# Protocol

TD-P Revision 3.0

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# Fluorometric Assay for Protease Inhibitors in Plant Tissues

### utilizing MUG

## 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. The enzyme activity can then 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. In addition, GUS-marked plants are considered healthy and fertile post-transformation, and tissue samples show high GUS activity after prolonged storage. GUS activity can be determined by using the substrate 4-methylubelliferyl β-D-glucuronide (MUG), which upon hydrolysis by GUS, produces a measurable fluorochrome, 4-methylumbelliferone (MU). In this protocol we detail a rapid and extremely sensitive fluorometric assay, which can be used to determine the kinetics of MU accumulation and give insight into β-glucuronidase activity in transformed and non-transformed plants.

## Materials

- 1mM 4-Methylumbelliferyl beta-D-glucuronide (MUG) (GoldBio Catalog # MUG)
- 0.2M $Na_2CO_3$
- 1 ml Dithiothreitol DTT (GoldBio Catalog # DTT)
- Phenylmethylsulfonyl fluoride (PMSF) (GoldBio Catalog # P-470) or preferred protease inhibitors
- Triton X-100
- Phosphate buffer 200mM
- 0.33 ml 30 % Sodium Lauryl Sarcosine (final concentration 0.1%)
- 0.5M $Na_2EDTA$
- Spectrofluorometer with excitation at 365 nm and emission at 455 nm

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

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For Extraction Buffer:

- 5 ml phosphate buffer 1M (Final concentration 50mM, pH 7.0)
- 0.2 ml 0.5M $Na_2EDTA$ (final concentration 1mM)
- 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

## Method

In this procedure, plant tissue from transformed or non-transformed plants is homogenized in buffer containing protease inhibitors, then incubated. Fluorescence is then measured at different time points and kinetics of MU accumulation is determined by plotting fluorescence versus time.

1. Prepare homogenates of plant tissue in Extraction buffer containing protease inhibitors (PMSF) and 1mM MUG in a total volume of 1 ml.

2. Remove a 200 µl aliquot at zero time and terminate the reaction with 0.8 ml of 0.2M $Na_2CO_3$.

3. Incubate the remaining solution at 37°C for 4-16 hours. During this incubation remove 200 µl aliquots and terminate each with 0.8 ml of 0.2M $Na_2CO_3$. Continue until desired number of samples have been collected.

4. Create a blank containing freshly prepared MU standards (100nM and 1µM) in the same buffer as your sample and use it to calibrate the fluorimeter.

5. Measure fluorescence of all aliquots with excitation at 365 nm and emission at 455 nm.

6. Use these measurements to generate a plot of MU fluorescence versus time and determine MU accumulation kinetics giving insight into β-glucuronidase activity in transformed or non-transformed plants.

## Associated Products

- 4-Methylumbelliferyl beta-D-glucuronide (MUG) (GoldBio Catalog # MUG)
- Phenylmethylsulfonyl fluoride (PMSF) (GoldBio Catalog # P-470)
- Dithiothreitol DTT (GoldBio Catalog # DTT)

## References

Jefferson, R. A. (1987). Assaying chimeric genes in Plants: The GUS gene fusion system. *Plant Molecular Biology Reporter*, *5*:387-405.

Jefferson, R. A., Kavanagh, T. A., & Bevan, M. W. (1987). GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal*, *6*(13), 3901-3907. Doi:10.1002/j.1460-2075.1987.tb02730.x