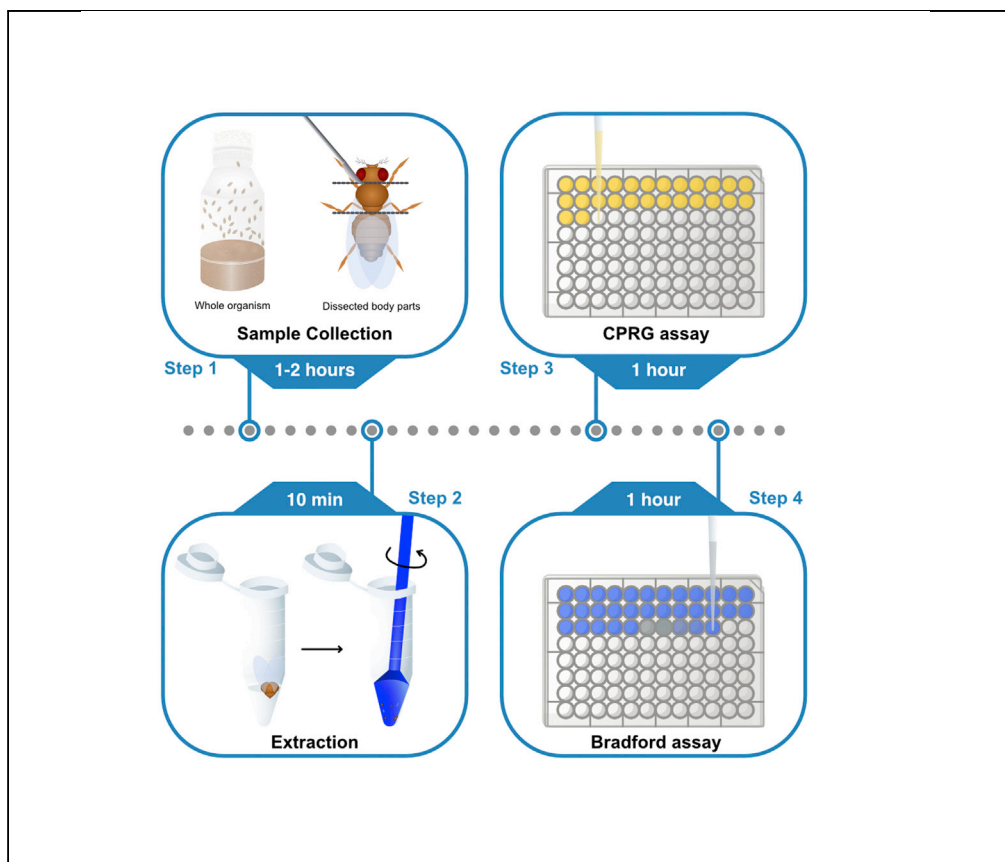


Protocol

Protocol to measure β -galactosidase in *Drosophila* extracts using a CPRG assay



The quantification of β -galactosidase activity is routinely required by laboratories worldwide. We present a cost-effective, highly replicable, simple technique for quantifying β -galactosidase-specific activity from crude extracts made from whole organisms or dissected tissues or cells. Extracts are prepared and measured without the need for any specialized equipment, and tissue is ground manually by pestle and measured by colorimetric CPRG and Bradford assays. This protocol describes the assay using *Drosophila* extracts but could be applied to any biological system of interest.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Taylor Barwell,
Laurent Seroude

9teb4@queensu.ca (T.B.)
seroude@queensu.ca
(L.S.)

Highlights

A detailed and quick protocol to prepare extracts

A step-by-step procedure to measure β -galactosidase activity and protein content

A comprehensive guide for data analysis with examples of reproducibility

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Protocol

Protocol to measure β -galactosidase in *Drosophila* extracts using a CPRG assayTaylor Barwell^{1,2,*} and Laurent Seroude^{1,3,*}¹Queen's University, Kingston, ON K7L 3N6, Canada²Technical contact³Lead contact*Correspondence: 9teb4@queensu.ca (T.B.), seroude@queensu.ca (L.S.)
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SUMMARY

The quantification of β -galactosidase activity is routinely required by laboratories worldwide. We present a cost-effective, highly replicable, simple technique for quantifying β -galactosidase-specific activity from crude extracts made from whole organisms or dissected tissues or cells. Extracts are prepared and measured without the need for any specialized equipment, and tissue is ground manually by pestle and measured by colorimetric CPRG and Bradford assays. This protocol describes the assay using *Drosophila* extracts but could be applied to any biological system of interest.

