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β-GLUCURONIDASE (GUS) FLUORESCENT REPORTER GENE ACTIVITY DETECTION KIT

Product No. **GUS-A**

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TECHNICAL BULLETIN

Reporter genes are "markers" widely used for analysis of mutationally altered genes as well as gene regulation and localization. The expressed reporter genes are detected by biochemical activity assay, immunological assay or by histochemical staining of tissue sections or cells.[1]

The *E. coli* GUS (β-glucuronidase) gene is extensively used as a gene fusion marker for analysis of gene expression in transformed plants. The GUS reporter gene system has many advantages including the stability of the expressed *E. coli* GUS enzyme and the low intrinsic activity of GUS in higher plants. The enzyme does not interfere with normal plant metabolism, and it remains active when fused to other proteins at its amino terminus making it useful for study of organelle transport in plants.[2,3,4,5] GUS transformed plants develop normally and are healthy and fertile.

The *E. coli* β-glucuronidase enzyme hydrolyzes D-glucuronic acid conjugated through a β-O-glycosidic linkage to an aglycone. The compound, 4-methylumbelliferyl glucuronide (4-MUG) is a GUS substrate, which upon hydrolysis produces the fluorescent 4-methylumbelliferone (4-MU). It is used in this fluorescent activity detection test designed for plant tissues expressing the *E. coli* GUS enzyme.

Wild-type and transformed plants are separately extracted with phosphate-EDTA buffer, pH 7.0, supplemented with detergents. The extracted β-glucuronidase hydrolyzes the 4-MUG to glucuronic acid and the fluorescent compound 4-MU. The reaction is stopped with sodium carbonate solution; raising the pH also serves to enhance the fluorescence of the 4-MU. The 4-MU can be excited at 365 nm; it's emission maximum is at 455 nm. *E. coli* β-glucuronidase serves as a positive control enzyme.

This kit includes all buffers, substrate and reagents required for highly sensitive, easy to perform quantitative activity assays.

Unit Definition

One unit of β-glucuronidase will release one pmole of 4-MU from 4-MUG per minute at pH 7.0 and 37°C.
Note: This unit definition is different from Sigma's Fishman units for Product No. G 5897.

Reagents Provided

This kit is sufficient for 200 tests.

- 5X Extraction Buffer, Product No. E 4398 25 ml
  250 mM sodium phosphate, pH 7.0, containing 50 mM EDTA, 50 mM β-mercaptoethanol, 0.5% sodium n-lauroylsarcosine and 0.5% Triton X-100
- 4-Methylumbelliferyl β-D-Glucuronide, 25 mg
  (4-MUG Substrate), Product No. M 5664
- 4-Methylumbelliferone Standard, 25 mg
  (4-MU), Product No. M1508
- β-Glucuronidase Positive Control 1 vial
  From *E. coli,* Product No. G 5897
  Minimum 1000 Fishman units per vial
- 5X Stop Solution, Product No. S 5930 100 ml
  1 M sodium carbonate

Reagents and Equipment Required but not Provided

Sigma product numbers are given where appropriate

- Liquid nitrogen
- Temperature controlled 37 °C water bath
- 10 mm x 75 mm glass test tubes
- Microcentrifuge tubes
- Mortar and pestle
- Overhead stirrer
- Disposable pestles
- Glass beads, Product No. G 4649
- Fluorimeter and cuvets

Precautions and Disclaimer
Sigma's β-Glucuronidase Activity Detection Kit is for laboratory use only. Not for drug, household or other use.

Storage
Store kit at –20 °C.

Preparation Instructions
Preparation of working solutions
1. Extraction buffer:
   Mix thawed 5X extraction buffer thoroughly before diluting 1:5 with deionized water.
2. Substrate solution:
   Dissolve 4-MUG at a concentration of 0.7 mg/ml (2 mM) in extraction buffer.
3. Calibration standard:
   Dissolve 2.0 mg 4-MU in 10 ml deionized water to make a 1 mM stock solution. Dilute 100 µl of the stock solution to a volume of 10 ml with deionized water (1:100) to make a 10 µM intermediate stock solution. Dilute 50 µl of the intermediate stock solution to a volume of 5 ml with 1X stop solution to make the 100 nM working solution. Use freshly prepared solutions.
4. Positive control enzyme solution:
   Reconstitute β-glucuronidase with 10 ml of a 50% glycerol solution in water. Aliquot and store enzyme stock solution at –70 °C. Dilute enzyme stock solution 1:20 with extraction buffer by adding 50 µl enzyme stock solution to 950 µl extraction buffer.
5. Stop solution:
   Mix thawed 5X stop solution thoroughly before diluting 1:5 with deionized water.

Fluorimeter settings:
Set fluorimeter at 365 nm excitation, 455 nm emission, 5 nm slide width. Zero the instrument using 2 ml stop solution

Procedure
A. Plant Extraction
This procedure may be modified, or replaced with a preferred procedure.
1. Weigh 50 mg plant tissue into a vial.
2. Freeze with liquid nitrogen.
3. Grind frozen tissue with mortar and pestle.
4. Transfer ground tissue as a suspension to a microcentrifuge tube.
5. Allow the liquid nitrogen to evaporate.
6. Add 500 µl extraction buffer and a few glass beads to the microcentrifuge tube.
7. Homogenize tissue with a disposable pestle connected to an overhead stirrer for 5 minutes.
8. Centrifuge for 10 minutes at 14,000 rpm. Remove supernatant and discard the pellet.
9. Aliquot the supernatant in 50 µl portions into several vials.
10. Determine the protein concentration in mg/ml of material in one of the vials.
11. Either use the plant extract immediately or freeze at –70 °C. **Do not freeze the extract at –20 °C**; enzyme activity is lost at –20 °C.

