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# Real-Time Quantitative PCR to Demonstrate Gene Expression in an Undergraduate Lab

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## Real time quantitative PCR to demonstrate gene expression in an undergraduate lab.

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**Abstract**

The objective of this teaching note is to develop a laboratory exercise, which allows students to get a hands-on experience of a molecular biology technique to analyze gene expression. The short duration of the biology laboratory for an undergraduate curriculum is the biggest challenge with the development of new labs. An important part of cell biology or molecular biology undergraduate curriculum is to study gene expression. There are many labs to study gene expression in qualitative manner. The commonly used reporter gene expression studies are primarily qualitative. However, there is no hands-on experience exercise to quantitatively determine gene expression. Therefore, it is necessary to design a laboratory exercise that enables the students to carry out cell or molecular biological assays in the desired time. Here we report a laboratory where we can introduce students to gene expression using the real time Quantitative Polymerase Chain Reaction (RT-qPCR) by comparative $C_T$ method to analyze expression of genes in *Drosophila* tissues. Keywords: *Drosophila melanogaster*, eye, real time quantitative PCR, gene expression.

**Introduction**

A challenging situation emerging with fast paced growth on the research front in various disciplines of Biology is to introduce emerging new concepts into the undergraduate curriculum too (Puli and Singh, 2011; Tare *et al*., 2009; Tare and Singh, 2008; Usman and Singh, 2011; Wood, 2009). Interestingly, central dogma of molecular biology is an age old and time-tested concept that has been delivered in the undergraduate classroom. Even though the basic concept about central dogma is that genetic information of an organism or a cell is stored in nucleic acid DNA, which is then transcribed into single stranded RNA, and finally translated to protein but the strategies to study gene expression (qualitatively and quantitatively) have been evolving to date. The conventionally used approaches to deliver this curriculum in laboratory class are to use reporter gene expression, immunohistochemistry, or using protein trap lines. However, the majority of these techniques are qualitative, or to some extent semi-quantitative, in nature. Therefore, there are not many quantitative approaches to determine or compare levels of gene expression among different tissues that can be used for classroom demonstration.

*Drosophila melanogaster* has not only served as a workhorse for research but has also been exploited to develop undergraduate laboratory classes. The short life cycle of 12 days, high reproductive ability of fly, and a long repertoire of genetic tools have made this a very useful model for undergraduate classroom (Puli and Singh, 2011; Singh *et al*., 2012; Tare *et al*., 2009; Tare and Singh, 2008). *Drosophila* can be used to visualize gene expression by employing techniques like enhancer trapping, epitope tagging, antibody staining, or gene trapping. All these methods are qualitative in nature and are laborious and time consuming (Puli and Singh, 2011; Tare *et al*., 2009; Tare and Singh, 2008). In multicellular organisms, including flies, differential gene(s) expression along spatial levels of gene(s) expression in different cells/ tissues and temporal axis generates diversity in cell types and patterning. Furthermore, the gene expression also varies under different experimental conditions. Therefore, quantification of gene expression has been a crucial aspect of modern day biological research. There are different ways to quantify gene expression: as a validation of protein levels (Kim *et al*., 2008), as a validation of the extent of transcription of a gene (Pal *et al*., 2007), to study differences in gene expression between the diseased and the normal state (Ren *et al*., 2007), change in expression of cells exposed to chemical substances (Woods *et al*., 2008), quantification of non-coding RNA gene expression (Calin *et al*., 2007), and as a diagnostic tool (Paik *et al*., 2004). Real time quantitative PCR (RT-qPCR) has served as one of the modern day workhorses to perform such quantitative analysis. Hence, we are introducing here the use of Real time quantitative Polymerase Chain Reaction (RT-qPCR) technique to help students to investigate gene expression quantitatively along the temporal as well as spatial axes.

**Protocol**

This RT-qPCR laboratory designed for undergraduate curriculum has been divided into two labs/classes. The first day, students extract total RNA from the tissue, run RNA on a gel, and determine quality and concentration of RNA. They also prepare cDNA from mRNA in the first lab. On the second day, they run qPCR and analyze data (Figure 1). Thus, we have divided this qPCR into four steps: (1) Sample preparation and RNA isolation, (2) RNA to cDNA conversion, (3) qPCR, and (4) Analysis.