For complete details on the use and execution of this protocol, please refer to Seroude et al. (2002),¹ Poirier et al. (2008),² and Barwell et al. (2017).³

BEFORE YOU BEGIN

Since the breakthrough discovery of the lac operon by Jacob and Monod,⁴ *lacZ* has been a pillar of many techniques in molecular biology. The *lacZ*-encoded β -galactosidase enzyme is used universally as a reporter across biological systems for measuring increases in transcription or translation, yeast two-hybrid screens, or monitoring cellular senescence. In *Drosophila*, *lacZ* is frequently used to measure manipulations of gene expression. The turnover of β -galactosidase in flies has been reported to be between 5–7 h,⁵ making it ideal to be able to measure temporal changes in expression during aging. In comparison to traditional techniques like western blotting, a β -galactosidase assay is completed within an hour instead of days. Although genomic approaches allow for measurements of gene expression, the sophisticated equipment and elevated cost to execute these experiments often hampers the accessibility to perform such experiments in third world countries or laboratories with limited funding. Here, we present a fast and cheap method (~\$0.08CAD/reaction in reagents; \$2.07CAD including cost of a microplate) that can be performed with standard laboratory equipment. This protocol describes the steps for measuring *Drosophila* larvae, pupae, and whole or dissected adults. This assay uses chlorophenol red- β -D-galactopyranoside (CPRG) as substrate, which is reported to be more sensitive than o-nitrophenyl- β -D-galactopyranoside (ONPG), and for fly extracts has the advantage that the product absorbs at a wavelength that does not interfere significantly with eye pigments.⁶ This technique can be used to measure whole animal, dissected tissues or cells. Extracts can be stored at any temperature and enzyme activity perdures for up to three days after extraction (Figure 1).

Preparation of extraction buffer and substrate

© Timing: 10 min



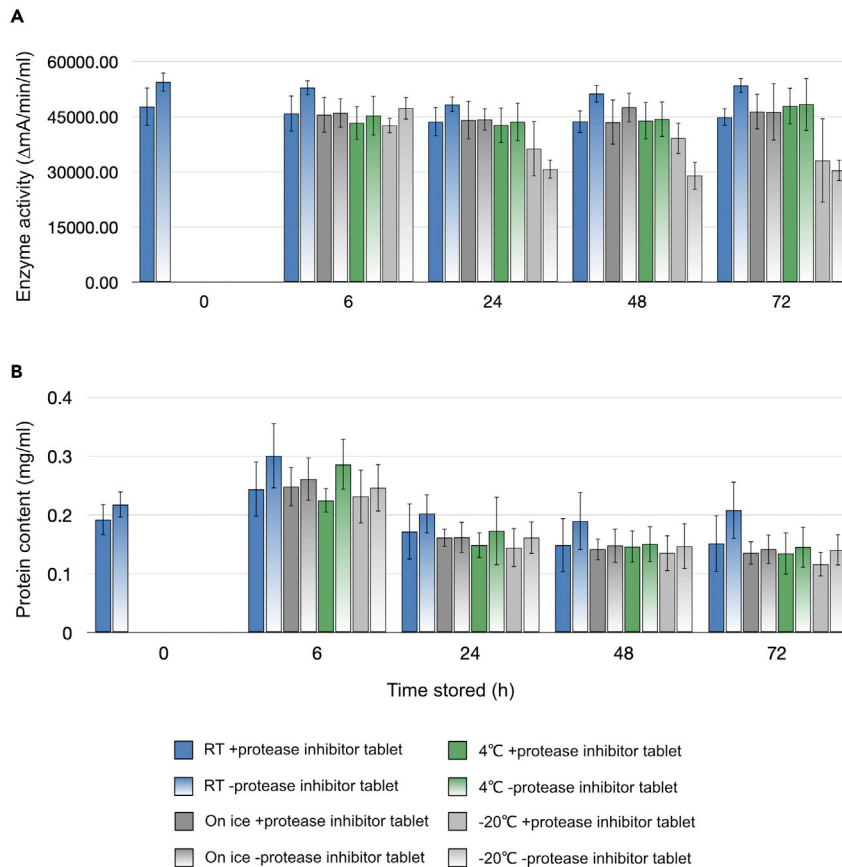


Figure 1. Effect of storage conditions on enzyme activity and protein concentration

The effect of the protease inhibitor tablet and storage temperature on enzyme activity and protein content were assessed. Extraction buffer omitting the protease inhibitor was prepared and 1 protease inhibitor tablet was dissolved in 20 mL. The extracts were prepared as follows: six flies expressing *lacZ* (DJ694, Bg2 recombinant strain) were each ground in 50 μ L of buffer without protease inhibitor. The extracts were centrifuged and 40 μ L of supernatant was recovered from each fly and pooled (240 μ L total). The extract was split into two tubes (120 μ L each) and 120 μ L of +protease inhibitor buffer was added to one tube and 120 μ L of -protease inhibitor buffer was added to the other tube. Each extract condition was measured at 0 h. From each extract tube 60 μ L was removed and stored on ice, 60 μ L was removed and stored at 4°C, and 5 aliquots of 12 μ L each were removed and stored at -20°C. The remaining extract from each tube was stored at room temperature (23°C–25°C). Measurements from each temperature condition were taken at 6, 24, 48, 72 h. The extract preparations and measurements were done in triplicate. Independent extract preparations were made to measure enzyme activity (CPRG assay) and protein content (Bradford assay). Bars represent the average of the 3 independent replicates. Error bars represent \pm SD.

(A) Effect of protease inhibitor and temperature on enzyme activity over time.

(B) Effect of protease inhibitor and temperature on protein content over time. The data set used for this Figure is provided in Data 1 (Mendeley Data: <https://doi.org/10.17632/vx8x5fx95n.1>).

1. Prepare the Extraction buffer. See recipe and storage conditions in the [materials and equipment](#).
 - a. Prepare 0.1 mL for every fly being measured, plus the volume needed to prepare the CPRG.
2. Prepare the stock solution of CPRG from powder at 20 mM using Extraction buffer as solvent.

