Designing TaqMan® MGB Probe and Primer Sets for Allelic Discrimination Assays Using Primer Express® Software Version 2.0

Overview

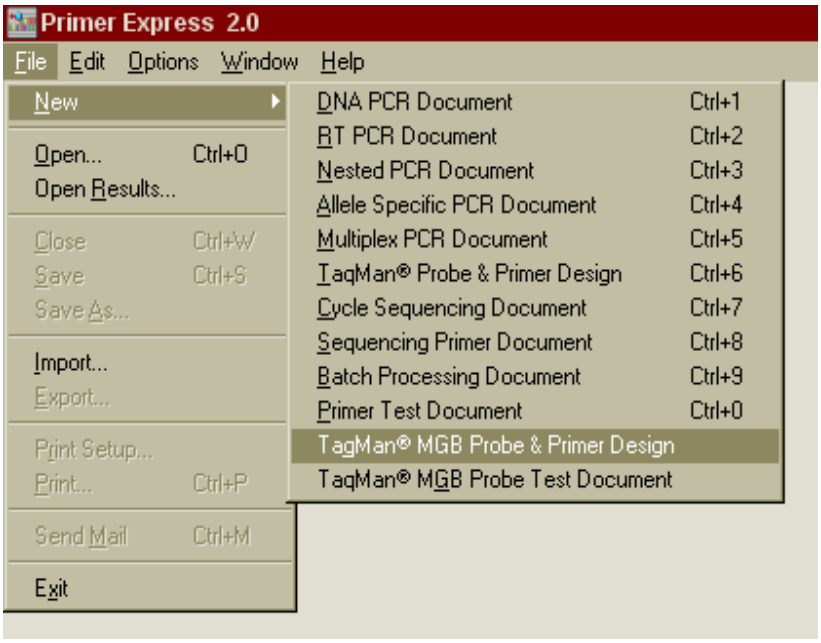
This tutorial details how to design primers and TaqMan® MGB probes for allelic discrimination assays. The tutorial assumes a basic working knowledge of the Primer Express® Software version 2.0. If you are unfamiliar with the software, please first review the following documents.

- 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

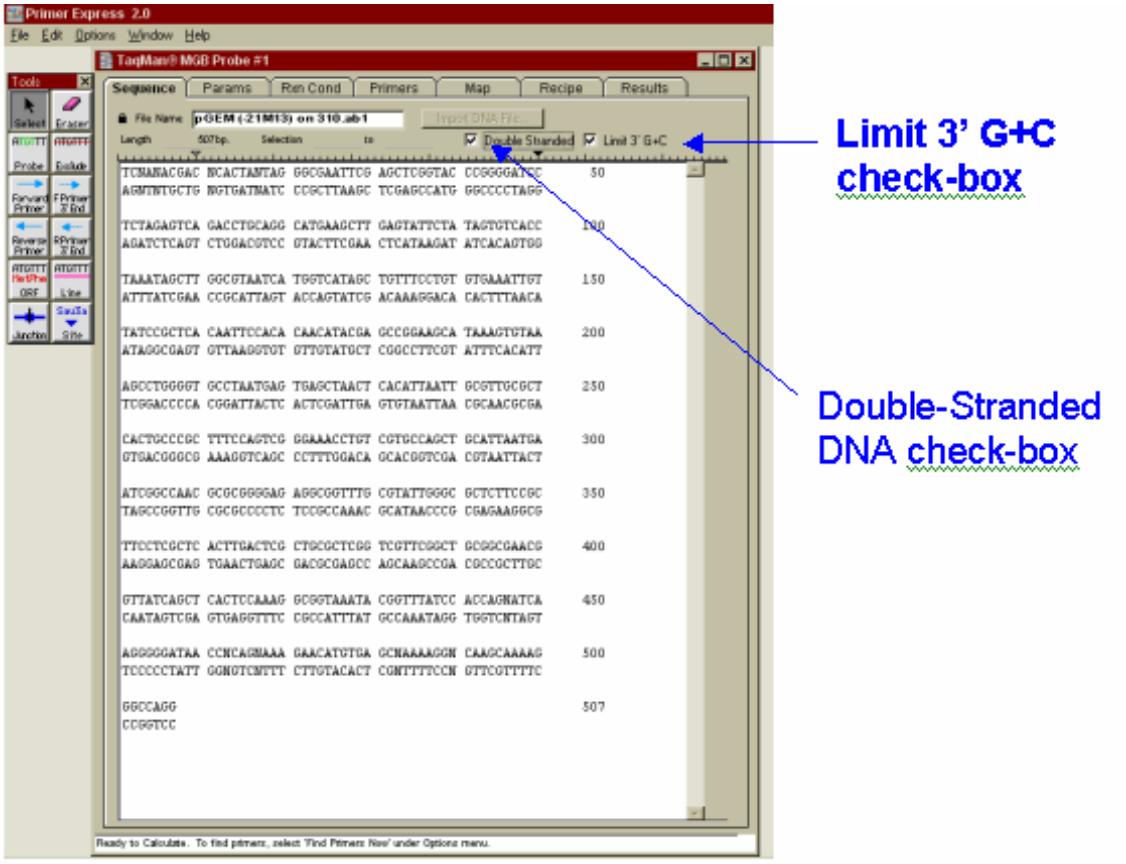
Starting the Design and Entering the Sequence

For an allelic discrimination assay, select the **TaqMan® MGB Probe and Primer Design** document from the **File/New** menu.

