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An Undergraduate Cell Biology Lab: Western Blotting to Detect Proteins from Drosophila Eye

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An undergraduate cell biology lab: Western Blotting to detect proteins from *Drosophila* eye.

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Abstract

We have developed an undergraduate laboratory to allow detection and localization of proteins in the compound eye of *Drosophila melanogaster*, a.k.a fruit fly. This lab was a part of the undergraduate curriculum of the cell biology laboratory course aimed to demonstrate the use of Western Blotting technique to study protein localization in the adult eye of *Drosophila*. Western blotting, a two-day laboratory exercise, can be used to detect the presence of proteins of interests from total protein isolated from a tissue. The first day involves isolation of proteins from the tissue and SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) gel electrophoresis to separate the denatured proteins in accordance to their molecular weight/s. The separated proteins are then transferred to the Nitrocellulose or Polyvinylidene difluoride (PVDF) membrane in an overnight transfer. The second day lab involves detection of proteins (transferred to the membrane) using Ponceau-S stain, followed by immunochemistry to detect the protein of interest along the total protein transferred to the membrane. The presence of our protein of interest is carried out by using a primary antibody against the protein, followed by binding of secondary antibody which is tagged to an enzyme. The protein band can be detected by using the kit, which provides substrate to the enzyme. The protein levels can be quantified, compared, and analyzed by calculating the respective band intensities. Here, we have used fly eyes to detect the difference in level of expression of Tubulin (Tub) and Wingless (Wg) proteins in the adult eye of *Drosophila* in our class. The idea of this laboratory exercise is to: (a) familiarize students with the underlying principles of protein chemistry and its application to diverse areas of research, (b) to enable students to get a hands-on-experience of this biochemical technique. Keywords: *Drosophila melanogaster*, eye, Western Blot, protein estimation. localization of proteins, SDS-PAGE gel electrophoresis.

Introduction

Recent educational research on teaching biology to undergraduates has raised concerns about how traditional approaches in large classes fail to reach many students and thereby emphasized on the need for more hand-on experiential learning instructions (Puli and Singh, 2011; Tare *et al.*, 2009; Tare and Singh, 2008; Uman and Singh, 2011; Wood, 2009; Woodin *et al.*, 2009). One of the hallmarks of the modern day science education is experiential learning, which allows students to get a hands-on-experience to understand latest scientific research and concepts. In modern day undergraduate curriculum, research is an important component of habits of inquiry and learning (Puli and Singh, 2011; Tare *et al.*, 2009; Tare and Singh, 2008; Uman and Singh, 2011). Efforts have been channeled to develop a repertoire of laboratory courses to expose undergraduates to modern day biology concepts and techniques used in biomedical research. The new text books provide exhaustive and detailed information through movies and illustrations on how proteins play a role in a biological function and what approaches can be used to determine their localization as well as

quantitate them using Western Blot approach. Despite the utility of animations and videos the best way of learning is through hands-on experiential learning (Puli and Singh, 2011; Tare *et al.*, 2009; Tare and Singh, 2008; Wood, 2009). However, it comes with a cost of time and resources. We devised a laboratory to introduce students to the Western Blot technique, its principle and applications, which will allow students to determine presence or absence of a protein in a particular tissue and how to semi-quantitatively estimate a protein in a sample. Furthermore, this exercise can be finished in two laboratory sessions with some preparation done prior to the demonstration to the students (Figure 1).