Note: For long term storage, aliquot and store at -20°C. Aliquoting by 0.5 mL is the precise amount needed to prepare 10 mL of 1 mM CPRG, which is just enough to fill a standard 96-well microplate.

3. Dilute the CPRG to a working concentration of 1 mM using Extraction buffer as solvent.
 - a. Prepare 0.1 mL for every fly being measured.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Chlorophenol red-β-D-galactopyranoside (CPRG)	Sigma Aldrich	Cat#59767
cComplete, Mini, EDTA-free protease inhibitor cocktail	Roche	Cat#11836170001
Na ₂ HPO ₄	BioShop	Cat#SPD307
NaH ₂ PO ₄	BioShop	Cat#SPM306
MgCl ₂	BioShop	Cat#MAG510
Bovine serum albumin (BSA)	BioShop	Cat# ALB001
Critical commercial assays		
Bio-Rad protein assay dye reagent concentrate	Bio-Rad	Cat#5000006
Software and algorithms		
SoftMax Pro	Molecular Devices	https://www.moleculardevices.com/products/microplate-readers/acquisition-and-analysis-software/softmax-pro-software
Microsoft Excel		
Other		
SpectraMax 190	Molecular Devices	Cat#190
Micropestles	Sigma Aldrich	Cat#Z359947
Corning® 96-well EIA/RIA Clear Flat Bottom Polystyrene Not Treated Microplate	Corning	Cat#9017
Thermo Scientific Medifuge Small Benchtop Centrifuge	Fisher Scientific	Cat#13-101-501
PIPETMAN Classic Starter Kit P20, P200, P1000	Gilson	Cat#F167300

MATERIALS AND EQUIPMENT

Extraction buffer		
Reagent	Final concentration	Amount
Na ₂ HPO ₄ /NaH ₂ PO ₄ (100 mM pH 7.2) ¹	50 mM	25 mL
MgCl ₂ (10 mM) ²	1 mM	5 mL
cComplete™ protease inhibitors cocktail tablet (Roche) ³	1 tablet/50 mL	1 tablet
ddH ₂ O	N/A	20 mL
Total	N/A	50 mL

Extraction buffer can be stored at 4°C up to 1 week. 1: Na₂HPO₄ and NaH₂PO₄ are stored as 1 M at 23°C–25°C up to 2 years. The 100 mM solution can be stored at 23°C–25°C up to 1 month. 2: MgCl₂ is stored at 1 M at 23°C–25°C up to 2 years. The 10 mM solution is stored at 23°C–25°C up to 1 month. 3: Tablets are stored at 4°C up to expiration date.

△ **CRITICAL:** MgCl₂ cannot be omitted. In our experience, most accidental omissions did not impact the assay but in few occurrences the enzyme activity was reduced or abolished.

Note: Protease inhibitor is not strictly required but prolongs the enzyme activity over time if stored at –20°C. The effect of storage temperature and protease inhibitor on the longevity of enzyme activity and protein content was assessed (Figure 1). It can be seen that only after 24 h at –20°C does the enzyme activity start to decline (Figure 1A). The addition of the protease inhibitor has a tendency to slow this decline slightly, at least until 48 h. In terms of protein content, no matter the storage temperature the protein content starts to decline after 24 h (Figure 1B).

Alternatives: The SoftMax Pro software and SpectraMax 190 microplate reader can be substituted with any microplate reader and associated software. The Corning 96-well microplates used here can also be substituted with any clear, untreated microplate. Microsoft Excel is used here for data processing, but any spreadsheet software could be used instead such as Apple Numbers or Linux LibreOffice. Any benchtop microcentrifuge can be used instead of the one from Thermo Scientific. Other brands of micropipettes may be used instead, but using Gilson is strongly recommended for their uncontested accuracy and durability (and easily repairable).

Optional: A Microsoft Excel template is provided which if desired can be used to calculate the preparation of all solutions used, provides a blank table in which to copy raw data, graphs the data, and calculates specific activity automatically. Two versions of the template are provided in [supplemental information](#), a version which is empty ([Data S1](#)) and ready to be used, and another which has been filled with sample data ([Data S2](#)) to demonstrate how the template works.

STEP-BY-STEP METHOD DETAILS

Extract preparation

⌚ **Timing:** 1–2 h

Collect and grind fly in extraction buffer.

1. Aliquot 100 μL of Extraction buffer into 1.5 mL microcentrifuge tubes. Prepare as many tubes as the number of organisms to be processed.

Note: Generally, five individuals of a given genotype or treatment are measured per experimental replicate. The variability resulting from pipetting error is negligible (<1.5%), however performing reactions in duplicate or triplicate may be necessary for inexperienced experimentalists, or when using low grade or homemade micropipettes. At least four experimental replicates are measured and presented data represent the average of all individuals across all four replicates ([Figure 2](#)).

2. Collect samples to be measured and deposit one organism into each microcentrifuge tube of Extraction buffer.

Note: Whole organisms may be used, or specific body parts may be dissected. Here, whole L3 larvae and early pupae were used and adult thorax dissections were performed as follows.

- a. Use anesthesia to knock out the flies.
 - b. Use forceps to grasp the legs and position the fly.
 - c. Use a scalpel to sever the head and abdomen.
 - d. Using the forceps to deposit one dissected thorax into each microcentrifuge tube of Extraction buffer.
3. Grind the tissue.
 - a. Using a micropestle grind the thorax by hand in the Extraction buffer until it is clearly broken apart.