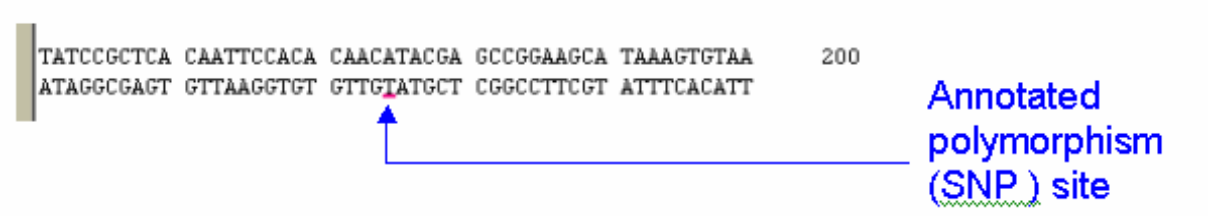


Enter the sequence for Allele 1 either by going to **File**/**Import**, using the **Import DNA File** button in the **Sequence** Tab, or by copying and pasting the sequence through the **Edit** menu.

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



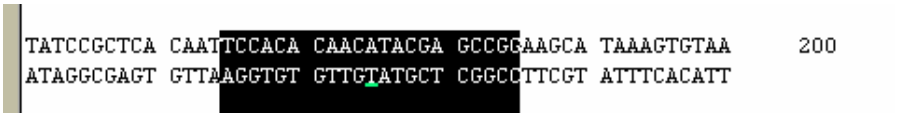
Label the polymorphism site within the sequence using the Line tool.



Open a second **TaqMan® MGB Probe and Primer Design** document. Following the steps above, enter the sequence for Allele 2, and label the polymorphism site.

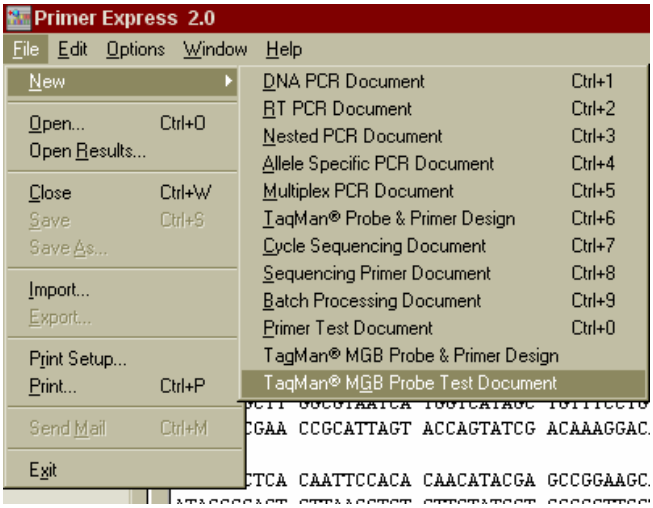
Designing the Allele 1 Probe

Click the **Sequence** tab. Using the mouse, click and drag to highlight the SNP and approximately 10 nucleotides in both the 5' and 3' directions.



From the **Edit** menu, select **Copy**.

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



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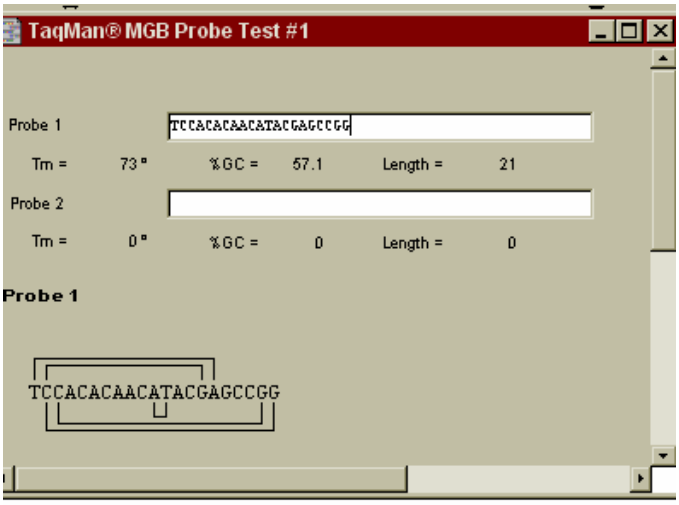
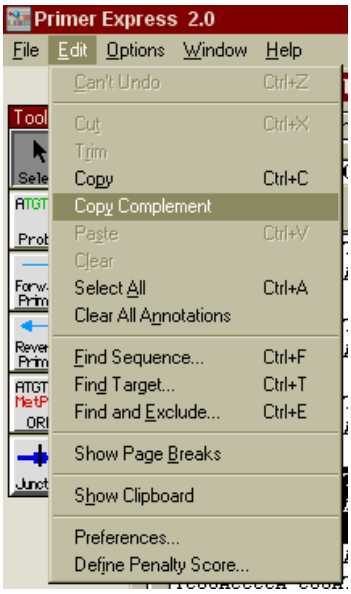
Click in the **Probe 1** text box and then select **Paste** from the **Edit** menu.



