TECHNICAL MANUAL

UMP/CMP-Glo™ Glycosyltransferase Assay

Instructions for Use of Products
VA1130, VA1131 and VA1132

10/17
TM506

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UMP/CMP-Glo™ Glycosyltransferase Assay

All technical literature is available at: www.promega.com/protocols/
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E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The UMP/CMP-Glo™ Glycosyltransferase Assay[a] is a bioluminescent assay for detecting the activity of glycosyltransferases that use CMP-, CDP- or UDP-sugars as donor substrates and release CMP or UMP. Glycosylating reactions catalyzed by glycosyltransferases (GTs) are central to many biological processes, including cell-cell interactions, cell signaling and bacterial cell wall biosynthesis (1). Because of the importance of this class of enzymes, there is a need for biochemical assays to monitor their activity, their mode of regulation, and to search for their selective and potent inhibitors. Phosphoglycosyltransferases (PGTs) transfer the phospho-sugar from a nucleotide diphosphate sugar donor (e.g., UDP-diNAcBac, UDP-GlcNAc) to an acceptor molecule such as polyprenol-phosphate or polysaccharide-phosphate (2). In a PGT reaction, the UMP moiety is released as a product. Sialyltransferases (STs) transfer the sugar from a nucleotide monophate-sugar donor (e.g., CMP-sialic acid) to an acceptor molecule. In a sialyltransferase reaction, the CMP moiety is released as a product (3). Therefore, an assay that detects UMP or CMP as products of these reactions would be suitable for monitoring the activity of all the UMP- or CMP-releasing glycosyltransferases.

1. Description (continued)

The UMP/CMP-Glo™ Glycosyltransferase Assay is a homogeneous, one-step-reagent-addition method to rapidly detect UMP or CMP formation in glycosyltransferase reactions. After the glycosyltransferase reaction, an equal volume of UMP/CMP Detection Reagent is added to simultaneously convert the UMP or CMP product to ATP and generate light in a luciferase reaction. The light generated is detected using a luminometer (Figure 1). Luminescence can be correlated to UMP or CMP concentration by using a UMP or CMP standard curve. The light output is proportional to the concentration of UMP or CMP from low nM to 50μM (Figures 2 and 3, Panel A). The assay is easy to use and highly sensitive (Figures 2 and 3, Panel B), two features that are desirable and essential for measuring the activity of different UMP- or CMP-releasing glycosyltransferases such as sialyltransferases (Figure 4) or PGTs. Therefore, the UMP/CMP detection assay requires less glycosyltransferase enzyme in the reactions. This assay is fast and simple (Figure 5). The UMP/CMP-Glo™ Assay is performed in a single well of a multiwell plate and can be used to detect glycosyltransferase activity in as little as a 5μl reaction.

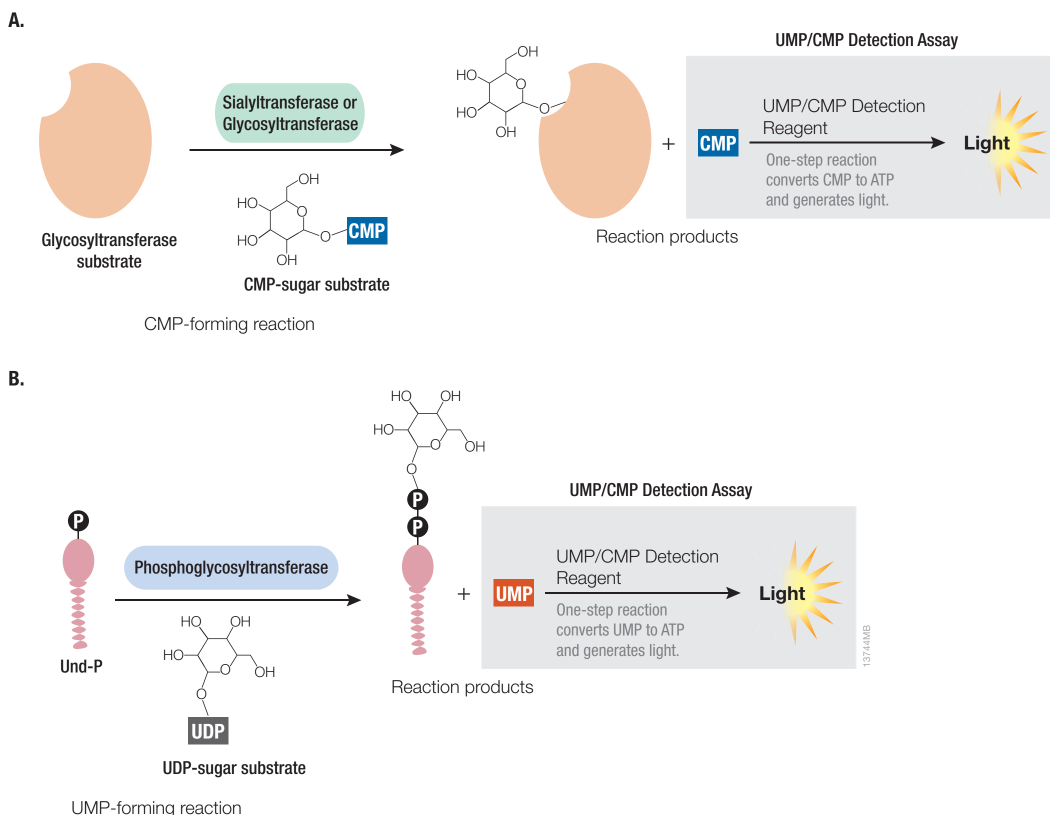

Figure 1. UMP/CMP-Glo™ Glycosyltransferase Assay principle. The assay is performed in one step. After the glycosyltransferase reaction, UMP/CMP Detection Reagent is added to convert CMP (**Panel A**) or UMP (**Panel B**) to ATP and measure the newly synthesized ATP using a luciferase/luciferin reaction. The light generated correlates to the amount of UMP or CMP produced by the glycosyltransferase, which indicates glycosyltransferase activity.

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The UMP/CMP-Glo™ Glycosyltransferase Assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that is formulated to generate a stable glow-type luminescent signal and improve performance across a wide range of assay conditions. The signal produced by the luciferase reaction initiated by adding the UMP/CMP Detection Reagent is stable for more than 3 hours (Figures 2 and 3, Panel C). This extended stability eliminates the need for a luminometer equipped with injectors and allows batch-mode processing of multiple plates. Furthermore, the combination of Ultra-Glo™ Recombinant Luciferase (4) and proprietary formulation of the UMP/CMP Detection Reagent results in luminescence that is much less susceptible to interference from library compounds than other luciferase- or fluorescence-based assays (5). In addition to providing biochemical values (e.g., K_m of UMP- or CMP-sugars or K_m of acceptor substrates) comparable to those reported in the literature, the UMP/CMP-Glo™ Glycosyltransferase Assay can be used in screening for specific glycosyltransferase inhibitors and the study of their mode of action.

