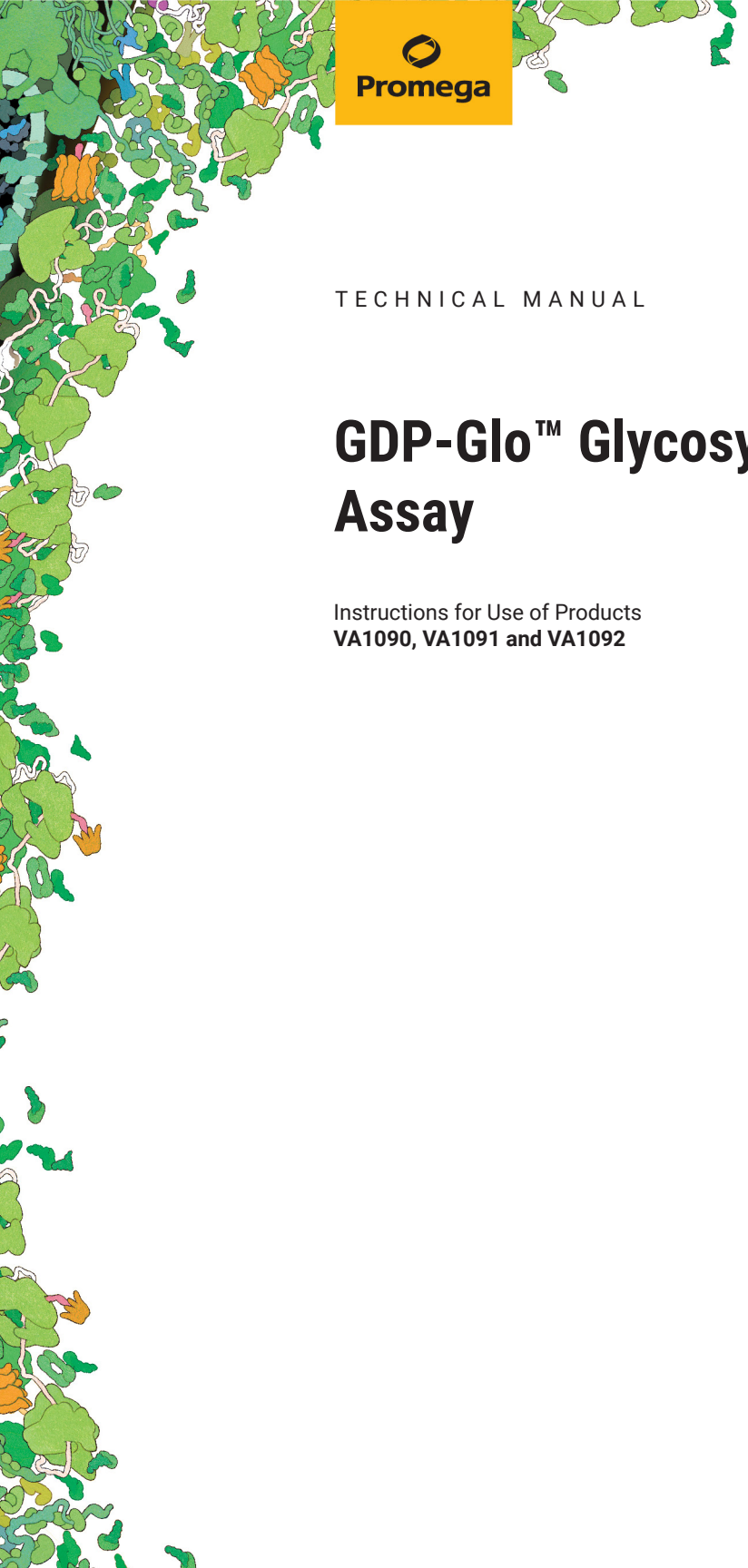


TECHNICAL MANUAL

GDP-Glo™ Glycosyltransferase Assay

Instructions for Use of Products
VA1090, VA1091 and VA1092

Revised 7/25
TM505

GDP-Glo™ Glycosyltransferase Assay

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The GDP-Glo™ Glycosyltransferase Assay[a] is a bioluminescent assay for detecting the activity of glycosyltransferases that use GDP-sugars as donor substrates and release GDP as a product. 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. Glycosyltransferases transfer sugar from a nucleotide-glycosyl donor to an acceptor molecule. GDP-sugar is one of the most utilized sugar donors for glycosylating enzymes after UDP-sugars (e.g., GDP-fucose and GDP-mannose; 2). In a glycosyltransferase reaction, the GDP moiety is released as a product. Therefore, an assay that detects GDP as product of these reactions would be suitable for monitoring the activity of all the GDP-sugar-utilizing glycosyltransferases.