Return to the **Sequence** tab and select **Copy Complement** from the **Edit** menu (when designing probes for a SNP assay, it is important to test probes on both strands in order to select probes that satisfy all design guidelines).

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.



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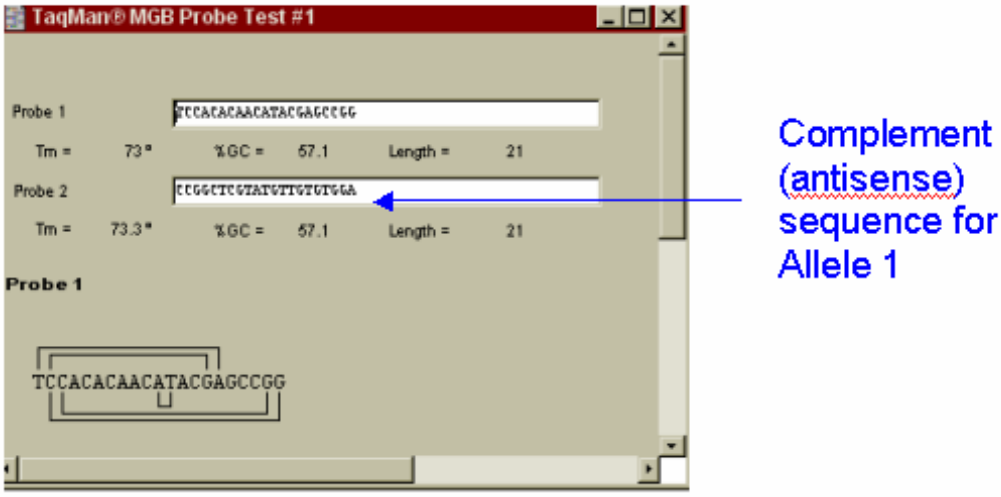
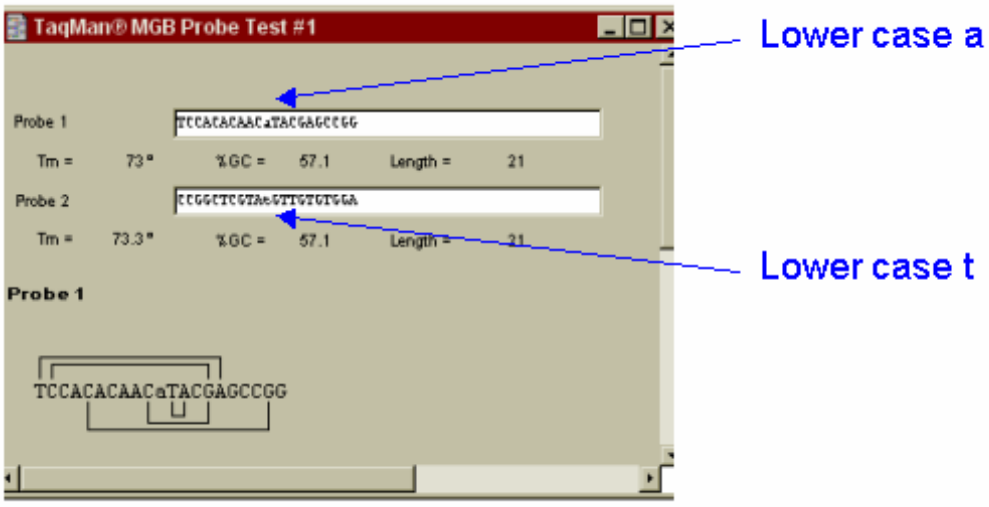
Return to the **TaqMan MGB® Probe Test Document**. Click in the **Probe 2** text box, and select **Paste** from the **Edit** menu.



For a visual aid, you can label the polymorphism site in each probe sequence. For the sense probe, select the appropriate base using the mouse to highlight it. Make sure caps lock is turned off, and then press the key corresponding to the letter of the polymorphic base. The polymorphism will now be displayed in lower case.

