Glycosyltransferase Activity Kit

Catalog Number EA001

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

PRINCIPLE OF THE ASSAY

The Glycosyltransferase Activity Kit provides a simple, non-radioactive high-throughput compatible format for assaying the enzyme activity of all glycosyltransferases that use diphosphonucleotide sugars as donor substrates. This kit takes advantage of a specific phosphatase to remove inorganic phosphate quantitatively from the leaving nucleotide diphosphate, such as UDP or GDP, of glycosyltransferase reactions (Figure 1). The released inorganic phosphate is then detected by Malachite Green phosphate detecting reagents. The amount of inorganic phosphate released by the coupling phosphatase is equal to the nucleotide sugar consumed or glycoconjugate product generated; therefore, the rate of inorganic phosphate production reflects the kinetics of a glycosyltransferase reaction.

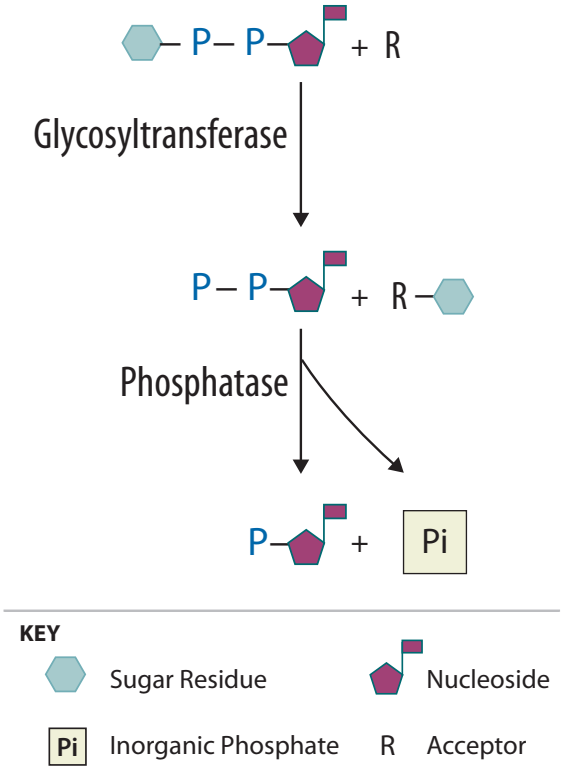

Figure 1: Glycosyltransferase activity assay principle. The released inorganic phosphate is detected using Malachite Green reagents. Examples of glycosyltransferases assayed using this method have been described by Wu *et al.* (1).

MATERIALS PROVIDED & STORAGE CONDITIONS

The protocol presented is for a 96-well format. Materials provided are sufficient for two 96-well microplates or equivalent.

Store UDP and Coupling Phosphatase 1 at ≤ -20 °C until use. All other components may be stored at ambient temperature. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Coupling Phosphatase 1	895404	200 µL of 100 ng/µL of Coupling Phosphate 1 in 20 mM Tris, 120 mM NaCl, 4 mM $CaCl_2$, 20% glycerol, pH 7.5.	Use fresh for each assay. Discard after use.
UDP	895406	100 µL of 5 mM UDP in 10 mM sodium borate, pH 9.0.	
Phosphatase Buffer 1	895405	2 vials (1.5 mL/vial) of a 10X solution of 250 mM Tris, 100 mM $CaCl_2$, pH 7.5.	May be stored at room temperature.*
$MnCl_2$	895407	2 vials (1.5 mL/vial) of 100 mM $MnCl_2$ in deionized water.	
Phosphate Standard	895408	200 µL of 1 mM phosphate (KH_2PO_4) in deionized water.	
Malachite Green Reagent A	895855	3 vials (3 mL/vial) of ammonium molybdate in 3 M sulfuric acid.	
Malachite Green Reagent B	895856	3 vials (3 mL/vial) of malachite green oxalate and polyvinyl alcohol.	

* Provided this is within the expiration date of the kit.

OTHER MATERIALS REQUIRED

- Glycosyltransferase.
- Donor substrate for glycosyltransferase.
- Acceptor substrate for glycosyltransferase.
- Assay buffer (if different than provided).
- Deionized or distilled water
- Microplate reader capable of measuring absorbance at 620 nm.
- 37 °C incubator.
- Microplate (R&D Systems®, Catalog # DY990).
- Microcentrifuge tubes or equivalent.
- Pipettes and pipette tips.
- Plate sealers (R&D Systems®, Catalog # DY992).