The GDP-Glo™ Glycosyltransferase Assay is a homogeneous, one-step-reagent-addition method to rapidly detect GDP formation in glycosyltransferase reactions. After the glycosyltransferase reaction, an equal volume of GDP Detection Reagent is added to simultaneously convert the GDP 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 GDP concentration by using a GDP standard curve. The light output is proportional to the concentration of GDP from low nM to 25µM (Figure 2, Panel A). The assay is easy to use and highly sensitive (Figure 2, Panel B), two features that are desirable and essential for measuring the activity of different GDP-sugar-utilizing glycosyltransferases such as fucosyltransferases (Figure 3). Therefore, the GDP detection assay uses less enzyme in glycosyltransferase reactions. This assay is fast and simple (Figure 4). The GDP-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.

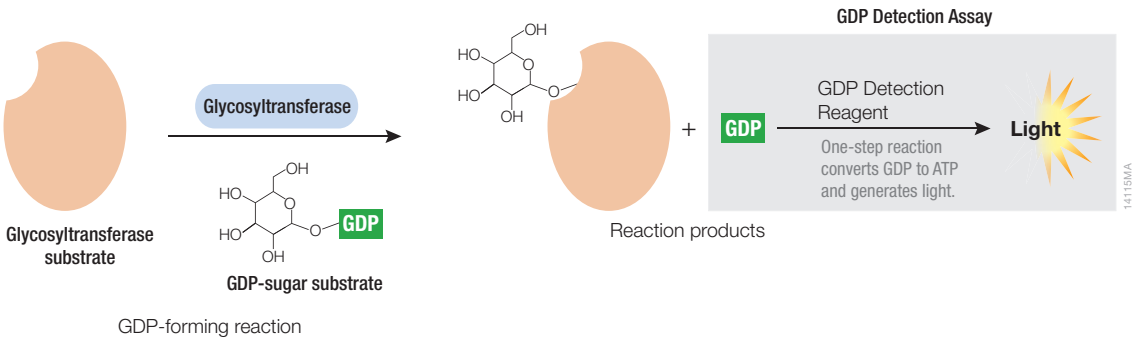
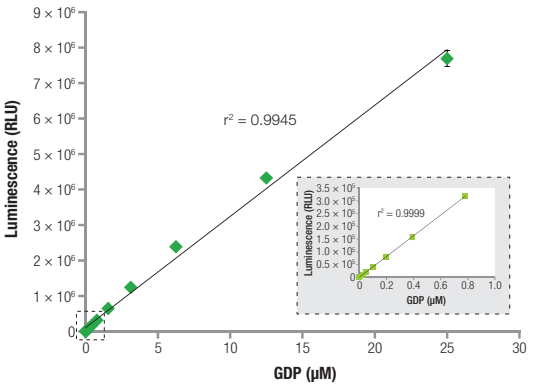


Figure 1. GDP-Glo™ Glycosyltransferase Assay principle. The assay is performed in one step. After the glycosyltransferase reaction, GDP Detection Reagent is added to convert GDP to ATP and measure the newly synthesized ATP using a luciferase/luciferin reaction. The light generated correlates to the amount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

The GDP-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 GDP Detection Reagent is stable for more than 3 hours (Figure 2, 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 (3) and proprietary formulation of the GDP Detection Reagent results in luminescence that is much less susceptible to interference from library compounds than other luciferase- or fluorescence-based assays (4). In addition to providing biochemical values (e.g., K_m of GDP-sugars or K_m of acceptor substrates) comparable to those reported in the literature, the GDP-Glo™ Glycosyltransferase Assay can be used in screening for specific glycosyltransferase inhibitors and the study of their mode of action (5).

1. Description (continued)

A.



B.

	GDP (µM)											
	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	0.05	0.02	0
Signal-to-Background Ratio at 60 minutes	35,124	19,758	10,898	5,682	2,965	1,457	715	369	183	96	46	1

C.

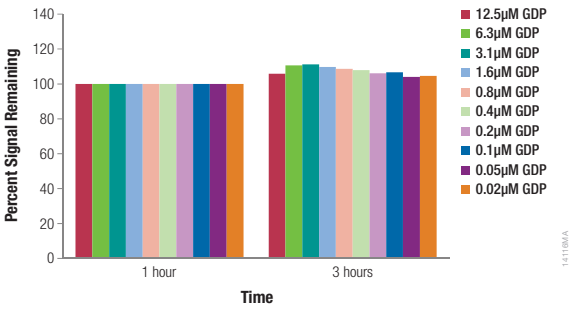


Figure 2. Linearity and sensitivity of the GDP-Glo™ Glycosyltransferase Assay. Panel A. GDP standard curve was prepared over the indicated range of GDP concentrations in 25µl of 1X glycosyltransferase reaction buffer in a solid white 96-well plate. (Standard curve preparation is described in Section 3.B.) GDP-Glo™ Glycosyltransferase Assay was performed using 25µl of GDP Detection Reagent at room temperature as described in Section 4. Luminescence was recorded using a GloMax® 96 Microplate Luminometer. Values represent the mean of four replicates. **Panel B.** Luminescence was measured 1 hour after adding the GDP Detection Reagent, and signal-to-background ratios calculated for each concentration of the GDP standard curve. **Panel C.** To determine signal stability, luminescence was recorded at 1 hour and 3 hours after adding the GDP 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.

