Chapter 17

Quantification of Transcript Levels with Quantitative RT-PCR

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Abstract

Differential gene expression is a key factor driving phenotypic divergence. Determining when and where gene expression has diverged between organisms requires a quantitative method. While large-scale approaches such as microarrays or high-throughput mRNA sequencing can identify candidates, quantitative RT-PCR is the definitive method for confirming gene expression differences. Here, we describe the steps for performing qRT-PCR including extracting total RNA, reverse-transcribing it to make a pool of cDNA, and then quantifying relative expression of a few candidate genes using real-time or quantitative PCR.

Key words: Gene expression, Messenger RNA, Quantitative RT-PCR, Real-time RT-PCR

1. Introduction

One of the goals of evolutionary genetics is to determine the molecular basis for differences in phenotypes. The two most common molecular mechanisms driving diversification and adaptation involve either differences in gene coding sequences, causing a change in protein function (1–3), or differences in gene expression, altering the timing, location, and amount of a particular gene and its associated protein (4–6). Numerous debates have arisen as to the relative importance of these two mechanisms for phenotypic evolution (7–9). However, these mechanisms are part of a continuum of molecular change (10, 11), requiring an integrative genomic approach (12).

In this chapter, we discuss one facet of these approaches, quantifying the relative amount of expressed transcripts using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). This method, also called real-time RT-PCR, relies on PCR to quantify the relative amounts of a few genes and has been nicely
described in several review articles (13–15). This method is more
limited than those that provide data for hundreds to thousands
of genes, including microarray experiments (16, 17) or next-
generation transcriptomics (18–20). However, qRT-PCR is the
gold standard by which differences in transcript levels identified by
other methods are confirmed (21, 22).

The key steps in this method are to isolate RNA (typically as
total RNA) from a tissue and developmental stage of interest,
quantify the RNA, reverse-transcribe the messenger RNA to make
cDNA, and perform quantitative PCR (qPCR) on the expressed
genes of interest (Table 1). This chapter describes each of these
steps in detail.

There are several important decisions to make in designing a
qRT-PCR experiment. The first decision is whether to do a one-
step process where reverse transcription occurs in the same tube as
the subsequent qPCR or whether to do a two-step experiment
with reverse transcription done first, followed by qPCR. The one-
step process is possible if the number of genes to be quantified is
four or fewer, and if a multicolor qPCR machine is available that
can separately follow all four transcripts. However, quantifying
more than four transcripts typically requires a two-step process
where cDNA is first prepared and then multiple genes are quanti-
fied in multiple wells.

The second decision is how to normalize the transcripts to make
comparisons between different individuals or different treatments.

| Table 1
| Key steps in the qRT-PCR process |
|-------------------------------|----------------------------------|
| **Task**                     | **Steps**                        |
| Total RNA isolation          | Dissect tissue                   |
|                               | Tissue disruption using micropesite tube |
|                               | Tissue homogenization using QIAshredder column |
|                               | Purification on RNA-binding column |
| RNA quantification of total RNA | Prepare RNA dilution            |
|                               | Measure $A_{260}$ and $A_{280}$ and calculate RNA concentration |
| Reverse transcription to make cDNA | Add primer to total RNA; heat to 65°C and quench on ice |
|                               | Add RT enzymes and incubate at 42°C |
| qRT-PCR                       | Prepare cDNA master mixes        |
|                               | Prepare gene specific primer–probe master mixes |
|                               | Pipette to plate and run on qPCR machine |
| Data analysis                 | Extract $C_b's$ for each sample  |
|                               | Determine PCR efficiencies from standard or dilution series |
|                               | Calculate relative gene expression |
Some methods normalize gene expression relative to a housekeeping gene such as beta-actin or GAPDH. However, Bustin (14) argues that expression levels of housekeeping genes are often not constant, biasing the normalized results. Instead, he suggests that the best normalization method is to quantify total RNA and then to reverse-transcribe the same amount of total RNA for each individual or treatment for subsequent analyses.