Note: This assay detects only the activity of glycosyltransferases that release UMP or CMP as a product and can only be used with purified glycosyltransferases, not whole cells or cell extract. However, glycosyltransferases can be purified from cell extract using immunoprecipitation or affinity tag pull down then used in the UMP/CMP-Glo™ Glycosyltransferase Assay.

The sensitivity of the UMP/CMP-Glo™ Glycosyltransferase Assay means it can detect low UMP or CMP concentrations with high dynamic range. Therefore, whether assaying for a low-activity glycosyltransferase whose sugar transfer rate is low or using a low concentration of enzyme that produces a small amount of UMP or CMP, a high signal-to-background ratio is obtained with the UMP/CMP-Glo™ Glycosyltransferase Assay. See Figure 2, Panel B.

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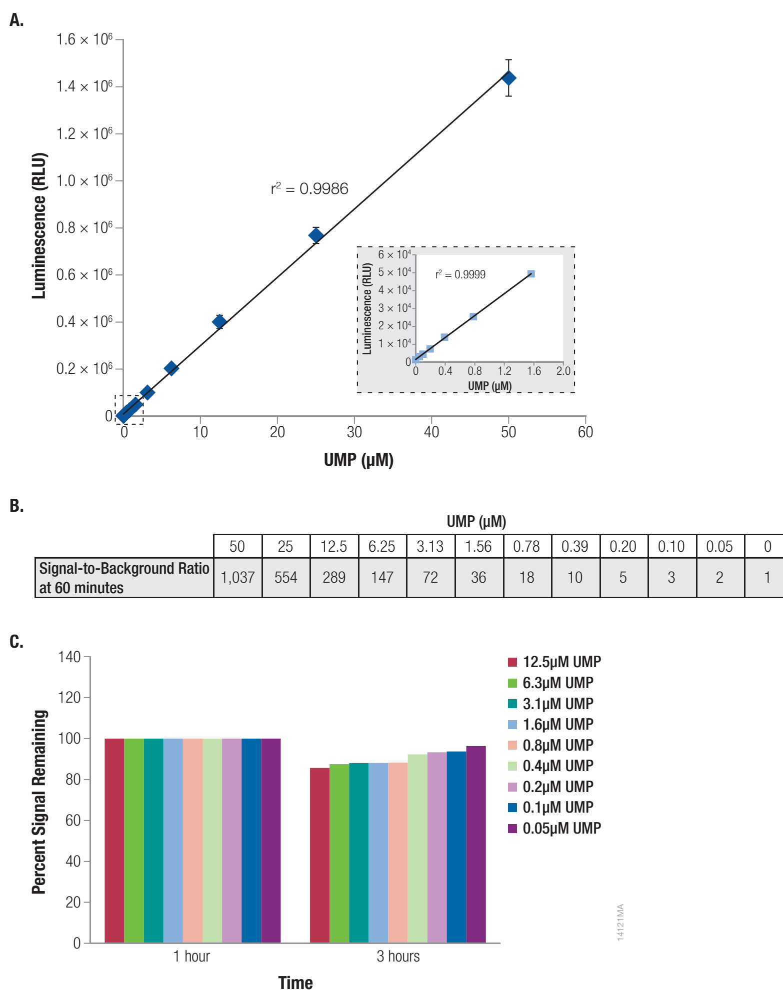
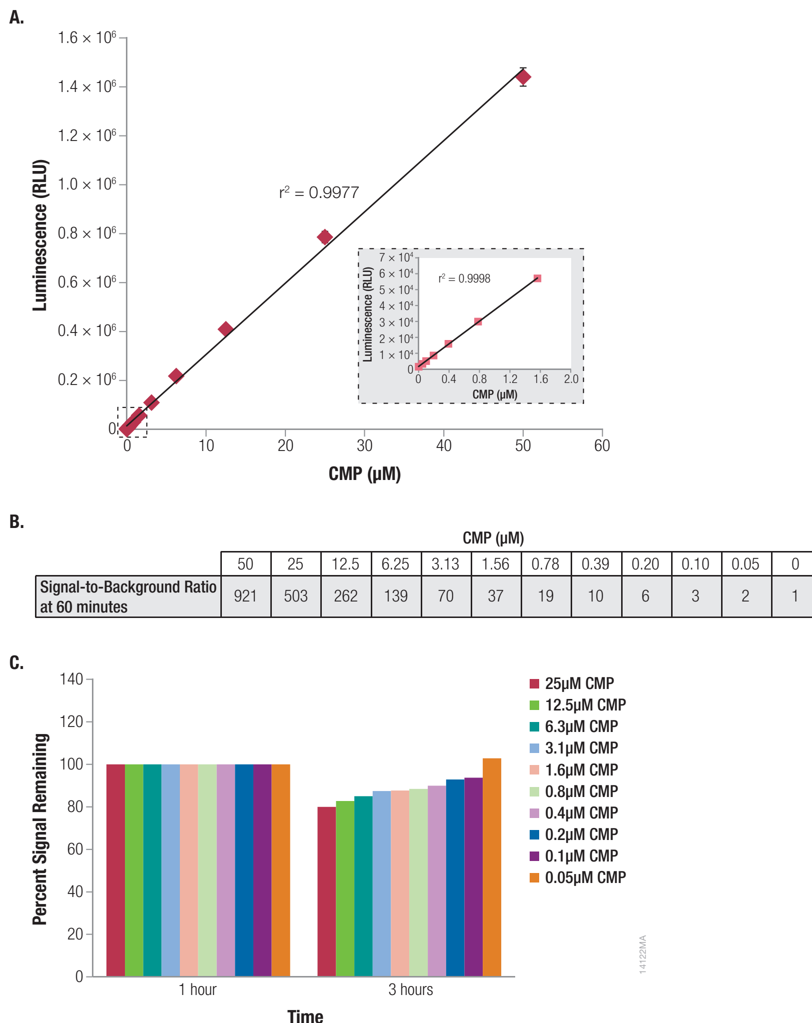
1. Description (continued)



	UMP (µM)											
	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	0.05	0
Signal-to-Background Ratio at 60 minutes	1,037	554	289	147	72	36	18	10	5	3	2	1

Figure 2. Detection linearity and sensitivity for UMP using the UMP/CMP-Glo™ Glycosyltransferase Assay. Panel A. UMP standard curve was prepared over the indicated range of UMP concentrations in 25µl of 1X glycosyltransferase reaction buffer in a solid white 96-well plate. (Standard curve preparation is described in Section 3.B.) UMP/CMP-Glo™ Glycosyltransferase Assay was performed using 25µl of UMP/CMP Detection Reagent at room temperature as described in Section 4. Luminescence was recorded using a GloMax® 96 Microplate Luminometer (Cat.# E6501). Values represent the mean of two replicates. **Panel B.** Luminescence was measured 1 hour after adding the UMP/CMP Detection Reagent, and signal-to-background ratios were calculated for each concentration of the UMP standard curve. **Panel C.** To determine signal stability, luminescence was recorded at 1 and 3 hours after adding the UMP/CMP Detection Reagent. Values represent the mean of two replicates. The signal-to-background ratio did not change over the time measured (data not shown). RLU = relative light units.