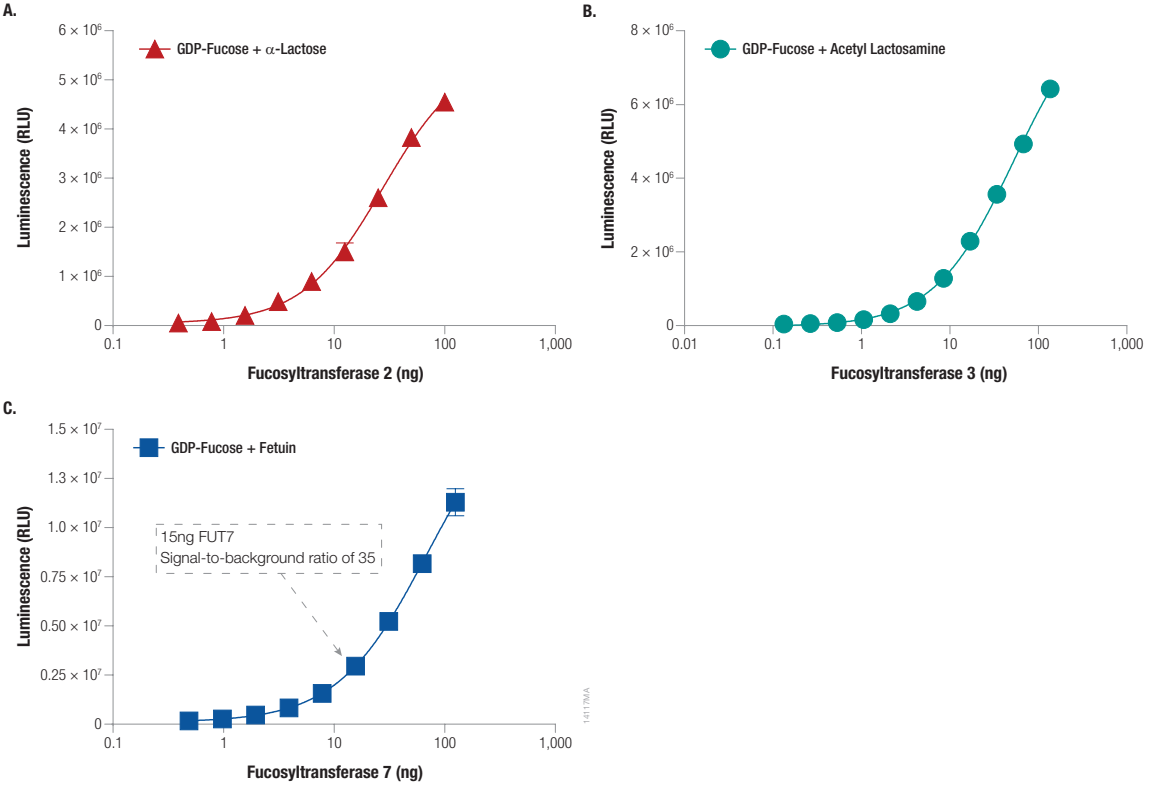


Figure 3. Detection of the activity of various GDP-sugar-utilizing enzymes. Panel A. FUT2, (R&D Systems Cat.# 7770-GT) was titrated in 1X FUT2 reaction buffer the presence of 40µM Ultra Pure GDP-Fucose (Cat.# VA1097) and 10mM α-Lactose (Sigma Cat.# L2643) as an acceptor substrate. **Panel B.** FUT3 (R&D Systems Cat.# 4950-GT) was titrated in 1X FUT3 reaction buffer in the presence of 40µM Ultra Pure GDP-Fucose and 100mM Acetyl Lactosamine (Carbosynth Cat.# OA08244), as an acceptor substrate. **Panel C.** FUT7, (R&D Systems Cat.# 6409-GT) was titrated in 1X FUT7 reaction buffer in the presence of 40µM Ultra Pure GDP-Fucose and 20µM Fetuin (Sigma Cat# F2379) as an acceptor substrate. All enzyme reactions were performed in a 25µl volume in a solid white 96-well plate. FUT 2 and FUT7 were incubated at 37°C for 30 minutes and FUT3 was incubated at 23°C for 60 minutes. The GDP-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. The insert highlights FUT activity at nanogram amounts with a high signal-to-background ratio. RLU = relative light units.