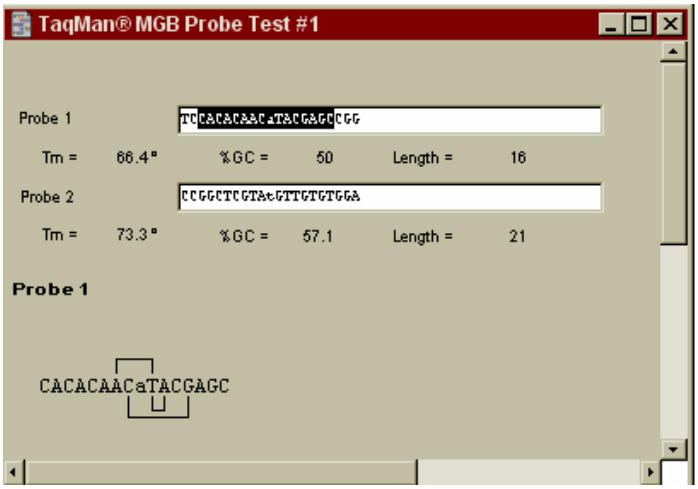
Repeat for the complement (antisense) sequence.



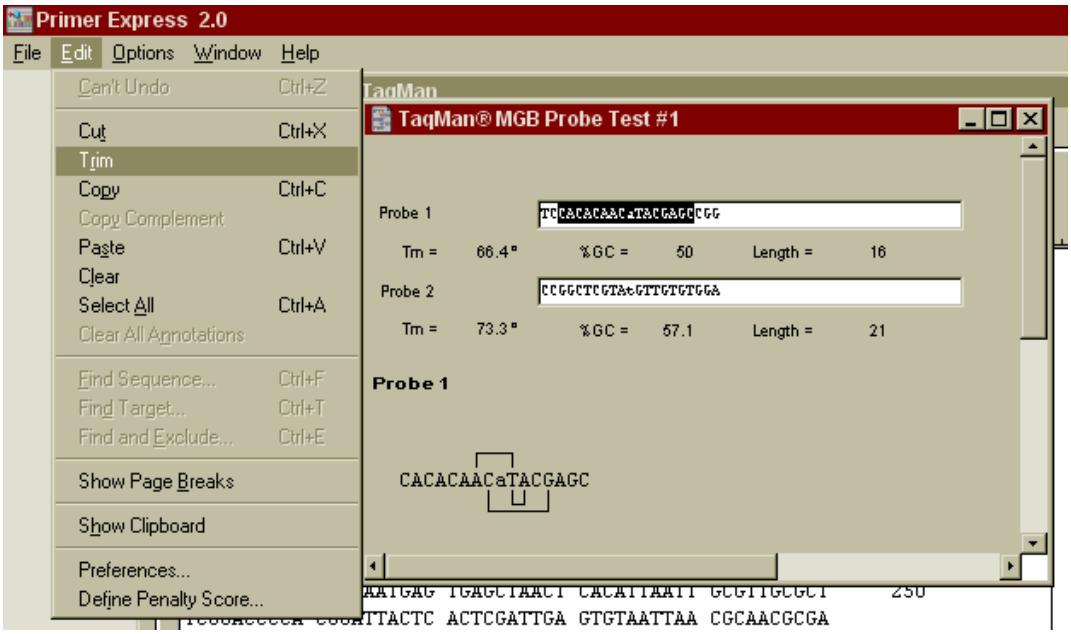
Check the melting temperature (Tm) of each potential allele 1 probe (sense and complement). TaqMan® MGB Probes for allelic discrimination should have a Primer Express Software-estimated T_m of 65-67°C. TaqMan® MGB probes for allelic discrimination should also be designed following the guidelines listed below.

1) Avoid probes with a guanine residue at the 5' end of the probe.
2) Select probes with a Primer Express® software-estimated T_m of 65-67°C.
3) Make TaqMan® MGB Probes as short as possible without being shorter than 13 nucleotides.
4) Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more should be avoided.
5) Both the Allele 1 and Allele 2 probes must anneal to the same strand.
6) Keep the Allele 1 and Allele 2 probe T_ms within one degree of each other.
7) Position the polymorphic site in the central third of the probe. The polymorphic site can be shifted toward the 3' end to meet the above guidelines; however, the site must be located more than 2 nucleotides upstream from the 3' terminus.

