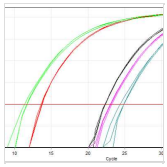

Version 2

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qPCR Primer Design V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.br4hm8t6

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ABSTRACT

Protocol for designing qPCR primers for analysis of transcript abundance using SYBR Green chemistry.

There are many good guides about how to do this online such as this, from which the following protocol is derived.

Reference for mfold/UNAfold
https://doi.org/*10.1093/nar/gkg595*

DOI

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PROTOCOL CITATION

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GUIDELINES

Preferred properties of qPCR primers:
(Note: These guidelines represent the ideal situation which may not always be achievable)

- each primer should be between 15–30 bp in length
- theoretical Tm of the two primers should be within 2°C of each other.
- Try to avoid G/C clamps at the 3´ ends of the primers to prevent these oligos from folding on themselves or annealing nonspecifically.
- The five bases at the 5´ terminal end generally should contain no more than two guanines and cytosines, although it is acceptable to have three in the final 5 bases if no two are adjacent.
- If possible have dA nucleotides near the 3' end to allow for UNG activity to prevent primer-dimers
- Since thymidine tends to mis- prime more readily than the other bases, a 3´ terminal T should be avoided if possible.
- The 5´ end of the primers should not contain an inverted repeat sequence that would allow it to fold on itself.

Identify target sequence

1 Decide on a gene of interest, download the transcript sequence in .gb format and upload to a new folder on Benchling for analysis.

If analyzing an endogenous (as compared to transgene), gene sequences can be obtained from public databases such as NCBI. For many plant genes, my preference is to use Phytozome, but you should choose whichever database has the most recent and accurate transcriptome models.

One thing to be aware of is that genes can have multiple splice forms. You may, or may not wish to perform splice form specific transcript quantification. In most instances, you are likely interested in "gene expression", or the sum of transcripts originating from a gene. In this case, perform an alignment of known splice form sequences using clustal omega and retain only the parts which are common to all.

Use Primer-BLAST to test for specificity

2 Navigate to NCBI Primer-BLAST in your browser

3 Adjust parameters to suit qPCR assays and record the search conditions in your Lab book.

- Amplicon size to 70-150 bp
- Minimum of 4 mismatches
- RefSeq mRNA database
- Under the organism tab, enter the name of the species of interest e.g. Solanum tuberosum

You can also include information about intron-exon boundaries, however, this is often too stringent and will yield no positive results.

4 Paste in the transcript sequence against which you want to design primers (from step 1) and click "Get Primers".

5 From the results choose primers which have a single perfect match using BLAST (this should be against record the following details in a table for the purpose of reporting according to the format above:

- Primer forward sequence
- Primer reverse sequence

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- Amplicon size

Check primer properties

6 Open IDT's OligoAnalyzer Tool in a new tab for QC analysis of primer properties.

7 Change parameter settings to "qPCR" on the dropdown menu on the right and adjust concentration settings:
[Oligo] = 0.33 nM,
[Na+] = 50 mM,
[Mg^{2+}] = 3 mM,
[dNTP] = 1.2 mM

Note these parameter settings are adapted for use with SSoAdvanced Universal SYBR Green Supermix. The precise parameters will vary depending on the buffer components in the PCR reaction mix being used.

8 Enter the first primer sequence from Primer-BLAST and run

9 Analyze hairpin formation and record the highest Tm. Record the results in the table.

Tm value should be lower than the annealing temperature of the primers (e.g. 60 oC if that is what the assay was designed at). Otherwise, primer sequence may be prone to forming hairpin structures under assay conditions resulting in inaccurate results.

10 Analyze self-dimer free energy and record thelowest dG in a table. Record the results in the table.

dG value should be > -9. Anything lower than this fails the quality control check as the sequence has too high a chance of forming primer-dimer amplification products leading to inaccurate results.

11 Analyze heterodimer free energy by entering the reverse primer as a secondary sequence. Record the results in the table.

dG value should be > -9. Anything lower than this fails the quality control check as the sequence has too high a chance of forming primer-dimer amplification products leading to inaccurate results.

12 If the forward primer passes the QC checks in steps 9-11, repeat for the reverse primer.

In the case that the primer sequence fails QC, return to the Primer-BLAST results and select another pair of primers

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for analysis. It is common to need to do this, in most instances, it should be possible to find suitable primers.

Check amplicon properties

13 Open the software UNAfold in a new tab on your browser to test for secondary structure formation by the amplicon sequence to be amplified by primers.

Ideally, your primers should pass this QC as well, but in the event, it is not possible to find a pair that passes both the primer and amplicon QC it is worth progressing to test experimentally.

14 Set UNAfold parameters for analysis of assay amplicon:
- Chose to fold DNA
- Set the concentration of Mg++ to 3 mM

15 Run UNAfold

16 Inspect the predicted structures formed by the amplicon.

The highest Tm for any of the predicted structures should be less than the annealing temperature of the primers (e.g. >60 oC if that is what they were designed at).

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