*Revised: 18–March–2001*

# *ImaGene Green™ $C_{12}$FDGlcU GUS Gene Expression Kit (I-2908)*

**Quick Facts**

***Storage upon receipt:***
- –20°C
- Desiccate
- Protect from light

***Abs/Em of the reaction product:*** 495/518 nm

## *Introduction*

New genetic information may be introduced into plants both by *Agrobacterium tumefaciens*–mediated gene transfer and by direct transformation of protoplasts.[1,2] Jefferson *et al.* have proposed the *E. coli* β-glucuronidase (GUS) gene as a fusion marker for analysis of gene expression in transformed plants.[3] The GUS gene has been cloned and sequenced, and encodes a stable enzyme that has desirable properties for the construction and analysis of gene fusions.[4] In comparison with other reporter systems, GUS has several advantages: 1) There is no detectable background level of β-glucuronidase activity in most higher plants including tobacco, potato, soybean, wheat, rice and *Brassica*; 2) The enzyme is very stable and can be assayed at any physiological pH, with an optimum pH between 5.2 and 8.0; and 3) β-Glucuronidase catalyzes the cleavage of a wide variety of β-glucuronides, including many chromogenic and fluorogenic substrates, thus allowing the histochemical, spectrophotometric and fluorometric measurements of GUS gene fusion expression.[4,5]

Fluorescence measurements of enzymatic activity are usually orders-of-magnitude more sensitive than methods based on absorption, particularly in cells. Molecular Probes offers several fluorogenic substrates for detecting β-glucuronidase activity in homogenates and extracts. These include 4-methylumbelliferyl β-D-glucuronide (M-1490) and fluorescein di-β-D-glucuronide (F-2915). These substrates are relatively membrane impermeant, limiting their use to *in vitro* assays, although plant cells may be somewhat tolerant to substrate loading under weakly acidic conditions. In any case, the fluorescent hydrolysis products from these substrates are not well retained by viable cells.

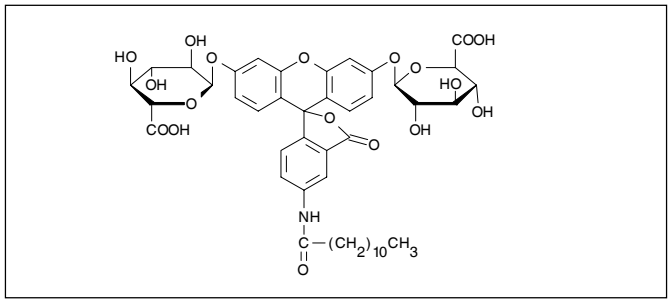


***Figure 1.*** *ImaGene Green $C_{12}$FDGlcU.*

The most common substrate for measuring GUS expression in single plant cells has been X-glucuronic acid. This chromogenic substrate tends to be membrane impermeant and forms a relatively cytotoxic product. Although very useful in tissues, it is not suitable for flow cytometric analysis and separation of viable plant cells and does not have the sensitivity to detect very low levels of expression.

Scientists at Molecular Probes have developed a fluorogenic β-glucuronidase substrate that is relatively membrane permeant in normal culture medium and that forms a nontoxic green fluorescent product that is well retained in viable plant cells. This lipophilic analog of fluorescein di-β-D-glucuronic acid is provided in our ImaGene Green™ GUS Gene Expression Kit (I-2908). This lipophilic substrate has shown to freely diffuse across the membranes of viable tobacco leaf cells and protoplasts[6] and the shoot apical meristem of tomatoes[7] under physiological conditions. It has also been used to demonstrate endophyte-associated GUS activity of *Acremonium*-infected ryegrass.[8] The green fluorescent enzymatic hydrolysis product is retained for hours to days in the membrane of those cells that actively express the GUS reporter gene.

The substrate used in our ImaGene Green GUS Gene Expression Kit is $C_{12}$FDGlcU, a lipophilic analog of fluorescein di-β-D-glucuronic acid containing a 12-carbon aliphatic chain (Figure 1). The colorless, lipophilic, fluorogenic substrate enters the cells by embedding in the outer layer of the cell membrane as a glycolipid and then transferring to the inner membrane by a "flip-flop" mechanism. Once inside the cell, the substrate is cleaved by β-glucuronidase, generating the yellow-colored, green-fluorescent, 5-dodecanoylaminofluorescein, which is apparently retained in the cell membrane because of its lipophilic hydrocarbon chain.

The *E. coli* GUS operon has also been used as a transgenic marker for bacteria because there is almost no β-glucuronidase background activity in most bacteria and fungi tested. Therefore the ImaGene Green $C_{12}$FDGlcU GUS Gene Expression Kit may be useful for the detection of GUS gene expression in bacteria as well.