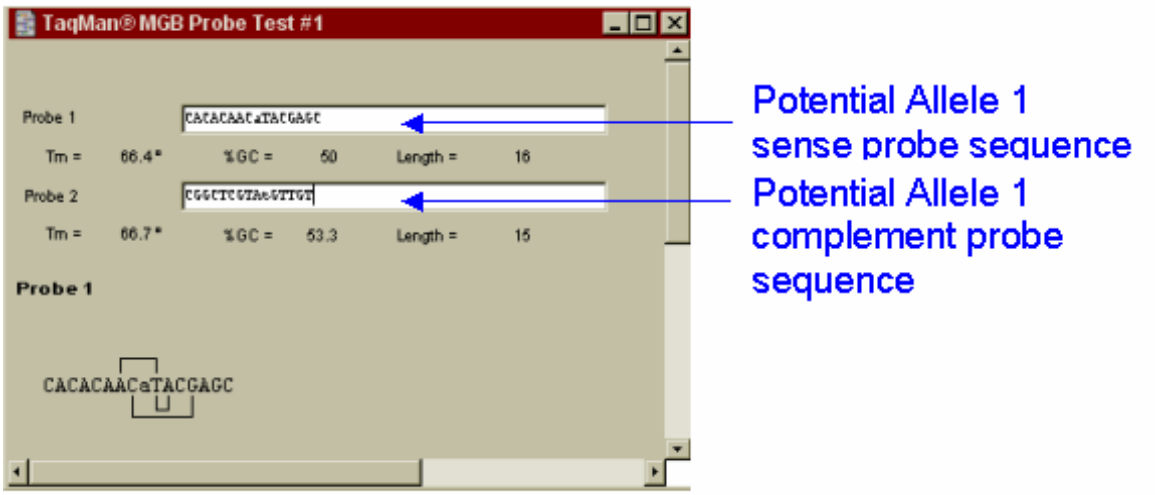
If the Tm of the potential allele 1 probe sense sequence is too high, use the mouse to highlight a portion of the sense sequence in the **TaqMan® MGB Probe Test Document**. The **TaqMan® MGB Probe Test Document** will now report the Tm of the highlighted portion of the sequence. Highlight potential sense probe sequences until you find a sequence that meets the design guidelines above.



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.



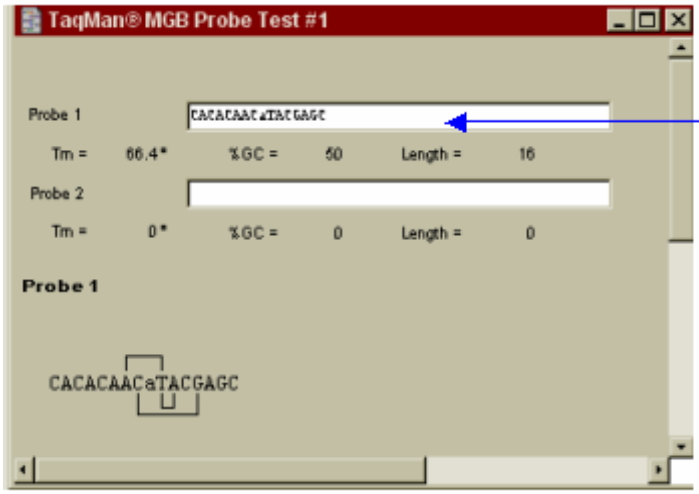
Repeat this process for the potential complement allele 1 probe sequence making sure that the TaqMan® MGB probe satisfies the additional parameters that are presented on page 6.



If it is not possible to select a sense-strand probe that satisfies all of the design guidelines (ex. if it is not possible to select a probe without a guanine residue at the 5' end), you will need to consider selecting the complement probe sequence. Please note that both the Allele 1 and Allele 2 probes must anneal to the same strand.

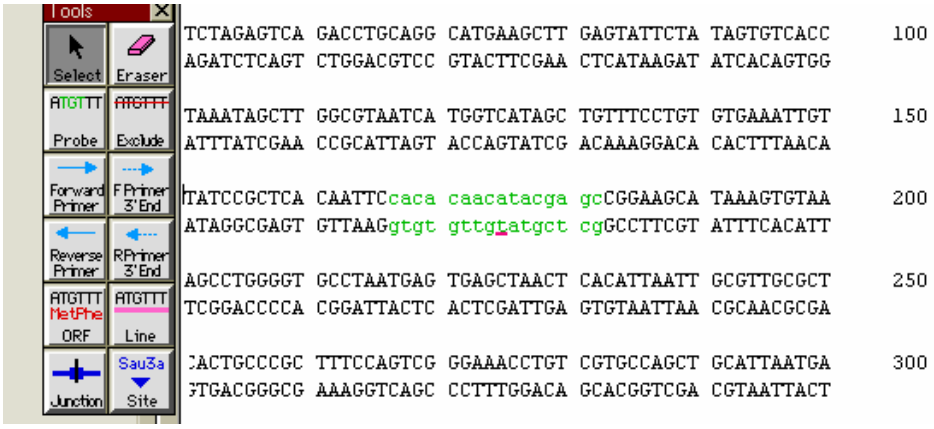
Copy and Paste the selected Allele 1 probe into a text document.

Double click on the unused probe sequence and press the Backspace key to remove it from the **TaqMan® MGB Probe Test Document**.



Note: Both the sense and complement probe sequences satisfied all design guidelines, and the sense probe sequence was picked arbitrarily.

Return to the **TaqMan® MGB Probe and Primer Design** document for Allele 1. Click the Probe tool, and highlight the final Allele 1 probe sequence.