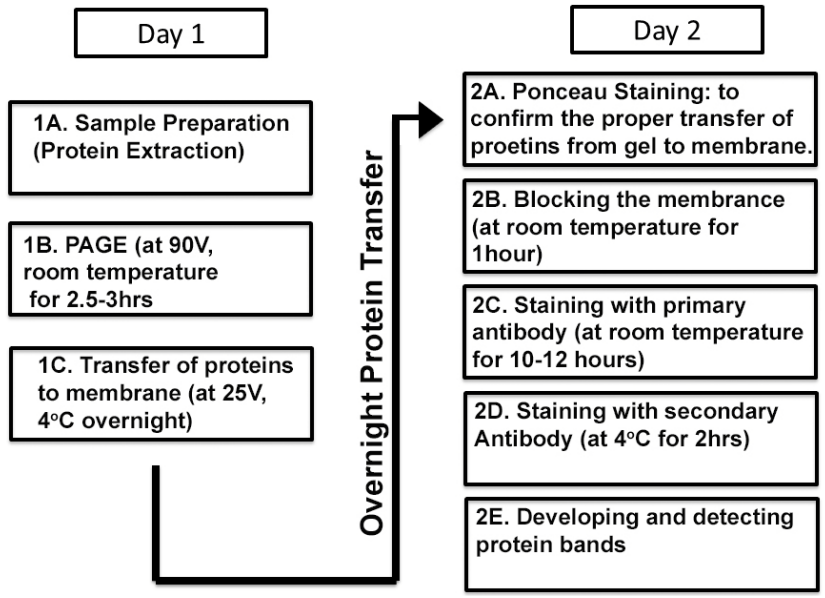

Figure 1. Schematic presentation of time line for Western Blot analysis. We have developed a two-day western blot protocol for undergraduate laboratory course. This strategy will allow demonstration of this modern day technique to undergraduate students.

We have developed this laboratory exercise to study the ubiquitously expressed Tubulin (Tub) and Wingless (Wg) protein in the adult eye of *Drosophila*. The *Drosophila* model is highly versatile as it is easy to rear flies in masses in a short period of time (Puli and Singh, 2011; Singh *et al.*, 2012; Tare *et al.*, 2009; Tare and Singh, 2008). Furthermore, there are several eye specific mutants available in flies if you want to show comparison of gene expression among various genetic backgrounds. *Drosophila* model can be easily used to demonstrate protein isolation, detection and quantitation.

Western Blotting technique (or immunoblotting) was first described by Towbin *et al.* (Towbin *et al.*, 1979). Since then, this technique has become one of the widely used techniques in the field of basic sciences. Western blot is a highly sensitive biochemical technique, which uses the property of monoclonal/polyclonal antibodies (highly specific in nature) to bind to their respective antigens. It is mainly used for the detection, presence/absence, and finding differences in the expression level of a particular protein, or in characterization of proteins (Kim, 2017). Western blot involves isolation/identification of specific proteins of interest from tissue samples, or mixture of proteins extracted from cells, which are later quantified, normalized, denatured (in order to convert their complex structure of protein into its simpler forms). The denatured polypeptides in the protein sample are then separated on the gel based on their size, molecular weight (Kilo-Daltons (kDa), using SDS-PAGE (denatured) gel electrophoresis (Kim, 2017; Mahmood and Yang, 2012). The gel containing the separated protein bands is then placed onto the nitrocellulose or PVDF membrane, and the protein bands are then electrophoretically transferred from the gel on to the membrane. These membranes are then subjected first to the blocking step (5% Bovine Serum Albumin, BSA, in order to prevent non-specific binding of antibodies onto the membrane). After the blocking step, the membranes are treated and incubated with both primary (specific to the target protein), and secondary antibodies (specific to the primary antibody, covalently bound/labeled with enzymes). The enzyme becomes active upon availability of its chromogenic substrate and causes a color reaction. The development of a colored product (using these set of specific enzyme and substrate reactions) is detected and analyzed using gel documentation (BioSpectrum 500) system.

Table 1. List of reagents and solutions used in the Western Blotting lab.