	CMP (µM)											
	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	0.05	0
Signal-to-Background Ratio at 60 minutes	921	503	262	139	70	37	19	10	6	3	2	1

Figure 3. Detection linearity and sensitivity for CMP using the UMP/CMP-Glo™ Glycosyltransferase Assay. Panel A. CMP standard curve was prepared over the indicated range of CMP concentrations in 25µl of 1X glycosyltransferase reaction buffer in a solid white 96-well plate. (Standard curve preparation is described in Section 3.B.) UMP/CMP-Glo™ Glycosyltransferase Assay was performed using 25µl of UMP/CMP Detection Reagent at room temperature as described in Section 4. Luminescence was recorded using a GloMax® 96 Microplate Luminometer (Cat.# E6501). Values represent the mean of two replicates. **Panel B.** Luminescence was measured 1 hour after adding the UMP/CMP Detection Reagent, and signal-to-background ratios were calculated for each concentration of the CMP standard curve. **Panel C.** To determine signal stability, luminescence was recorded at 1 and 3 hours after adding the UMP/CMP Detection Reagent. Values represent the mean of two replicates. The signal-to-background ratio did not change over the time measured (data not shown). RLU = relative light units.

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1. Description (continued)

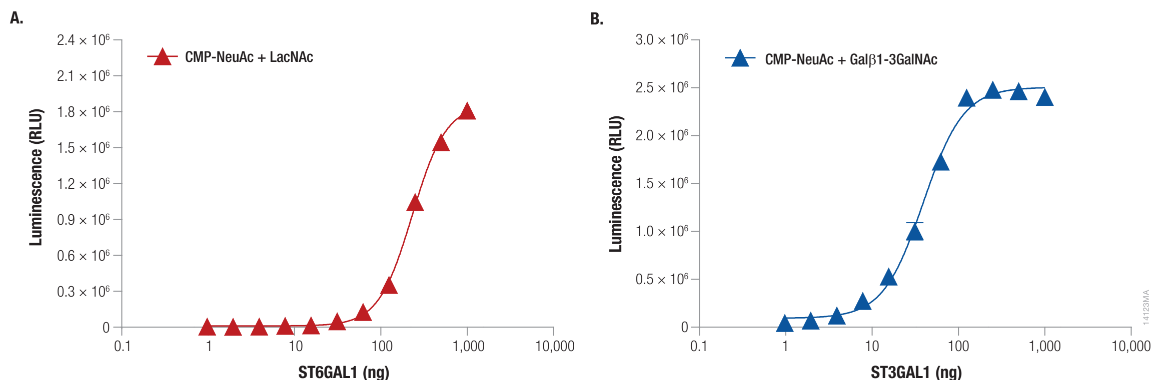

Figure 4. Detection of the activity of various sialyltransferases. Panel A. ST6GAL1 (R&D Systems Cat.# 7620-GT) was titrated in 1X ST6GAL1 reaction buffer in the presence of 100µM of CMP-NeuAc (Sigma Cat.# C8271) and 1mM LacNAc (Dextra Cat.# GN204) as an acceptor substrate. **Panel B.** ST3GAL1 (R&D Systems Cat.# 6905-GT-020) was titrated in 1X ST3GAL1 reaction buffer in the presence of 200µM of CMP-NeuAc (Sigma Cat.# C8271) and 0.5mM β-1,3-galactosyl-N-acetyl galactosamine (Dextra Cat.# GN213), as an acceptor substrate. All enzyme reactions were performed in 25µl volume in a solid white 96-well plate and incubated at 23°C for 60 minutes. The UMP/CMP-Glo™ Glycosyltransferase Assay was performed as described in Section 4.A. Each point is an average of two experiments, and the error bars represent the standard deviations. Curve fitting was performed using GraphPad Prism®, version 6, sigmoidal dose-response (variable slope) software. RLU = relative light units.

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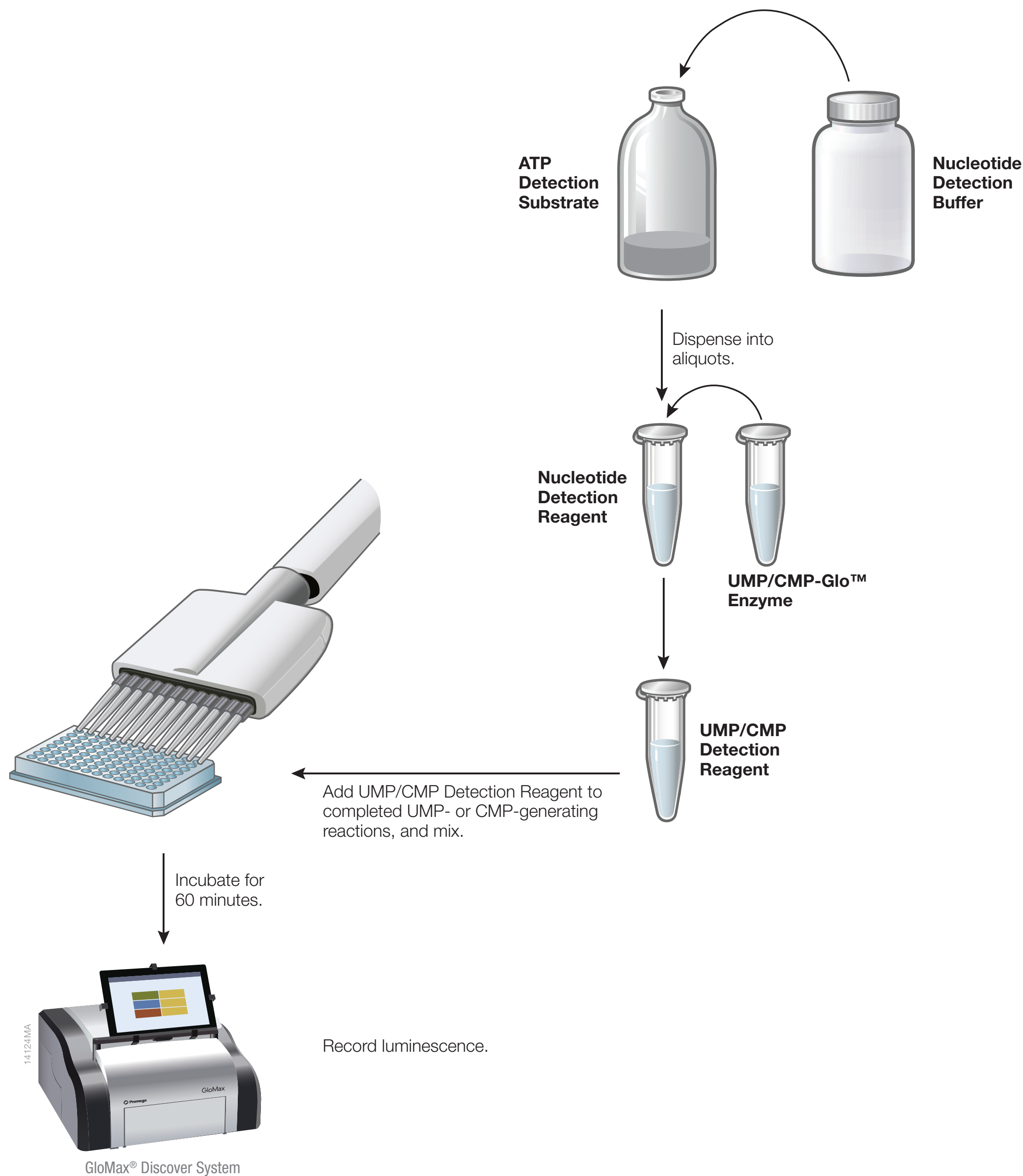

