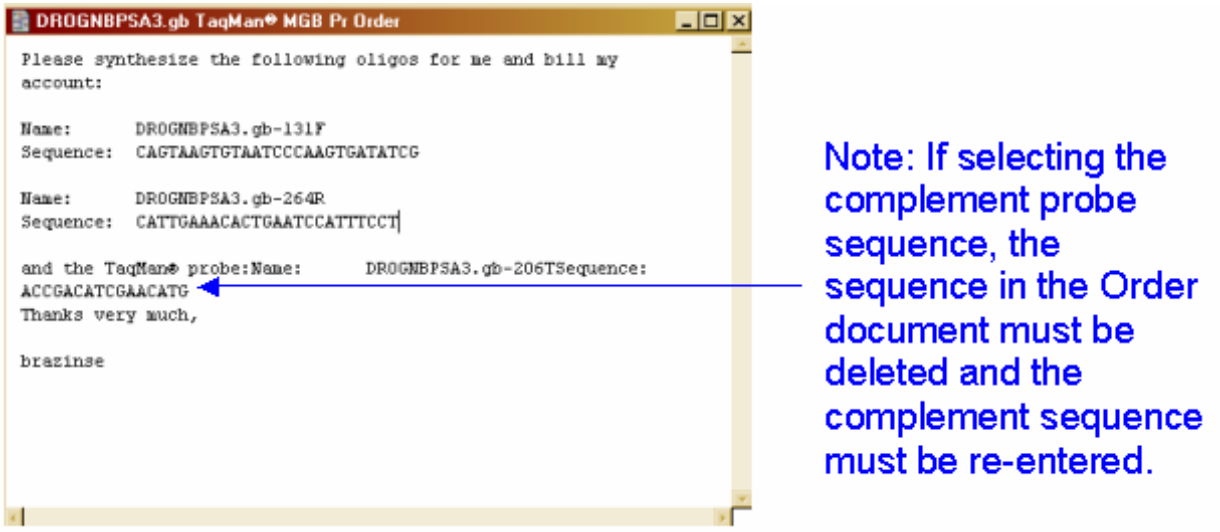
Click on the line containing the chosen primer and probe set.



Click on the **Order** button at the bottom of the **Primers** tab.



This order form does not actually place electronic orders. The **Order** document is a text file that enables the editing of the sequence information for ordering. If the TaqMan® MGB probe was designed as a complement sequence, the TaqMan® MGB probe **must** be re-entered in as the complement sequence. To edit the **Order** document, copy the final probe sequence (complement) from the **TaqMan® MGB Probe Test Document**. Return to the **Order** document and delete the current probe sequence. Select **Paste** from the **Edit** menu.



The sequences within the order document can now be copied and pasted into electronic orders.

There may be times when the Primer Express® Software is unable to find primer sets in conjunction with your manually designed probe. If no acceptable primer sets were found, you will need to perform a manual design within the Primer Express® Software.

Manually designing primers through the Primer Express® Software

NOTE: When selecting sequences for the forward and reverse primers, consider the guideline for amplicon size: 50-150 bases. Select primers close enough in proximity to the probe to stay within this guideline.

First, copy the final probe sequence from the **TaqMan® MGB Probe Test Document** and paste it into a text document.

From the **Sequence** page, use the mouse to click and drag over a portion of sequence upstream of the probe, approximately 30 bp in length. Make sure the last 5 bases of the 3' end of the sequence contain no more than 2 (total) G+C. Select **Copy** from the **Edit** menu.

Open a **Primer Test Document** through the **File/New** Menu. Paste the sequence into the **Forward Primer** text box. If the Tm is too high, use your mouse to highlight a portion of a putative primer sequence in the **Primer Test Document** until you find a primer that meets the design guidelines as described on page 4-10 of the Primer Express® v2.0 User's Manual. These guidelines are also listed below.

1) Avoid runs of an identical nucleotide. This is especially true for guanine (G), where runs of four or more Gs should be avoided.
2) Design primers as close as possible to the probe without overlapping the probe.
3) Keep the G - C content within 30-80%.
4) Select primers with a T_m of 58-60^{o}C.
5) The five nucleotides at the 3' end should have no more than two G and/or C bases.

When you have a sequence that meets the guidelines, select **Trim** from the **Edit** menu. This will delete the unhighlighted portion(s) of the sequence, and only a primer sequence that satisfies the guidelines will remain.

Copy the new primer sequence. Paste it in to a text document containing the probe sequence and label it as the forward primer.

The process can be repeated for the reverse primer, this time selecting a sequence region downstream of the probe and using **Copy Complement** to copy the sequence into the **Primer Test Document**.

NOTE: Remember that the reverse primer will fall on the antisense DNA strand. With that in mind, be sure to use the **Copy Complement** function from the **Edit** menu (in place of the Copy function) when manually selecting a reverse primer sequence.

Ordering Primers and TaqMan® Probes

Ordering instructions for North America customers only, international customers should contact their local Applied Biosystems sales office. International contact information can be found at http://www.appliedbiosystems.com/about/offices.cfm.

To order Applied Biosystems reagents including primers and TaqMan® probes, go to the Applied Biosystems store at http://store.appliedbiosystems.com. Note: you must register to be able to login and order Applied Biosystems reagents via the web. Once the registration has been filled-out, Applied Biosystems order administration will send an e-mail within 48 hours confirming your registration and you will then be able to place an order.

To order primers and TaqMan® probes click on "ABI PRISM® Primers/Probes" in the catalog column (left-hand column). Then click on "TaqMan® Primers & Probes" in the catalog column. Scroll-down to locate the items that will be purchased. For the products that are to be ordered, check the boxes located on the left-hand side of the product names. Once all of the products have been selected, click on the "Add to Shopping Basket" button. The products will now be itemized in the shopping basket. It is important to enter the sequences of the primers and/or probes. Click the "not customized" button next to the product name for each custom primer and TaqMan® probe. Follow the instructions to enter in the sequence of the primer or TaqMan® probe. Repeat this process for all custom primers and TaqMan® probes. To process the order, click on the "Process Order" button and fill out the requested information to complete the order.

Ordering questions for primers and TaqMan® probes can be directed to the Applied Biosystems Custom Oligo ordering group at 800-327-3002. Follow the touch-tone menu to speak with an Order Administration representative regarding primers and TaqMan® probes. Ordering questions for SDS/Real Time PCR reagents and consumables can be directed to the Applied Biosystems Order Administration group at 800-327-3002. Follow the touch-tone menu to speak with an Order Administration representative regarding reagents and consumables. Technical questions about Applied Biosystems SDS/Real Time

PCR reagents and consumables, including primers and TaqMan® probes, can be directed to the Applied Biosystems PCR & SDS Technical Support group at 800-762-4001.

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