PRECAUTION

Malachite Green Reagent A supplied with this kit is an acidic solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

REAGENT PREPARATION

1X Assay Buffer - Add 500 µL of Phosphatase Buffer 1 and 500 µL of the 100 mM $MnCl_2$ to 4.0 mL of deionized water in a tube. Mix well.

Note: *For best glycosylation activity, buffer optimization may be required.*

Enzymes and Substrates - As a starting point, prepare working solutions of the glycosyltransferase (1-100 ng/µL), Coupling Phosphatase 1 at 20 ng/µL, donor and acceptor substrates at 0.5-5 mM in 1X Assay Buffer. Optimal conditions may vary and need to be experimentally determined.

0.1 mM UDP - Add 5 µL of 5 mM UDP to 245 µL of 1X Assay Buffer and mix well.

PHOSPHATE STANDARD CURVE DETERMINATON

It is recommended that the standards be assayed in duplicate and a standard curve be generated in each laboratory for each application.

1. Add 40 µL of the 1 mM Phosphate Standard to 360 µL of 1X Assay Buffer in a microcentrifuge tube and mix well. Transfer 200 µL of the dilution into 200 µL of 1X Assay Buffer in a second tube. Repeat the process to prepare a 2-fold serial dilution. The eighth tube contains only 1X Assay Buffer and serves as the zero standard.
2. Transfer 50 µL of each dilution into a well of a microplate.
3. Add 30 µL of Malachite Green Reagent A to each well. Mix by gently tapping the plate.
4. Add 100 µL of deionized or distilled water to each well.
5. Add 30 µL of Malachite Green Reagent B to each well. Mix by gently tapping the plate.
6. Incubate the plate for 20 minutes at room temperature to stabilize the color development. The yellow background will fade during incubation.
7. Determine the optical density (OD) of each well using a microplate reader set to 620 nm.
8. Average the duplicate readings for each standard and subtract the average zero standard optical density. Plot phosphate input (pmol/well) vs. the corrected OD (see Table 1 and Figure 2 for an example).

Table 1.

Std. Conc. (µM)	Phosphate Input (pmol/well)	Optical Density (620 nm)		Average O.D.	Corrected O.D.
100	5000	1.575	1.576	1.576	1.490
50	2500	0.834	0.861	0.847	0.761
25	1250	0.459	0.463	0.461	0.375
12.5	625	0.272	0.280	0.276	0.190
6.25	313	0.184	0.184	0.184	0.098
3.13	156	0.132	0.132	0.132	0.046
1.56	78	0.110	0.112	0.111	0.025
0	0	0.085	0.087	0.086	———

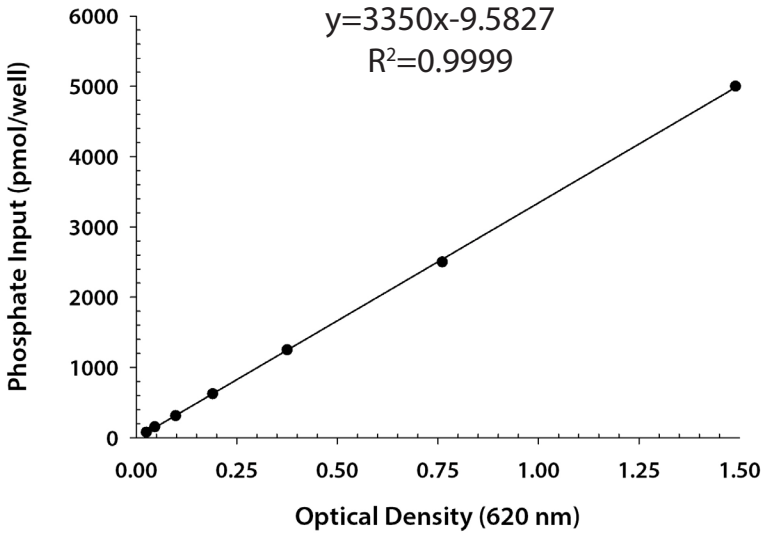

Figure 2: A phosphate standard curve determined using 1X Assay Buffer. The slope of the linear regression line, 3350 pmol/OD, represents the amount of phosphate corresponding to a unit of absorbance at 620 nm. It is referred to as the phosphate conversion factor (CF) in subsequent calculations. This standard curve is provided for demonstration only.