Figure 5. Schematic representation of the UMP/CMP-Glo™ Glycosyltransferase Assay protocol.

1. Description (continued)

Advantages of the UMP/CMP-Glo™ Glycosyltransferase Assay

- **Positive linear response in the nM to µM range:** Assay signal increases linearly with increasing product formation. Uses low concentrations of nucleotide-sugars, decreasing feedback glycosyltransferase inhibition issues.
- **High dynamic range:** High signal-to-background ratios at lower concentrations of UMP or CMP means using less enzyme during the phosphoglycosyltransferase or glycosyltransferase reactions.
- **High sensitivity:** Detect 1.25–2.5pmol UMP/CMP with a more than twofold difference over background.
- **Reliable, reproducible data:** Routinely obtain Z´ factor values >0.7 even with low UMP or CMP production rates.
- **Universal assay:** Use any UMP- or CMP-releasing glycosyltransferase and acceptor substrates of various chemical natures.
- **Luminescence-based UMP or CMP detection:** Experience less overall assay interference from chemical compounds compared to fluorescence-based assays.
- **Batch plate processing:** Highly stable luminescent signal with >80% signal remaining after 3 hours.
- **Homogeneous non-radioactive assay.**

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
UMP/CMP-Glo™ Glycosyltransferase Assay	**200 assays**	**VA1130**

This system is sufficient for 200 assays performed in 96-well plates using a 25µl glycosyltransferase reaction and 25µl of UMP/CMP Detection Reagent. This system also can be used in 384-well plates using 5µl:5µl for a total of 1,000 assays. Includes:

- 100µl UMP, 10mM
- 100µl CMP, 10mM
- 200µl UMP/CMP-Glo™ Enzyme
- 5ml Nucleotide Detection Buffer
- 1 vial ATP Detection Substrate (lyophilized)

PRODUCT	SIZE	CAT.#
UMP/CMP-Glo™ Glycosyltransferase Assay	**400 assays**	**VA1131**

This system is sufficient for 400 assays performed in 96-well plates using a 25µl glycosyltransferase reaction and 25µl of UMP/CMP Detection Reagent. This system also can be used in 384-well plates using 5µl:5µl for a total of 2,000 assays. Includes:

- 100µl UMP, 10mM
- 100µl CMP, 10mM
- 400µl UMP/CMP-Glo™ Enzyme
- 10ml Nucleotide Detection Buffer
- 1 vial ATP Detection Substrate (lyophilized)

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PRODUCT	SIZE	CAT.#
UMP/CMP-Glo™ Glycosyltransferase Assay	**4,000 assays**	**VA1132**

This system is sufficient for 4,000 assays performed in 96-well plates using a 25µl glycosyltransferase reaction and 25µl of UMP/CMP Detection Reagent. This system also can be used in 384-well plates using 5µl:5µl for a total of 20,000 assays. Includes:

- 100µl UMP or CMP, 10mM
- 100µl CMP, 10mM
- 4 × 1ml UMP/CMP-Glo™ Enzyme
- 100ml Nucleotide Detection Buffer
- 1 vial ATP Detection Substrate (lyophilized)

Storage Conditions: Store the UMP/CMP-Glo™ Glycosyltransferase Assay kit at less than –65°C. Alternatively, store UMP/CMP-Glo™ Enzyme at less than –65°C and the other components at –10°C to –30°C. Before use, completely thaw all components at room temperature except the UMP/CMP-Glo™ Enzyme, which should be thawed just before use and any remaining volume should be returned immediately to less than –65°C. Once thawed, all components should be thoroughly mixed before use. Any remaining Nucleotide Detection Reagent (Nucleotide Detection Buffer + ATP Detection Substrate) should be dispensed into aliquots and stored at less than –65°C. For best results, prepare only the amount of UMP/CMP Detection Reagent (Nucleotide Detection Reagent + UMP/CMP-Glo™ Enzyme) needed. If smaller amounts of UMP/CMP Detection Reagent are needed for each use, the UMP/CMP-Glo™ Enzyme should be dispensed in single-use aliquots and stored at less than –65°C.

3. Preparing for the UMP/CMP-Glo™ Glycosyltransferase Assay

Materials to Be Supplied by the User

- solid white multiwell plate (do **not** use black plates or clear plates)
- enzyme reaction buffers; used for enzyme, substrate and compound dilution
- multichannel pipette or automated pipetting station
- glycosyltransferase (e.g., sialyltransferase or phosphoglycosyltransferase)
- sugar acceptor substrate
- luminometer capable of reading multiwell plates (e.g., GloMax® Discover System [Cat.# GM3000])
- plate shaker

3.A. Preparing the UMP/CMP Detection Reagent

Calculate the required volumes of each reagent needed for your experiment, and increase or decrease the volumes appropriately. For example, prepare 2.5ml of UMP/CMP Detection Reagent for each 96-well plate.