Note: Try and be as consistent as possible with grinding the tissue samples. For example, apply the same number of turns (or strokes) of the micropestle. It does not take great effort or time to grind fly samples, and only requires visual inspection to confirm that the sample has been broken apart. Using mechanical homogenizers are not necessary, unless you are working with another organism that has more robust tissue.

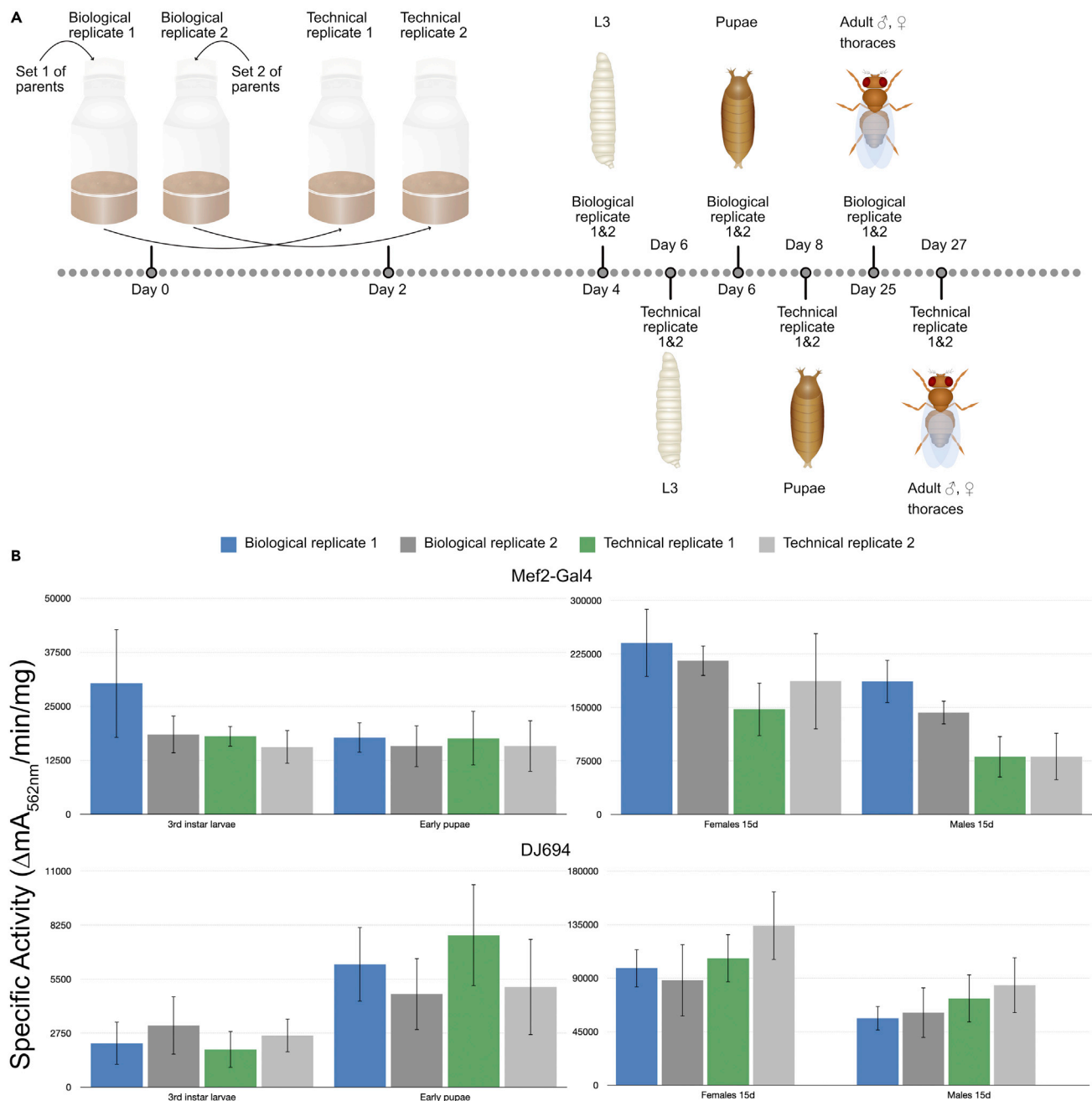


Figure 2. Reproducibility of specific activity across replicates

(A) Schematic timeline showing how the 4 different replicates (Biological 1 & 2, Technical 1 & 2) were produced and workflow of when measurements were taken. Flies were raised at 29°C.

(B) The graphs compare the level of specific activity ($\Delta mA_{562nm}/min/mg$) between the 4 replicates. Two different muscle-specific GAL4 drivers were used, Mef2-Gal4 (top graphs) (BDSC: 27390) and DJ694 (bottom) (BDSC: 8176), which were crossed with a second chromosome insertion of UAS-lacZ (Bg2) (BDSC: 1776). Measurements were performed on whole third instar larvae (L3) and early pupae (left graphs), and adult male and female thoraces (15d old) (right graphs). Bars represent the average of 5 individuals from a given replicate. Error bars represent \pm SD. The data set used for this Figure is provided in Data 2 (Mendeley Data: <https://doi.org/10.17632/k2m2m3jgmn.1>).

