

A TBS-380 Mini-Fluorometer Method for

4-methylumbelliferone



1. INTRODUCTION

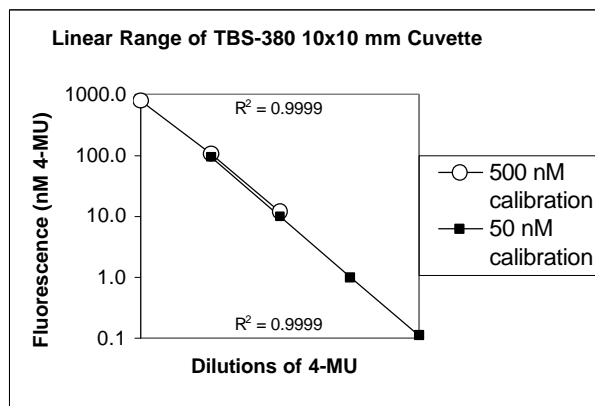
The gene for β -galactosidase is commercially available in a variety of configurations for reporter gene studies. The recombinant β -galactosidase enzyme can be routinely manipulated and assayed to study promoter function, tissue specific expression, developmental regulation, mRNA stability, and signal 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 TBS-380 Mini-Fluorometer.

2. 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 460 nm when excited by 365 nm light.

The Turner BioSystems TBS-380 Mini-Fluorometer provides sensitive, reliable measurements of 4-MU. Using standard 10x10 mm cuvettes, the TBS-380 has a linear detection range from 750 nanomolar down to 0.1 nanomolar, or 20 fg/mL 4-MU. Sensitivity levels for the minicell adaptor range from 200 nanomolar down to 1.0 nanomolar.

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 10x10 mm cuvettes.

4-MU detection using the TBS-380 Mini-Fluorometer.

Replicate fluorescence measures of 4-MU serial dilutions were averaged and plotted. Two overlapping curves were generated using two different calibration values, 500 nM and 50 nM, 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 TBS-380 Mini-Fluorometer is 0.1 nM using a 99.5% confidence limit (Ziebold, 1967).

3. MATERIALS REQUIRED

- ◆ TBS-380 Mini-Fluorometer with UV optical configuration (P/N 3800-003)
- ◆ 10x10 mm Methacrylate Fluorescence Cuvettes (P/N 7000-959)
- ◆ Minicell Adaptor Kit, optional, (P/N 3800-928)
- ◆ Minicell Borosilicate Cuvettes, optional, (P/N 7000-950)
- ◆ 4-methylumbelliferone, sodium salt, MW=198.20 (Sigma, P/N 1508)
- ◆ Distilled water
- ◆ Sodium carbonate, anhydrous, MW=105.99

4. 4-MU SOLUTION PREPARATION

- ### 4.1 4-MU stock solution A (1mM):
- 19.8 mg 4-methylumbelliferone (sodium salt), MW = 198.20
 - Add distilled water to 100 mL
 - Store at 4°C, away from light.

4.2 4-MU stock solution B (1 μ M):

10 μ L 4-MU stock solution A
Add 10 mL Distilled water
Store at 4 $^{\circ}$ C, away from light.

4.3 Carbonate stop buffer (0.20 M):

2.12 g Sodium carbonate, anhydrous,
MW = 105.99
Add DIH₂O to 100 mL.

5. 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 TBS-380's sensitivity to detect 4-MU fluorescence.

5.1 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.

5.2 Add 100 μ L of cell lysis incubation to 1.9 mL Carbonate Stop buffer to stop β -Gal enzyme activity and prepare sample for measurement.

6. 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.

6.1 Make sure the TBS-380 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.

6.2 Prepare blank by adding Carbonate Stop Buffer to the 10x10 mm cuvette or minicell cuvette.

6.3 Add 100 μ L stock solution B to 1.9 mL Carbonate Stop Buffer (final conc. 50 nM). Add 5.0 μ L solution B to 45 μ L Carbonate Stop Buffer when using the minicell.

6.4 Set the standard value to 50 by pressing [STD VAL]. Use the arrow keys to raise and lower the values.

6.5 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.

6.6 Read remaining samples in dilution series.

Stock A Dilution	10x10 (2 mL total)	Minicell (100 mL total)	Final conc. (nM)
1:100	100 μ L	5.0 μ L	500
1:500	100 μ L	5.0 μ L	100
Stock B	100 μ L	5.0 μ L	50
1:5	100 μ L	5.0 μ L	10
1:50	100 μ L	5.0 μ L	1.0

7. MEASURING UNKNOWN SAMPLE

7.1 Calibrate the instrument with a dilution near the average fluorescence of your samples, typically 100 nM to 500 nM (steps 5.3 through 5.5).

7.2 Measure unknown sample by transferring 2 mL from step 4.2 into 10x10 mm cuvette, or transfer 100 μ L into minicell cuvette. Insert cuvette and press [READ].

7.3 Record results or use spreadsheet interface to import data into an Excel spreadsheet.

8. REFERENCES

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

9. ABOUT TURNER BIOSYSTEMS, INC.

Orders for Turner BioSystems' products may be placed by:

Phone: (408) 636-2400 or
Toll Free: (888) 636-2401 (US and Canada)
Fax: (408) 737-7919

Web Site: www.turnerbiosystems.com

E-Mail: sales@turnerbiosystems.com

Mailing Address:

Turner BioSystems, Inc.
645 N. Mary Avenue
Sunnyvale, CA 94085 USA