Considerations when adopting published sequences for your own qPCR assay

Increase sensitivity by converting probe sequences to double-quenched ZEN™ or TAO™ probes

Lab tip: Published papers that use qPCR applications are a resource of vetted assay sequences. These can be co-opted and converted to more sensitive, double-quenched probes for use in your own experiments. Before ordering, though, go through this checklist to ensure that these sequences will work well in your assays, and consider up-to-date PrimeTime Predesigned qPCR Assays with guaranteed efficiencies of >90%.

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Researchers often find probe and primer sequences for a specific qPCR assay in the literature, which they want to use for their own experiments. And why not? These sequences have already been vetted by other scientists and used successfully to generate publishable data.

Check published sequences against current sequence databases

However, before you order assays published in the literature, consider whether they were based on outdated sequence information. With the increasing amount of cataloged sequencing data, there has been a concomitant increase in the number of identified single nucleotide polymorphisms (SNPs) and updated transcript structure and annotation. While the frequency of SNP occurrence may make them unrealistic to avoid when choosing PCR primer and probe sequences (the human genome contains a SNP approximately once every 22 bases), it is important to take their position into consideration. See *Considering SNPs when designing PCR and qPCR assays (/pages/decoded/decoded-articles/pcr-qpcr/decoded/2017/01/31/considering-snps-when-designing-pcr-and-qpcr-assays)*, for data on SNP frequency, and a discussion of the impact SNPs can have on PCR and qPCR assay results. The article also provides recommendations for managing SNP impact on your assay results.

Convert literature-derived probe sequences to more sensitive, double-quenched probes

Converting literature-derived probe sequences to double-quenched probes that contain an internal quencher has become popular over the past few years, due to the lower background fluorescence, increased signal, and reduced crosstalk double-quenched probes afford. This adaptation is easily done. IDT provides 2 types of internal quenchers, ZEN and TAO Internal Quenchers, that are compatible with a wide range of 5′ fluorescent dyes and 3′ quenchers (see Figure 1).

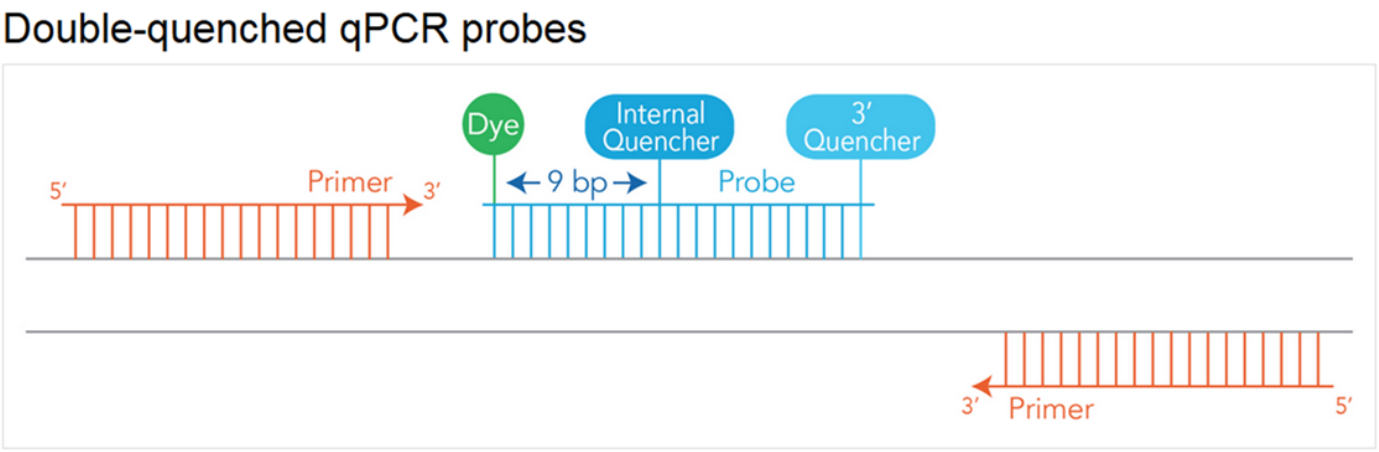

Figure 1. Positioning of internal quencher within a probe sequence. While traditional probes have approximately 20–30 bases between the fluorophore and the quencher, the internal ZEN or TAO quencher decreases that length to only 9 bases. This shortened distance, particularly when combined with the traditional 3′ end quencher, leads to more thorough quenching with much lower background and enables the use of much longer probes for designing in AT-rich target regions. In addition to the significantly decreased background, the Double-Quenched Probes also have consistently reduced Cq values and improved precision when compared to traditional probes. Use of the Double-Quenched Probes can allow users to experience both increased sensitivity and precision in their qPCR experiments.