Nucleotide Detection Reagent Preparation

1. Equilibrate the Nucleotide Detection Buffer and ATP Detection Substrate to room temperature before use.
2. Transfer the entire volume of Nucleotide Detection Buffer into the amber bottle containing ATP Detection Substrate to reconstitute the lyophilized luciferase enzyme/substrate mixture. This forms the Nucleotide Detection Reagent.
3. Mix to homogeneity by gently vortexing, swirling or by inverting the contents. The ATP Detection Substrate should go easily into solution in less than 1 minute.
4. Use Nucleotide Detection Reagent immediately or dispense into aliquots and store at less than –65°C.

UMP/CMP Detection Reagent Preparation

1. Equilibrate an aliquot of Nucleotide Detection Reagent to room temperature.
2. Prepare UMP/CMP Detection Reagent by adding 40µl of UMP/CMP-Glo™ Enzyme to each 1ml of Nucleotide Detection Reagent immediately before use.
3. Mix contents to homogeneity by gently pipetting or vortexing.

   Note: Make only enough UMP/CMP Detection Reagent required for the experiment. Return the remaining UMP/CMP-Glo™ Enzyme to less than –65°C immediately after use.

3.B. Generating a Standard Curve for UMP or CMP

To estimate the amount of UMP or CMP produced in the glycosyltransferase reaction, we recommend creating a standard curve of 0–50µM UMP or CMP.

The UMP or CMP standards can be prepared in a separate 96-well or 384-well plate. Once the standards are prepared, transfer the appropriate amount to the same assay plate where the glycosyltransferase reaction is being performed. We recommend assaying each UMP or CMP standard concentration in triplicate. Figures 2 and 3 show representative data from UMP and CMP standard curves.

1. Prepare 200µl of 50µM UMP or CMP solution in preferred 1X glycosyltransferase reaction buffer using the provided 10mM standard. Then add all 200µl of the 50µM solution to well A1 of a preparative 96-well plate.
2. Add 100µl of 1X glycosyltransferase buffer to wells A2 through A12 of the preparative 96-well plate.

   Note: Depending on the requirements of your system, you can use glycosyltransferase buffer containing UMP or CMP and other appropriate substrate or only UMP or CMP.
3. Perform a serial twofold dilution as shown in Figure 6 by transferring 100µl from well A1 to well A2, pipetting to mix. Transfer 100µl from well A2 to well A3, pipetting to mix. Repeat for wells A4 through A11. Discard the extra 100µl from well A11. Do not add UMP or CMP to the no-UMP/CMP control reactions in well A12.

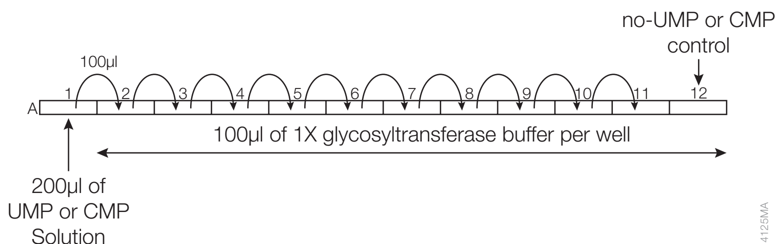

Figure 6. Dilution scheme for creating a UMP or CMP standard curve.

4. Transfer the desired volume of each UMP or CMP standard from the 96-well plate to the wells reserved for the standard curve on your assay plate.
5. Proceed immediately to the assay protocol (Section 4).

We recommend the following volumes for different plate formats:

96-well assay plate: Transfer 25µl of UMP or CMP standards.
384-well assay plate: Transfer 10µl of UMP or CMP standards.
Low-volume 384-well assay plate: Transfer 5µl or less of UMP or CMP standards.

The luminescence output of the assay is proportional to the concentration of UMP or CMP in the standard curve. This means luminescence readout can be directly compared to those UMP or CMP concentrations generated in a glycosyltransferase reaction sample as long as the volume of the UMP or CMP standards used is the same as the volume of the glycosyltransferase reaction.

4. UMP/CMP-Glo™ Glycosyltransferase Assay Protocols

Prior to performing the UMP/CMP-Glo™ Glycosyltransferase Assay, prepare the reagents and UMP or CMP standards as described in Section 3. Equilibrate the volume of Nucleotide Detection Reagent to room temperature before use. The UMP/CMP Detection Reagent is stable for 2 hours at 22°C with minimal loss of signal and up to 4 hours with ~20% loss of signal. However the signal-to-background ratio is stable for at least 5 hours.

Note: The reconstituted Nucleotide Detection Reagent remains stable for at least 9 months with no loss of signal observed after 5 freeze-thaw cycles.

4.A. UMP/CMP-Glo™ Glycosyltransferase Assay Protocol

The UMP/CMP-Glo™ Glycosyltransferase Assay consists of a single reagent added to the completed glycosyltransferase reaction as outlined in Figures 1 and 5. For 96-well plates, we recommend a 25µl glycosyltransferase reaction and 25µl of UMP/CMP Detection Reagent for a total volume of 50µl. For 384-well plates, volumes may be reduced fivefold to a 5µl glycosyltransferase reaction and 5µl of UMP/CMP Detection Reagent for a total volume of 10µl. Other volumes may be used provided the 1:1 ratio of glycosyltransferase reaction volume to UMP/CMP Detection Reagent volume is maintained. The UMP/CMP-Glo™ Glycosyltransferase Assay protocol for 96-well plates is described below.

1. Perform a 25µl glycosyltransferase reaction using the 1X glycosyltransferase buffer of your choice. (See Section 7 for buffer examples.)

   If the glycosyltransferase reaction was not incubated at room temperature, equilibrate the plate to room temperature before adding the UMP/CMP Detection Reagent.

2. Prepare the UMP/CMP Detection Reagent as instructed in Section 3.A.

3. Add 25µl of UMP/CMP Detection Reagent to each well of the assay plate.

   The UMP/CMP Detection Reagent terminates the glycosyltransferase reaction; therefore there is no need to add an inhibitor to terminate the glycosyltransferase reaction (e.g., EDTA, acid, etc.). However, if a glycosyltransferase-termination reagent is added to the glycosyltransferase reaction, do not use a magnesium-chelating agent such as EDTA because the UMP/CMP-Glo™ Glycosyltransferase Assay requires magnesium. The optimal pH for this assay is pH 6–9.

4. Mix assay plate with a plate shaker for 30 seconds, and incubate at room temperature for 60 minutes.

5. Measure the luminescence with a plate-reading luminometer or charge-coupled device (CCD) camera.

   Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline. The long half-life of the UMP/CMP-Glo™ Glycosyltransferase Assay signal allows plates to be left longer at room temperature before reading, if desired.