1. Description (continued)

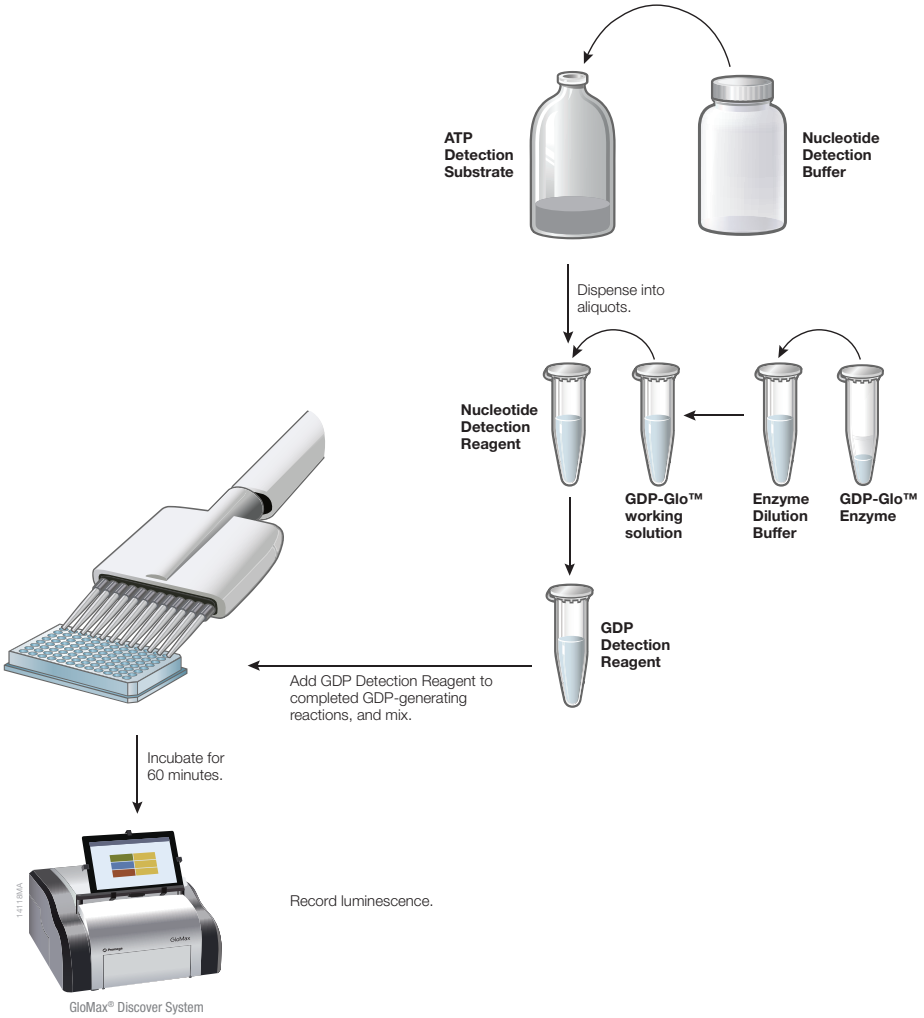


Figure 4. Schematic representation of the GDP-Glo™ Glycosyltransferase Assay protocol.

Note: This assay detects only the activity of glycosyltransferases that use GDP-sugar as a donor substrate 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 GDP-Glo™ Glycosyltransferase Assay.

The sensitivity of the GDP-Glo™ Glycosyltransferase Assay means it can detect low GDP 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 GDP, a high signal-to-background ratio is obtained with the GDP-Glo™ Glycosyltransferase Assay. See Figure 2, Panel B.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
GDP-Glo™ Glycosyltransferase Assay	**200 assays**	**VA1090**

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

- 100µl GDP, 10mM
- 20µl GDP-Glo™ Enzyme
- 1.5ml Enzyme Dilution Buffer
- 5ml Nucleotide Detection Buffer
- 1 vial ATP Detection Substrate (lyophilized)

PRODUCT	SIZE	CAT.#
GDP-Glo™ Glycosyltransferase Assay	**400 assays**	**VA1091**

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

- 100µl GDP, 10mM
- 40µl GDP-Glo™ Enzyme
- 2 × 1.5ml Enzyme Dilution Buffer
- 10ml Nucleotide Detection Buffer
- 1 vial ATP Detection Substrate (lyophilized)

2. Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
GDP-Glo™ Glycosyltransferase Assay	**4,000 assays**	**VA1092**

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

- 100µl GDP, 10mM
- 200µl GDP-Glo™ Enzyme
- 15ml Enzyme Dilution Buffer
- 100ml Nucleotide Detection Buffer
- 1 vial ATP Detection Substrate (lyophilized)

Storage Conditions: Store the GDP-Glo™ Glycosyltransferase Assay kit at less than –65°C. Alternatively, store GDP-Glo™ Enzyme at less than –65°C and the other components at –30°C to –10°C. Before use, completely thaw all components at room temperature except the GDP-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 GDP Detection Reagent (Nucleotide Detection Reagent + GDP-Glo™ working solution) needed. If smaller amounts of GDP Detection Reagent are needed for each use, the GDP-Glo™ Enzyme should be dispensed in single-use aliquots and stored at less than –65°C.

GDP-Sugar Substrates

PRODUCT	SIZE	CAT.#
Ultra Pure GDP-Fucose, 50mM	**50µl**	**VA1097**
	5 × 50µl	**VA1098**
Ultra Pure GDP-Mannose, 100mM	**50µl**	**VA1099**
	5 × 50µl	**VA1100**

GDP-Glo™ Glycosyltransferase Assay + GDP-Sugar Substrates

PRODUCT	SIZE	CAT.#
GDP-Glo™ Glycosyltransferase Assay (VA1090) + Ultra Pure GDP-Fucose, 50mM (VA1097)	**200 assays**	**VA1093**
GDP-Glo™ Glycosyltransferase Assay (VA1091) + Ultra Pure GDP-Fucose, 50mM (VA1097)	**400 assays**	**VA1094**
GDP-Glo™ Glycosyltransferase Assay (VA1090) + Ultra Pure GDP-Mannose, 100mM (VA1099)	**200 assays**	**VA1095**
GDP-Glo™ Glycosyltransferase Assay (VA1091) + Ultra Pure GDP-Mannose, 100mM (VA1099)	**400 assays**	**VA1096**

3. Preparing for the GDP-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., fucosyltransferase or mannosyltransferase)
- sugar acceptor substrate
- luminometer capable of reading multiwell plates (e.g., GloMax® Discover System [Cat.# GM3000])
- plate shaker

3.A. Preparing the GDP 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 GDP 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.