The third decision is which chemistry to use for monitoring PCR products in the qPCR. The simplest chemistry is to use two primers and a dye that binds to double-stranded DNA (e.g., SYBR Green) to monitor PCR progress. While this is often successful, it can require optimization to minimize unwanted signal. Background signal can arise either from primer dimers or from any carried-over genomic DNA. (Nearly all RNA extraction methods carry over some amount of genomic DNA.) Careful checks need to be run to test for genomic DNA contamination by performing a control reaction using a sample that has not undergone reverse transcription. An alternative is to treat the RNA sample with DNase prior to reverse transcription. To avoid these potential problems, a qPCR chemistry such as TaqMan® can be used. In this chemistry, a dual labeled probe binds to a specific DNA sequence located between the two amplification primers. This probe is hydrolyzed during PCR, releasing a fluorescing dye from its quenching partner. The fluorescence of the released dye is proportional to the amount of PCR product generated. The binding site of this probe typically spans an exon–exon junction, preventing the probe from binding to genomic DNA. Therefore, only cDNA amplification is detected. Typically, this is sufficient to discriminate against any background genomic DNA so that DNase treatment is not required prior to reverse transcription. Further, there should be minimal background signal from primer dimers, since the TaqMan probe will not bind to the primer dimers.

The fourth and final decision that needs to be made is the number of biological and experimental replicates to perform. Biological replicates account for individual variation and should include two to four samples. This would, therefore, include multiple individuals for each comparison, be it developmental time points, tissues, treatments, or populations sampled. For experimental replicates, qPCR quantification is done two or three times for each biological sample. This is necessary because of the inherent exponential nature of PCR and the resulting errors that can accumulate through many rounds of PCR. Studies sometimes also repeat the reverse transcription step as well. In this case, a given biological RNA sample would be reverse-transcribed twice and each of these would then be quantified by qPCR at least twice. In our experience, most of the error is in the qPCR step, rather than the reverse transcription step, since the former is exponential and the latter is a linear process.
Quantification of the relative amounts of transcripts by qPCR assumes that products accumulate exponentially during the earlier cycles of PCR amplification. During this exponential phase, the amount of PCR product of gene i, $P_i$, is related to the initial amount of gene transcript, $T_i$, the PCR amplification efficiency of that gene, $E_i$, and the number of PCR cycles, $n$, by the following equation:

$$ P_i = T_i (1 + E_i)^n $$  \hspace{1cm} (1)$$

In the ideal situation, efficiency is 1 and PCR product doubles with each amplification cycle. In practice, it is necessary to quantify efficiency for each gene.

While it is possible to determine the absolute amount of initial gene transcript by comparison to a standard curve of samples with known copy number, this is not necessary if all that is desired is a comparison of one gene between two treatments or of two genes within an individual. In this case, it is possible to monitor PCR product, $P_i$, and then set a threshold level somewhere in the exponential amplification phase. At this point, the two samples have the same amount of PCR product ($P_i = P_j$) and the relative amounts of initial transcripts can be determined:

$$ P_i = T_i (1 + E_i)^{C_i} = P_j = T_j (1 + E_j)^{C_j} $$  \hspace{1cm} (2)$$

$$ \frac{T_i}{T_j} = \frac{(1 + E_i)^{C_i}}{(1 + E_j)^{C_j}} $$  \hspace{1cm} (3)$$

Here, $i$ and $j$ are either two treatments or two genes, and $C_i$ is the number of cycles at which each PCR reaches threshold, or the critical cycle number. The ratio of initial transcripts is then determined from the critical cycle numbers and the PCR efficiencies.

Determining PCR efficiencies can be done by many different methods. One of the easiest methods is to prepare a dilution curve of the template of interest and perform qPCR on this series of samples. The dilution series can be prepared from the actual cDNA sample itself containing the expressed gene, or it can use an amplified PCR product from the gene, or a plasmid containing the cloned gene of interest. In the latter two cases, the gene should be amplified from cDNA and not genomic DNA so that it has the same exonic structure as the expressed transcript. In considering Eq. 1, we note that at threshold, $P$ is equal to the constant threshold, and $n$ is equal to $C_i$. Therefore, we can take the natural log of both sides, set the log of the threshold level to be $K$, and rearrange Eq. 1 to get:

$$ \ln T = K - C_i \ln (1 + E) $$  \hspace{1cm} (4)$$

Therefore, a plot of $\ln T$ versus $C_i$ for the dilution series will yield a line with a slope of $-\ln(1 + E)$. Here, $T$ is the relative transcript concentration (e.g., 1x, 10x, 500x), and need not be an absolute amount.
transcripts by qPCR during the earlier potential phase, the initial amount ency of that gene, swing equation:

(1)

duct doubles with γ to quantify efficiency of total amount of initial γe of samples with that is desired is a x or of two genes to monitor PCR where in the exposure samples have the relative amounts of genes, and \( C_i \) is the fold, or the critical then determined ciencies. by many different re a dilution curve this series of samplings the actual cDNA can use an amplification the cloned be amplified is the same exonic ng Eq. 1, we note fold, and \( n \) is equal both sides, set the Eq. 1 to get:

(2)

n series will yield a n absolute amount.

The slope is determined from a linear least squares fit to the data and used to calculate efficiency from the following equation:

\[
E = e^{-\text{slope}} - 1
\]

(5)

The following method is an example from our work to illustrate how these steps might proceed as well as some specific variations. Much of our work involves expression of opsin genes in fish retina (23, 24). Like most other fishes and vertebrates, cichlid fishes have cone opsin genes from the classes of short wavelength sensitive (SWS), rhodopsin-like (RH2) and long-wavelength-sensitive (LWS) opsin genes (25, 26). Specifically, cichlids have seven different genes, which fall in six different classes and cover the full spectral range: SWS1 (ultraviolet), SWS2B (violet), SWS2A (blue), RH2B (blue-green), RH2A (green), and LWS (red). To quantify the relative expression of these six opsin gene classes, we use TaqMan® chemistry, with a forward and reverse primer for each gene as well as a gene-specific probe. Although there are different forms of the RH2A gene, RH2Aα and RH2Aβ genes (24) are genetically so similar that the same set of primers and probe amplify both forms. They can be distinguished by moving the forward primer further upstream where the gene sequences diverge (27), though we typically quantify them together and deal only with six cone opsin gene classes.

We use the dilution method to obtain the absolute efficiency of one gene of interest, for example the RH2A gene. This typically involves making a dilution series covering three orders of magnitude in concentration. Comparisons are then made between samples with \( T \) set to nine different relative concentrations: 1x, 2x, 5x, 10x, 20x, 50x, 200x, 500x, and 1,000x. These nine samples are then run to obtain \( C_i \)'s and estimate \( E \) for this gene from the slope of the lnT versus \( C_i \) plot.

In order to determine the relative PCR efficiencies of the other five gene classes, we made a gene construct in which the key parts of each of the six gene classes are ligated together (24). This construct, therefore, contains each of six genes in a fixed 1:1:1:1:1:1 ratio (see Note 1). Therefore, we can get the relative efficiency of each gene to any other. To solve for this efficiency relationship, we start with Eq. 2 where we know that at threshold, the amount of each gene PCR product is equal. Then, we note that in the construct, the initial amount of each gene is identical, since they are ligated together. By setting \( T_i = T_γ \), we can simplify Eq. 2 to get:

\[
(1 + E_γ)^{C_i} = (1 + E_γ)^{C_i}
\]

(6)

Then, we use Eq. 6 to solve for the efficiency of any of the other genes from the RH2A expression, determined from the dilution series. In Eq. 6, we substitute \( E_γ = E_{RH2A} \) and \( C_i = C_{RH2A} \) so that:

\[
(1 + E_{RH2A})^{C_i} = (1 + E_{RH2A})^{C_{RH2A}}
\]

(7)
We are typically most interested in the expression level of each opsin gene relative to the total opsin levels present in the retina so that we can determine which opsins are present and which are not. We are not so concerned with the absolute levels. We, therefore, determine the amount of transcript of each opsin relative to the total opsin genes from:

\[
\frac{T_i}{\sum T_i} = \frac{\frac{1}{1+E_i}^{s_i}}{\sum \frac{1}{1+E_i}^{y_i}}
\]  

(8)

Using the efficiencies determined from the construct, we can solve for the relative expression of any of the genes in a sample normalized to total cone opsin expression using the C_i's determined in the qPCR step.