GLYCOSYLTRANSFERASE ASSAY PROTOCOL

This is a standard protocol for glycosyltransferase assay using a 50 µL volume.* The reaction may be scaled up proportionally. 1X Assay Buffer can be used in most glycosyltransferase reactions. Individual glycosyltransferase reactions can also be optimized by adjusting the pH, the concentrations of glycosyltransferase, donor and acceptor substrates, salt and metal ions, and incubation time. It is recommended that all samples be assayed in duplicate. For more information, see the Technical Hints and Limitations section.

1. Combine the working solutions of donor and acceptor substrates and Coupling Phosphatase 1 in a final volume of 25 µL/well.

	Volume/well
Substrate	10 µL
Acceptor Substrate	10 µL
Coupling Phosphatase 1	5 µL
Total Volume	25 µL

2. Initiate the reaction by adding 25 µL/well of the working glycosyltransferase solution.
3. For a negative control, use 1X Assay Buffer in place of the glycosyltransferase solution.
4. For the Coupling Phosphatase 1 control, add 20 µL of 1X Assay Buffer, 5 µL of Coupling Phosphatase 1 (20 ng/µL), and 25 µL of 0.1 mM UDP in a final volume of 50 µL/well.
5. Use 50 µL/well of the 1X Assay Buffer as the assay blank.
6. Cover the microplate with a plate sealer, and incubate at 37 °C or at room temperature for desired length of time (15 minutes to 20 hours).
7. Add 30 µL of Malachite Green Reagent A to each well. Mix gently by tapping the plate.
8. Add 100 µL of deionized or distilled water to each well.
9. Add 30 µL of Malachite Green Reagent B to each well. Mix gently by tapping the plate.
10. Incubate the plate for 20 minutes at room temperature to stabilize the color development.
11. Determine the optical density of each well using a microplate reader set to 620 nm, and adjust the OD by subtracting the reading of the negative control.
12. Calculate product formation using the conversion factor determined from the phosphate standard curve.

*For larger reaction volumes, it is necessary to maintain the ratio of reaction volume plus water : Malachite Green Reagent A : Malachite Green Reagent B at 5:1:1.

DETERMINATION OF GLYCOSYLTRANSFERASE SPECIFIC ACTIVITY

An example of a glycosyltransferase activity assay is provided. In this assay, varying amounts of recombinant human B4GalT1 (R&D Systems®, Catalog # 3609-GT) were assayed in the presence of 5 nmol of UDP-Galactose, 500 nmol of glucosamine, 50 ng Coupling Phosphatase 1 in 1X Assay Buffer using the protocol provided. The reaction was stopped after 15 minutes of incubation at room temperature. The corrected ODs were plotted against the amounts of the glycosyltransferase (Figure 3). The specific activity of the enzyme was calculated using the following equation.

$$\text{Specific Activity} = \frac{\text{S (OD/µg) x CF(pmol/OD)}}{\text{Time (minutes)}}$$

S=Slope of the line (Figure 3)
CF=Conversion Factor (determined in Figure 2)