GDP Detection Reagent Preparation

The following instructions will prepare 1–30ml of GDP Detection Reagent.

1. Equilibrate an aliquot of Nucleotide Detection Reagent to room temperature.
2. Prepare 300µl of GDP-Glo™ working solution by adding 4µl of GDP-Glo™ Enzyme to 296µl Enzyme Dilution Buffer. Mix well.
3. Prepare GDP Detection Reagent by adding 10µl of GDP-Glo™ working solution to each 1ml of Nucleotide Detection Reagent immediately before use.
4. Mix contents to homogeneity by gently pipetting or vortexing.

   Notes:

   a. Make only enough GDP Detection Reagent required for the experiment. Return the remaining GDP-Glo™ Enzyme to less than –65°C immediately after use.

   b. To prepare more than 30ml of GDP Detection Reagent, increase the volume of GDP-Glo™ working solution by adjusting volumes of both the GDP-Glo™ Enzyme and Enzyme Dilution Buffer to accommodate the volume of GDP Detection Reagent needed for your experiment.

   c. Because there is sufficient GDP-Glo™ Enzyme and Enzyme Dilution Buffer for the number of reactions listed for the assay size, discard any unused GDP-Glo™ working solution.

3.B. Generating a Standard Curve for GDP

To estimate the amount of GDP produced in the glycosyltransferase reaction, we recommend creating a standard curve of 0–25µM GDP.

The GDP 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 GDP standard concentration in triplicate. Figure 2 shows representative data from a GDP standard curve.

1. Prepare 200µl of 25µM GDP solution in preferred 1X glycosyltransferase reaction buffer using the provided 10mM GDP standard. Then add all 200µl of the 25µM GDP 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 GDP and other appropriate substrate or only GDP.
3. Perform a serial twofold dilution as shown in Figure 5 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 GDP to the no-GDP control reactions in well A12.

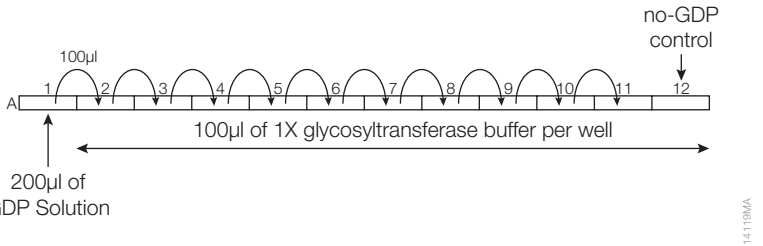


Figure 5. Dilution scheme for creating a GDP standard curve.

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

We recommend the following volumes for different assay plate formats:

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

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

4. GDP-Glo™ Glycosyltransferase Assay Protocols

Prior to performing the GDP-Glo™ Glycosyltransferase Assay, prepare the reagents and GDP standards as described in Section 3. Equilibrate the volume of Nucleotide Detection Reagent to room temperature before use. The GDP Detection Reagent is stable for 2 hours at 22°C with minimal loss of signal and up to 5 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. GDP-Glo™ Glycosyltransferase Assay Protocol

The GDP-Glo™ Glycosyltransferase Assay consists of a single reagent added to the completed glycosyltransferase reaction as outlined in Figures 1 and 4. For 96-well plates, we recommend a 25µl glycosyltransferase reaction and 25µl of GDP 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 GDP Detection Reagent for a total volume of 10µl. Other volumes may be used provided the 1:1 ratio of glycosyltransferase reaction volume to GDP Detection Reagent volume is maintained. The GDP-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 GDP Detection Reagent.

2. Prepare the GDP Detection Reagent as instructed in Section 3.A.

3. Add 25µl of GDP Detection Reagent to each well of the assay plate.

   The GDP 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 GDP-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 GDP-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 GDP-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.

Notes:

a. Use only the provided Ultra Pure GDP-sugar substrates when performing the GDP-Glo™ Glycosyltransferase Assay. Other sources of GDP-sugar may contain free GDP that could result in high background. Due to the sensitivity of the GDP-detection assay, there is no need to use higher amounts of GDP-sugars in the glycosyltransferase reactions. We typically use 100µM GDP-sugars or lower.

b. We recommend optimizing the glycosyltransferase reaction conditions at room temperature to ensure uniform temperature across the plate during the GDP-Glo™ Glycosyltransferase Assay.