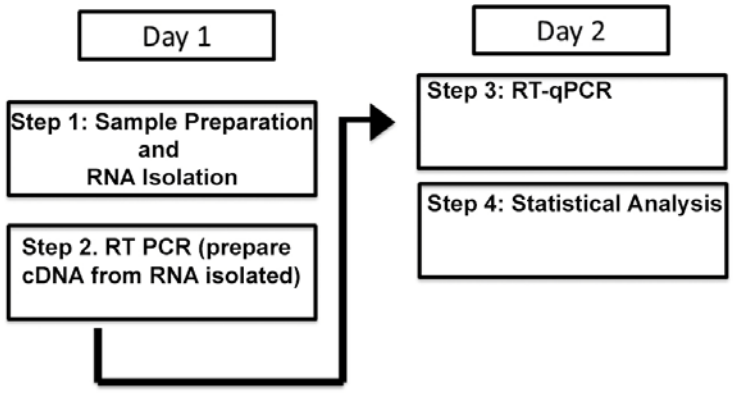


Figure 1. Schematic presentation of time line for Real Time Quantitative Polymerase Chain Reaction (RT-qPCR). We have developed a two-day RT- qPCR protocol for undergraduate laboratory course. This strategy will allow demonstration of this modern day technique to undergraduate students.

1. Sample Preparation and RNA Isolation

*Drosophila melanogaster*, flies, were obtained from the Bloomington Stock Center, Indiana; http://flystocks.bio.indiana.edu. The third instar larvae were selected and dissected in Phosphate Buffered Saline (PBS, containing 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4; Dulbecco and Vogt, 1954) using sharp forceps (Electron Microscopy Sciences Cat. No. # 72707-01). We isolated the eye-antennal imaginal discs to study the expression level of desired genes. Twenty pairs of third instar eye-antennal imaginal disc (n = 40) were collected in microcentrifuge tubes upon dissection. The imaginal discs were stored in RNAlater (Thermo Fisher, Cat. No. # AM7024) solution.

The tubes were briefly centrifuged and RNAlater solution was completely removed. We added 500 µl of TRIzol Reagent (Thermo Fisher, Cat. No. # 15596926), which is used to isolate good quality RNA from tissue samples. TRIzol is a monophasic solution, primarily consisting of phenol and guanidine isothiocyanate along with other proprietary components, which was used for homogenization of tissue. Chloroform was

added and homogenate was allowed to separate into a clear upper aqueous layer. 200 µl of the aqueous phase was transferred to RNA clean and Concentrator™ (Zymo research, Cat. No. R1080) columns, and the recommended protocol was followed, *i.e.*, solution was passed through the RNA binding buffer, which binds RNA to the desired columns. Then RNA wash buffer was added to remove all the impurities from the column. Finally RNA was eluted in 20 µl of molecular grade water (DNase/RNase free) and collected in a separate tube. The molecular grade water serves as the elution buffer. It releases the RNA from the column. Quality of RNA as well as concentration of RNA was determined by calculating absorbance at 260 nm (A260) and 280 nm (A280) wavelengths using Nanodrop 2000 spectrophotometer (Thermo Scientific). Good quality samples had A260/ A280 ratio greater than 2 and a peak at 260 nm.

2. RNA to cDNA Preparation

On Day 1 of the lab, the reverse transcription (RT) reaction is used to prepare cDNA from RNA. RNA concentration for each sample was calculated using the absorbance values obtained from Nanodrop. For RT reactions, 200 ng total RNA was used for each reaction. RNA was heated at 65°C for 10 minutes. Then, it was snap chilled on ice for 3 minutes, followed by a short spin of 10 seconds. It was tapped five times, after which again a short spin of 3 minutes was given, and RNA was ready for RT PCR reaction. We used first-strand cDNA synthesis kit (GE healthcare, Cat# 27926101) to generate cDNA from the isolated RNA. This kit has NotI-(dT)18 bi-functional primer, which can be used to selectively prime mRNA with poly(A) tail and is designed to generate full-length first strand cDNA. The kit master mix is comprised of: RNA 9 µl (for 200 ng concentration), first strand mix- 5 µl, DTT- 1 µl, oligo (dt) primer-1 µl. The mix is incubated at 37°C for 1 hour, then 135 µl molecular grade water is added and solution is incubated again for 5 min at 98°C for enzyme inactivation. Half of the volume(s) are used for negative RT reaction without using oligo(dt) primers, *i.e.*, first strand mix- 2.5 µl, DTT- 0.5 µl, molecular grade water: 68 µl. Solution was incubated for 5 min at 98°C for enzyme inactivation to which 4.5 µl of RNA was added.

