

BIO-RAD LABORATORIES

Probe/Primer Design Session: Useful Websites

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Primer design:

- Targets an amplicon length of 75 to 150 bp
- 50 to 60% GC content
- Limit secondary structure
- Limit stretch of G or C's longer than 3 bases
- No stable interaction between forward and reverse primers (primer/dimer pairs)
- Place C's and G's on ends of primers, but no more than 2 in the last 5 bases on 3' end
- Melting Temperature (T_m) above 50 °C
- Verify specificity

Design probe by hand (eye):

1. Scan gene of interest for g/c rich regions. They will have higher annealing temperatures than the average sequence and we want our probe to anneal about 8-10 degrees before our primers anneal.
2. Find a likely probe sequence (has T_m of approximately 68-70 degrees) then go to the DNA folding site to see what kind of secondary structure this probe has.
3. Please also be aware of what kind of probe you are designing.

TaqMan probes should have a minimum of secondary structure to work well. You should also consult the PE site for guidelines (no G near the reporter and length). These are not as well quenched when the probe gets long.

Molecular Beacon probes have secondary structure in the stem sequences. You should calculate the probe annealing T_m without the stem sequence, then add the sequence when you go to the folding site.

4. After you have an appropriate probe sequence, design primers per the above rules that incorporate the probe

Useful bookmarks for probe and primer design:

<http://www.operon.com/oligos/toolkit.php>

Use free online T_m calculators to see what the T_m for primer and probe sequences are. We

use the Operon calculator, as it also has a good tool to check possible primer-dimerization sequences. Reach it from www.operon.com then select DNA Synthesis, then select Oligo Toolkit.

http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

This is a link to Primer3 software. It is software that allows for primer design and also helps picking an internal oligo sequence to these primers (a probe sequence). Like most primer design algorithms, it has the disadvantage of not taking into account secondary structure issues that are paramount in primer/probe design for real-time PCR. With that caveat in mind, it is a good place to start the design process, if you are not inclined to do it by eye (i.e. scanning the sequence yourself). Then you can check your sequences at the folding site (described below).

<http://www.ncbi.nlm.nih.gov/BLAST/>

This is the site for the **B**asic **L**ocal **A**lignment **S**earch **T**ool from the National Center for Biotechnology Information. Use this site for checking specificity of probe and primer sequences.

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<http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi>

1. Give a name to your sequence – example: bactin1
2. Copy the sequence or retype it into the big text box for sequence
3. Scroll down the page to the part marked "ionic conditions": change the units to mM. Set Na^{++} to 50 and Mg^{++} to 3
4. Scroll down further to enter your e-mail address - it does not e-mail to you but it will not proceed without it.
5. Click on the button marked Fold DNA - it has a smiley face next to it.
6. Now you'll get a list of structures - ideally you see only one. Probes with lots of possible secondary structures are bad choices.
7. You also get a free energy value (ΔG). The larger the negative value of the free energy, generally the better. Positive free energy values are not a good sign.
8. You can look at the .gif diagram of the structure. If there is a lot of self-complementarity then you can assume that the probe won't open up and anneal when you want it to, therefore, it's probably not a good sequence.

The best indicator is the T_m that is presented with the .gif which represents the T_m for melting of the secondary structure. This should be 2-5 degrees below target T_m (calculated at the Operon site) yet still 2 - 5 degrees above any T_m for the primers. Generally you go through 3 - 10 different sequences before finding one that looks best.

Please also be aware of what kind of probe you are designing.

TaqMan probes should have a minimum of secondary structure to work well. You should also consult the PE site for guidelines - no G near the fluorophore and length of probe. These are not as well quenched when the probe gets long.

Molecular Beacon probes have secondary structure in the stem sequences. You should calculate the probe annealing T_m without the stem sequence, then add the sequence when you go to the folding site.