4. Centrifuge the extracts at 10,000 g for 1 min.

△ CRITICAL: Positive and negative controls. It is critical to include a sample that is known to have no activity as a negative control. The residual activity that may be observed in some

situations should be subtracted from the activity of the experimental extracts. The detection of significant activity in the negative control would happen if the pH of the Extraction buffer is incorrect (See troubleshooting, [problem 2](#)). When the bipartite UAS/GAL4 system is used to express *lacZ*, negative control genotypes are generated in which one component is missing (UAS alone). It is also important to include a positive control sample. In *Drosophila* there are a multitude of genotypes that are easy to obtain or generate. We routinely use *lacZ* enhancer trap strains that do not require any crosses, such as *arm-lacZ* (BDSC: 7370). In other systems one could acquire purified β -galactosidase preparations, *E. coli* extracts or saliva.

Note: Cold temperature for extraction is not critical. Both enzyme activity and protein content perdure for hours at 23°C–25°C. Freezing diminishes enzyme activity and protein content declines no matter the temperature after 24 h ([Figure 1](#)).

Alternatives: Since *lacZ* expression is restricted to the adult muscle in the samples used, thorax dissections were performed in order to enrich extracts and reduce variability caused by the inclusion of non-expressing tissues. Dissection procedures may be altered or omitted depending on specific needs.

Enzyme measurement

⌚ Timing: 1 h

Fill the microplate and measure using the spectrophotometer with microplate reader.

5. Set the microplate reader.
 - a. Kinetic measurements (every 30 s, for 30 min) at wavelength 562 nm.
6. For each extract, pipette the volume of extract to be tested (2–10 μ L) into a well of a microplate.

Note: This range of volumes is appropriate for 100 μ L *Drosophila* extracts, but the exact volume may have to be adjusted for samples with very high activity. The extraction volume and the volume to measure will be determined in a pilot experiment when extracts are made from other sources.

7. Distribute 100 μ L of 1 mM CPRG into each well.

Note: Since the volume of CPRG is at least ten times the volume of extract, mixing is not strictly required. When the option is available the microplate reader can be configured to shake the microplate to ensure reactions are well combined.

8. Place the microplate in the reader and hit Start.
9. After the measurements have all been taken, the data can either be exported as a plain text (.txt) format and copied into a desired spreadsheet software and proceed to steps 10 and 11.

Optional: Alternatively, export the data as a plain text (.txt) format and copy the data into the optional LacZ template empty.xls file ([Data S1](#)) and proceed to Optional steps for automated data processing.

10. Plot the readings for each extract (Absorbance vs Time).
11. Calculate SAV, the enzyme specific activity per volume of extract ($\Delta A/\text{time}/\text{volume}$). The calculations are done to get the activity in $\Delta \text{mA}/\text{min}/\text{mL}$ (change in milli-absorbance per minute per mL of extract). In all below equations units of each variable are indicated in square brackets.

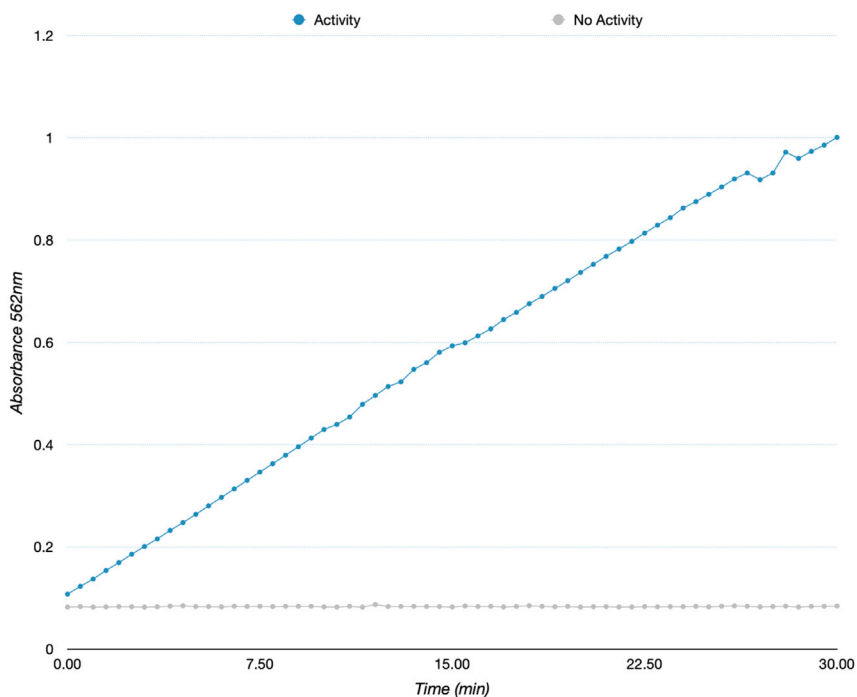


Figure 3. β -galactosidase enzymatic activity

Typical example of activity measurement plotted as absorbance over time from an extract with activity (blue) and with no activity (grey).

