Sep 03, 2024

qPCR to measure mRNA expression in S. rosetta

DOI
dx.doi.org/10.17504/protocols.io.n2bvjnjongk5/v1

Fredrick leon[1], David Booth[2]

[1]UCSF; [2]University of California, San Francisco

BioBooth
Tech. support phone: **+1 (719) 429-6547** email: **dbooth@berkeley.edu**

Fredrick leon
UCSF

DOI: dx.doi.org/10.17504/protocols.io.n2bvjnjongk5/v1

Protocol Citation: Fredrick leon, David Booth 2024. qPCR to measure mRNA expression in S. rosetta. **protocols.io https://dx.doi.org/10.17504/protocols.io.n2bvjnjongk5/v1**

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

Created: August 06, 2024

Last Modified: September 03, 2024

Protocol Integer ID: 104839

Abstract

This protocol tailors common qPCR methods for quantifying transcript abundance in *S. rosetta.* The protocol covers primer design, synthesis of ssDNA standards, and assembling the qPCR reaction. It builds upon the preferential lysis and cDNA synthesis protocols and links to relevant guides and calculators for ease of use.

Protocol materials

Qubit ssDNA kit **Thermo Fisher Scientific** **Catalog #**Q10212	Step 6
Lambda Exonuclease - 1,000 units **New England Biolabs** **Catalog #**M0262S	Step 5
Luna Universal qPCR Master Mix - 2,500 rxns **New England Biolabs** **Catalog #**M3003E	In 2 steps
Magnetic mRNA Isolation Kit - 25 isolations **New England Biolabs** **Catalog #**S1550S	Step 7.3

Primer design

1 Search for your gene of interest here
https://protists.ensembl.org/Salpingoeca_rosetta_gca_000188695/Info/Index
After opening the gene page, click the "Sequence" tab on the left-hand column to display the gene's exons. From there click download sequence and select "cDNA". Reference the exon sequences on this page as you design primers or manually annotate exons in the cDNA file downloaded.

2 To begin qPCR primer design, primers must span exon-exon junctions, which should be previously annotated from the step above. A good source on qPCR primer design can be found here https://www.bio-rad.com/en-us/applications-technologies/qpcr-assay-design-optimization?ID=LUSO7RIVK
Generally, select 19-20 bp spanning an exon junction, with a GC content of 50-60% and a tm of 50-65°C. The final amplicon should be 75-200 bp, so pick your second primer accordingly, which should also span an exon junction.

> **Note**
>
> If your qPCR is to test the effects of a KO mutation, you'll want the entirety of the amplicon to be downstream of the PTS insertion because transcription will likely read through until the PTS. If the amplicon is before the PTS insert, the qPCR will likely result in no or less of a decrease in expression.

> **Note**
>
> qPCR primers must span exon-exon junctions to avoid genomic DNA amplification.

Primer validation

3 **Amplification validation:**
Select your qPCR reaction mix (we used NEB luna qPCR master mix), and use cDNA (previously synthesized, protocols for **preferential lysis**, **RNA extraction and cDNA synthesis**) as template, test amplification in the qPCR master mix by following the manufacturer's protocols. Run the completed reaction on a 1% agarose gel and check that the amplicon is the expected size and that there is only one amplicon.

Luna Universal qPCR Master Mix - 2,500 rxns **New England Biolabs Catalog #**M3003E

4 **Amplification efficiency:**
To test the efficiency of amplification, you'll need to make a standard curve that as closely as possible matches your input material for the final qPCR reaction. In our case, this was ssDNA from cDNA synthesis. Therefore, our standard curve was composed of ssDNA matching the template or antisense strand of our gene since that's the product of cDNA synthesis.

5 **ssDNA standard synthesis:**
ssDNA standards were generated from PCR products amplified from cDNA with a forward primer that was 5' phosphorylated to promote Lambda exonuclease digestion of that strand and a reverse primer with phosphorothioate bonds between the first four 5' nucleotides to block digestion. Those PCR products were then digested with Lambda exonuclease following NEB's protocol.

Components	50 µl REACTION
DNA	up to 5 µg
Lambda Exonuclease Reaction Buffer (10X)	5 µl (1X)
Lambda Exonuclease	1 µl (5 units)
Nuclease-free H2O	up to 50 µl

Lambda Exonuclease - 1,000 units **New England Biolabs Catalog #**M0262S

6 **ssDNA standard concentration determination:**
ssDNA concentrations were determined by Qubit ssDNA Assay Kit by creating a standard curve with the provided standards (0 ng/µl and 20 ng/µl, making serial dilutions of the 20 ng/µl standard to generate the in-between concentrations). 50 µL of standards and digested ssDNA template were placed in a clear bottom 96 well plate and then measured for fluorescence (Ex = 500 nm / Em=540 nm) on a plate reader.
Once the sample concentration was determined, DNA copy number determined by this online calculator https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html

Qubit ssDNA kit **Thermo Fisher Scientific Catalog #**Q10212

7 **Standard dilutions:**
Serially dilute the ssDNA standards from 10^5 to 10^0 copies/µl in a solution with 10 ng/µl of *S. rosetta* total RNA (after removing mRNAs) to account for any matrix effects.

7.1 **Making S. rosetta total RNA without mRNAs for resuspending qPCR standards:**
Wild type cells were grown and lysed according to the **preferential lysis protocol**,

7.2 The lysate was then purified via the total RNA clean up kit according to the **RNA extraction and cDNA synthesis protocol**

7.3 Using NEBs magnetic mRNA isolation kit: 10m

1. 50 µL beads were equilibrated in 200 µL binding buffer provided in the kit
2. Place the equilibrated beads on a magnet and remove the supernatant
3. Resuspend the beads with 250 µL total purified RNA and incubate at Room temperature for 00:10:00
4. Place the incubated beads on a magnet, taking and keeping the supernatant. This is total RNA without mRNAs.
5. Spec the total RNA by nanodrop or qubit and dilute in RNase free water to 10 ng/µl.

Magnetic mRNA Isolation Kit - 25 isolations **New England Biolabs Catalog #**S1550S

qPCR

8 Using the NEB Luna qPCR master mix, set up the following reaction according to the manufacturer, with 3 µL of sample cDNA and of each standard previously made, all in triplicate. Scale accordingly

	A	B	C
	COMPONENT	20 µl REACTION	FINAL CONCENTRATION
	Luna Universal qPCR Master Mix	10 µl	1X
	Forward primer (10 µM)	0.5 µl	0.25 µM
	Reverse primer (10 µM)	0.5 µl	0.25 µM
	Template DNA	variable	< 100 ng
	Nuclease-free Water	to 20 µl	

Luna Universal qPCR Master Mix - 2,500 rxns **New England Biolabs Catalog #**M3003E

9 Cover the plate with an optically clear cover and then cycle the reactions according to the manufacturer on a qPCR machine (We use an Applied Biosystems Quantstudio 3). Measure fluorescence in the FAM/SYBR Green Channel when using the NEB Luna qPCR Master Mix.

	A	B	C	D
	CYCLE STEP	TEMPERATURE	TIME	CYCLES
	Initial Denaturation	95°C	60 seconds	1
	Denaturation	95°C	15 seconds	40-45
	Extension	60°C	30 seconds (+ plate read)	
	Melt Curve	60-95°C*	various	1

*The melt curve step is optional but is useful to confirm that only a single target was amplified.