## *Materials*

- **ImaGene Green $C_{12}$FDGlcU substrate reagent** (Component A), 1 mL of 10 mM substrate in 50% DMSO/50% water (v/v). The substrate reagent is to be diluted with buffer or culture medium immediately before use to obtain a working solution. *Do not keep the substrate working solution at 37°C for extended periods because spontaneous hydrolysis*

*may occur*. Hydrolysis will be accompanied by an increase in color and fluorescence of the reagent. When used at 50 µM (a 1:200 dilution) in accordance with the instructions herein, this substrate will prepare 200 mL of culture medium, which is sufficient for approximately 100 tests, depending on the volume used for each experiment.

- **D-Glucaric acid-1,4-lactone** (Component B), 1 mL of 20 mM solution in water. This reagent is an inhibitor of β-glucuronidase that permits one to stop the reaction for later analysis. It has a reported $K_i$ of 2.5 µM.[8]

### Storage

Upon receipt, store the reagents at -20°C. To avoid frequent freezing and thawing, the substrate should be divided into small aliquots and stored frozen until use. This will minimize decomposition of the substrate. The reagents are stable for at least six months, provided that they are stored frozen.

## Experimental Protocols

The ImaGene Green $C_{12}$FDGlcU GUS Gene Expression Kit permits detection of β-glucuronidase in cells by measuring the fluorescence of the ImaGene Green $C_{12}$FDGlcU substrate hydrolysis product. In this manner, a highly fluorescent lipophilic fluorescein derivative (5-(*N*-dodecanoyl)aminofluorescein) is produced by enzymatic cleavage of the ImaGene Green $C_{12}$FDGlcU substrate in cells that express the marker gene for β-glucuronidase.

### Cell Staining

For detection of β-glucuronidase in living cells, cells are grown in suitable medium. The ImaGene Green $C_{12}$FDGlcU substrate (Component A) is diluted into the medium and the cells are incubated for a suitable period (from 20 minutes to several hours). The optimal concentration for cell staining must be determined by experimentation and may vary with the cell membrane permeability, the incubation temperature and other conditions. Staining of tissues may depend on the access of the substrate to the cells. An initial concentration of 50 µM is suggested, although the lowest concentration that gives a detectable signal in GUS-positive cells may be preferred to reduce nonspecific background. If desired, the cells can be washed with substrate-free medium; however, this is usually not required. Background may result from the slow hydrolysis of the substrate in the relatively acidic plant cell environment, the hydrolysis by other enzymes or the extreme sensitivity of detection. Fluorescence of stained cells is typically visualized by fluorescence microscopy using a fluorescein filter set.

Individual cell populations expressing β-glucuronidase can be selectively isolated by flow or imaging cytometry. Because we have observed that the ImaGene GUS substrate can sometimes yield fluorescence in GUS-negative cells, it is recommended that the β-glucuronidase activity of the cloned cells be confirmed using X-glucuronic acid (B-1691).

Detection of GUS activity in cell extracts using the ImaGene Green $C_{12}$FDGlcU substrate can be performed using either a standard fluorometer or a fluorescence microplate reader. Unlike the more polar substrates, permeability of the ImaGene Green $C_{12}$FDGlcU substrate may permit this activity to be measured in viable cells.

### Tissue Staining

For histochemical assay of GUS expression in plant tissue, unfixed cross-sections (4–6 cell layers thick) may be incubated in medium containing approximately 50 to 100 µM $C_{12}$FDGlcU at room temperature for 30 minutes to 2 hours depending on the level of GUS gene expression and the permeability of the cell membranes. We have not yet investigated the effect of permeabilization reagents or conditions such as detergents, hypotonic shock or electroporation.

### Use of the β-Glucuronidase Inhibitor

Prepare cells and stain them with $C_{12}$FDGlcU as described above. At a predetermined time (usually 30, 60 or 120 minutes, depending on the degree of GUS activity of the cells), add D-glucaric acid-1,4-lactone[9] (Component B) to the medium and mix thoroughly. The final concentration of the inhibitor in your staining system should be no less than 0.1 mM in order to completely inhibit the GUS activity.

## References

**1.** Science 227, 1229 (1985); **2.** Herrera-Estrella, L. *et al.* in *Plant Molecular Biology Manual, Volume B1,* Kluwer Academic Publishers (1988) pp 1–22; **3.** EMBO J 6, 3901 (1987); **4.** Plant Mol Biol Rep 5, 387 (1987); **5.** The *E. coli* β-glucuronidase has a monomer molecular weight of about 68,000 daltons and exists *in vivo* as a tetramer. The enzyme is very stable and will tolerate many detergents and widely varying ionic conditions.; **6.** J Cell Biol 115, 151a, abstract 872 (1991); **7.** Plant J 10, 745 (1996); **8.** Microbiology 143, 267 (1997); **9.** Zollner, H. in *Handbook of Enzyme Inhibitors*, VCH, New York (1989).

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