

MAR 08, 2023



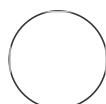
qPCR Primer Design

Forked from [qPCR Primer Design](#)

Steven J Burgess¹

¹University of Illinois at Urbana-Champaign

UIUC Long Lab



Lynn Doran

Realizing Increased Photosynthetic Efficiency (RIPE)

OPEN ACCESS

Protocol Citation: Steven J Burgess 2023. qPCR Primer Design. [protocols.io](https://protocols.io/view/qpcr-primer-design-cqrkv4w) <https://protocols.io/view/qpcr-primer-design-cqrkv4w>

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working
We use this protocol and it's working

Created: Mar 08, 2023

Last Modified: Mar 08, 2023

PROTOCOL integer ID:
78348

Keywords: qPCR, primer design

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>

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 T_m 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.

Note

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*

Note

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

- 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,
[Mg²⁺] = 3 mM,
[dNTP] = 1.2 mM

Note

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 T_m. Record the results in the table.

Note

T_m value should be lower than the annealing temperature of the primers (e.g. 60 °C 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 the lowest dG in a table. Record the results in the table.

Note

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.

Note

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.

Note

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

Note

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.

Note

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