FluorAce™ ß-glucuronidase Reporter Assay Kit

Instruction Manual

Catalog Number
170-3151

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BIO-RAD

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Section 1
Introduction

ß-glucuronidase (GUS)[1] from *E. coli*, encoded by the *gusA* gene, has become a widely used reporter gene in plants and animal cells due to its stability and high sensitivity levels (*i.e.*, low background levels of endogenous activity). We have developed a quantitative assay to determine the level of enzymatic activity using 4-methylumbelliferyl-glucuronide (MUGluc) as substrate. ß-glucuronidase hydrolyzes the substrate resulting in release of the fluorescent molecule, 4-methylumbelliferone (4MU). Fluorescence of the 4-methylumbelliferone is then measured on a fluorometer using an excitation wavelength of 360 nm and emission wavelength of 460 nm.

The assay is initiated by adding 20–40 µg of total protein to 500 µl of assay buffer. Samples are incubated in a 37 °C water bath for 30 minutes, during which time the ß-glucuronidase hydrolyzes the colorless substrate to a highly fluorescent compound. The reaction is terminated by addition of 1x Stop Buffer. The amount of product formation is measured in a fluorometer.

Section 2
Kit Components

2.1 Contents

The FluorAce ß-glucuronidase Reporter Assay Kit contains reagents for 200 x 1.5 ml or 2000 x 150 µl reactions, 4MU calibration curve, and purified ß-glucuronidase as the positive control. Measurements require a fluorometer (cuvette or microplate) using 360 nm excitation and 460 nm emission filters.

Component	Concentration	Volume
1x Reaction Buffer (green cap)	1x	100 ml
4-methylumbelliferyl ß-D-glucuronide (MUGluc)	100 mM	1200 µl
10x Stop Buffer (red cap)	10x	50 ml
4-methylumbelliferone (4MU)	1.0 mM	400 µl
ß-glucuronidase	6–11,000 Units*	100 µl

*One unit of ß-glucuronidase hydrolyzes 1 nanomole of 4-methylumbelliferyl ß-D-glucuronide per minute at pH 7.5 at 37 °C.

2.2 Materials Required

ß-mercaptoethanol (catalog number 161-0710).

2.3 Storage and Stability

All components are guaranteed for 12 months from the date of purchase when used as described in this manual and stored as described in the table below:

Component	Storage Temperature
1x reaction buffer	Room temperature
Stop buffer (10x or 1x)	Room temperature
4-methylumbelliferyl ß-D-glucuronide (MUGluc)	4 °C
4-methylumbelliferone (4MU)	4 °C
ß-glucuronidase	4 °C

Section 3
Preparation of Stock Solutions

1x Stop Buffer

Prepare 1x Stop Buffer by mixing 50 ml of 10x Stop Buffer with 450 ml of distilled water. Store solution at room temperature.

Section 4
Experimental Procedures

The FluorAce ß-glucuronidase Reporter Assay Kit has been designed for use with any fluorometer system. The following section describes the procedure to be used with the Bio-Rad VersaFluor™ Fluorometer. If another instrument is used, refer to the instruction manual regarding specific operation details.

4.1 Fluorometer Set-up

1. Turn on the fluorometer to allow instrument to warm up for at least 20 minutes. If the instrument is not fully warmed up the readings may be irreproducible.
2. Insert a 360 nm excitation filter and a 460 nm emission filter into the fluorometer.
3. Set the gain to LOW and adjust the range to read 00000.

4.2 Sample Measurement Guidelines

Follow the guidelines below to insure accurate results.

- Always use calibrated pipets to insure accurate pipetting
- Mix standards and sample completely by using a disposable transfer pipet
- Remove any air bubbles in the cuvette
- Hold fluorometer cuvettes by the upper edges; avoid handling the four optically-clear sides
- Clean the cuvette sides with a lint-free tissue
- Read all standards and samples at ambient temperature
- While taking a reading, keep the samples in the fluorometer only. This helps reduce photobleaching.

4.3 Standard Curve Preparation

Generate a calibration curve of 4-methylumbelliferone (4MU) from 10 nM to 10,000 nM in 1x Stop Buffer. Add 1x Stop Buffer and 4MU to each cuvette as described in the table below. Mix the solutions using a new disposable pipet for each cuvette or by placing parafilm on the top of the cuvette and inverting a few times.