- Evaluate the 'Absorbance vs Time' plots. Positive controls should show activity and negative controls should show little or no activity. [Figure 3](#) shows an example of an extract with no activity.
- For all measurements, select points from the 'Absorbance vs Time plot' where the curve is linear (close to the start of the reaction). [Figure 4](#) shows an example of selecting points from a plot where curve plateaus. Calculate the enzyme activity (EA) as the change in absorbance per minute ($\Delta A/\text{min}$) using the slope between the two points:

$$EA [\Delta A / \text{min}] = \frac{\text{Absorbance at time 2 [A]} - \text{Absorbance at time 1 [A]}}{\text{time 2 [min]} - \text{time 1 [min]}}$$

We convert the unit of EA from $\Delta A/\text{min}$ to the change in milli-absorbance per minute ($\Delta \text{mA}/\text{min}$) by multiplying the EA by 1000. This is optional but is done to avoid working with values with a large number of decimal places.

$$EA [\Delta \text{mA} / \text{min}] = EA[\Delta A / \text{min}] \times 1000$$

- Calculate the specific enzyme activity per volume (SAV) in $\Delta \text{mA}/\text{min}/\text{mL}$:

$$\text{SAV} [\Delta \text{mA} / \text{min} / \text{mL}] = \frac{EA[\Delta \text{mA}/\text{min}]}{\text{volume of extract measured [mL]}}$$

- Average the SAV from the negative controls. This represents the background activity BSAV.
- Subtract the background activity from each experimental measurement to obtain the corrected enzyme activity CSAV.

Note: To ensure that the rate of the reaction is not influenced by the concentration of substrate and instead is only influenced by the concentration of enzyme in the extract, 1 mM

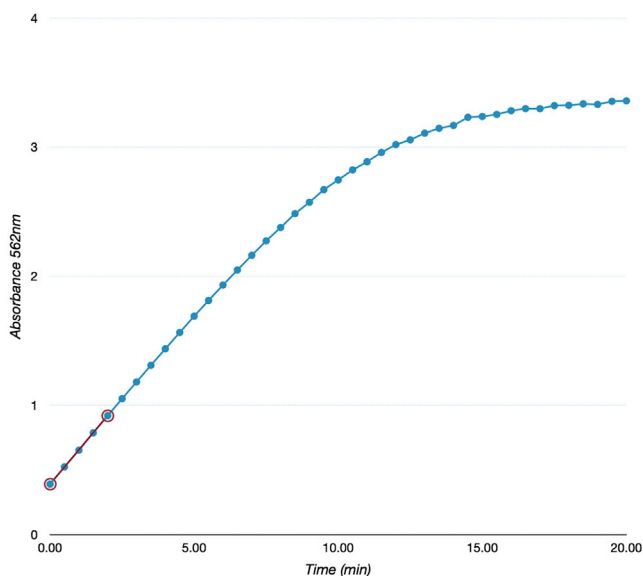


Figure 4. Selecting data points to calculate enzyme activity

Typical example of activity measurement plotted as absorbance over time from an extract with high activity. Since the plot of the activity is not linear, points are selected near the start of the reaction where the slope is linear (highlighted in red).

CPRG (step 7) is used.⁷ The reaction is initiated as soon as CPRG is added. For a full microplate one would perform pilot measurements to determine the volume of extract appropriate to set reaction rates tailored to the speed of the experimentalist.

When measuring genotypes where no activity is expected, as in negative controls, it is important to be able to distinguish between a reaction that yields low levels of activity, versus no activity. It is important to take a sufficient number of readings (step 5a) to be sure of the shape of the curve. Especially, at such low levels there can be “noise” or slight fluctuations in the readings that may appear like activity when only looking at very few points. Figure 3 demonstrates an example of a reaction with activity versus one without.

It is also very important to ensure the selection of the points used in the activity calculations is done accurately (step 11b). With high concentrations of enzyme, it is possible for the reaction to plateau. It would be inappropriate to take the linear regression through the entire curve, as it would underestimate the true level of activity. Figure 4 demonstrates how to select points in a situation where the curve plateaus. It is most accurate to take points as close to the start of the reaction as possible.

Optional: The length of measurement time, and the number of readings taken can be revised for specific needs (step 5a). The volume of extract measured (step 6) can be revised depending on the concentration of samples being measured. The following steps describe how to use the optional Lac template empty.xls file (Data S1) (step 9):

- i. The empty template file and a filled template file with example data are provided in the [supplemental information](#). In the provided template, all cells that require information to be filled are highlighted in orange. Enter sample names how they will be loaded in the plate in the ‘Raw data table.’ The number of samples will be calculated automatically and used to automatically calculate the preparations of the Extraction buffer, Substrate, and Bradford dye. All cells with values to be pipetted are highlighted in green.
- ii. Enter the mass of BSA weighed for stock preparation. The volume of solvent to add will be automatically calculated.

- iii. Enter names for the BSA standard curve dilutions and the volume prepared of each. The volumes to pipette will be automatically calculated.
- iv. Once the CPRG assay has run, export the data in a plain text format (.txt). Copy the absorbance readings into the Raw data table in the template. The data will be plotted automatically in graphs at the bottom of the table.
- v. At the bottom of the Raw data table there are three additional rows 'Start,' 'End,' and 'R².' These rows are used to determine the appropriate data points which will be used to calculate the slope of the enzyme activity curves. Enter time points in the 'Start' and 'End' rows. Looking at the graphs should give an idea of where the curves are linear and where they start to plateau. The R² value (square of Pearson product-moment correlation coefficient) is calculated to test the linearity between the selected 'Start' and 'End' points. Perfect linearity will give a value of 1. The R² value will be calculated automatically. Non-adjacent points must be selected to assess linearity with an R² value. The cut-off value of 0.999 is used. Conditional formatting has been applied to highlight R² values that are <0.999, which would indicate the slope is changing (plateauing). If the returned R² value is highlighted, select new time points.
- vi. Below the Raw data table are the CPRG and Bradford data analysis tables. The activity will automatically be calculated under the 'ΔmA/min/mL' heading using the time points selected in the 'Start' and 'End' rows.