2. Materials

In performing RNA isolation and reverse transcription, it is important to take precautions to minimize the presence of RNase which can degrade RNA. Chemicals should be set aside and used just for RNA procedures. Typically, this means using newly opened bottles of chemicals and wearing gloves whenever handling them. Do not use general lab chemicals, which are not assured of being RNase-free. RNase-free water can either be purchased or be prepared by treatment with diethylpyrocarbonate (DEPC). All plastics and glassware should be RNase-free. Plastics can be purchased that are guaranteed RNase-free, while glassware can be acid-washed and baked at 180°C for four hours prior to use. Gloves should be worn and changed frequently to prevent contamination with RNases. Once past the reverse transcription step (Subheading 3.3), it is no longer necessary to maintain RNase-free conditions. At that point, plasticware and water should be DNase-free as is typical for most other molecular biology experiments.

2.1. RNA Extraction

1. 1.5-ml micropestle tubes and pestles (Kontes through VWR) (see Note 2).
2. RNeasy mini kit (QIAGEN, Valencia, CA). This kit includes buffers RLT, RWI, and RPE, as well as RNA binding columns. Ethanol needs to be added to buffer RPE prior to its use.
3. QIAshredder columns (QIAGEN, Valencia, CA). These columns provide tissue homogenization following disruption, which reduces solution viscosity.
4. β-Mercaptoethanol (Sigma Aldrich, St. Louis, MO).
5. 70% Ethanol, made up fresh from ethanol and RNase-free water.

### 2.2. RNA Quantification
1. RNase-free water.
2. Small volume (50 μl) UV transmissive cuvette.

### 2.3. Reverse Transcription
1. Total RNA, extracted and quantified.
2. Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA).
3. RNaseOUT, recombinant ribonuclease inhibitor (Invitrogen, Carlsbad, CA).
4. polyT primer, 10 μM primer made up in RNase-free water. We use a polyT<sub>17</sub> primer with a unique sequence on the 5' end: GCGAATTCGTGACAGGCT<sub>17</sub>.
5. dNTPs, 10 mM each. These are made from 100 mM stocks (USB, Cleveland, OH). Add 30 μl of each of ATP, CTP, GTP, and TTP to 180 μl of RNase-free water. Keep frozen in an RNase-free O-ring-sealed tube at −20°C.

### 2.4. qRT-PCR
1. qPCR machine (see Note 3).
2. PCR plate that fits the qPCR machine along with sealing tape.
3. TaqMan universal PCR master mix (Applied Biosystems, Carlsbad, CA).
4. Primers and probes for each gene to be studied (see Note 4). This includes a forward primer (3 μM), a reverse primer (3 μM), and a dual labeled probe (2 μM). These gene-specific primers and probes can be designed using Primer Express (Applied Biosystems, Carlsbad, CA) with the probe spanning an exon–exon junction. If using a single-color qPCR machine, the probe is typically 5' labeled with 6FAM and 3' labeled with TAMRA and then HPLC-purified. The primers need to be desalted.
5. cDNA mixture from reverse transcription step.
6. Positive control, which could be a plasmid containing the gene or genes of interest.

### 3. Methods

#### 3.1. RNA Extraction
There are many methods for RNA isolation (see Note 5). The following RNA extraction method follows QIAGEN's instructions for the RNeasy kit with the addition of the QIAshredder column.
3.1.1. Tissue Disruption and Homogenization

1. Prepare the tissue of interest either from a fresh dissection or by removing it from an RNA preserving solution (e.g., RNALater).

2. Place in a micropestle tube containing 600 μl of buffer RLT and 6 μl of β-mercaptoethanol (see Notes 6 and 7). β-Mercaptoethanol and buffer RLT are harmful and should be used in a hood while wearing gloves and protective clothing.

3. Disrupt the tissue by fine grinding using the micropestle (see Note 2).

4. Homogenize the solution by pipetting it onto the QIAshredder column and spinning at 12,000 × g for 2 min.

5. Remove the QIAshredder column and cap the tube. Spin for an additional 3 min at maximum speed.

3.1.2. Purification on RNA Column

1. Add 600 μl of 70% ethanol to a clean 1.5-ml tube. Transfer the solution spun through the QIAshredder column to the 70% ethanol and mix by pipetting (see Note 8).