Preparation of Glycosyltransferase Titration Components

This protocol is written for a fucosyltransferase 7 (FUT7) enzyme titration as an example to select an optimal enzyme concentration for use in subsequent experiments such as substrate K_m determination. The FUT7 reaction is performed in a 96-well plate using 1X FUT buffer, 40µM GDP-Fucose as a sugar donor, 20µM Fetuin as an acceptor and a serial dilution of FUT7 enzyme from 0–100ng/reaction in 25µl volume. A GDP standard curve is performed in the same assay plate to correlate luminescence to the GDP concentrations generated in each FUT7 reaction.

Note: Different FUTs or other glycosyltransferases have varying specific activities. Therefore, the useful enzyme dilution range may vary greatly and should be determined experimentally prior to inhibitor potency determinations.

1. **Substrate Mix Preparation:** Prepare 400µl of 2.5X GDP-Fucose/Fetuin 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 FUT7 reaction buffer	80µl
50mM GDP-Fucose	0.8µl
1mM Fetuin	20µl
ATP-free water	299.2µl

2. **FUT7 Solution Preparation:** Prepare 90µl of FUT7 enzyme solution as described below (15µl/reaction/well). This will give 100ng of FUT7/reaction starting concentration.

Component	Volume
5X FUT7 reaction buffer	18µl
FUT7 (909ng/µl)	0.66µl
ATP-free water	71.34µl

   a. Prepare 1ml of 1X FUT7 reaction buffer by mixing 200µl of 5X FUT7 reaction buffer with 800µl of ATP-free water.

   b. Add 90µl of FUT7 enzyme solution to well A1 of a 96-well plate.

   c. Add 45µl of 1X FUT7 reaction buffer to wells A2 through A12 of the 96-well plate.

   d. Perform a serial twofold dilution by transferring 45µl from well A1 to well A2, pipetting to mix as described in Table 1. Transfer 45µl from well A2 to well A3, pipetting to mix. Repeat for wells A4–A11. Discard the extra 45µl from well A11. Do **not** add FUT7 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 FUT7.

Well Number	FUT7 (ng/reaction)	Starting Volume of Each Well	Volume to Transfer
A1	100	90µl	45µl
A2	50	45µl	45µl
A3	25	45µl	45µl
A4	12.5	45µl	45µl
A5	6.25	45µl	45µl
A6	3.12	45µl	45µl
A7	1.56	45µl	45µl
A8	0.78	45µl	45µl
A9	0.39	45µl	45µl
A10	0.19	45µl	45µl
A11	0.09	45µl	0µl; No transfer
A12	0	45µl	Buffer only

4.B. Optimizing Glycosyltransferase Reaction Conditions (continued)

FUT7 Reaction and GDP Standard Curve Experiment

1. Transfer 25µl of the GDP serial dilution in duplicate into the standard curve-designated wells of the 96-well assay plate.
2. Transfer 15µl of FUT7 dilution samples in duplicate from the wells of the FUT7 titration plate to the wells of the assay plate.
3. Transfer 10µl of the 2.5X GDP-Fucose/Fetuin Substrate Mix to the rows of the FUT7 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 GDP-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 chemical compound screens and IC_{50} determinations is the amount that produces luminescence within the linear range of the FUT7 titration curve and generates an adequate signal-to-background ratio.

Because the GDP-Glo™ Glycosyltransferase Assay is very sensitive, it can detect a very small amount of GDP with a high signal-to-background ratio. As a result, a small amount of enzyme that produces low amount of GDP is sufficient for use with the GDP-Glo™ Glycosyltransferase Assay. Figure 6 shows that the amount of GDP produced with a small amount of enzyme results in high signal-to-background ratios (15ng FUT7 produced a signal-to-background ratio of 35-fold).

4.C. Determining K_m Value for FUT7 Substrate GDP-Fucose

The following protocol is an example of a substrate titration in FUT7 reaction to determine the K_m value for GDP-Fucose Substrate. Representative substrate titration data is shown in Figure 6. The FUT7 reaction is performed in a 96-well plate using 1X FUT7 buffer, 15ng FUT7/reaction, 20µM Fetuin as an acceptor substrate and a serial dilution of GDP-Fucose as a sugar donor from 0–200µM/reaction in a 25µl volume. A GDP standard curve is performed in the same assay plate to convert luminescence to GDP concentration generated in each FUT7 reaction. This protocol is designed for a 96-well plate using a 25µl:25µl ratio of glycosyltransferase reaction volume to GDP 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 GDP Detection Reagent volume is maintained.