4.B. Optimizing Glycosyltransferase Reaction Conditions

For optimal performance when using the UMP/CMP-Glo™ Glycosyltransferase Assay, optimize the amounts of glycosyltransferase and glycosyltransferase substrates in the reaction. If the amount of glycosyltransferase or its substrates has been determined, proceed to Section 4.A.

Note: We recommend optimizing the glycosyltransferase reaction conditions at room temperature to ensure uniform temperature across the plate during the UMP/CMP-Glo™ Glycosyltransferase Assay.

Preparation of Glycosyltransferase Titration Components

UMP/CMP-Glo™ Glycosyltransferase Assay has been used successfully with phosphoglycosyltransferases such as PglC from *C. jejuni* and *H. pullorum*, and WecA from *T. maritima,* and shown to be a valuable assay for characterizing PGT enzymes that produce UMP as a product (2). The following protocol is written for the α(2,6)-sialyltransferase-1 (ST6GAL1) enzyme titration shown in Figure 4, Panel A, as an example to select an optimal enzyme concentration for use in subsequent experiments such as substrate K_m determination. The ST6GAL1 reaction is performed in a 96-well plate using 1X ST buffer, 100µM CMP-sialic acid (CMP-NeuAc; Sigma Cat.# C8271) as a sugar donor, 1mM LacNAc (Dextra Cat.# GN204) as an acceptor, and a serial dilution of ST6GAL1 enzyme from 0–1µg/reaction in 25µl volume. A CMP standard curve is performed in the same assay plate to correlate luminescence to the CMP concentration generated in each ST6GAL1 reaction.

Note: Different STs or other glycosyltransferases have varying specific activities. Therefore, the useful enzyme dilution range may vary greatly and should be determined experimentally.

1. **Substrate Mix Preparation:** Prepare 400µl of 2.5X CMP-NeuAc/LacNAc Substrate Mix (10µl/reaction/well) in a 1.5ml tube as described below and keep on ice until ready to dispense in the assay plate.

Component	Volume
5X ST reaction buffer	80µl
10mM CMP-NeuAc	10µl
100mM LacNAc	10µl
ATP-free water	300µl

4.B. Optimizing Glycosyltransferase Reaction Conditions (continued)

2. **ST6GAL1 Solution Preparation:** Prepare 100µl of ST6GAL1 enzyme solution as described below (15µl/reaction/well). This will give 1µg of ST6GAL1/15µl starting concentration.

Component	Volume
5X ST reaction buffer	20µl
ST6GAL1 (0.549µg/µl)	12.14µl
ATP-free water	67.86µl

a. Prepare 1ml of 1X ST reaction buffer by mixing 200µl of 5X ST reaction buffer with 800µl ATP-free water.

b. Add 100µl of ST6GAL1 enzyme solution to well A1 of a 96-well plate.

c. Add 50µl of 1X ST reaction buffer to wells A2 through A12 of the 96-well plate.

d. Perform a serial twofold dilution by transferring 50µl from well A1 to well A2, pipetting to mix as described in Table 1. Transfer 50µl from well A2 to well A3, pipetting to mix. Repeat for wells A4–A11. Discard the extra 50µl from well A11. Do **not** add ST6GAL1 to the no-enzyme control reaction in well A12.

Note: Do not create bubbles while preparing the dilution series.

Table 1. Performing Serial 1:1 Dilutions of ST6GAL1.

Well Number	ST6GAL1 (ng)	Starting Volume of Each Well	Volume to Transfer
A1	1,000	100µl	50µl
A2	500	50µl	50µl
A3	250	50µl	50µl
A4	125	50µl	50µl
A5	62.5	50µl	50µl
A6	31.2	50µl	50µl
A7	15.6	50µl	50µl
A8	7.8	50µl	50µl
A9	3.9	50µl	50µl
A10	1.9	50µl	50µl
A11	0.93	50µl	0µl; No transfer
A12	0	50µl	Buffer only

ST6GAL1 Reaction and CMP Standard Curve Experiment

1. Transfer 25µl of the CMP serial dilution in duplicate into the standard curve-designated wells of the 96-well assay plate.
2. Transfer 15µl of ST6GAL1 dilution samples in duplicate from the wells of the ST6GAL1 titration plate to the wells of the assay plate.
3. Transfer 10µl of the 2.5X CMP-NeuAc/LacNAc Substrate Mix to the rows of the ST6GAL1 dilutions.
4. Centrifuge the plate and mix with a plate shaker for 2 minutes. Incubate the reaction at room temperature for 60 minutes or the desired time.
5. Follow the UMP/CMP-Glo™ Glycosyltransferase Assay protocol described in Section 4.A, starting at Step 2.
6. Record luminescence.

   Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

Note: The optimal amount of a glycosyltransferase to use in subsequent experiments including substrate K_m determination is the amount that produces luminescence within the linear range of the ST6GAL1 titration curve and generates an adequate signal-to-background ratio.

4.C. Determining K_m Value for ST6GAL1 Substrate CMP-NeuAc

The following protocol is an example of a substrate titration in ST6GAL1 reaction to determine the K_m value for CMP-NeuAc substrate. Representative substrate titration data is shown in Figure 7, Panel A. The ST6GAL1 reaction is performed in a 96-well plate using 1X ST buffer, 0.2µg ST6GAL1/reaction, 1mM LacNAc as an acceptor substrate and a serial dilution of CMP-NeuAc as a sugar donor from 0–2mM/reaction in a 25µl volume. A CMP standard curve is performed in the same assay plate to convert luminescence to the CMP concentration generated in each ST6GAL1 reaction. This protocol is designed for a 96-well plate using a 25µl:25µl ratio of glycosyltransferase reaction volume to UMP/CMP Detection Reagent. To perform the assay in a 384-well plate, reduce volumes fivefold. Other volumes may be used, provided the 1:1 ratio of glycosyltransferase reaction volume to UMP/CMP Detection Reagent volume is maintained.