Table 1. Recipe for PCR reaction.

| Ingredient | Per reaction (µl) |
|---|---|
| SYBR green reagent | 12.5 |
| Forward/reverse primer mix (50 mM each) | 0.625 |
| Molecular biology grade water | 9.25 |

3. Quantitative Real Time Polymerase Chain Reaction

On Day 2 of the lab, we performed the RT-qPCR reaction. It was performed using iQ ™ SYBR® Green Supermix (Bio-Rad) and Bio-Rad iCycler (Bio-Rad). Following master mix was prepared, accounting to one to two additional reactions per gene (Table 1). To each well of 96-well plate, 23 µl of master mix (Bio-Rad, Cat # 223-9941) was added, followed by addition of 2 µl of cDNA (generated in Step 2). Plate was sealed using sealing strip (MSBI001 Bio-Rad). A brief spin (up to 1,500 rpm) was performed on a centrifuge equipped with a 96-well plate adapter. Then PCR plate was placed in iCycler and PCR was performed as per the manufacturers' protocol. Typically, step 1: 95°C for 3 minutes (1 cycle). This step is performed for initial denaturation of the double stranded cDNA to the single strands and loosen secondary structures in single stranded DNA. The step 2 comprise of 40 cycles of 30 seconds at 95°C and 30 seconds at 60°C. This is an annealing step during which DNA amplification occurs, and as DNA keeps on amplifying so does SYBR® Green get intercalated to (binds into) the DNA double helix. This alters the structure of the dye and causes it to fluoresce more. Thus, along the course of time as the concentration of DNA increases, so does the intensity of fluorescence. An instrument that combines thermal cycling with fluorescent dye scanning capability can measure this change in the fluorescent intensity. Fluorescence is plotted against the cycle number, and finally RT-qPCR generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction. This way real time quantification of gene product is done, and Ct values were recorded and then using pre designed template (Figure 3A) fold change was calculated.

4. Analysis

There are various methods in which RT-qPCR data can be reported including absolute or relative expression level. Absolute expression provides the exact copy number of data *via* a standard curve (Chen *et*

*al*., 2005). The data are presented as copy number per cell. As in relative PCR, data are presented in reference to another gene often represented as the internal control. We perform absolute quantification only when precise copy number of gene is required, for example, calculation of viral load (Niesters, 2001). The disadvantage of absolute PCR is the increased effort to generate a standard curve.

Various strategies have been developed to represent the relative gene expression level data such as efficiency correction method, sigmoid curve fitting method, and comparative $C_T$ method (also known as the $2^{-\Delta\Delta C_T}$ method). $C_T$ method is best among all (Livak and Schmittgen, 2001). Though despite being the best method so far, one should not forget that the comparative $C_T$ method makes several assumptions, including that the efficiency of the PCR is close to 1 and the PCR efficiency of the target gene is similar to the internal control gene (Livak and Schmittgen, 2001).