Solutions	Volume	Composition	Preparation/Catalog No.
4X Separation Buffer	500ml	Tris Base – 90g Sodium dodecyl sulfate (SDS) – 2g	Adjust the pH to 8.8 with HCl and make up the volume to 500ml with autoclaved water. Store at room temperature.
4X Stacking buffer	500ml	Tris Base- 30.25g Sodium dodecyl sulfate (SDS) - 2g	Adjust the pH to 6.8 with HCl and make up the volume to 500ml with autoclaved water. Store at room temperature.
1X Tris / Glycine / SDS buffer (Running Buffer)	1Liter	10X Tris/Glycine/SDS buffer - 100ml Autoclave Water - 900ml	100ml of 10X Tris/Glycine/SDS buffer is dissolved in 900 ml autoclaved water. Store at room temperature.
1X Tris / Glycine buffer (Transfer buffer)	1Liter	10X Tris/Glycine buffer - 100ml Methanol - 200ml Autoclaved water - 700ml	100ml of 10X Tris/Glycine buffer and 200ml of methanol is dissolved in 700ml of autoclaved water. Store at 4°C.
1X TBST (TBS Tween-20 buffer)	1Liter	20X TBS Tween-20 buffer (readymade) - 50ml Autoclave Water – 950ml	50ml of 20X TBS Tween-20 buffer is dissolved in (Make up the volume with) 950ml autoclaved water. Store at room temperature.
70% Ethanol	100ml	Reagent Alcohol - 70ml Autoclave Water - 30ml	70ml Reagent Alcohol is dissolved in 30ml of Autoclave Water and stored at room temperature.
5% BSA (Bovine Serum Albumin)	10ml	BSA – 0.5g 1X TBS Tween-20 buffer (TBST) – 10ml	0.5g BSA is dissolved in 10ml of 1XTBST and stored at 4°C.
10% APS (Ammonium persulfate) (freshly prepared)	1ml	APS – 0.1g Autoclave water – 1ml	0.1g APS dissolved in 1 ml of autoclave water in a sterile tube.
TEMED (Tetramethylethyl enediamine)	20gm (26ml)	Ready to use	Fischer Scientific, Cat. #BP150-20
2X Sample Buffer (Laemmli buffer) Concentrate	1 vial	Ready to use	SIGMA, Cat. #S3401
Phenylmethanesulfonyl fluoride (PMSF)	1ml	PMSF – 0.035g Isopropanol – 1ml	0.035g PMSF is dissolved 1 ml isopropanol in a sterile tube and stored at room temperature.
1% Glacial Acetic Acid	100ml	Glacial Acetic Acid – 1ml Autoclave Water – 99ml	1ml Glacial Acetic Acid is dissolved in 99 ml autoclaved water and stored at room temperature.
40%Acrylamide / bisacrylamide (29:1)	1Liter	Ready to use	Fischer Scientific, Cat. #BP1406-1

Protocol

The entire methodology of Western Blotting can be divided into four major steps: (1) Sample preparation, (2) SDS-PAGE gel electrophoresis, (3) Transfer of proteins to the membrane, and (4) Identification of a protein from a total protein sample using immunochemistry.

DAY I

1. Sample Preparation

We have used *Drosophila* adult eyes as the tissue source for the total protein isolation. We used fruit flies as they are easy to rear and large number of flies can be generated in a small time window as life cycle if just 12 days long at room temperature (Singh *et al.*, 2012; Tare *et al.*, 2013). The biological samples, ~25 adult fly heads are first separated from their respective adult fly bodies using sterilized tweezers and are collected in labelled tubes that are kept on ice. To each tube, 50 µl of Laemmli 2× Concentrate Sample Buffer, (SIGMA, Cat.# S3401-1VL) and 3 µl of Phenyl methane sulfonyl fluoride (PMSF) (SIGMA, Cat. #P7626-5G), a protease inhibitor, is added. The tubes are labelled and the samples are macerated thoroughly with a sterilized pestle. They are then boiled at 100°C for 10-15 minutes and are immediately kept on ice for 10 minutes. The samples are then subjected to centrifugation for 10 minutes at 10,000 rpm and then snap chilled on ice again for 10 minutes. The supernatant is transferred into a labelled fresh tube, which is later stored at a low temperature of -20°C. The total protein concentration in a sample is determined by calculating absorbance at 280 nm wavelength using spectrophotometer (Nanodrop) along with the control (2× sample buffer can be used as control. The samples are then normalized by calculating the amount of protein required for a total concentration of 30 or 40 µg/ml per well and diluting it by adding 2× sample buffer (loading a total volume of 10 µl per well).

Table 2. Recipe for preparation of SDS-PAGE gel. The reagents required for preparation of a 10% gel are mentioned below.