2. Transfer half of this new mixture to the RNA binding column in a 2-ml collection tube. Centrifuge at 8,000 × g for 15 s.

3. Pour off the flow-through and add the other half of the solution to the RNA column and spin again for 15 s. Discard flow-through.

4. Add 500 μl of buffer RW1 to the column and spin at 8,000 × g for 15 s.

5. Transfer the RNA column to a clean collection tube. Add 500 μl of buffer RPE and spin at 8,000 × g for 15 s. Discard flow-through and add another 500 μl of buffer RPE and spin at 8,000 × g for 1 min. Discard the flow-through.

6. Place the RNA column back in the collection tube and spin for another 2 min to dry the column and remove any traces of ethanol.

7. Place the RNA column into a clean, RNase-free tube. Add 30–50 μl of RNase-free water and let the column sit for 1 min. Spin at 12,000 × g for 1 min. Add another 30–50 μl of water and repeat letting it sit and then spin to elute the RNA.

3.2. RNA Quantification

Quantify the total RNA isolated with the QIAGEN RNeasy kit by measuring the ratio of absorbance at 260 nm, A_{260}, to that at 280 nm, A_{280} (see Note 9).

1. Zero the spectrophotometer at 260 and 280 nm by taking a blank using pure water.

2. Dilute a small quantity of the RNA, such as 4 μl diluted to 60 μl of RNase-free water, and place in a 50-μl cuvette.
3. Measure the absorbance of the diluted RNA sample at 260 and 280 nm.

4. Calculate the RNA concentration. In the ideal case, the $A_{260}/A_{280}$ ratio is 2 and there is no protein contamination. RNA concentration can then be determined using $A_{260} = \varepsilon C$ where $A_{260}$ is the absorption at 260 nm, $\varepsilon$ is the RNA extinction coefficient which is 25 µl/µg at 260 nm, and $C$ is the concentration. (This all assumes a 1-cm cell path length.) However, for other $A_{260}/A_{280}$ ratios, a correction should be made for contributions of protein to the absorption, using the method of Glasel (28). In this case:

$$N = \frac{R(e_{280,n} - e_{260,p})}{(e_{260,n} - e_{260,p}) - R(e_{280,n} - e_{280,p})}$$

$$C_N = \frac{NA_{260}}{Ne_{260,n} + Pe_{260,p}}$$

where $N$ is the ratio of nucleic acids (RNA in this case) to the sum of nucleic acids plus protein, $P$ is the ratio of protein to the sum (so that $P = 1 - N$), $C_N$ is the concentration of nucleic acid (RNA), $R$ is the $A_{260}/A_{280}$ ratio, and the $\varepsilon$ are the extinction coefficients of RNA and protein at the two wavelengths ($e_{260,n} = 25$ µl/µg, $e_{260,p} = 0.57$ µl/µg, $e_{280,N} = 12.5$ µl/µg, $e_{280,p} = 1$ µl/µg; (28)). $N$ can be calculated from Eq. 9 using $R$ and the extinction coefficients. $P$ is then calculated as $1 - N$. These are then used in Eq. 10 with the absorption at 260 nm ($A_{260}$) to get the RNA concentration, $C_N$.

5. The concentration of the initial RNA solution is calculated from that of the diluted sample by multiplying by one over the dilution factor. For the dilution above, where 4 µl is diluted to 60 µl, the measured (diluted) concentration should be multiplied by 15 to obtain the initial concentration.

6. Calculate the volume of RNA required to add 0.5 µg of RNA to the reverse transcription (RT) reaction.

1. Prepare the initial additions to the reverse transcription reaction including 0.5 µg of total RNA, 2.5 µl of 10 µM polyT primer, 1.25 µl of 10 mM each dNTPs, and enough RNase-free water to bring the volume to 15.6 µl in an RNase-free tube. It is easiest to add the water, then 3.75 µl of a master mix of polyT primer and dNTPs to each tube, and finally the individual total RNA.