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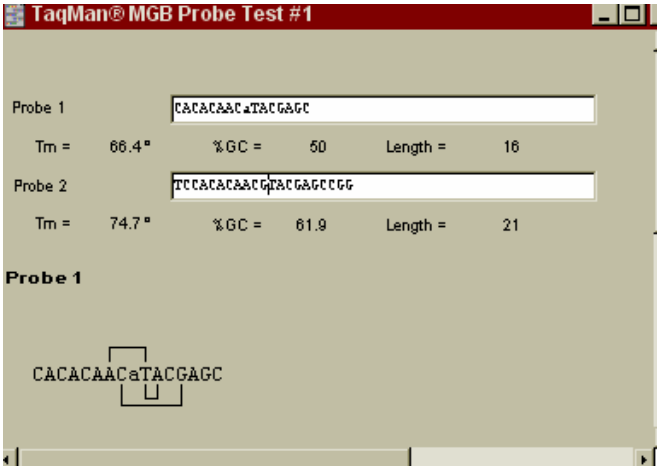
Designing the Allele 2 Probe

Click the **Sequence** tab in the **TaqMan® MGB Probe and Primer Design** document for Allele 2.

Using the mouse, click and drag to highlight the SNP and approximately 10 nucleotides in both the 5' and 3' directions.

NOTE: It is important that both probes in the assay come from the same strand (both sense or both complement). If the Allele 1 probe was from the sense strand, select **Copy** from the Edit menu. If the Allele 1 probe was from the complementary strand, select **Copy Complement** from the Edit menu.

Return to the **TaqMan® MGB Probe Test Document**, which still contains the Allele 1 probe sequence. Click in the empty box, and select Paste from the Edit menu.



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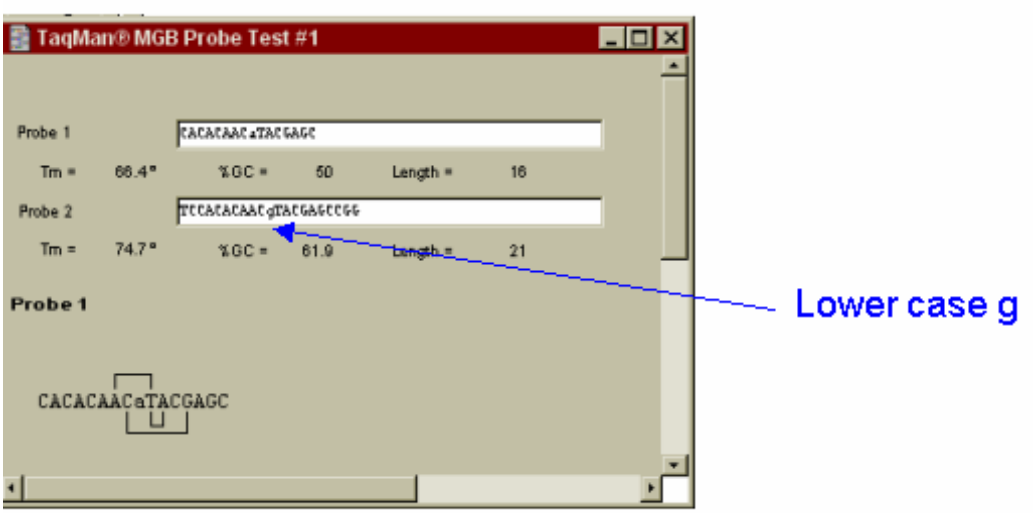
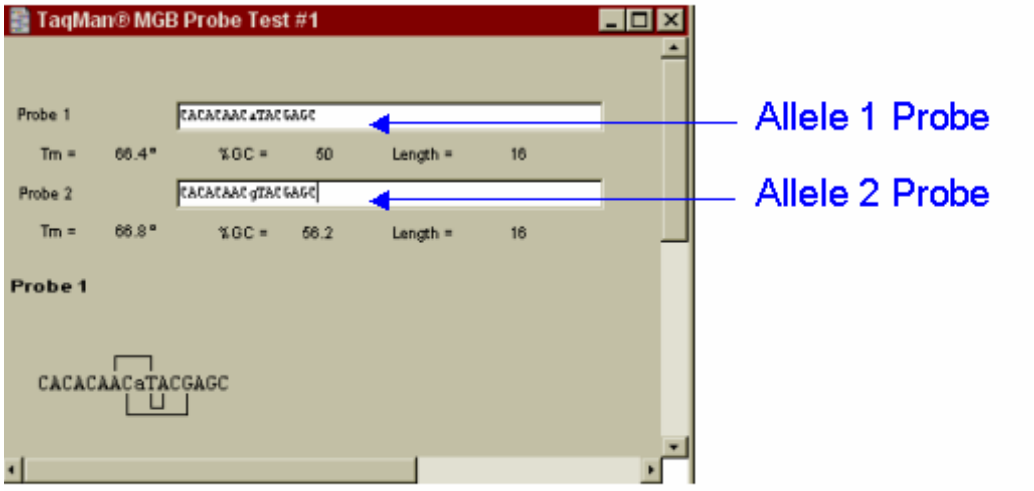
For a visual aid, label the polymorphic site as done for Allele 1. Select the appropriate base using the mouse to highlight it. Make sure caps lock is turned off, and then press the key corresponding to the letter of the polymorphic base. The polymorphism will now be displayed in lower case.



