Protocol

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Gus Gene Assay in Transformed Tissues

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Introduction

Gene reporter systems have become an invaluable tool for the study of gene expression regulation in plant research. In these systems, a gene reporter, usually an enzyme, is fused to a specific gene promoter, leading to transcription of the gene reporter under control of the promoter. Then, the enzyme activity can be measured and used as indication of gene expression levels. Of the many reporters in use today, β-glucuronidase (GUS) is the most popular and has been particularly useful in helping identify transgenic events in plants due in part to its stability in various conditions and use in various sensitive assays. *E. coli* βglucuronidase catalyzes the cleavage of various β-glucoronides, has a molecular weight of 68.2 kDa, and appears to function as a tetramer. It is very stable in a variety of conditions and is most active in the presence of thiol reducing agents such as β-mercaptoethanol (βME) or DTT. In addition, it may be assayed at any physiological pH and it has optimal activity between pH 5.2 and 8.0. The original GUS reporter gene reporter was developed as a gene fusion marker in E. coli and in C. elegans. However, currently it is used extensively to monitor chimeric gene expression in plants. Interestingly, there is little or no detectable β-glucuronidase activity of yeast, *Drosophila*, *C. elegans*, *Dictyostelium*, or in almost any higher plant. In *Agrobacterium*, however, some of the GUS plasmids showed significant GUS activity even in the absence of a promoter. Thus, in order to use this system in *Agrobacterium*-mediated transformations and accurately measure gene expression regulation by specific promoters, one laboratory constructed GUS genes carrying an intron, which must be processed before expression takes place. Thus, allowing the monitoring of Agrobacterium-mediated transfer and expression of foreign genes in plants. Here, we describe how to use the GUS system in a histochemical assay to perform a qualitative analysis of β-glucuronidase activity in tissues and cells from transformed organisms. In addition, we describe a fluorimetric assay, which allows us to quantify β-glucuronidase activity in tissues and cells transformed using the GUS reporter gene system.

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Materials

- X-Gluc (GoldBio Catalog # G1281)
- Tissue Fixative Buffer
- Extraction Buffer
- Assay Buffer
- STOP Buffer
- MES (GoldBio Catalog # M-091)
- Mannitol
- Dithiothreitol, DTT (GoldBio Catalog # DTT)
- MUG (GoldBio Catalog # MUG)
- dH_2O
- Formaldehyde
- Dimethylformamide (DMF)
- Phosphate buffer
- Na_2EDTA
- Sodium Lauryl Sarcosine
- Triton X100
- Na_2CO_3

Preparation of Solutions and Buffers
For MES Stock Solution

- Dissolve 10.66 g MES (GoldBio Catalog # M-091) in 80 ml dH_2O.
- Adjust pH with NaOH and fill to 100 ml.
- Store at room temperature.

For Tissue Fixative Buffer

- 1.6 ml Formaldehyde (0.6% final concentration).
- 4 ml 0.5M MES Stock Solution (20mM final concentration, pH 5.6).
- 10.93 g Mannitol
- Fill to 100 ml with dH_2O

For 200mM Phosphate Buffer, pH 7.0

- Stock A: 200mM NaH_2PO_4 (24.00 g/L) in dH_2O.
- Stock B: 200mM Na_2HPO_4 (28.39 g/L) in dH_2O.
- For pH 7.0, combine 38 ml Stock A with 62 ml Stock B.

For X-GLUC Stain

- Dissolve 5 mg X-Gluc in 1 ml DMF.
- Add 9 ml of 50mM Phosphate Buffer, pH 7.0.

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For Extraction Buffer

- 25 ml phosphate buffer 200mM (Final concentration 50mM, pH 7.0).
- 0.2 ml 0.5M Na_2EDTA (final concentration 1mM).
- 1 ml DTT (final concentration 10mM)
- 0.33 ml 30 % Sodium Lauryl Sarcosine (final concentration 0.1%)
- 1 ml of 10% Triton X-100 (final concentration 0.1%)
- Total volume is 100 ml

Assay Buffer

- Dissolve 17.6 mg MUG in 50 ml extraction buffer in a 50 ml disposable polypropylene tube (final concentration is 1mM MUG).
- Store at 4°C for up to two weeks.