Protein quantification

⌚ Timing: 1 h

Perform Bradford assay for quantification of protein content in extracts.

12. Prepare a standard curve using BSA.
 - a. Prepare a stock solution of BSA at 2 mg/mL.
 - b. Perform 2-fold serial dilutions to prepare 1, 0.5, 0.25 and 0.125 mg/mL BSA solutions that will be used to generate the standard curve. Use Extraction buffer as the 0 mg/mL BSA.
13. Prepare the Bradford dye from concentrate (Bio-Rad Protein Assay Dye Reagent Concentrate).
 - a. Dilute concentrate 5-fold. Prepare 0.2 mL for each concentration in the standard curve, plus each extract to be quantified.
14. For each extract, pipette the volume of extract to be measured (2–10 μL) into a well of a microplate.
15. For each concentration of the standard curve, pipette 10 μL into a well of a microplate.
16. Distribute 200 μL of diluted Bradford dye into each well.
17. Incubate at 23°C–25°C for at least 5 min before measuring.

⚠ **CRITICAL:** Incubation time must not exceed 1 h, as the dye-protein complex will precipitate.

18. While incubating, set the microplate reader.
 - a. Single measurement at wavelength 595 nm.
 - b. After incubation, place the microplate in the reader and hit Start.
19. If using a preferred spreadsheet software, export the data in a plain text format (.txt) and use steps 20 and 21 to calculate the specific activity.

Optional: If using the optional LacZ template empty.xls file ([Data S1](#)), export the data in a plain text format (.txt) and proceed to Optional steps below for automated data processing.

20. Determine the protein concentrations of the extracts. The below calculations compute the concentration in mg/mL.

- a. Correct the absorbance readings of the Bradford assay. Subtract the absorbance of the 0 mg/mL BSA sample from all measurements. The new values represent the corrected absorbance readings. The 0 mg/mL BSA sample should have an absorbance of 0.
- b. Create a plot of the standard curve with the corrected absorbance readings of the BSA samples vs concentration (0, 0.125, 0.25, 0.5, 1 mg/mL).
- c. Fit the corrected absorbances from each of the extracts to the standard curve to determine the concentration (FC).
- d. Obtain the protein concentration (PC) by multiplying with the ratio of BSA sample volume to extract volume:

$$PC[\text{mg} / \text{ml}] = FC[\text{mg} / \text{ml}] \times \frac{\text{volume of BSA measured} [\text{ml}]}{\text{volume of extract measured} [\text{ml}]}$$

21. Calculate the specific activity ($\Delta\text{mA}/\text{min}/\text{mg}$).

$$\text{Specific activity} [\Delta\text{mA} / \text{min} / \text{mg}] = \frac{\text{CSAV}[\Delta\text{mA}/\text{min}/\text{ml}]}{\text{PC} [\text{mg}/\text{ml}]}$$

Optional: The volume of extract measured (step 14) can be revised depending on the concentration of samples being measured. All absorbances should plug between experimental points of the standard curve. The following steps describe how to use the optional Lac template empty.xls file ([Data S1](#)) (step 19):

- vii. In the Bradford data analysis table, the loading order can be entered by filling the sample names in the 'Sample' column in correspondence with the 'Well' column. The names of the samples in the BSA standard curve should match the names given at the top in the BSA dilutions preparation table.
- viii. Once the Bradford has run, export the data in a plain text format (.txt). Copy the absorbance readings into the Bradford data analysis table under the heading 'Unblanked.'
- ix. At the very bottom of the Bradford data analysis table is the Standard curve. The template will automatically extract the absorbance readings for the BSA dilutions from the 'Unblanked' column based on the assigned names of the BSA dilutions in the 'Sample' column. The absorbances for the BSA dilutions will be entered into the Standard Curve table automatically.
- x. The 'Corrected' column takes into consideration the blank (BSA 0 mg/mL), subtracting this absorbance from the absorbance of every sample.
- xi. A graph of the Standard Curve is produced automatically using the corrected absorbances vs protein concentration.
- xii. In the 'Plug to curve' column of the Bradford analysis table, the corrected absorbances for each sample are plugged into the Standard Curve. The formula will test between which pair of points in the standard does the point plug. Then using the trend function, the concentration is obtained by plugging the absorbance to the equation of the line between said points.
- xiii. The final concentration is given in the 'mg/mL' column, where the last step is to take the value that was given by fitting to the curve and adjust based on the ratio of the volume of BSA standard loaded to the volume of sample that is loaded.
- xiv. Specific activity will be automatically calculated (' $\Delta\text{mA}/\text{min}/\text{mg}$ ' column) by looking up the sample by name and dividing the activity in $\Delta\text{mA}/\text{min}/\text{mL}$ by the concentration in mg/mL.