Evaluate your probe sequence

Adding ZEN or TAO Internal Quenchers into standard hydrolysis probe sequences with 5' fluorescent dyes and 3' quenchers is straightforward and will not appreciably affect probe T_m. However, note that probes from the literature containing MGB (or other T_m boosting modifiers) will have a reduced T_m when ordered without these modifications. Therefore, before ordering, it is a good idea to assess whether the published probe sequence has the ideal T_m to function optimally in your assays.

Use these steps to evaluate the published probe sequence before you order:

1. **Check the T_m of the probe sequence**. If the probe T_m is not provided in the literature reference, use our free OligoAnalyzer Tool (/calc/analyzer) to calculate it. We recommend that the probe T_m be at least 4°C higher than primer T_m. Note that MGB (or other T_m boosting modified) probes from the literature will have a reduced T_m when ordered without these modifications.
2. **Determine whether you can modify your probe sequence**. If the probe vs primer T_ms do not meet the above criteria, you may need to either change the position of your assay or modify the probe sequence. Consider a predesigned assay for that exon location as the best primer probe combination is chosen (see the below sidebar, ***An alternative to published sequences***). If the location of your probe cannot be moved, here are a few ways to modify your assay so that probe and primers will work optimally together during PCR.
    - Change the length or position of your probe and/or primers so that primer and probe T_ms meet the criteria in step 1 above.
    - Increase the T_m of a short probe by adding bases onto the 5′ or 3′ end. Use the OligoAnalyzer Tool (/calc/analyzer) to assess T_m, and to rule out dimer formation between probe and primers, as well as secondary structure formation resulting from presence of the added bases.
    - If you do not want to modify your probe sequence, another way to increase probe T_m is to replace 2–4 bases spaced throughout the sequence with LNA bases. LNA (locked nucleic acid) bases are extremely stable and will raise probe T_m. Our Application Support Group can provide guidelines on where in the probe sequence to integrate LNA bases. Contact them at applicationsupport@idtdna.com (mailto:applicationsupport@idtdna.com). Note that LNA probes are not available as double-quenched probes; order them as single-quenched probes here (/pages/products/qpcr-and-pcr/custom-probes/lna-primetime-probes).

Order the published probe sequence as a double-quenched probe

Now that you have confirmed your primer and probe sequences are up to date, and determined that the T_m of the probe sequence is optimal for use in your assay, convert a probe of your own design or one from the literature to one containing a ZEN or TAO Internal Quencher. Go to our qPCR probes design tool (/site/order/qpcr/primetimeprobes) (Figure 2), and follow these steps to order the double-quenched version of the probe. To enter complete assays of both primer and probe sequences, use our qPCR assay design tool (/site/order/qpcr/assayentry) and follow these same steps):

Step 1. Provide a name for this sequence entry. (See the Step 1 blue arrow in Figure 2.)

Step 2. Select a synthesis scale. (See the Step 2 blue arrow in Figure 2.)

Step 3. Enter your sequence (e.g., copy and paste), without modifications. (See the Step 3 blue arrow in Figure 2.)

Step 4. Choose your 5' dye + 3' quencher combination from the dropdown list. (See the Step 4 blue arrow in Figure 2.) The dropdown list of modifications is shown in Figure 3. Note that with the 5′ dye choice, a compatible ZEN or TAO internal quencher will be automatically integrated into the appropriate position in your probe sequence.

Note that LNA probes are not available as double-quenched probes; order them as single-quenched probes here (/pages/products/qpcr-and-pcr/custom-probes/lna-primetime-probes).