Stop Buffer

- Dissolve 21.2 g of Na_2CO_3 in dH_2O (final concentration 0.2M).
- Fill to 1 L with dH_2O

Method

Histochemical Assay

Currently, the substrate X-Gluc is used for the histochemical localization of β-glucuronidase activity in tissues and cells. This substrate is highly effective and yields a blue precipitate at the site of enzyme activity. Notably, there are numerous variables that affect the quality of βglucuronidase histochemical localization, including all aspects of tissue preparation and fixation, as well as the reaction itself. Thus, it is necessary to understand the nature of the reaction. The initial product of glucuronidase hydrolysis of X-Gluc has no color. Instead, the initial indoxyl derivative produced must undergo an oxidative dimerization to form the insoluble and highly colored indigo dye. This dimerization is stimulated by atmospheric oxygen, and can be enhanced by using an oxidation catalyst such as a K+ ferricyanide/ferrocyanide mixture. Without a catalyst, the results are often very good, but one must consider the possibility that localized peroxidases may enhance the apparent localization of glucuronidase. In addition, fixation conditions will vary with the type of tissue and its permeability to the fixative. For example, if glutaraldehyde is used, one must note that it does not easily penetrate the leaf cuticle, but it does penetrate stem cross sections well. Alternatively, formaldehyde seems to be a more gentle fixative than glutaraldehyde and can be used for longer times. Furthermore, whole tissues, callus, suspension culture cells and protoplasts, whole plants or plant organs can be stained, but survival of the stained cells is not certain. After staining, clearing the tissue with 70% ethanol seems to improve contrast in many cases.

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Procedure

1. Take one or two fresh leaf disks directly from a selection plate. Cut fresh disks into quarters.

2. Transfer sections to 0.5 ml of X-Gluc stain in 24-well plates and incubate for 1 hour to overnight at 37°C.

3. After staining, rinse sections in 70% ethanol for at least 5 minutes.

   Note: If green color persists, clear the tissue of chlorophyll by soaking in 70% ethanol for at least 4 hours.

4. Examine for GUS stain under dissecting microscope.

   Note: Immerse tissue in Tissue Fixative Buffer if fresh tissues are not used.

5. Store in fume hood at room temperature for up to 3 months (or until precipitates appear).

Fluorimetric Assay

Although various spectrophotometric substrates for GUS are available, GUS activity in solution is usually measured with the fluorometric substrate 4-methylumbelliferyl-β-D-glucuronide (MUG). Fluorometry is preferred over spectrophotometry because of its greatly increased sensitivity and wide dynamic range. The assay is highly reliable and simple to use. Occasionally, endogenous compounds will interfere with the assay, either by quenching or by producing a high background fluorescence. In these situations, fluorometric substrates with differing excitation and emission wavelengths are recommended (the most popular being resorufin-β-Dglucuronic acid). The substrate 4-trifluoromethylumbelliferyl-β-D-glucuronic acid (4-TFMUG) allows continuous monitoring of GUS activity because, unlike MUG, it becomes fluorescent upon hydrolysis at the assay pH. In contrast, after hydrolysis of MUG by GUS, the reaction must be terminated with a basic solution. This stops the enzyme reaction and causes the fluorescence.

Procedure

1. Homogenize approximately 100 mg of callus or one fresh leaf disk in 100 µl Extraction Buffer in a 1.5 ml centrifuge tube. Use a small amount of sand or glass beads in the mixture.

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2. Centrifuge for 5 minutes at 4°C at 25,000 g. Proceed to Step 1 of the Fluorogenic Assay Protocol.

Note: Extracts can be stored at -70°C with no loss of activity for a long time, or at 4°C with little loss of activity. Avoid storage at -20°C, which kills the enzyme in lysis buffer.

Note: If the extract is high in endogenous fluorescent compounds or produces high levels of polyphenolics, they may be extracted in extraction buffer with polyclar (insoluble polyvinyl pyrollidone) followed by a brief spin column of Sephadex G 25 to eliminate almost all polyphenolics and low molecular weight fluorescent contaminants from the extract.

Fluorogenic Assay
Protocol Procedure

1. Incubate 0.5 ml aliquots of Assay Buffer at 37°C to pre-warm the buffer.

2. Add 50 µl of extract to 0.5 ml Assay buffer.

3. Mix thoroughly with pipette tip or vortex.

4. At regular time intervals (30 minutes for high GUS activity or 1 hour to overnight for low GUS activity) remove successive 100 µl aliquots into labeled 1.5 ml centrifuge tubes containing 0.9 ml Stop Buffer.

Note: Take 3-4 time points if possible and one overnight incubation.

Typical preliminary results can be obtained by placing the 1.5 ml centrifuge tubes on a UV transilluminator used for observing stained ethidium stained gels.

Associated Products

- X-Gluc (GoldBio Catalog # G1281)
- MES (GoldBio Catalog # M-091)
- Dithiothreitol DTT (GoldBio Catalog # DTT)
- MUG (GoldBio Catalog # MUG)

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