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Quantitative (q)PCR and Differential Expression Analysis

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Protist Research to Optimize Tools in Genetics (PROT-G) Lewis Lab

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ABSTRACT

A generalizable protocol for measuring relative changes in gene expression via qPCR (cDNA synthesis, qPCR primer optimization, and qPCR analysis), with specific optimization for the budding yeast *Saccharomyces cerevisiae*.

GUIDELINES

While this protocol has been optimized for *Saccharomyces cerevisiae*, the protocol should be broadly adaptable to both prokaryotic and eukaryotic gene expression analysis.

MATERIALS

NAME	CATALOG #	VENDOR
Microseal® 'B' Adhesive Seals	MSB-1001	BioRad Sciences
Sodium hydroxide	S8045	Sigma – Aldrich
UltraPure 0.5M EDTA, pH 8.0	15575-038	Thermo Fisher Scientific
RNase-Free Water	10977015	Thermo Fisher Scientific
Zymo DNA Clean & Concentrator - 5	D4014	Zymo Research
SuperScript™ III Reverse Transcriptase	18080085	Thermo Fisher
TE, pH 8.0, RNase-free	AM9858	Thermo Fisher
Maxima SYBR Green/Fluorescein qPCR Master Mix (2X)	K0241	Thermo Fisher
Random hexamers	51-01-18-25	Integrated DNA Technologies
Oligo d(T)20VN	12577011	Thermo Fisher Scientific
RNase-Free dNTPs	95057-688	VWR International
Multiplate low-profile 96-well unskirted PCR plates pack of 25	MLL-9601	BIO-RAD

SAFETY WARNINGS

DNA-binding dyes such as SYBR Green have the potential to be carcinogenic.

BEFORE STARTING

1. Make stock solutions: 2.5 mM dNTPs (RNase-free), 1M NaOH, and 0.5M EDTA.
2. Pre-label 1 set of RNase-free 0.2 mL microcentrifuge tubes and RNase-free 1.7 mL microcentrifuge tubes for each sample.
3. Have RNase-Free Barrier tips ready for all pipetting steps.

qPCR Primer Design

1 1. Primer Design

- We use Primer3 to design primers: http://biotools.umassmed.edu/bioapps/primer3_www.cgi
- Primers are designed to have a Tm as close to 58°C as possible. This helps to ensure that primer annealing will be similar for all reactions.
- Primers should amplify a product of ~100 - 200 bp. Longer products can have reduced PCR efficiency, and are more susceptible to differences in RNA degradation levels.
- For gene expression analysis, primers are designed within the 3' end of an ORF. This mitigates against partial cDNA synthensis, especially if only oligo-dT is used for cDNA generation.

Validating PCR Primer Specificity

2 1. To validate specificity of the primers, perform PCR on genomic DNA template from both the wild-type strain and a deletion strain for the gene of interest. Use the same polymerase (e.g. Taq) and cycling conditions as you would for qPCR:

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95°C x 3 min
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95°C x 15 sec
55°C x 1 min      30 cycles
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2. Perform gel electrophoresis (2% agarose, ~120V for 30-60 min) with the expectation of a single band (100 - 200 bp) in the wild-type and no band in the negative control (deletion strain).

You should see a single band for the wild-type control and no band for the negative control. If you observe a band in the negative-control sample and/or multiple bands in your wild-type sample, the primers are likely annealing to other locations in the genome and should be redesigned.

Validating qPCR Primer Efficiency

3 1. Dilute cDNA to a range of concentrations: 0.01 ng, 0.05 ng, 0.1 ng, 1 ng, 5 ng, 10 ng, 25 ng and 50 ng (can adjust higher or lower if necessary)

Use a cDNA sample where you are certain that your gene of interest is being expressed. We standardly use a wild-type strain grown under "control" conditions.

Note: the recommendations for concentrations here assume that cDNA was generated using oligo-dT and not random hexamer. Higher concentrations of cDNA may be necessary if using random hexamer (see Step 4).

2. Perform qPCR (Step 6) on new primers (plus the control gene) on the range of cDNA concentrations.

3. Determine your Ct (aka Cq on BioRad qPCR machines) values for your test gene and control gene at each cDNA concentration.

4. Log_{10} transform your cDNA concentrations (e.g. log_{10} of 0.01 = -2 and log_{10} of 50 = 1.69)

5. Plot the data (e.g. in GraphPad Prism or Excel) with Ct-values on the y-axis and log_{10}-transformed cDNA concentrations on the x-axis.

- Carefully examine that plot for cDNA concentrations that fall out of the linear range of the assay. If necessary repeat the cDNA dilutions (step 1) to exclude cDNA concentrations outside of the linear range, and to include more cDNA concentrations that fall within that range.

6. Perform linear regression analysis to calculate the slope and r^2 of the line.

- The r^2 of the regression is typically close to 0.99.

7. Calculate efficiency of each primer set using the equation: Efficiency(%) = $(10^{(-1/slope)} - 1) \times 100$

Example:
Slope of regression line: -3.353
$(10^{(-1/-3.353)} - 1) \times 100 = 98.7\%$

8. Generally, primer-pair efficiencies >90% are acceptable (closer to 100% is best), and all primer pairs (experimental and control genes) should have efficiencies within 5% of each other.