Cuvette	4MU	1x Stop Buffer (ml)	[4MU] (nM)
1	20 µl of 1 mM 4MU stock	1.98	10000
2	200 µl of cuvette 1	1.80	1000
3	200 µl of cuvette 2	1.80	100
4	200 µl of cuvette 3	1.80	10
5	0	2.00	0

4.4 ß-glucuronidase Assay

Homogenized crude tissue samples or cell extracts prepared with extraction buffer (see Appendix 5.3 for formulation) may be assayed. In addition to the negative (no enzyme) and positive (purified ß-glucuronidase) controls, it is recommended that a mock cell lysate (non-transformed preparation) be included in the assay to detect endogenous ß-glucuronidase activity.

Note: For accurate results, samples must be kept on ice until all enzyme mixtures have been added.

1. Keep ß-glucuronidase and ß-mercaptoethanol on ice. Thaw 4MU and MUGluc at room temperature. Do not thaw at elevated temperatures as this may cause precipitation. Vortex 4MU and MUGluc into solution.
2. Prepare fresh assay buffer by diluting 100 mM MUGluc and 14.2 M ß-mercaptoethanol to a final concentration of 1.2 mM MUGluc and 10.0 mM ß-mercaptoethanol in 1x Reaction Buffer. Vortex to mix.

   Example: 100 samples would require 50 ml of assay buffer (500 µl/sample). To prepare 50 ml of assay buffer, add 600 µl of 100 mM MUGluc and 35.2 µl of 14.2 M ß-mercaptoethanol to 50 ml of 1x Reaction Buffer.
3. Add 500 µl assay buffer prepared above to each 1.5 ml microfuge tube.
4. Add 2.5 µl of distilled water to the negative control tube.
5. Add 2.5 µl of purified ß-glucuronidase (supplied with the kit) to the positive control tube.
6. Add sample. As a guideline, 20–40 µg of total protein should fall within the linear range of 4MU detection.
7. Incubate reactions in a 37 °C water bath for 30 minutes.
8. While samples are incubating, label fluorometer cuvettes and add 1.0 ml 1x Stop Buffer to the cuvettes.
9. Stop the reactions by pipetting the entire reaction volume into the cuvettes containing 1.0 ml 1x Stop Buffer. Pipet samples up and down to mix.
10. Read samples as soon as possible; prolonged incubation may lead to a decrease in fluorescence signal.

4.5 Reading Samples

A. Standard Curve

Note: Use cuvettes prepared in Section 4.3.

1. Zero the VersaFluor by placing the cuvette containing 1x Stop Buffer (cuvette 5) in the cuvette holder. Close sample compartment lid. Wait approximately 2–3 seconds for the detector to stabilize. Press the SET ZERO button.
2. Insert cuvette 1, highest concentration cuvette, into the cuvette holder. Close the sample compartment lid. Wait approximately 2–3 seconds for the detector to stabilize.
3. Set the range to 14,000. Remove cuvette.

   Note: Do not change range settings after this point.

4. Check zero by placing the cuvette containing 1x Stop Buffer (cuvette 5) in the instrument. Re-zero the instrument if necessary.
5. Read the standard curve samples. Record or print relative fluorescence units for data interpretation. Fluorescent measurements can be made continuously. It is not necessary to zero the instrument before each measurement.

B. Assay Samples

1. Read samples. Record or print relative fluorescence units for data interpretation. If sample readings fall outside the standard curve (RFU value greater than 14,000), reduce the amount of extract in the reaction and/or shorten the incubation period. If the level of expression is low, increase the amount of extract in the reaction and/or lengthen the incubation period.

4.6 Data Analysis

The relative fluorescence units (RFU) are used to compare the activity level between samples or inserted into the least squares regression equation to determine the amount of substrate hydrolyzed.

1. If samples were performed in duplicate or triplicate, use the average RFU readings.
2. Subtract the non-transformed RFU values from the samples to correct for endogenous ß-glucuronidase activity.
3. From the data, prepare a calibration curve by plotting 4-methylumbelliferone concentration (nM) versus relative fluorescence units (RFU). An example of the 4MU standard curve is shown in Figure 1.