2. Heat this mixture to 65°C for 5 min and then quench on ice for 1 min. This reduces secondary structure to ensure transcription of full-length cDNAs.
3. Prepare a master mix of 5 µl of 5x first-strand buffer, 2.5 µl of 0.1 M DTT, and 0.65 µl of RNaseOUT (25 U) for all the samples and then add 8.15 µl of master mix to the total RNA mixture.

4. Spin down and incubate at room temperature for 2 min.

5. Add 1.25 µl (250 U) of Superscript III, bringing the total volume to 25 µl.

6. Briefly vortex, spin down, and incubate at room temperature for 10 min.

7. Incubate at 42°C for 50 min.

8. Heat-inactivate at 70°C for 15 min (see Note 10) and then store at -20°C. In the subsequent qPCR step, we typically use 0.5 µl of this cDNA mixture in each qPCR reaction. If each gene is quantified in a separate qPCR, and each gene is measured twice, the 25 µl of cDNA mixture is enough to quantify 25 different genes, with replicates. If genes are multiplexed with more than one primer–probe mixture per tube, more genes could be measured from this volume of cDNA.

3.4. qRT-PCR

The qRT-PCRs are prepared using master mixes. These include one master mix for each individual or cDNA and one for each gene’s primer–probe combination (see Note 11). One method of arranging things is for the individuals to be divided across the rows and then each gene added down a column of a plate.

1. For each individual or cDNA, make a master mix containing 10 µl of 2x TaqMan universal PCR buffer, 3.5 µl of water, and 0.5 µl of cDNA mixture. This is multiplied by however many genes are to be quantified plus 10% for pipetting losses. Mix the three reagents in one tube, vortex, spin down, and aliquot 14 µl into each well of the qPCR plate, possibly along one row.

2. Make a master mix for each gene containing 2 µl each of the forward primer, reverse primer, and probe. This is multiplied by however many individuals are to be quantified plus 10% for pipetting losses. The primer master mix is vortexed, spun down, and then 6 µl is aliquoted into each well of the qPCR plate, possibly along one column. This step can be nicely done with a multichannel pipettor if the primers lay out in a simple fashion. It is helpful to mix with the pipettor as the primer–probe mixture is added to the TaqMan/cDNA mixture in each well.

3. Seal the qPCR plate with sealing tape and then lightly spin down in a centrifuge.

4. Run the plate on a quantitative or real-time PCR machine for at least 40 cycles (see Note 12 for cycling parameters, Note 13 for replicate plate layouts, and Note 14 for factors affecting qPCR reproducibility).

5. Determine the critical cycle numbers for each sample (see Note 15).
It is common to first perform a dilution curve for a particular primer–probe combination. This confirms that the qPCR result is proportional to template concentration and also provides the PCR efficiency for a particular primer–probe combination. This PCR efficiency is specific to the actual primer–probe concentrations and is typically measured every time a new batch of primers and probes are made up.

1. Prepare a set of dilutions. The DNA used can be a plasmid containing the gene of interest or cDNA from an individual known to express the gene. The dilutions should cover at least three orders of magnitude in concentration (e.g., 1x, 2x, 5x, 10x, 20x, 50x, 200x, 500x, and 1,000x).

2. Prepare a qPCR plate containing a dilution series for each gene being studied and run them together on one plate.

3. Extract the critical plate containing a dilution series for each gene being studied and run them together on one plate.

4. Plot lnT versus Ct, obtain the slope, and calculate the PCR efficiency for each gene, using Eq. 5. For the data shown in Fig. 1, the RH2A slope is \(-0.708 \pm 0.023\). Here, 0.023 is the standard error in the slope, obtained from the linear regression. The resulting PCR efficiency is \(1.03 \pm 0.1\) (see Note 16). Larger PCR efficiencies suggest the gene of interest is amplifying well and should provide data proportional to transcript concentration. The other two genes shown in Fig. 1, SWS1 and LWS, have efficiencies that are not quite so high. Their slopes are \(-0.620 \pm 0.021\) and \(-0.605 \pm 0.16\), which give PCR efficiencies of \(0.87 \pm 0.08\) and \(0.83 \pm 0.06\), respectively. These are typical values for short 70–80 bp amplicons. Longer amplicons typically have lower efficiencies.

