

A QUANTIFLUOR™-ST FLUOROMETER METHOD FOR 4-methylumbelliferone

INTRODUCTION

The gene for β -galactosidase is commercially available in a variety of configurations for reporter gene studies. The recombinant β -galactosidase enzyme can be routinely manipulated and assayed to study promoter function, tissue specific expression, developmental regulation, mRNA stability, and signal sequences that target proteins for various organelles. The advantages of using β -galactosidase to report the activity of promoters and genes are two-fold; assays are straightforward, and substrates for enzymatic analysis are readily available. β -galactosidase activity in solution can be revealed by the fluorogenic substrate 4-methylumbelliferone in a sensitive, quantitative assay using the QuantiFluor™-ST Fluorometer.

HOW IT WORKS

Esters of 4-methylumbelliferone (4-MU) do not fluoresce unless cleaved to release the fluorophore. Fluorometric enzyme assays are based on the hydrolysis of 4-MU-containing substrates such as β -4-MU-glucuronide by β -glucuronidase (GUS), or β -4-MU-galactose by β -galactosidase (GAL). Cleavage of 4-methylumbelliferyl- β -D-galactoside by β -galactosidase enzyme yields the fluorescent molecule 4-MU that emits light at 460nm when excited by 365nm light.

The QuantiFluor™-ST Fluorometer provides sensitive, reliable measurements of 4-MU. Using standard 10x10mm cuvettes, the QuantiFluor™-ST has a linear detection range from 750 nanomolar down to 0.1nanomolar, or 20fg/ml 4-MU. Sensitivity levels for the minicell adaptor range from 200nM down to 1.0nM.

The average coefficient of variance (CV) for three replicates of 15 dilution points was 3.5%.

The variability seen in replicate measures is associated with photo-bleaching and inherent fluctuations in 4-MU assays. This result includes measurements from minicell and 10x10mm cuvettes.

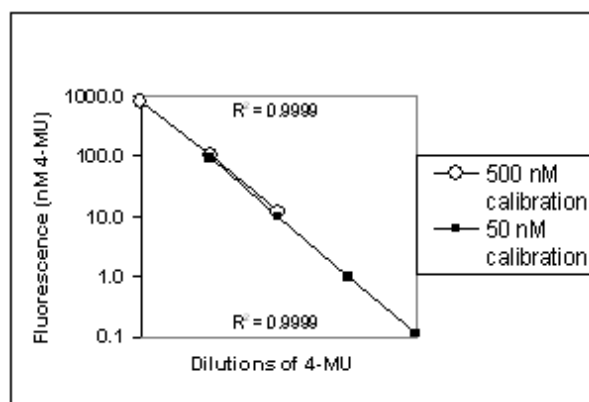


Figure 1. 4-MU detection using the QUANTIFLUOR™-ST Fluorometer. Replicate fluorescence measures of 4-MU serial dilutions were averaged and plotted. Two overlapping curves were generated using two different calibration values, 500nM and 50nM, for greater accuracy. R-square values are shown for both curves and indicate linear relationship between fluorescence and 4-MU concentration. The Minimum Detection Limit (MDL) for 4-MU using the QuantiFluor™-ST Fluorometer is 0.1nM using a 99.5% confidence limit (Ziebold, 1967).

MATERIALS REQUIRED

- ◆ QuantiFluor™-ST Mini-Fluorometer
- ◆ 10x10 mm Methacrylate Fluorescence Cuvettes
- ◆ Minicell Adaptor Kit, optional,
- ◆ Minicell Borosilicate Cuvettes, optional
- ◆ 4-methylumbelliferone, sodium salt, MW=198.20
- ◆ Distilled water
- ◆ Sodium carbonate, anhydrous, MW=105.99

4-MU SOLUTION PREPARATION

4-MU stock solution A (1mM)

19.8mg 4-methylumbelliferone (sodium salt),
MW = 198.20

Add distilled water to 100ml. Store at 40°C, and away from light.

4-MU stock solution B (1µM)

10µl 4-MU stock solution A

Add 10ml Distilled water. Store at 40°C, and away from light.

Carbonate stop buffer (0.20M)

2.12g Sodium carbonate, anhydrous,
MW = 105.99

Add Distilled water to 100ml.

EXPERIMENTAL PROTOCOL

In order to measure 4-MU for reporter gene assays, the β-Gal producing cells needs to be lysed and incubated with the appropriate substrate. Commercial kits using β -Gal reporter genes typically include treatment protocols and signal enhancers specific to tissue and recombinant enzyme. These application notes are based on *E. coli* β -galactosidase activity that is active at neutral pH. However, the vertebrate form of β -galactosidase is a lysosomal enzyme, which has optimal activity at pH 4.5 in acetate buffer. Buffer conditions during incubation should not affect the QuantiFluor™-ST's sensitivity to detect 4-MU fluorescence.

1. Lyse cells and incubate with 4-MU containing substrate according to reagent manufacturer directions. Incubate all samples for the same period of time, generally 2 minutes.
2. Add 100µl of cell lysis incubation to 1.9ml Carbonate Stop buffer to stop β -Gal enzyme activity and prepare sample for measurement.

1. Generating a Standard Curve

Free 4-MU can be used as a standard to calibrate β -galactosidase activity in cell cultures or tissues. Generating a standard curve verifies the linearity of the assay within a particular concentration range. It is recommended that you perform this at least once when working with a new instrument or performing the assay for the first time. Also, you may want to generate a standard curve every few weeks as a quality check on the standard, a reliability check on the instrument, and a consistency check on technique.

1. Make sure the QuantiFluor™-ST is set to UV optical configuration. If you are using the minicell adaptor, make sure it is placed in the chamber with "UV" indicator visible from front.
2. Prepare blank by adding Carbonate Stop Buffer to the 10x10mm cuvette or minicell cuvette.
3. Add 100µl stock solution B to 1.9ml Carbonate Stop Buffer (final concentration 50nM). Add 5.0µl solution B to 45µl Carbonate Stop Buffer when using the minicell.
4. Set the standard value to 50 by pressing [STD VAL]. Use the arrow keys to raise and lower the values.
5. Press [CAL] to calibrate. Press [ENTER] to continue. Insert the blank and measure as directed. Insert the dilution and measure. Press [ENTER] to accept calibration values.
6. Read remaining samples in dilution series.

Stock A Dilution	10x10 (2ml total)	Minicell (100µl total)	Final concentration (nM)
1:100	100µl	5.0µl	500
1:500	100µl	5.0µl	100
Stock B	100µl	5.0µl	50
1:5	100µl	5.0µl	10
1:50	100µl	5.0µl	1.0

4. Sample Analysis

1. Calibrate the instrument with a dilution near the average fluorescence of your samples, typically 100nM to 500nM.
2. Measure unknown sample by transferring 2ml from Step 2 of the Experimental Protocol into a 10x10mm cuvette, or transfer 100µl into minicell cuvette. Insert cuvette and press [READ].
3. Record results or use spreadsheet interface to import data into an Excel spreadsheet.

REFERENCES

1. Ziebold, T. O. (1967) *Anal. Chem.* **39**, 858.

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