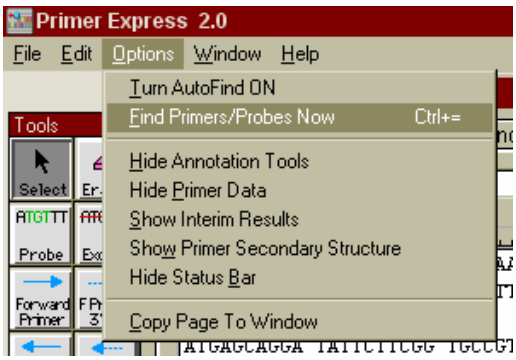
Highlight potential probe sequences until you identify an Allele 2 probe that meets the guidelines listed on page 6. With the desired probe sequence highlighted, select **Trim** from the **Edit** menu.



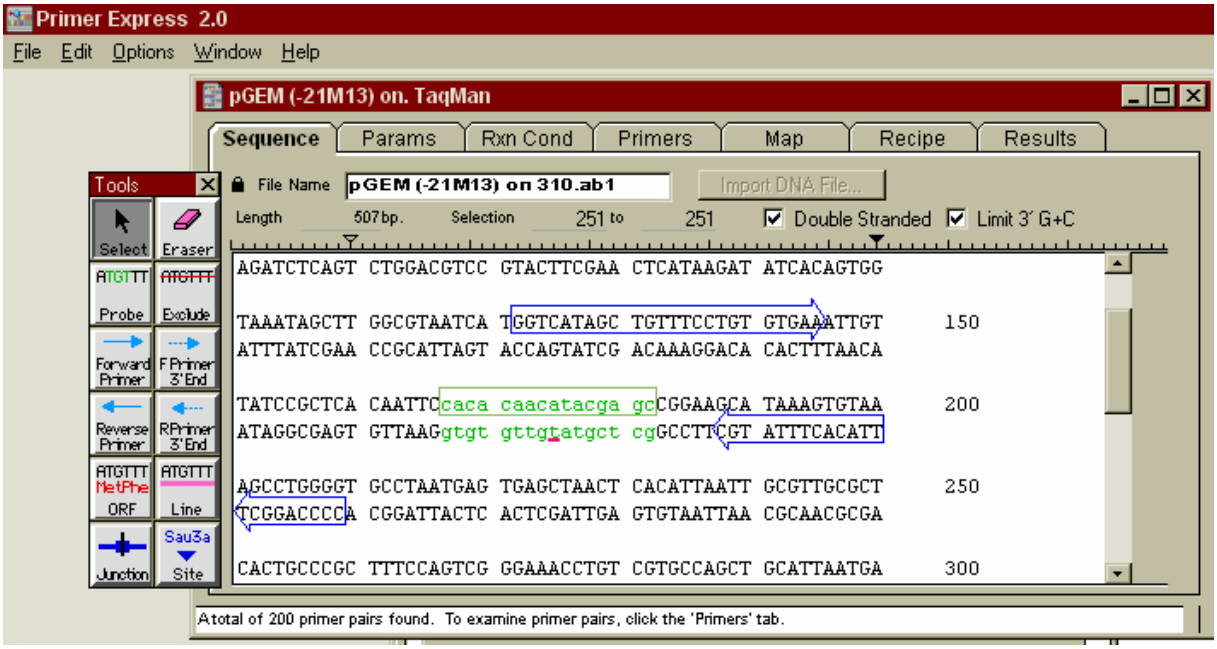
Copy and paste the final sequences for the Allele1 and Allele 2 probes into a text document for ordering.

Designing the Primers

Return to the **Sequence** Tab of the **TaqMan® MGB Probe and Primer Design** document for Allele 1. 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.**



