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Primer design protocol for qPCR primers when testing the natural variation in expression

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"Natural variation in plasticity of roots in salt", Magdalena Julkowska, Iko Tamar Koevoets, Selena Mol, Huub CJ Hoefsloot, Richard Feron, MarkTester, Joost J.B. Keurentjes, Arthur Korte, Michel A Haring, Gert-Jan de Boer, Christa Testerink, The Plant Cell Nov 2017, tpc.00680.2016; DOI: 10.1105/tpc.16.00680

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

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Abstract

How to design primers for qPCR when testing the expression in different Arabidopsis accessions. A simple protocol - suggestions welcome.

Go to 1001 genome SALK Genome DB and enter the AtXgABCDE identifier @ Search http://signal.salk.edu/atg1001/3.0/gebrowser.php

1 If you have multiple splice variants of your gene check @ TAIR which gene models were observed (Gene specific website under “protein data”) and use most commonly observed splice variant observed for your primer designing

At 1001 genome SALK Genome DB choose “Col-0.MPI” from the “tracks” menu and at “Display” menu change “View:” from “Graphic” to “Text”.

2

Make sure you have your 5′ UTR (the end of your gene) in the view window and that the correct splice variant is purple.

3

Copy the Col-0 sequence to word file

4

Remove introns, indicated with lower case letters if you clicked on the gene

5 Make sure to write down the positions of nucleotides flanking the intron in your CDS

Paste the sequence in primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/)

6

At “General Settings” in option “Load and Save – Please select special setting here:” choose qPCR and click on “Activate settings”.

7 At General Settings -
“Primer Tm” = 58 / 60 / 58 ;
“max Tm diff.” = 2 ;
“Primer GC%” = 40 / 50 / 60 ;

At Advanced Settings -
“Max Poly-X” = 4 ;
“Max Self Complementarity” = 4;
“Max 3′ Self Complementarity” = 2;
“GC clamp” = 1,
“Product Size' = 70-100-150

Paste your CDS sequence in “Main”, click “Pick Primers” (green button – cannot miss it) and pick the best primers (usually highlighted on top).

8 Preferentially pick primers of which the product is spanning the intron sequence (the location of which you wrote down at step 5), but that the primer sequences themselves are NOT overlapping the intron/exon boundary.

Preferentially chose primers that are closest to 5′ end of your CDS sequence, as they are going to be closest to polyA tail. If the primers are designed too far into 3′ end the chances are that you are not going to get any signal due to poor cDNA synthesis.

FYI – if you go back & forth in one window at Primer3PLUS and design multiple primers for multiple target genes the settings will be saved!

Check for primer specificity by BLASTING the primers against TAIR10 Transcripts (https://www.arabidopsis.org/Blast/).

9 If significant alignment is found with other than your target gene using BOTH primers then go back to step 8 and pick other primer pair

Check for the polymorphisms in the annealing primer sites by going back to 1001 genomes SALK Genome DB and selecting the sequences of all the accessions that are used for expression experiment (+ sequenced).

10 The sequences of the accessions are constantly added.

Check whether your primer is found in all selected accessions by clicking “cmd” + “F” and pasting your primer sequence in the “Search” window.

11 As soon as you enter the sequence the search function will tell you the number of the occurrence of the sequence in the window you are currently in ⇒ make sure that the number is matching the number of selected accession sequenced.

Make sure to reverse-compliment the rev. primers before searching the sequence (http://www.bioinformatics.org/sms/rev_comp.html).

If the primer annealing site is not found in all accessions selected go back to step 8 and select new primers.