4X Separating Gel	Volume req. for preparing 1 gel	Volume req. for preparing 2 gels
40% Acryalmide/bisacrylamide (29:1)	1.25 ml	2.5 ml
4X separation buffer	1.25 ml	2.5 ml
Autoclaved water	2.5 ml	5 ml
10%APS (freshly prepared, stored at 4°C)	50 µl	100 µl
TEMED (stored at 4°C)	5 µl	10 µl
4X Stacking Gel	**Volume req. for preparing 1 gel**	**Volume req. for preparing 2 gels**
40% Acryalmide/bisacrylamide (29:1)	0.25 ml	0.5 ml
4X stacking buffer	0.625 ml	1.25 ml
Autoclaved water	1.625 ml	3.25 ml
10%APS (freshly prepared, stored at 4°C)	25 µl	50 µl
TEMED (stored at 4°C)	2.5 µl	5 µl

2. SDS-PAGE Gel electrophoresis

In order to save time, we sometimes use precast gels (Mini Protean stain free precast gels from Bio-Rad). For casting gels, the two glass plates are washed first with autoclaved water and cleaned with 70%

ethanol (Reagent Alcohol, Fisher Scientific, Cat. #A962-4) for setting them up in the gel apparatus. The polyacrylamide gels are formed by polymerization of acrylamide and bis-acrylamide (bis, N,N'-methylene-bis-acrylamide, Fischer Scientific, Cat. #BP1406-1). Polymerization is initiated by Ammonium Persulfate (APS) (Fisher Scientific, Cat. #BP179-100) and TEMED (Tetramethylethylenediamine) (Fisher Scientific, Cat. #BP150-20). TEMED accelerates the rate of formation of free radicals from persulfate and these in turn catalyze polymerization. Therefore, the 4× separating gel mixture is prepared first and mixed well before addition of both APS and TEMED. APS is added first followed by addition of TEMED (Table 2). The gel components are mixed thoroughly to ensure homogenous solution. The gel mixture (without any further delay) is poured inside the space (up to 70% of total size of glass plate) present between the two glass plates. Around 350 µl of 70% ethanol (or just needed enough to cover the surface) is poured on top of the polymerizing gel to prevent the gel from coming in contact with air, which may trigger rapid polymerization of only the upper part of the gel. After the gel has polymerized (after ~ 35 minutes), the 70% ethanol is poured out, and the top of the gel is washed thoroughly with autoclaved water.

The 4× stacking gel mixture is prepared in a similar fashion as 4× separating gel mixture, and the volume and concentration of chemicals required to prepare a 4× stacking gel is mentioned in Table 2. Once prepared, 4× stacking gel mixture is poured on top of the polymerized 4× separating gel using a micropipette. The combs are inserted slowly just to make sure no bubbles are trapped inside and the gel is left undisturbed to complete the polymerization process. Once the gel has completely polymerized, the gel plates are fitted inside (lower glass plate facing inside) the gel cassette (containing red-positive and black-negative electrodes). The gel cassette is then lodged inside the electrophoresis unit that contains 1× Tris/Glycine/SDS (1× TG-SDS) (10× Tris/Glycine/SDS Buffer, BIO-RAD, Cat. #161-0732) buffer (Table 1). The top of the gel cassette unit is also filled with the 1× TG-SDS buffer, which is required to complete the circuit. The combs are then taken out and wells are washed nicely with 1× TG-SDS buffer (to remove any loose pieces of acrylamide, which if left untreated, can block the wells during the gel run). The normalized protein samples are mixed with 2× Laemmli Concentrate Sample Buffer to make up the total volume to 10 µl, which is then loaded into the respective wells of the gel. A molecular weight marker (Precision Plus Protein Standards Kaleidoscope (BIO-RAD, Cat. #161-0375) is loaded (~4.5 µl) adjacent to the experimental samples in order to get an idea about the size or molecular weight of the protein of interest (Kim, 2017; Mahmood and Yang, 2012; Weber and Osborn, 1969), which is measured in Kilo-Daltons (kDa). The gel is then subjected to electrophoresis using power supply unit at 90V for 2.5-3 hours.