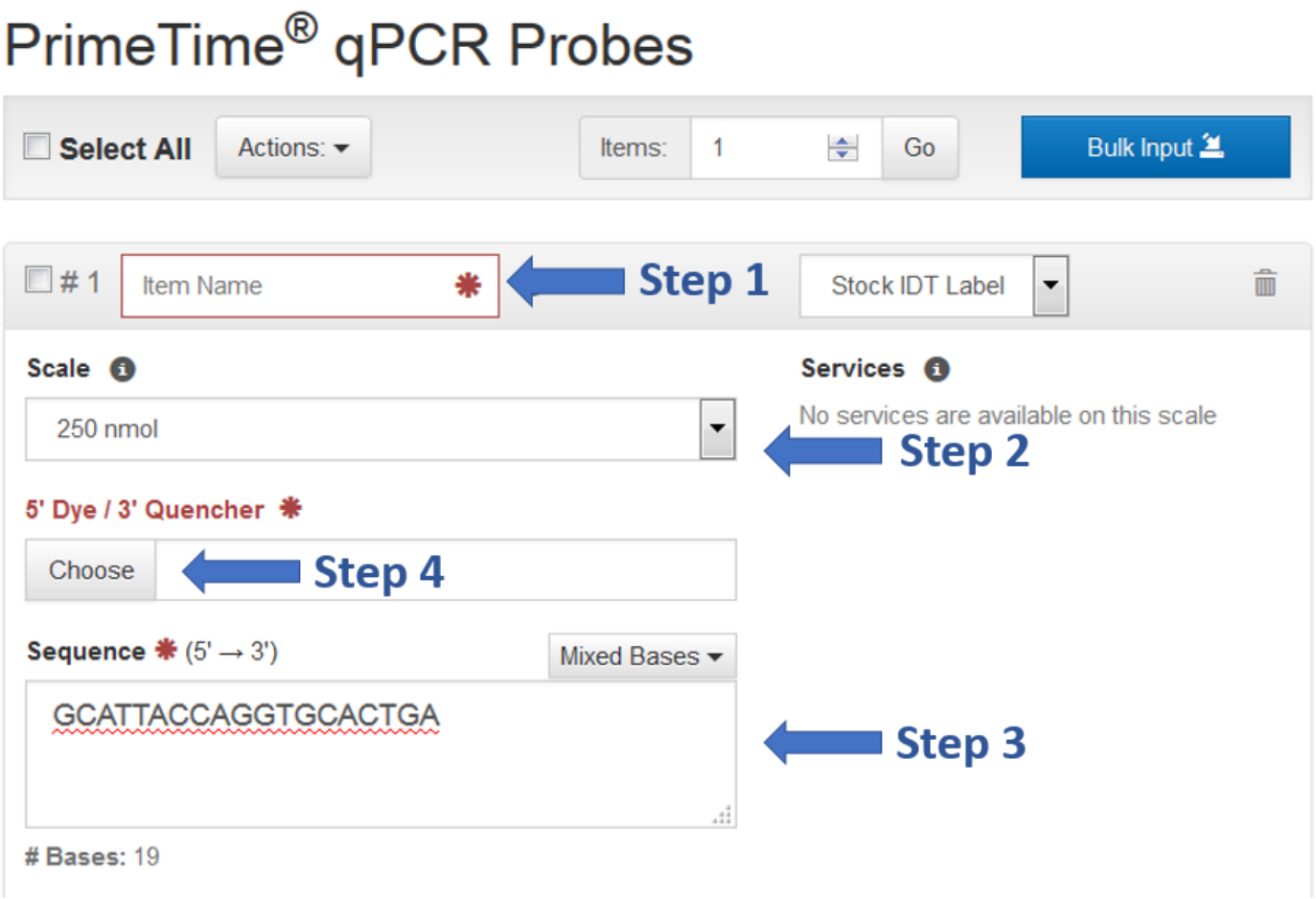

Figure 2. Order probes through the manual entry form. Access this form here.