The reaction is carried out either as a single reaction assay or as a multiple reaction assay from which aliquots are removed at various time points. The reaction is performed in extraction buffer containing 1 mM 4-MUG at 37 °C. The reaction is initiated by the addition of plant tissue extracts or positive control, and is stopped by transfer of aliquots from the reaction assay to 2 ml of cold stop solution.

B. Calibration Procedure[6]

Single reaction assay: Since the activity of β-glucuronidase in the extract is unknown, perform a preliminary single reaction assay to find the proper dilution factor so that the measured values fall within the fluorimeter's detection range.
1. Prepare one vial containing 2.0 ml stop solution and place in an ice bath.
2. Pipet 100 µl substrate solution into a tube.
3. Add 50 µl extraction buffer.
4. Pre-incubate 1-2 minutes at 37 °C.
5. Initiate reaction with the addition of 50 µl of plant tissue extract (sample volume). Note the reaction volume is 200 µl.
6. Cap tube and incubate at 37 °C for 1 hour.
7. Transfer 100 µl of the reaction assay (volume per test) to a vial with 2 ml of 1X stop solution. Keep the samples on ice.
8. Measure the fluorescence intensity (FI) of the samples.

If the values obtained are higher than the upper limit of the instrument, dilute the samples in stop solution until measurements are on scale. Determine the dilution factor. According to the dilution factor, reduce the plant tissue extract volume, the duration of the reaction, the volume per test or a combination of these variables. For example, if the dilution factor is 5, use 10 µl of sample volume instead of 50 µl, or use a shorter time of assay with 20 µl as volume per test. If values obtained are below the normal range of the instrument, allow the reaction to proceed for a longer period of time.

C. Multiple Reaction Kinetic Assay Procedure[6]

Complete Calibration Procedure in order to optimize the sample volume (X µl), reaction time and volume per test. Note that the kinetic assay reaction volume is 1 ml while the calibration assay reaction volume is 200 µl.

REAGENT	SAMPLE ASSAY	POSITIVE ENZYME CONTROL ASSAY	NEGATIVE CONTROL ASSAY
Extraction buffer	500 µl – X µl	480 µl	500 µl
Substrate solution	500 µl	500 µl	500 µl
Sample extract	X µl	----	----
Diluted β-glucuronidase positive control	-----	20 µl	----

1. Prepare one vial containing 2.0 ml stop solution for each dilution level and place in an ice bath.
2. Pipet 0.5 ml substrate solution into each assay tube.
3. Add the amount of extraction buffer indicated in the above table to each assay tube.
4. Mix and equilibrate for 1-2 minutes at 37 °C.
5. Add plant tissue sample extract to sample assay tube and diluted positive control to positive control assay tube.
6. Immediately remove 100 µl aliquots (volume per test) from all three reaction mixes into 2 ml stop solution as the zero minute test points. Volume per test is determined according to calibration procedure B. Keep the tubes on ice.
7. Cap tubes and incubate at 37°C.
8. Transfer 100 µl aliquots at 10 minute intervals to vials containing 2 ml cold stop solution.
9. Prepare 4-MU standard dilutions in stop solution. A concentration of 10 nM-100 nM is suitable; include a minimum of 5 data points in order to generate a standard curve. 10 nM equals 20 pmol in a 2 ml cuvet.
10. Measure fluorescence intensity (FI) of all samples, positive enzyme and negative controls and 4-MU standard dilutions.

D. Calculations

1. Draw a calibration curve of 4-MU standards FI versus pmol 4-MU.
2. Calculate FI per pmol 4-MU.
3. Plot sample, negative control and positive enzyme control FI versus time.
4. Calculate FI per minute for sample, negative control and positive enzyme control.
5. Subtract the negative control FI per minute value from the sample and positive enzyme control values.
6. Calculate β-glucuronidase activity of extract in pmol 4-MU per minute per µg protein (units per µg protein), and positive control in pmol 4 MU per minute per ml (units per ml), according to the following equations
7. Positive control enzyme activity should be about 100,000 units per ml.

Sample calculations

$$\text{Activity of extract (pmol MU/min/mg protein)} = \frac{\text{Fl/min}}{\text{Fl/pmole MU}} \times \frac{\text{Reaction volume (ml)}}{\text{Sample volume (ml)}} \times \frac{1}{\text{Vol.per test (ml)}} \times \frac{1}{\text{Extract conc., (mg protein/ml)}}$$

$$\text{Activity of positive control enzyme (pmol MU/min/vial)} = \frac{\text{Fl/min}}{\text{Fl/pmole MU}} \times \frac{\text{Reaction volume (ml)}}{\text{Sample volume (ml)}} \times \frac{\text{Dilution factor}}{\text{Vol.per test (ml)}}$$

Using the above assay conditions:

X = Sample volume
2.1 = Reaction volume
0.1 = Vol. per test

Note that the volume per test may be optimized using the calibration (single reaction) assay; the reaction volume will be 2.0 ml plus the volume per test (ml).

References

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6. Jefferson, R.A., *et al.,* EMBO J., **6**, 3901-3907 (1987)

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