3. Transfer of proteins to the membrane

The 1× Tris Glycine buffer (or TG, transfer buffer) (10× Tris/Glycine Buffer, BIO-RAD, Cat. #161-0734) is prepared according to Table 1 and is kept at 4°C for pre-cooling. Both Nitrocellulose or PVDF membranes (Immun-Blot PVDF: BIO-RAD, Cat. #162-0177) can be used during the transfer process, but PVDF membranes are more durable, hydrophobic, chemically more inert (as compared to nitrocellulose membranes), which increases their potential to bind more to protein (Bass *et al.*, 2017). The PVDF membrane is cut to the size of the gel and is soaked in methanol for 5-10 mins. The membrane, 2 filter papers, 2 sponges are then transferred into 1× Tris/Glycine buffer (Table 1) in order to equilibrate them before the transfer process. The glass plates (containing polymerized gel) are taken out from the gel cassette. The upper plate is removed slowly followed by removal of stacking gel gently from the rest of the gel and the gel is poured with 1× Tris/Glycine buffer to equilibrate.

Preparation of transfer sandwich (to be carried out overnight): It is performed in a tray containing 1× Tris/Glycine (transfer buffer, Table 1). The sequence for sandwich formation is as follows - The black side of the sandwich apparatus is placed down in the tray (containing 1× transfer buffer), followed by a sponge (wetted in 1× transfer buffer) and a rectangular piece of white filter paper (wetted in 1× transfer buffer). The gel is placed and is covered by placing the nitrocellulose or PVDF membrane onto the gel (make sure no bubbles are trapped inside). The bubbles are removed by rolling a glass rod on the gel and membrane. Onto the gel and the membrane, another piece of white filter paper (wetted in 1× transfer buffer) is placed, followed

by another sponge (wetted in 1× transfer buffer). The sandwich is locked afterwards. The sandwich is then placed hinge down, with its black side towards the black side (cathode-negative) of the transfer apparatus. The transfer apparatus is filled with a small ice pack and pre-cooled 1× transfer buffer filled up to the brim. The transfer process is performed in a cold room at 25 Volts (V), at 4°C for overnight or at 60V, 4°C for 2 hours.

DAY II

After the transfer process is done, the membrane is carefully taken out from the transfer electrophoresis unit and washed three times with autoclaved water (2 minutes each). The membrane is then treated two times with 1% Glacial acetic acid solution (ARISTAR, Cat# BDH3094-2.5LG) for 5 minutes each. The membrane is then stained with Ponceu-S staining solution (SIGMA, CAT. #P7170-1L), while shaking for 5-10 minutes, and is further de-stained with 1% Glacial acetic acid (protein bands are clearly visible at this stage). Ponceau-S stain marks the protein bands. However, if the bands are not clearly visible, it doesn't always mean that it won't show any signal during the developing process, because West Dura developing kit is a lot more sensitive than Ponceau-S stain. It can detect approximately 100 ng of protein per band (Ness *et al.*, 2015). The membrane is then washed three times with autoclaved water (10 minutes each) and is then equilibrated with 1× TBST (20× TBS Tween-20 Buffer, Thermo Scientific, Cat. #28360) solution three times (10 minutes each).

4. Identification of a protein from a total protein sample using immunochemistry

Primary and Secondary Antibodies: The membrane is first blocked with 5% Bovine Serum Albumin (BSA) (Fisher Scientific, Cat. # BP1600-100) prepared in 1× TBST for 1 hour at room temperature and is then incubated with primary antibody-Monoclonal Anti-α-Tubulin antibody (1:12000) produced in mouse (SIGMA, Cat. # T5168), Monoclonal Anti-Wingless (1:500) produced in mouse (DSHB, 4D4) prepared in 5% BSA, 1× TBST, overnight at 4°C or 3-4 hours at room temperature (depending on the time available to instructor). The membrane is then washed three times with 1× TBST (10 minutes each, more or less number of washes depends on the antibody used) and is further treated with secondary antibody (Goat anti-Mouse IgG-HRP 1:5000) (Santa Cruz Biotechnology, Cat. Sc-2005) prepared in 5% BSA, 1× TBST for 30 minutes at room temperature or for 2 hours at 4°C. After secondary antibody treatment, the membrane is washed three times with 1× TBST, 10 minutes each to remove any extra unbound antibody left on the membrane to avoid nonspecific signal.