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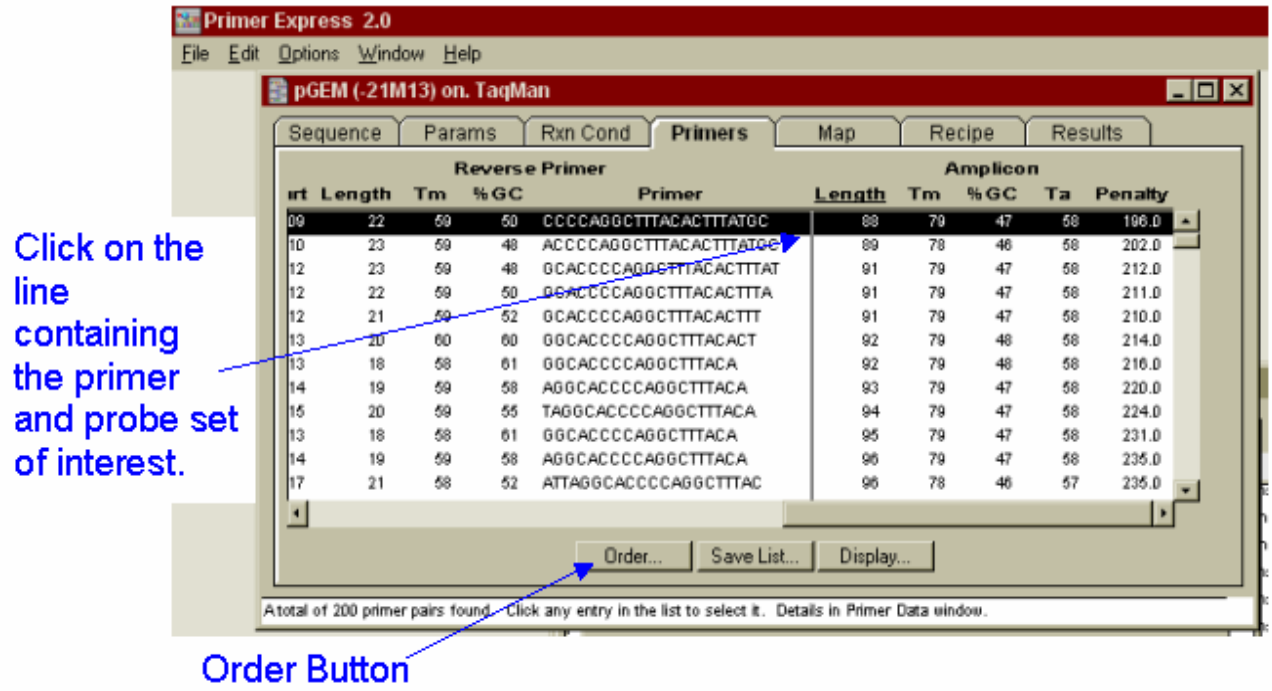
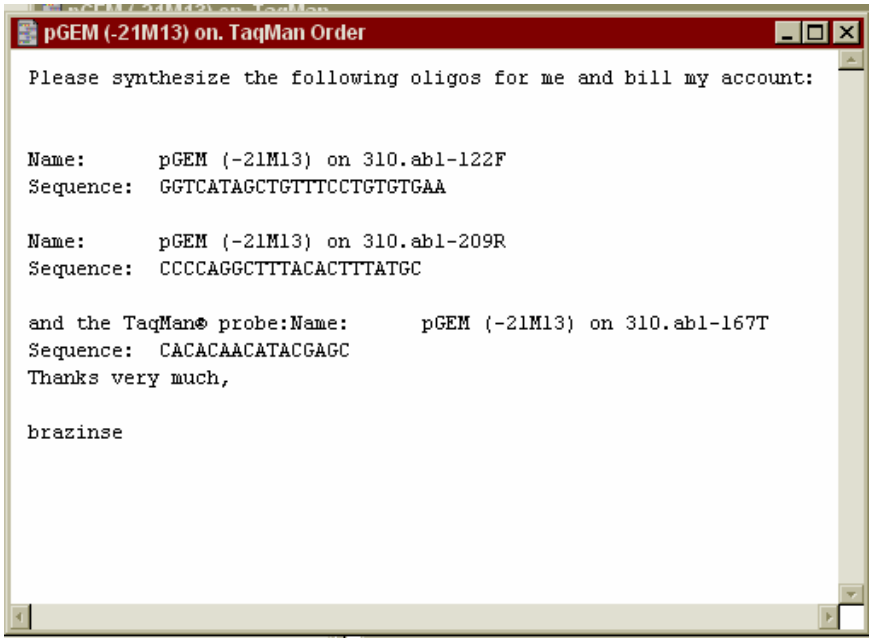


Select a primer pair from the list that will produce the shortest amplicon while satisfying all design guidelines.

Click on the line containing the chosen primer and probe set. Click on the **Order** button at the bottom of the **Primers** tab.



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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. Copy the forward primer sequence and paste the sequence into the text document that contains the probe sequences. Repeat for the reverse primer.

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.

If not done already, copy the final probe sequences from the **TaqMan® MGB Probe Test Document** and paste them into a text document for ordering.

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 into a text document containing the probe sequences 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 complementary 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.

If a TaqMan MGB® Probe manual design is unsuccessful

There may be cases where the manual design of a TaqMan® MGB Probe for allelic discrimination is unsuccessful. A possibility for sequence incompatibility with a TaqMan® MGB probe design would be a high G/C content immediately around the polymorphism. This high G/C content could cause a TaqMan® MGB probe Tm to be too high even with a probe length of 13 bases (The minimum length of a TaqMan® MGB probe is 13 bases). In a case such as this, one can try the design with a standard TaqMan TAMRA™ probe. Allelic Discrimination assays using TaqMan® TAMRA™ probes must be designed using the **TaqMan® Probe and Primer Design** document instead of the **TaqMan® MGB Probe and Primer Design**. Keep in mind that the primer/probe design guidelines are similar with the exception that TaqMan® TAMRA™ probes should be designed as having more Cs than Gs. And, the maximum recommended length of TaqMan® TAMRA™ probes is 30 bases. The Tms of putative TaqMan® TAMRA™ probe sequences must be evaluated in **Primer Test Document**.

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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