Efficiencies higher than 100% could be do to pipetting errors, or due to the presence of polymerase inhibitors (most likely in the cDNA).

cDNA Synthesis

4 1. Prepare RNA/Primer mixture:

10 µg total RNA
3 µg anchored oligo-dT (T20VN)
3 µg random hexamer (optional)

If you are looking solely at poly-adenylated transcripts (e.g. eukaryotic mRNA) only oligo-dT is needed. Random hexamer is necessary to generate cDNA from non-coding RNAs and prokaryotic mRNAs. **Note: cDNA synthesis using random hexamer generates more total cDNA, but with a smaller overall fraction of mRNAs represented, and you may need to add more cDNA to your qPCR reactions.**

2. Adjust volume to 7 µl with RNase-Free TE.

3. Denature RNA/Primer mixture at 70°C for 10 min, then immediately chill on ice for > 2 min.

4. For each reaction, prepare the Reverse Transcriptase Master Mix

3.0 µl 5x Superscript Buffer
1.5 µl 0.1M DTT
3.0 µl 10x RNase-Free dNTPs (2.5 mM stock solution, final concentration of 250 µM)
0.5 µl Superscript III RT

When making the Master Mix, prepare enough for the number of samples plus one (e.g. 25 reaction volumes for 24 samples). This ensures that there will be enough MasterMix for all aliquots.

If doing no reverse transcriptase (RT) control reactions, prepare a no RT control Master Mix by substituting Superscript III with an equal volume of RNase-Free water.

5. Add 8 µl Reverse Transcriptase Master Mix to the denatured RNA and incubate on bench (25°C) for 7 min.
- The room temperature inclubation is critical for allowing random hexamers to anneal to the RNA, but can be skipped if using oligo-dT alone.

6. Incubate at 50°C for 2 hrs in a thermal cycler.

RNA Hydrolysis and Cleanup

5 1. To each reaction, add

10 µl 1 M NaOH
10 µl 0.5 M EDTA, pH 8.0

2. Incubate at 65°C for 15 min.

3. Immediately proceed to cDNA cleanup.

We use the Zymo Clean and concentrator (D4014), according to the manufacturer's instructions. We also add the eluate back onto the column and repeat the elution to increase cDNA yield.

4. Analyze concentration via Nanodrop or Qubit.

Quantitative PCR

6 1. Design qPCR plate layout using only the interior 60 wells.

Do not use the outer edge wells of the PCR plate for your samples (no RT controls are okay), because uneven heating and/or evaporation increases variability. That means there are 60 usable wells for samples per PCR plate.

2. Dilute each primer to 10 µM.

3. Dilute each cDNA sample 5-fold (e.g. if you are using 1 ng cDNA per reaction, dilute the cDNA to 0.2 ng / µl).

- cDNA concentration should be based on primer efficiency testing (Step 3), using a concentration within the linear range of the assay. One ng of cDNA per qPCR reaction tends to work well for the majority of genes.

When making cDNA dilutions, prepare a large enough volume so that you are pipetting no less that 2 µl of cDNA. Pipetting volumes smaller than 2 µl will decrease the accuracy of your dilutions. (E.g. 2 µl (1 ng / µl) cDNA to 8 µl of water for a 0.2 ng / µl dilution).

4. Prepare a qPCR Mastermix for each primer pair (N+1). For each reaction you will add the following amounts:

0.4 µl Forward Primer (final concentration 200 nM)
0.4 µl Reverse Primer (final concentration 200 nM)
4.2 µl water
10 µl Maxima 2X SYBR Green Mix (1x final)

5. Pipette 5 µl diluted cDNA to the ***bottom*** of the appropriate wells of a BioRad RT-PCR plate. Be careful not to touch the tips to the side of the wells. Use a single pipette tip for pipetting the same cDNA sample into multiple wells.

6. Pipette 15 µl qPCR Mastermix to the ***side*** of the appropriate wells. Use a single tip for each mastermix. Change tips when switching to a new mastermix (different primers). Be careful to only touch the side of the well so as to not contaminate the pipet tip with cDNA already in wells.

7. Seal qPCR plate with clear Bio-Rad sealing membrane; use roller if applicable.

8. Briefly spin the plate in a microplate centrifuge. This ensures all the qPCR mastermix reaches the cDNA in the bottom of the well.

9. Reaction conditions will be primer specific. Based on a primer Tm of 58°C we use the following cycling conditions:

95°C x 3 min

95°C x 15 sec
55°C x 1 min 40 cycles

Melt curve analysis (55°C - 95°C in 5 sec steps; default instrument settings)

Melt curve analysis allows us to check for co-amplification of non-specific products. More than one peak can indicate more than one PCR product, which should be verified via gel elecrophoresis on those samples.

10: Use the following instrument settings:
Cq determination mode = regression
Baseline settings = Baseline subtracted by curve fit with no fluorescence drift corrected
Analysis mode = Fluoropore

qPCR Data Analysis

7 ΔΔCt Analysis

- The critical threshold cycle (Ct - also labelled as Cq on Bio-Rad instruments) is the cycle number where the fluorescence signal crosses a set threshold. Differences in Ct values between samples can be used to measure relative transcript abundance.
- We use the ΔΔCt method to quantify difference in relative trancript abundance, as described by Livak and Schmittgen (Methods. 2001. 25(4): 402-408.). This method assumes that both target and control (reference) genes are amplified with efficiencies near 100% and within 5% of each other (i.e. Step 3).

1. Normalize the Ct values by subtracting the Ct for your control gene from the Ct of the target gene.

ΔCt (sample) = Ct (target gene) - Ct (control gene)

2. Calculate the ΔΔCt using the normalized values from step 1.

ΔΔCt = ΔCt (treated sample) - ΔCt (untreated sample)

Therefore:

ΔΔCt = [Ct(target gene, treated) - Ct(control gene, treated)] - [Ct(target gene, untreated) - Ct(control gene, untreated)]

To convert ΔΔCt to fold changes, use the following equation: $2^{(-\Delta\Delta Ct)}$