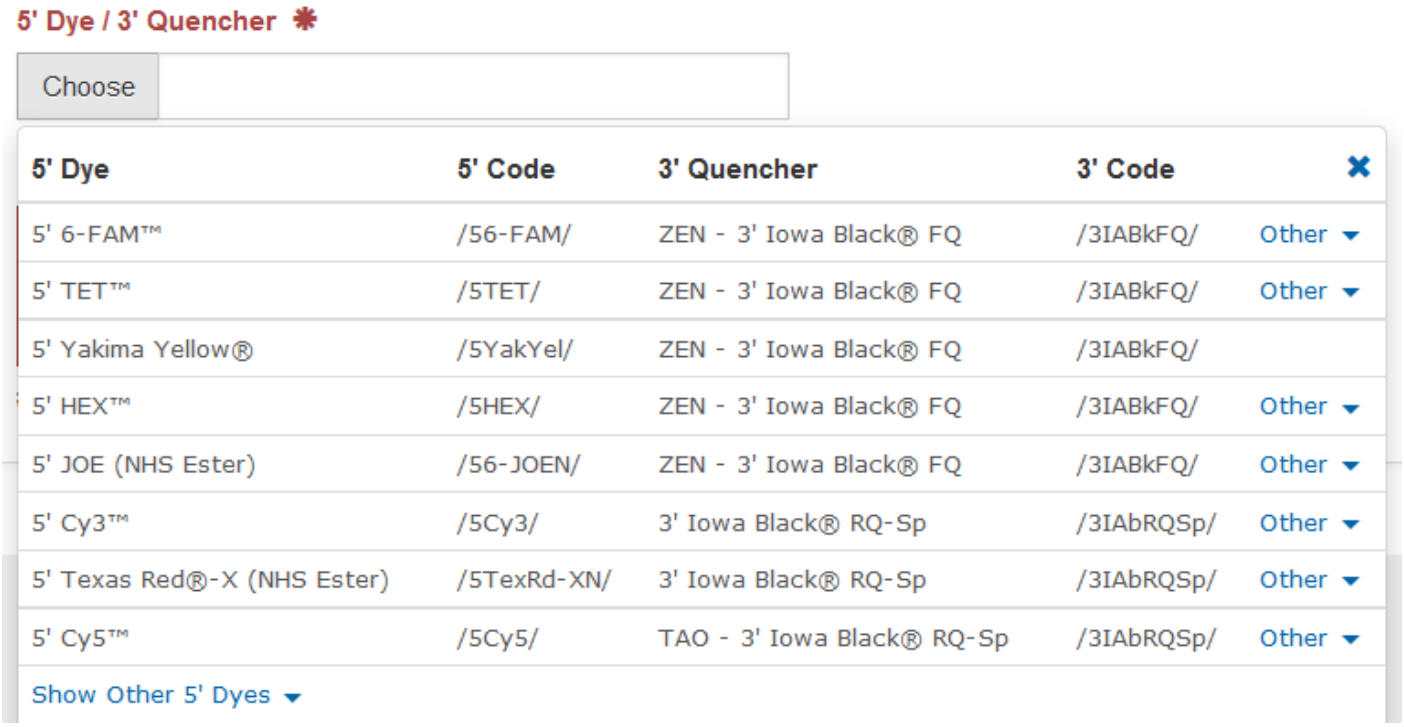

Figure 3. ZEN and TAO Internal Quenchers are compatible with a range of 5′ fluorescent dyes.

Checklist for adopting published primer and probe sequences for qPCR assays

- Check primer and probe sequences against current sequence databases
- Evaluate T_m of probe
- Modify probe to adjust T_m, if needed
- Add ZEN or TAO Internal Quencher to probe

An alternative to published sequences

A quick search of the IDT PrimeTime® Predesigned qPCR Assay database (/site/order/qpcr/predesignedassay) provides assays that cover the human, rat, and mouse transcriptomes that are guaranteed to perform with PCR efficiencies between 90–110% and R^2 >0.99. The database design engine uses complete target sequence information that is updated regularly.

Further questions about probe design and use of double-quenched probes?

You can always contact our Scientific Applications Specialists for help in designing your probes and assays. Reach them at applicationsupport@idtdna.com (mailto:applicationsupport@idtdna.com).

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