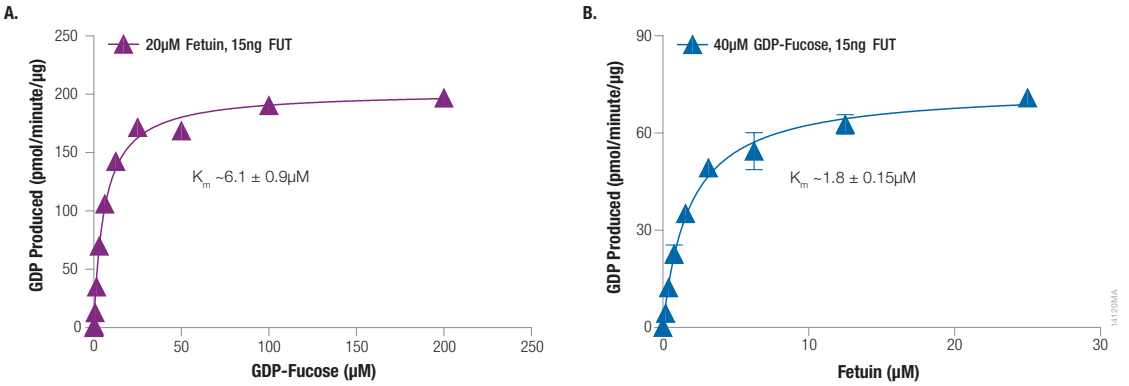


Figure 6. Determining K_m values for fucosyltransferase acceptor and donor substrates. Panel A. GDP-Fucose donor substrate was titrated from 0–200µM/reaction in a 25µl volume FUT7 reaction in the presence of 1X FUT7 buffer, 15ng FUT7/reaction and 20µM Fetuin as an acceptor substrate. **Panel B.** Fetuin acceptor substrate was titrated from 0–25µM/reaction in a 25µl volume FUT7 reaction in the presence of 1X FUT7 buffer, 15ng FUT7/reaction and 40µM GDP-Fucose as a donor substrate. A GDP standard curve is performed in the same assay plate to convert luminescence to the GDP concentration generated in each FUT7 reaction. Glycosyltransferase reactions were incubated for 30 minutes at 37°C. The GDP-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 nonlinear regression fit in GraphPad Prism® Version 6 analysis tool as described in Section 4.C. Error bars represent standard deviation. K_m values for GDP-Fucose and Fetuin determined using the GDP-Glo™ Glycosyltransferase Assay compare favorably to the K_m values reported for these substrates in the literature (6).

4.C. Determining K_m Value for FUT7 Substrate GDP-Fucose (continued)

Preparation of GDP-Fucose Titration Components

1. **GDP-Fucose Solution Preparation:** Prepare 100µl of 500µM GDP-Fucose solution as described below (final 10µl/reaction/well). This gives 200µM GDP-Fucose starting concentration in the assay.

Component	Volume
5X FUT7 reaction buffer	20µl
50mM GDP-Fucose	1µl
ATP-free water	79µl

   a. Prepare 1ml of 1X FUT7 reaction buffer by mixing 200µl of 5X FUT7 reaction buffer and 800µl of ATP-free water.

   b. Add 100µl of GDP-Fucose solution to well A1 of a 96-well plate.

   c. Add 50µl of 1X FUT7 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 GDP-Fucose solution to the no-substrate control reaction in well A12.

Table 2. Performing Serial 1:1 Dilutions of GDP-Fucose Solution.

Well Number	Final GDP-Fucose Concentration (µM)	Starting Volume of Each Well	Volume to Transfer
A1	200	100µl	50µl
A2	100	50µl	50µl
A3	50	50µl	50µl
A4	25	50µl	50µl
A5	12.5	50µl	50µl
A6	6.25	50µl	50µl
A7	3.13	50µl	50µl
A8	1.56	50µl	50µl
A9	0.78	50µl	50µl
A10	0.39	50µl	50µl
A11	0.20	50µl	0µl; No transfer
A12	No substrate	50µl	Buffer only

2. **FUT7 Solution Preparation:** Prepare 150µl of FUT7 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 15ng of FUT7 per reaction.

Component	Volume
5X FUT7 reaction buffer	30µl
FUT7 (909ng/µl)	0.5µl
ATP-free water	119.5µl

3. **Acceptor Substrate Mix Preparation:** Prepare 600µl of 2.5X Fetuin 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 20µM Fetuin final concentration in the reaction.

Component	Volume
5X FUT7 reaction buffer	120µl
1mM Fetuin	30µl
ATP-free water	450µl

GDP-Fucose Titration Experiment

1. Following the plate setup in Figure 7, transfer 25µl of GDP standard serial dilution in duplicate into the wells designated for the standard curve.
2. Transfer 10µl of GDP-Fucose 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 Fetuin solution to all the assay rows.
4. Transfer 5µl of the FUT7 solution in duplicate to wells A1–A12 and B1–B12 of the 96-well assay plate.

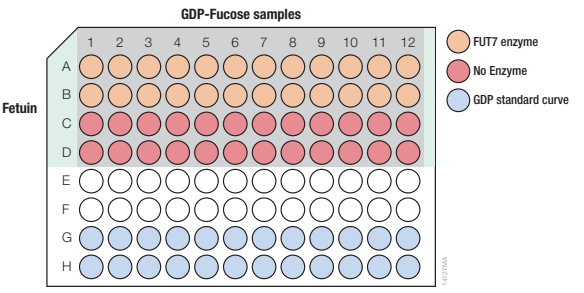


Figure 7. GDP-Fucose titration in a 96-well plate.

Note: Add 5µl of 1X FUT7 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 GDP-Fucose solution.