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4.C. Determining K_m Value for ST6GAL1 Substrate CMP-NeuAc (continued)

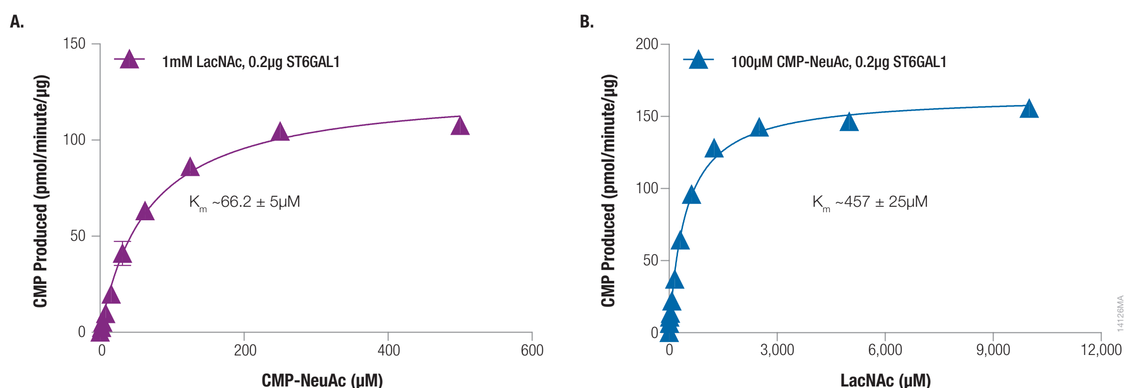

Figure 7. Determining K_m values for sialyltransferase acceptor and donor substrates. Panel A. CMP-NeuAc donor substrate was titrated from 0–0.5mM/reaction in a 25µl volume ST6GAL1 reaction in the presence of 1X ST6GAL1 buffer, 0.2µg ST6GAL1/reaction and 1mM LacNAc as an acceptor substrate. **Panel B.** LacNAc acceptor substrate was titrated from 0–10mM/reaction in a 25µl volume ST6GAL1 reaction in the presence of 1X ST6GAL1 buffer, 0.2µg ST6GAL1/reaction and 100µM CMP-NeuAc as a donor substrate. A CMP standard curve is performed in the same assay plate to convert luminescence to the CMP concentration generated in each ST6GAL1 reaction. The reactions were incubated for 60 minutes at 23°C. The UMP/CMP-Glo™ Glycosyltransferase Assay was performed as described in Section 4.A. Values represent the mean of two replicates. K_m was extracted from the data after fitting to the Michaelis-Menten equation using the non-linear regression fit in GraphPad Prism®, version 6, analysis tool as described in Section 4.C. Error bars represent standard deviation. K_m values for CMP-NeuAc and LacNAc determined using the UMP/CMP-Glo™ Glycosyltransferase Assay compare favorably to the K_m values reported for these substrates in the literature (6,7).

Preparation of CMP-NeuAc Titration Components

1. **CMP-NeuAc Solution Preparation:** Prepare 100µl of 5mM CMP-NeuAc solution as described below (final 10µl/reaction/well). This gives 2mM CMP-NeuAc starting concentration in the assay.

Component	Volume
5X ST reaction buffer	20µl
10mM CMP-NeuAc	50µl
ATP-free water	30µl

   a. Prepare 1ml of 1X ST reaction buffer by mixing 200µl of 5X ST reaction buffer and 800µl of ATP-free water.

   b. Add 100µl of CMP-NeuAc solution to well A1 of a 96-well plate.

   c. Add 50µl of 1X ST reaction buffer to wells A2–A12 of the 96-well plate.

   d. Perform a serial 1:1 dilution by transferring 50µl from well A1 to well A2 and pipetting to mix as described in Table 2. Transfer 50µl from well A2 to well A3 and pipet to mix. Repeat for wells A4–A11. Do **not** add CMP-NeuAc solution to the no-substrate control reaction in well A12.

Table 2. Performing Serial 1:1 Dilutions of CMP-NeuAc Solution.

Well Number	Final CMP-NeuAc Concentration (µM)	Starting Volume of Each Well	Volume to Transfer
A1	500	100µl	50µl
A2	250	50µl	50µl
A3	125	50µl	50µl
A4	63	50µl	50µl
A5	31	50µl	50µl
A6	16	50µl	50µl
A7	8	50µl	50µl
A8	4	50µl	50µl
A9	2	50µl	50µl
A10	1	50µl	50µl
A11	0.5	50µl	0µl; No transfer
A12	No substrate	50µl	Buffer only

2. **ST6GAL1 Solution Preparation:** Prepare 150µl of ST6GAL1 solution (excess amount of 30 reactions at 5µl/reaction/well) in a 1.5ml tube as described below and keep on ice until ready to dispense in the assay plate. This will give 0.2µg of ST6GAL1/reaction.

Component	Volume
5X ST reaction buffer	30µl
ST6GAL1 (0.549µg/µl)	10.9µl
ATP-free water	109.1µl

3. **Acceptor Substrate Mix Preparation:** Prepare 600µl of 2.5X LacNAc solution (excess amount of 60 reactions at 10µl/reaction/well) in a 1.5ml tube as described below and keep on ice until ready to dispense in the assay plate. This gives 1mM LacNAc final concentration in the reaction.

Component	Volume
5X ST reaction buffer	120µl
100mM LacNAc	15µl
ATP-free water	465µl

4.C. Determining K_m Value for ST6GAL1 Substrate CMP-NeuAc (continued)

CMP-NeuAc Titration Experiment

1. Following the plate setup in Figure 8, transfer 25µl of CMP standard serial dilution in duplicate into the wells designated for the standard curve.
2. Transfer 10µl of CMP-NeuAc samples from the substrate titration plate to the corresponding wells of the assay plate (e.g., well A1 from the 96-well titration plate to well A1–D1 of the 96-well assay plate, well A2 from the 96-well titration plate to well A2–D2 of the 96-well assay plate, etc.)
3. Transfer 10µl of the 2.5X LacNAc solution to all the assay rows.

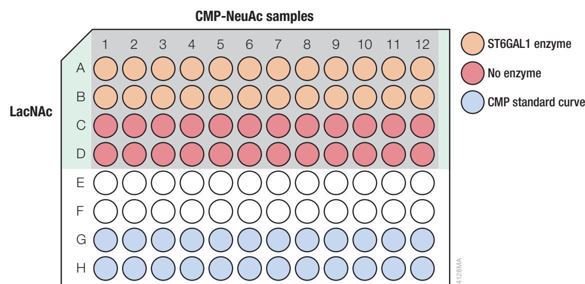

Figure 8. CMP-NeuAc titration in a 96-well plate.

4. Transfer 5µl of the ST6GAL1 solution in duplicate to wells A1–A12 and B1–B12 of the 96-well assay plate.

   Note: Add 5µl of 1X ST reaction buffer to wells C1–C12 and D1–D12 of the 96-well assay plate for the negative control (no-enzyme). This is the background from the CMP-NeuAc solution.
5. Centrifuge the plate. Mix with a plate shaker for 2 minutes. Incubate the reaction at room temperature for 60 minutes or the desired time.
6. Follow the UMP/CMP-Glo™ Glycosyltransferase Assay protocol described in Section 4.A, starting at Step 2.
7. Record luminescence.

   Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.
8. **Calculating K_m Value:** First, subtract the signal of the negative control (no-enzyme) wells from the corresponding sample signals. Using the CMP standard curve, calculate the CMP produced per minute per milligram enzyme for each point in the titration. K_m can be extracted from the data after fitting to the Michaelis-Menten equation using the non-linear regression fit in GraphPad Prism®, version 6, or similar data analysis tool.

Note: To determine K_m for the acceptor substrate LacNAc as shown in Figure 7, Panel B, the same procedure as in Section 4.C was followed, and LacNAc was titrated from 0–10mM in a reaction containing 1X ST buffer, 0.2ng FST6GAL1/reaction and 100µM CMP-NeuAc.

5. General Considerations

Temperature: The intensity and stability of the luminescent signal from the UMP/CMP-Glo™ Glycosyltransferase Assay depends on the rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction will result in a change in the intensity of light output and stability of the luminescent signal. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates and reagent to room temperature before adding the UMP/CMP Detection Reagent. Insufficient equilibration may result in a temperature gradient between the wells in the center and at the edge of the plate and, therefore, variability across the plate.

Solvents and Other Chemicals: The chemical environment in which the UMP/CMP-Glo™ Glycosyltransferase Assay is performed will affect the enzymatic rates and thus luminescence intensity. We recommend a pH of 6–9 for the glycosyltransferase buffer. Some vehicles used to resuspend the various test compounds or reagents used in the glycosyltransferase reaction buffer may interfere with the luciferase reaction and thus affect the light output of the assay. Various chemicals were shown to be compatible with or tolerated by the UMP/CMP-Glo™ Glycosyltransferase Assay (Table 3). Interference with the assay reaction can be detected by performing a UMP or CMP standard curve in the intended buffer compared with a simple buffer (Section 7).

Table 3. Solvents and Chemicals Compatible with the UMP/CMP-Glo™ Glycosyltransferase Assay.

Chemical	Maximum Concentration Tolerated[1]
NaCl	≤250mM
$CaCl_2$	≤20mM
DTT	≤10mM
Tween®-20	≤2%
Triton® X-100	≤2%
DMSO	≤5%
β-mercaptoethanol (BME)	≤250mM
$MgCl_2$	≤20mM
$MnCl_2$	≤10mM

[1]Higher concentrations of these chemicals will either decrease or increase the overall luminescence without affecting assay sensitivity. In some instances, higher concentrations might decrease the performance of the assay.

Plates and Instruments: We recommend using standard solid white, multiwell plates suitable for luminescence measurements (e.g., Corning Cat.# 3912, 3693, 4512). Luminescence can be recorded on a variety of plate readers although the relative light units will depend on the instrument. Assay well geometry and small dispensing volumes may affect the efficiency of mixing, thus poor assay homogeneity in individual wells may result in increased reaction noise or reduced signals or both. A UMP or CMP standard curve is useful for liquid handling and instrument optimization.

5. General Considerations (continued)

Testing for Compounds or Buffer Components that Interfere with the UMP/CMP-Glo™ Glycosyltransferase Assay: Compounds that interfere with the UMP/CMP-Glo™ Glycosyltransferase Assay are rare. We screened 1,280 compounds from the LOPAC chemical library using the UMP/CMP-Glo™ Assay reagents with 10µM compound; none of the compounds interfered with the UMP/CMP-Glo™ Glycosyltransferase Assay (5). When screening for glycosyltransferase inhibitors, compounds that inhibit only the glycosyltransferase will result in lower luminescence compared to vehicle-only controls and are easily distinguishable from compounds that inhibit other components of the assay. Test compounds that inhibit other components of the assay either alone or together with the glycosyltransferase might increase or decrease the luminescent signal, depending on the level of inhibition of the glycosyltransferase, luciferase or other enzyme components of the assay. To test hits from a glycosyltransferase screen for the possibility of chemical interference with enzymatic conversion of UMP or CMP, or generation of the luminescent signal, set up mock reactions without glycosyltransferase but with all other assay components present, including a concentration of UMP or CMP that mimics the glycosyltransferase reaction results. Add the appropriate concentration of test compound (usually 10µM) or vehicle control (e.g., 1% DMSO) to the mock reactions. A test compound that affects assay performance would alter luminescence by greater than 20% compared to vehicle control reactions without test compounds. Test compounds that inhibit luciferase may result in false hits, albeit rarely. However, the unique combination of Ultra-Glo™ Recombinant Luciferase and proprietary buffer compositions of the UMP/CMP-Glo™ Glycosyltransferase Assay will significantly reduce the number of false hits (4).

6. References

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7. Composition of Buffers and Solutions

1X ST6GAL1 reaction buffer

5mM	Tris (pH 7.5)
150µM	NaCl
5mM	$MnCl_2$
5mM	$CaCl_2$

1X ST3GAL1 reaction buffer

25mM	Tris (pH 7.5)
5mM	$MnCl_2$

8. Related Products

Product Name	Size	Cat.#
Endo H	10,000 units (500u/µl)	V4871
	50,000 units (500u/µl)	V4875
PNGase F	500 units (10u/µl)	V4831
Fetuin	500µg (10mg/ml)	V4961
Protein Deglycosylation Mix	20 reactions	V4931

GDP Glycosyltransferase Assay

Product Name	Size	Cat.#
GDP-Glo™ Glycosyltransferase Assay	200 assays	VA1090
	400 assays	VA1091
	4,000 assays	VA1092
Ultra Pure GDP-Fucose, 50mM	50µl	VA1097
	5 × 50µl	VA1098
Ultra Pure GDP-Mannose, 100mM	50µl	VA1099
	5 × 50µl	VA1100

8. Related Products (continued)

UDP Glycosyltransferase Assay

Product Name	Size	Cat.#
UDP-Glo™ Glycosyltransferase Assay	200 assays	V6961
	400 assays	V6962
	4,000 assays	V6963
Ultra Pure UDP-GlcNAc, 100mM	50µl	V7071
	250µl	V7072
Ultra Pure UDP-GalNAc, 100mM	50µl	V7081
	250µl	V7082
Ultra Pure UDP-Glucose, 100mM	50µl	V7091
	250µl	V7092
Ultra Pure UDP-Galactose, 100mM	50µl	V7171
	250µl	V7172
Ultra Pure UDP-Glucuronic Acid (UDP-GA), 100mM	50µl	V7321
	250µl	V7322

[a]U.S. Pat. Nos. 6,602,677, 7,241,584, 8,030,017 and 8,822,170, European Pat. No. 1131441, Japanese Pat. Nos. 4537573 and 4520084 and other patents pending.

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