![Diagram](image)

Fig. 1. Dilution series for several opsin gene templates. qPCRs with decreasing amounts of template are prepared and run. The resulting Ct values are then plotted versus the natural log of the relative concentration (lnT). The slope of this plot is related to PCR efficiency as given in Eq. 5.
5. For the opsin work, we can also use the multigene construct to obtain relative PCR efficiencies and then tie this to the RH2A efficiency determined from the dilution curve (Eq. 7). When we do this, we get slightly tighter estimates of PCR efficiencies as evidenced by smaller error bars. For example, PCR efficiencies estimated from the construct for the SWS1 and LWS genes are 0.909 ± 0.024 and 0.878 ± 0.035. These more accurate efficiencies enable a slightly better estimate of gene ratios.

6. Use the PCR efficiencies to determine the ratios between genes according to Eq. 3. If all of the opsin genes are measured, these can be normalized relative to the sum, using Eq. 8. Sample amplification curves are shown in Fig. 2a with the corresponding normalized gene expression ratios shown in Fig. 2b.

Fig. 2. (a) Sample amplification curves determined by TaqMan fluorescence monitored versus the number of PCR cycles. There can be significant variation in the overall shape because of random differences in amplification. The threshold method is used here to determine the critical cycle number for each gene. The threshold is set where amplification is in the exponentially increasing phase. If fluorescence were plotted on a logarithmic scale, this would be a linear region. (b) The relative gene expression for six opsin genes is determined from the critical cycle numbers using PCR efficiencies and Eq. 8. Note that even though \( C_{\text{LWS}} \) is higher than \( C_{\text{RH2A}} \), LWS gene transcripts are more abundant than RH2A transcripts because \( E_{\text{LWS}} \) is lower than \( E_{\text{RH2A}} \).
7. For final results, replicates of an individual sample can be averaged together. The individual results can then be averaged across individuals of a species (23), or developmental time points (27) or treatments (e.g., rearing temperature). Alternatively, the individuals might be used as separate data points in an ANOVA to compare species, developmental time points, or treatments (29).

4. Notes

1. The construct assures that the gene ratios are fixed. However, this approach could also use a mixture of gene products or plasmids made up with known relative concentrations.

2. The micropestle tubes work well for breaking up soft tissue such as retina. However, harder tissues should be disrupted using a rotor–stator tissue homogenizer to ensure that the tissue is thoroughly dissected and separated into cells.

3. There are numerous qPCR machines available. These differ primarily in the optical set up and in the software. Because PCR products are monitored by fluorescence, light must be sent to each well to excite a probe and then the emitted light is collected. In order to multiplex the detection of multiple genes, different dyes are used for each gene. These must then be spectrally separated during detection. Excitation typically uses either a spectrally broad lamp (tungsten–halogen or xenon) in combination with band-pass filters, or a spectrally narrower light-emitting diode or laser. Emitted light is typically separated by filters to isolate fluorescence from different dyes, which label different genes. Machines differ in how many emission signals they can separate from just one to four or more. Detection can be done one well at a time using a photomultiplier tube or photodiode. The detector is then scanned across each well of the qPCR plate to quantify each sample. Alternatively, light from the entire plate can be collected by a charge-coupled device (CCD) to detect and quantify PCR amplification in all wells simultaneously. Any of these machines can do the job as long as the fluorescent dyes are selected to match the excitation and emission wavelengths available for a given machine.

4. The primers and probes for qPCR can be made up using DNase-free water as these reactions work on cDNA and not RNA. We typically make primers up in TE/10 (1 mM Tris (pH 8.0) 0.1 mM EDTA). Some manufacturers, such as Applied Biosystems (ABI), recommend testing a range of primer–probe concentrations to determine the optimal concentrations. We have not found this necessary and have used the concentrations listed here, which occupy the midpoint of the range suggested by ABI.
5. There are cheaper methods than ones using a kit to extract total RNA. These use a reagent such as TRizol® (Invitrogen, Carlsbad, CA) or TRI reagent (Molecular Research Center, Cincinnati, OH) to isolate the RNA, followed by an RNA precipitation step. While these methods often work well, there can be problems with occasionally losing RNA pellets and difficulty with RNA resuspension. We have found the QIAGEN kit to be robust and reproducible across many different users in the lab.