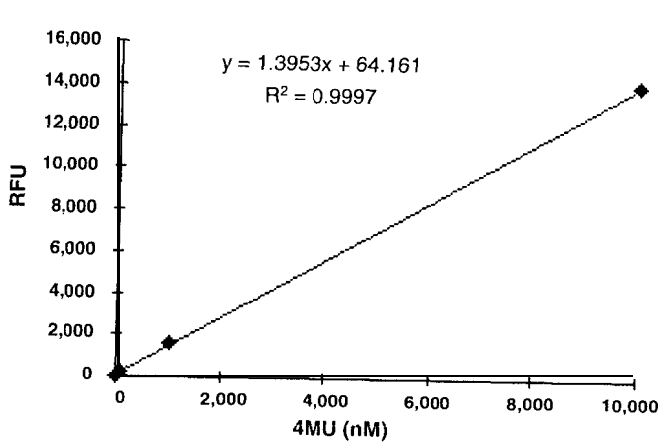

Fig. 1. Example plot of standard curve and linear equation.

4. To calculate the concentration of 4MU released, determine the least squares regression equation for the line generated by the standard 4MU samples. The equation for a line is y = mx + b, where:

   y is the instrument reading (RFU)
   x is the sample concentration (nM)
   m is the slope of the line
   b is the y-intercept

5. Determine the amount of MUGluc hydrolyzed in the sample by inserting the RFU values (y-value) into the linear equation and solving for x.

Section 5
Appendix

5.1 Microplate Assay

The 4MU standard curve (Section 4.3) and ß-glucuronidase assay (Section 4.4) may be scaled down 1:10 to be used in a 96-well microplate platform. The assay is performed directly in microtiter plates. Fluorescence units are determined in a microplate fluorometer.

1. Add 50 µl assay buffer (1x Reaction Buffer plus 1.2 mM MUGluc and 10.0 mM ß-mercaptoethanol) to each sample well.
2. Add samples (2.0–4.0 µg of total protein).
3. Incubate the microtiter plate for 30 minutes in a 37 °C incubator.
4. Stop the reactions by adding 150 µl 1x stop buffer. Pipet up and down to mix.
5. Read samples in a microplate fluorometer.

5.2 Microplate Fluorometer Guidelines

Use the following guide for making measurements on the Bio-Rad Fluoromark™. For other instruments, refer to the instruction manual regarding specific operation details.

Fluoromark settings:

Excitation filter	355 nm
Emission filter	460 nm
Gain	5
Flash	10

5.3 Extraction Buffer

Extraction Buffer[2]
50 mM Sodium phosphate, pH 7.0
10 mM Dithiothreitol (DTT)
1 mM Disodium EDTA
0.1% Sodium lauryl sarcosine
0.1% Triton X-100

Section 6
References

1. Gallagher, S. R., GUS Protocols, *Academic Press Inc.*, San Diego, California (1992).
2. Jefferson, R. A., *Plant Molecular Biology Reporter*, **5**, 387–405 (1987).

Section 7
Additional Supplies

Catalog Number	Product Description
	VersaFluor Fluorometer
170-2402	**VersaFluor Fluorometer 100/120/220**, includes a standard cuvette holder, 100 standard cuvettes, one excitation filter, and one emission filter
170-2420	**Optical Filter Excitation**, EX360/40 (340–380 nm)
170-2421	**Optical Filter Emission**, EM460/10 (455–465 nm)
	VersaFluor Disposable Cuvettes
170-2415	**Standard Cuvette**, 12.5 x 12.5 mm (OD) 4-sided optically clear disposable, polycarbonate, 3.5 ml, 100

Catalog Number	Product Description
	Fluoromark Microplate Fluorometer
170-6941	**Fluoromark Microplate Fluorometer 120V**, includes software, 5 excitation filters (355, 390, 485, 544 nm and time-resolved filter), and 5 emission filters (405, 460, 538, 590 nm, and time-resolved filter)
	Accessories
223-9522	**Disposable Transfer Pipet**
170-6963	**96-Well Fluorescence Microplate**, solid black, ZS
170-6964	**96-Well Fluorescence Microplate**, solid black with clear bottom, ZS