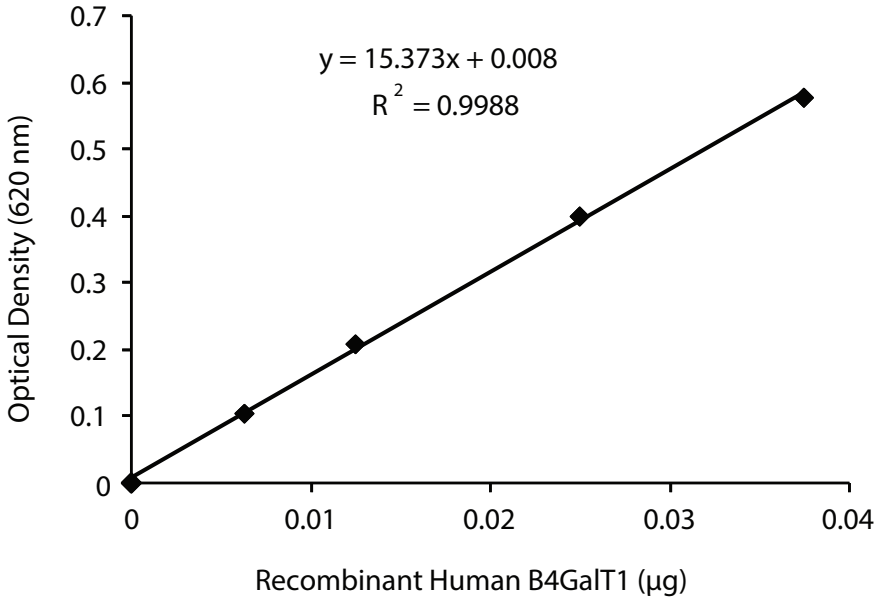

Figure 3: Recombinant human B4GalT1 (R&D Systems®, Catalog # 3609-GT) assay. Using the conversion factor of 3350 pmol/OD, the specific activity was calculated to be 3433 pmol/min/µg ((15.373 x 3350) ÷ 15).

TECHNICAL HINTS & LIMITATIONS

- Malachite Green Reagents are highly sensitive to phosphate. All reagents must be phosphate-free. In particular, **phosphate-containing buffers should be avoided at all times.** If a phosphate-containing enzyme preparation is to be assayed, its phosphate content should be removed using a dialysis or chromatography step.
- The best linear response region for phosphate detection is between 100-4000 pmol. Higher levels of phosphate may cause precipitation of the phosphate-malachite complex. To ensure that the phosphate content falls into this range, a portion of the glycosyltransferase reactions may be used for detection. Alternatively, the reactions can be diluted prior to detection.
- The provided Coupling Phosphatase 1 at 100 ng/well is sufficient to release phosphate from 10 nmol/well of UDP in 20 minutes at 37 °C using 1X Assay Buffer. Should different assay conditions be used, the amount of Coupling Phosphatase 1 may need adjustment.
- Coupling Phosphatase 1 shows the optimal activity from pH 7.0-8.5 and requires Ca^{2+} for activity. It loses ~30% activity at a pH of 5.5 and ~50% activity when the NaCl concentration increases to 0.3 M. The enzyme is stable at room temperature and loses ~20% of activity at 37 °C after overnight incubation. The enzyme is more active on GDP and ADP than UDP.
- If the reaction conditions for a glycosyltransferase and Coupling Phosphatase 1 are not compatible, a decoupled assay may be performed in which the phosphatase reaction can be carried out after the glycosyltransferase reaction. In this case, the strength of the phosphatase buffer is recommended to be 4X higher than that of the glycosyltransferase buffer.
- Pipetting concentrated proteins or polypeptides can cause foaming. Any foam which has formed should be eliminated.
- The assay blank is used to monitor phosphate contamination.

REFERENCES

1. Wu, Z.L. *et al.* (2011) Glycobiology **21**:727.

APPENDIX

The following detergents and common reagents were tested for interference with Malachite Green detection of phosphate. The effects occurred at concentrations above those listed.

Detergents	Level	Effect
Triton™ X-100	0.3%	Increased Blank
Tween® 20	0.1%	Reduced Sensitivity
NP-40 Alternative	1%	None
CHAPS	1%	None
SDS	≤ 0.01%	Increased Blank
Deoxycholate	≤ 0.01%	Increased Blank Precipitates

Common Reagents[1]	Level	Effect
Glycerol	5%	Reduced Sensitivity
DMSO	10%	Reduced Sensitivity
Ethanol	25%	Reduced Sensitivity
BSA	0.03 mg/mL	Reduced Sensitivity
EDTA	10 mM	None
Dithiothreitol	3 mM	Reduced Sensitivity
β-mercaptoethanol	10 mM	None
Na_3VO_4	1 mM	Reduced Sensitivity
NaF	10 mM	None
NaCl	100 mM	None
KCl	100 mM	None
$CaCl_2$	10 mM	None

[1]Tested using the microplate assay protocol in 25 mM Tris-HCl, pH 7.5, with or without 1 nmol phosphate (KH_2PO_4).

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