6. QIAGEN has also developed an alternate method that uses 2% dithiothreitol (12 μl of 2 M DTT in 600 μl of buffer RLT), instead of 1% β-mercaptoethanol (6 μl of β-ME per 600 μl of buffer RLT), during lysis.

7. No more than 30 mg of tissue should be used, as the mini column can only capture 100 μg of RNA. See the QIAGEN manual for estimated amounts of RNA obtained from various kinds of tissue. Larger kits are available that can handle more tissue.

8. After spinning retina through the QIAshredder, there may be a dark layer in the bottom of the tube. This layer may contain proteins and retinal pigment and is typically brown to black in color. It often is quite loose and stringy and so is easily caught up during pipetting. It should be avoided, as it decreases the quality of RNA (based on the $A_{260}/A_{280}$ ratio).

9. If there is significant pigment carryover into the RNA (e.g., from melanin in the retinal pigment epithelium), this will provide significant absorbance background such that the $A_{260}/A_{280}$ ratio will not provide a good estimate of RNA concentration. In this case, it is better to use a fluorescent quantification method based on a dye such as RiboGreen (Invitrogen, Carlsbad, CA).

10. We often skip the heat inactivation step following the reverse transcription reaction. The first step of the qPCR is a 95°C hold for 10 min which should easily inactivate any remaining RT enzyme.

11. Genes could be multiplexed, combining several primer–probe mixes in the same tube. This requires labeling probes for different genes with different dyes, and having a qPCR machine that can resolve multiple dyes.

12. The thermal cycling parameters depend somewhat on the primer–probe design characteristics. Often, the probe is designed to anneal 10°C higher than the primers. It is common to use a two-step cycle with a 95°C denaturation step and a 60°C annealing and extension step. However, we have also used 95°C denaturation step for 15 s, a 55°C annealing step for 30 s and a 65°C extension step for 1 min with good success.
13. In performing qPCR replicates, the best replicates will be obtained if the same individual is prepared using enough master mix for both replicates and then run on the same qPCR plate. However, this may not provide the best estimate of qPCR errors. It is probably more realistic to prepare separate master mixes for each replicate and run them on different qPCR plates to get an actual estimate of the instrumental and pipetting errors that go into estimates of transcript expression. This will provide better comparisons to the individual with each individual variation that is being quantified.

14. One factor that has a significant impact on \( C_t \) reproducibility is probe concentration, as it impacts PCR efficiency. A factor of two in probe concentration can cause a \( C_t \) shift of one cycle or more. We, therefore, make up a large enough batch of primer-probe mixture for an entire experiment. An experiment would include dilution curves for each gene plus all the individuals and replicates for that data set. So, if we wanted to examine ten individuals of ten species, we would make up enough primer-probe mixture (forward primer, reverse primer, and probe) for 2 replicates of 100 reactions plus another 20 reactions to measure the dilution series at the beginning and again at the end. This would then require 220 reactions worth of the primer-probe mixture for each gene. We have also noticed problems with extensive freeze thawing of primer-probe mixtures where \( C_t \) tends to increase over time. Therefore, we either make up a large primer-probe aliquot and store it in the refrigerator and use it over a 1- or 2-week period, or we make up aliquots of a size needed to prepare one qPCR plate and store them in the freezer so that each aliquot is thawed only once.

15. The critical cycle number is determined based on a threshold value, which is determined differently on different qPCR machines. The threshold value can be determined by setting a standard threshold for all reactions (ABI), by determining the noise level of the background signal and setting the threshold at several times above this noise level (Stratagene), or by using the second derivative to determine the cycles at which the PCR product is most rapidly increasing (Roche). We have repeated samples on an ABI machine using a set threshold and on a Roche machine using second derivatives and have gotten comparable results by these two methods.

16. Theoretically, the PCR efficiency can never be greater than 1, as PCR products can only double from one cycle to the next. However, there is always experimental error in determining the slope of the dilution curve. When we get PCR efficiencies slightly greater than 1, we typically set them to 1.
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