5. Centrifuge the plate. Mix with a plate shaker for 2 minutes. Incubate the reaction at 37°C for 30 minutes.
6. Equilibrate the plate to room temperature.
7. Follow the GDP-Glo™ Glycosyltransferase Assay protocol described in Section 4.A, starting at Step 2.
8. Record luminescence.

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

Calculating K_m Value: Subtract the signal of the negative control (no-enzyme) wells from the corresponding sample signals. Using the GDP standard curve, calculate the GDP pmol amount produced per minute per microgram 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 Fetuin as shown in Figure 6, Panel B, the same procedure as in Section 4.C was followed, and Fetuin was titrated from 0–200µM in a reaction containing 1X FUT7 buffer, 15ng FUT7/reaction and 40µM GDP-Fucose.

5. General Considerations

Temperature: The intensity and stability of the luminescent signal from the GDP-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 reagents to room temperature before adding the GDP 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 GDP-Glo™ Glycosyltransferase Assay is performed will affect the enzymatic rates and thus luminescence intensity. 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 GDP-Glo™ Glycosyltransferase Assay (Table 3). Interference with the assay reaction can be detected by performing a GDP standard curve in the same buffer as used in the glycotransferase reactions.

Table 3. Solvents and Chemicals Compatible with the GDP-Glo™ Glycosyltransferase Assay.

Chemical	Maximum Concentration Tolerated[1]
NaCl	≤200mM
$CaCl_2$	≤20mM
DTT	≤50mM
Tween®-20	≤2%
Triton® X-100	≤2%
DMSO	≤5%
β-mercaptoethanol (BME)	≤50mM
$MgCl_2$	≤20mM
$MnCl_2$	≤5mM

[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.

GDP-Sugar Substrates: GDP-sugars are prone to hydrolysis, releasing the GDP moiety and causing background in the assay, which could lead to reduced assay sensitivity when testing low-activity enzyme with high concentration GDP-sugar. The GDP-sugar substrates offered with GDP-Glo™ Glycosyltransferase Assay are highly pure, stabilized sugar donors that have less than 0.005% GDP contamination. To preserve the high sensitivity of the GDP-Glo™ Glycosyltransferase Assay, use only the provided Ultra Pure GDP-sugar substrates. Other sources of GDP-sugar may contain GDP that could result in high background and low assay sensitivity. Also, using the provided Ultra Pure GDP-sugar substrates will safeguard against GDP feedback inhibition of certain glycosyltransferases at the lower end of the enzyme activity curve. Using GDP-contaminated sugar donors might create erroneous results due to this inhibition.

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 GDP standard curve is useful for liquid handling and instrument optimization.

Testing for Compounds that Interfere with the GDP-Glo™ Glycosyltransferase Assay: Compounds that interfere with the GDP-Glo™ Glycosyltransferase Assay are rare. We screened 1,280 compounds from the LOPAC chemical library using the GDP-Glo™ Assay reagents with 10µM compound; none of the compounds interfered with the GDP-Glo™ Glycosyltransferase Assay (4). 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 GDP or generation of the luminescent signal, set up mock reactions without glycosyltransferase but with all other assay components present, including a concentration of GDP 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 GDP-Glo™ Glycosyltransferase Assay will significantly reduce the number of false hits (3).

6. References

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4. Zegzouti, H. *et al*. (2016) Homogenous detection of glycosyltransferase activities with universal bioluminescent assays. Poster presented in the 2016 Annual Conference of the Society for Glycobiology. November 19–22, 2016, New Orleans, Louisiana, U.S. Abstract in *Glycobiology* **26 (12)**, 1543.
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6. deVries T. *et al*. (2001) Production of Soluble Human alpha 3 fucosyltransferase (FUT VII) by membrane targeting and in vivo proteolysis. *Glycobiology* **11**, 711–7.

7. Composition of Buffers and Solutions

1X FUT2 reaction buffer

5mM	Tris (pH 7.5)
30mM	NaCl
2mM	$MnCl_2$
8mM	$MgCl_2$
2mM	$CaCl_2$

1X FUT3 reaction buffer

5mM	Tris (pH 7.5)
1mM	$MnCl_2$

1X FUT7 reaction buffer

25mM	HEPES (pH 7.5)
2.5mM	$MnCl_2$

8. Related Products

Product	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
UDP-Glo™ Glycosyltransferase Assay	200 assays	V6961
	400 assays	V6962
	4,000 assays	V6963

Product	Size	Cat.#
UMP/CMP-Glo™ Glycosyltransferase Assay	200 assays	VA1130
	400 assays	VA1131
	4,000 assays	VA1132
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

9. Summary of Changes

The following changes were made to the 7/25 revision of this document:

1. Updated the cover image and fonts.
2. Updated the patent statement.
3. Updated numerical values in and around Table 2, Section 4.C.
4. Added a new reference 5, changed existing reference 5 to 6.
5. Changed the serial dilution of FUT7 enzyme in Section 4.B from 0–500ng/reaction to 0–100ng/reaction.
6. Made miscellaneous text edits.

[a]U.S. Pat. Nos. 7,741,067 and 8,361,739.