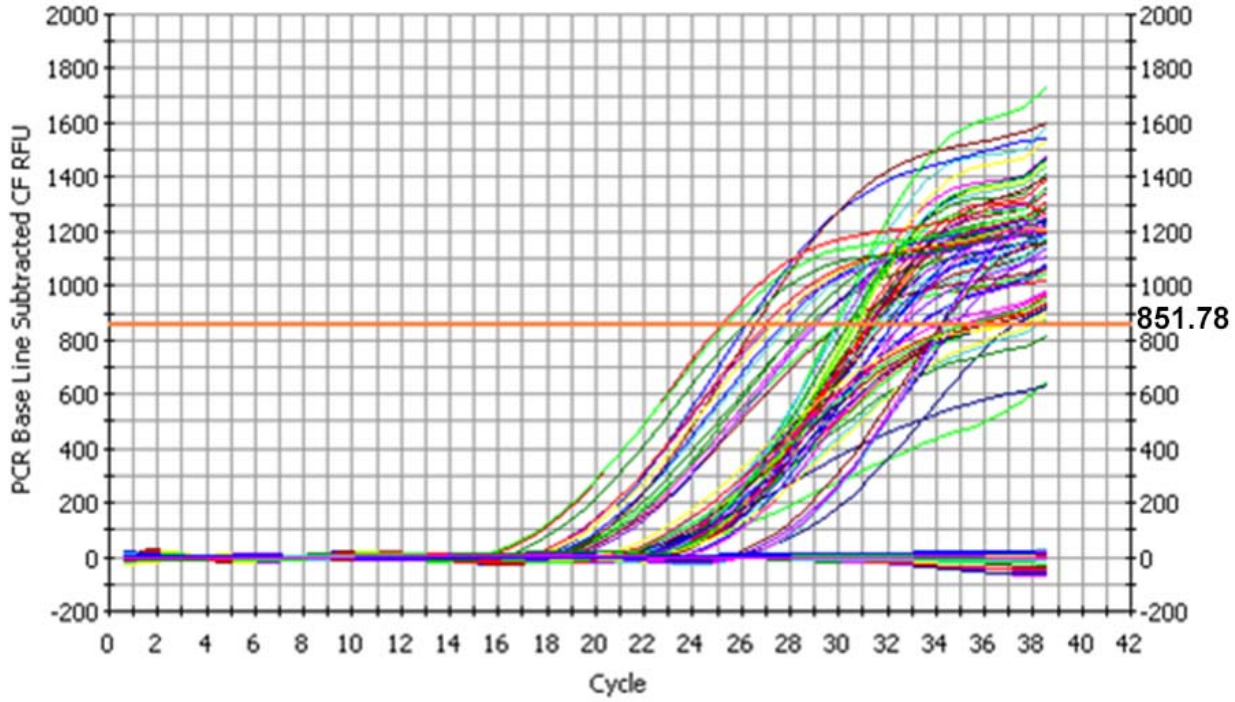


Figure 2. Demonstration of Real-time qPCR output by the calculation of $C_T$ values. Data from a typical real-time PCR output run (40 cycles) in our. The point at which the curve intersects the threshold (horizontal orange line), which corresponds to 851.78, is the $C_T$ value.

The quantitative endpoint for real-time quantitative PCR is the threshold cycle ($C_T$). The $C_T$ is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold (Figure 2). By presenting data as the $C_T$, one ensures that the PCR is in the exponential phase of amplification. The numerical value of the $C_T$ is inversely related to the amount of amplicon in the reaction (*i.e.*, the lower the $C_T$, the greater the amount of amplicon) (Schmittgen and Livak, 2008).

$C_T$ method presents data as a fold change in gene expression.

Equation 1

$$\text{Fold change} = 2^{-\Delta\Delta CT}$$

This equation is used to compare gene expression in two different samples (Sample A and Sample B); each sample is related to internal control gene, which could be a house keeping gene like GAPDH. Sample A can be the treated/ experimental sample, whereas Sample B is untreated (control). Also, Sample A can be the diseased (experimental) form and Sample B then can be normal/wild-type state (control). Sample A can be affected with Virus, and Sample B is not. Expanding the equation 1 in its full form:

Equation 2

$$2^{-\Delta\Delta CT} = [(C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ sample A} - (C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ sample B}]$$

Statistical analysis was performed using two-way analysis of variance (ANOVA) and Student's t-test for independent samples. Samples were run in triplicates ($n = 3$). Statistical significance was determined with 95% confidence ($p < 0.05$). Calculating such values for a variety of genes could be cumbersome at the undergraduate level, so we have developed a predesigned template for Microsoft Excel that can be used for undergraduates to calculate the final value with precision. It will also help them to calculate Standard deviation and P values. The results were graphed using Microsoft Excel (Figure 3A). We compared the expression levels of a gene *suppressor of stellate like protein* (*ssl*) between control and an experiment where we targeted the expression of a regeneration cascade gene in the third instar larval eye imaginal disc. We found that *ssl* levels were downregulated ~10 folds as compared to the wild-type third instar larva (Figure 3B). These results provided a quantitative estimate to our prior results from RNA Sequencing approaches. It further demonstrates that RT-qPCR can be an excellent tool for comparing gene expression levels.