Developing protein bands and detection: The Super Signal West Dura Extended Duration Substrate Kit (Thermo Scientific, Cat. #34076) (highly sensitive in nature) is used for developing the protein bands. The kit allows detection of even mid-femto gram of antigen by oxidizing luminol based chemiluminescent substrate for Horseradish peroxidase (HRP) detection. Equal volumes of SuperSignal West Dura Stable Peroxide Buffer (Prod. # 1859025) and SuperSignal West Dura Luminol/Enhancer Solution (Prod. #1859024) (~1 ml) are mixed together in a tube to form the developing solution and is applied on to the membrane. The membrane is then shaken manually, just to make sure the developing solution covers the entire surface of the membrane. The membrane is incubated with developing solution for 5 minutes at room temperature and the solution is drained afterwards. The membrane is then analyzed and imaged in a Gel Documentation System (UVP BioSpectrum 500 Imaging System with LM-26 and BioChemi 500 Camera f/1.2, S/N021110-001) with exposure time (limit range from 5 sec to 1 minute, and longer if necessary).

Advantages of using Western blotting technique

1. One of the challenges of teaching a laboratory course is the willingness of the institution to invest in setting up the lab. Therefore, the use of cost- and time-effective exercises can facilitate easy implementation of these laboratory programs. The solutions used for the Western Blot analysis are commercially available and are inexpensive.

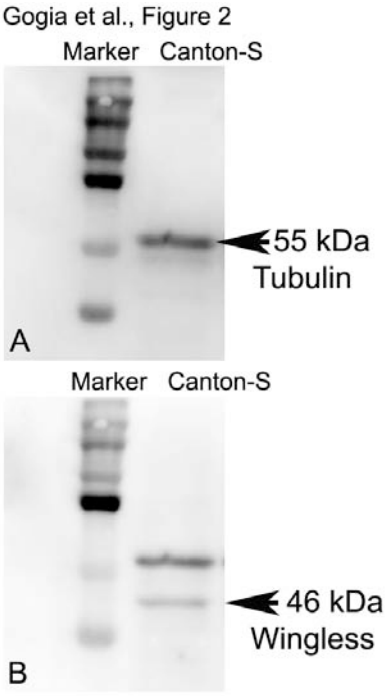

Figure 2. Western Blot Analysis to detect proteins. Total protein sample isolated from wild-type adult eye of *Drosophila* were separated by SDS-PAGE electrophoresis. The (A) Tubulin and (B) Wingless protein were detected using the Tubulin and Wingless antibody. (A) A band corresponding to 55 kDa molecular weight, was detected which corresponds to Tubulin. (B) A band corresponding to 46 kDa molecular weight, corresponding to Wingless (Wg) was detected. Images were captured using the BioSpectrum® 500 Imaging System. The same blot was first used to detect Tubulin. It was then stripped and used for detecting Wingless protein.

2. It is challenging for undergraduate students to learn these technique from books, animations, or tutorials. This teaching note will help develop experiential learning opportunities for students and learn this technique in an easy and effective manner.

3. The first step of Western blot involves separation of total proteins using SDS page electrophoresis. Therefore, this lab can be coupled with the SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) laboratory. Thus, in two days you can demonstrate two techniques as it just adds one more lab for identification of a protein using immunohistochemistry (as transfer of proteins from gel to membrane can be done overnight). Moreover, the procedure has incubation steps. The time between the incubations can be utilized for interaction with lab instructor, clarifying the concepts and class discussions.

4. The students get general overview of Western Blotting technique, which is highly sensitive and can detect as little as 0.1ng of protein. This exercise provides hands-on experience of this technique starting from sample preparation to visualizing the proteins onto the membrane.

5. The technique employs use of antigen-antibody reactions (highly specific in nature) and thus has the capability of detecting the protein of interest even from a mixture or a solution containing 300,000 diverse range of proteins. It can also help detect the immunogenic responses (caused by bacteria or viruses), or can be used to study regulation of genes known to cause asthma, allergy (García-Sánchez and Marqués-García, 2016), or for detection and diagnosis of deadly diseases like human immunodeficiency virus (HIV) (Feng *et al.*, 2017).

6. This Western blot analysis utilizes standard protein chemistry and is easy to demonstrate in a undergraduate laboratory setup as it does not need educational demonstration kits that minimize the exposure of students to details. It will add to the skill set of students and will help develop a core of trained individuals suitable for academics or industrial settings.

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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.