EXPECTED OUTCOMES

We present here a protocol for preparing extracts and quantifying β -galactosidase specific activity in a highly accessible, cost-effective manner. These assays have been performed routinely in our lab for over 20 years, by many different experimentalists. The data presented were collected by two different experimentalists and the results are reproducible even if they are measured decades apart. As previously reported, DJ694 expression is higher in females than males¹ and Mef2 expression is higher in the adults than in the third instar larvae and early pupae⁸ (Figure 2).

Here, we measure activity in whole animals or dissected thoraces, but this assay can certainly be used with any model organism, isolated tissue or cells. This assay has been successfully performed on tissues samples with a low number of cells such as *Drosophila* antennae.¹ When performing this assay on tissue with fewer cells, in order to concentrate the sample enough to see a signal, one can reduce the volume of Extraction buffer used to grind. However, this might make grinding more challenging. Alternatively, using more than one animal per extract works well. We have taken this approach when measuring first instar *Drosophila* larvae but it has the inconvenience that it increases variability due to the use of multiple individuals.³ Of course, when measuring any sample for the first time and the concentration of β -galactosidase is unknown, testing different volumes and/or concentrations of extract is required to obtain a measurable signal.

LIMITATIONS

There are two main contributors to variability in these measurements. The first comes from inconsistency in the grinding of the tissue samples, resulting in differences in the yield of extraction. It is critical to be as consistent as possible when grinding the tissue. A second source of variability depends on the genotypes being measured and how expression changes across time. If *lacZ* expression level changes greatly during certain time periods, in order to reduce variability between samples it would be pertinent to keep time windows for staged organisms as small as possible.

TROUBLESHOOTING

Problem 1

No activity is detected where there should be.

Potential solution

Having no activity is unlikely the result of a pH issue from an error with the preparation of the phosphate buffer, as the specific pH of 7.2 is used in order to differentiate activity from the endogenous *lacZ* versus activity from the exogenous *lacZ* expression.⁹ The original recipe for the Extraction buffer is derived from Simon and Lis.⁶ The potassium phosphate buffer was substituted with a sodium phosphate buffer to be amenable with procedures that involve sodium dodecyl sulfate (SDS), as potassium reacts with SDS to form flocculating potassium salt of SDS. A phosphate buffer is used as it has a pKa of 7.2. If the pH was more acidic such that the conditions were permissive to the endogenous activity, higher activity would be expected. Additionally, an incorrect pH would result in the detection of activity in the negative control.

The more likely cause of no activity would be if $MgCl_2$ had been omitted from the Extraction buffer. In our experience, most accidental omissions did not impact the assay but in few occurrences the enzyme activity was reduced or abolished. Prepare fresh Extraction buffer with $MgCl_2$ ([preparation of extraction buffer and substrate](#), step 1).

Problem 2

The negative control is positive.

Potential solution

It is important to ensure the smooth pipetting of the CPRG into the microplate to avoid splashing which may lead to contamination of the negative control ([enzyme measurement](#), step 7). Significant

activity in the negative control will occur if the pH of the Extraction buffer is incorrect and the sample is known to contain endogenous β -galactosidase (See troubleshooting, [problem 1](#)).

Problem 3

Concentration of β -galactosidase is too low to be detected.

Potential solution

Since the activity of extracts can perdure for several days even at 23°C–25°C ([Figure 1](#)), it is unlikely that the lack of activity would be attributed to sample degradation. The protocol does rely on availability of enough amount of tissue. For samples where the volume of tissue is relatively small (i.e., certain small, dissected body parts, *Drosophila* embryos, or L1 larvae) more than one organism can be measured per sample ([extract preparation](#), step 2).

Problem 4

The Absorbance vs Time curves plateau immediately.

Potential solution

The concentration of β -galactosidase is too high. Decrease the volume of extract measured or dilute the extract with Extraction buffer ([enzyme measurement](#), step 6).

Problem 5

A measurement that had been performed previously does not repeat as expected.

Potential solution

When using a frozen stock of CPRG, upon thawing some precipitation can occur (color gradient is clearly visible). Inexperienced experimentalists may neglect to vortex the thawed solution, resulting in a concentration of CPRG that is well below 1 mM ([preparation of extraction buffer and substrate](#), step 3). This would result in an enzyme activity that is much lower than expected since the binding of the substrate will influence the rate of the reaction.

Storage conditions of extracts may impact enzyme activity or protein content. At –20°C after 24 h, enzyme activity begins to decline ([Figure 1A](#)). Protein content is observed to be lower after 24 h no matter the storage temperature ([Figure 1B](#)). Lower protein content would result in elevated specific activity. Being consistent with storage of extracts is necessary for reproducible measurements.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Laurent Seroude (seroude@queensu.ca).

Materials availability

This study did not generate any unique reagents.

Data and code availability

[Figures 1](#) and [2](#) data have been deposited at Mendeley data and are publicly available Data 1 (Mendeley Data: <https://doi.org/10.17632/vx8x5fx95n.1>) and Data 2 (Mendeley Data: <https://doi.org/10.17632/k2m2m3jgm.1>) respectively.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101843>.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.B. and L.S.; Methodology, T.B. and L.S.; Validation, T.B. and L.S.; Writing - Original draft, T.B.; Writing - Review & Editing, T.B. and L.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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