**Advantages**

1. RT-qPCR is less time consuming and less cumbersome.

2. RT-qPCR has the ability to monitor the progress of PCR reaction as it occurs in real time. It also has the ability to precisely measure the amount of amplicon at each cycle. This allows highly accurate quantification of the amount of starting material in samples.

3. RT-qPCR can produce quantitative data with an accurate dynamic range and does not require post-amplification manipulation (Morrison *et al*., 1998).

4. RT-qPCR assays are 10,000- to 100,000-fold more sensitive than RNase protection assays (Wang and Brown, 1999), 1000-fold more sensitive than dot blot hybridization (Malinen *et al*., 2003), 10 times more sensitive then Agarose gel electrophoresis (Fellahi *et al*., 2016), and can even detect a single copy of a specific transcript (Palmer *et al*., 2003).

5. In addition, RT-qPCR assays can reliably detect gene expression differences as small as 23% between samples (Gentle *et al*., 2001) and have lower coefficients of variation (SYBR® Green at 14.2%; TaqMan® at 24%) than end point assays such as band densitometry (44.9%) and probe hybridization (45.1%) (Schmittgen *et al*., 2000).

**Conclusion**

The majority of the laboratory exercises taught in the undergraduate laboratories are either from commercially developed expensive kits or other conventional experimental labs that do not emphasize communication skills through the use of graphs. Our laboratory exercises (Puli and Singh, 2011; Tare *et al*., 2009; Tare and Singh, 2008), including this one, address this problem and are designed to expose students to

basic lab skill sets (a) involving RNA extraction and cDNA preparation, (b) Preparing sample for RT-qPCR, (c) Analyzing real time RT-qPCR data.

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A

| | *ssl* | | House keeping Gene |
|---|---|---|---|
| | control | Experiment | 18.9 |
| CT | 25 | 27.7 | 19.7 |
| | 24.7 | 27.6 | 22.1 |
| | 25.2 | 28 | 19.2 |
| HOUSE GEN | 20.23333 | 19.83333 | 19.9 |
| DIFFERENCE | -4.7666667 | -7.8666667 | 20.4 |
| | -4.4666667 | -7.7666667 | 18.2 |
| | -4.9666667 | -8.1666667 | 19.2 |
| 2^ΔΔCt | 0.03673587 | 0.00428447 | 19.8 |
| | 0.04522716 | 0.00459198 | 19 |
| | 0.03198043 | 0.00348007 | 19.5 |
| Average | 0.03798116 | 0.00411884 | 20 |
| Ratio | 0.9672131 | 0.11280522 | 17.7 |
| | 1.190779 | 0.12090164 | 17.9 |
| | 0.84200791 | 0.09162631 | 17.5 |
| Average | 1 | 0.10844439 | 16.5 |
| St Dev | 0.17668207 | 0.01511701 | 16.6 |
| P Value | | 0.01236584 | 18.3 |

B

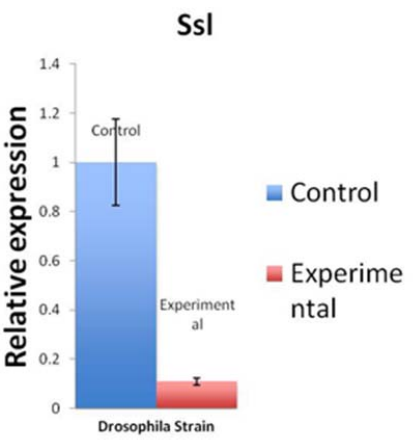


Figure 3. Graphical representation showing relative fold change in expression level of gene *suppressor of stellate like protein* (*ssl*) between control and experimental third instar larval eye imaginal disc. (A) A Microsoft Excel sheet template was designed with pre-inserted formulas to make necessary calculations, *i.e.*, fold change, Standard deviation, and P values. This will allow undergraduates to insert $C_T$ values in the template and the rest of the calculations will be done automatically. Using this excel sheet, gene *suppressor of stellate like protein* (*ssl*) levels in *Drosophila* third instar larval eye imaginal disc (Control: wild-type, Canton S larval eye imaginal disc) were compared to that of experiment where we targeted the expression of a regeneration cascade gene in the third instar larval eye imaginal disc. (B) Note that *ssl* levels were down-regulated ~10 folds as compared to the wild-type third instar larval eye imaginal disc.

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