bio-techne®

R&D SYSTEMS

Sialyltransferase Activity Kit

Catalog Number EA002

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

TABLE OF CONTENTS

Manufactured and Distributed by:

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

Distributed by:

Europe | Middle East | Africa Bio-Techne Ltd.
19 Barton Lane, Abingdon Science Park
Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info.emea@bio-techne.com

China Bio-Techne China Co., Ltd.
Unit 1901, Tower 3, Raffles City Changning Office,
1193 Changning Road, Shanghai PRC 200051
TEL: +86 (21) 52380373 (400) 821-3475
FAX: +86 (21) 52371001
E-MAIL: info.cn@bio-techne.com

PRINCIPLE OF THE ASSAY

The Sialyltransferase Activity Kit provides a simple, non-radioactive high-throughput compatible format for assaying all sialyltransferases regardless of different acceptor substrates. This kit takes advantage of a specific phosphatase to remove inorganic phosphate from the leaving nucleotide cytidine 5'-monophosphate (CMP) of sialyltransferase reactions and malachite green phosphate detection reagents that turn inorganic phosphate to a green colored complex. The amount of inorganic phosphate released by the phosphatase is equal to the CMP-sialic acid consumed or the sialyl-conjugate produced; therefore, the rate of inorganic phosphate produced reflects the kinetics of a sialyltransferase reaction.

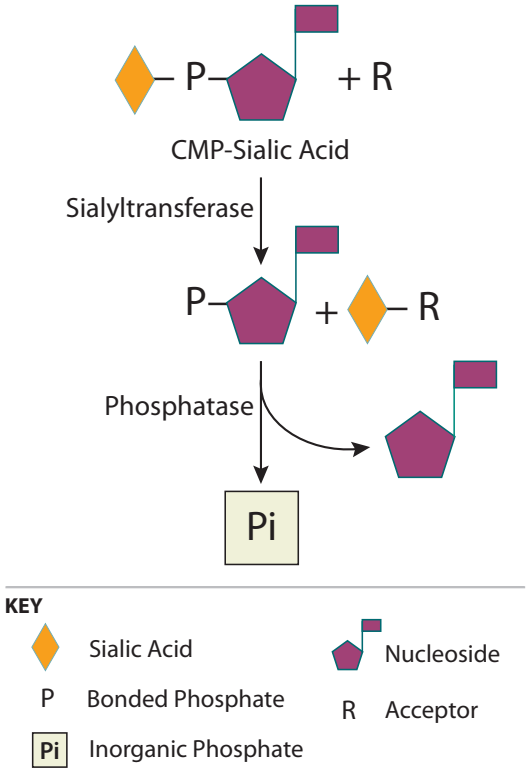

Figure 1: Sialyltransferase activity assay principle. The released inorganic phosphate is detected using malachite green phosphate reagents as described by Wu *et al.* (1).

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 CMP and Coupling Phosphatase 2 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 2	895409	100 µL of 100 ng/µL of coupling Phosphatase 2 in 20 mM Tris, 120 mM NaCl, 4 mM $CaCl_2$, 20% glycerol, pH 7.5.	Aliquot and store for up to 12 months at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
CMP	895411	100 µL of 5 mM CMP in 10 mM sodium borate, pH 9.0.	
Phosphatase Buffer 2	895410	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

- Sialyltransferase
- CMP-sialic acid (universal donor substrate)
- Acceptor substrate for sialyltransferase
- 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

The Malachite Green Reagent A provided with this kit is an acid solution.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

REAGENT PREPARATION

1X Assay Buffer - Add 500 µL of Phosphatase Buffer 2 and 500 µL of the 100 mM $MnCl_2$ to 4.0 mL of deionized water in a tube. **Mix well. Make fresh and discard after use.**

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

Enzymes and Substrates - As a starting point, prepare working solutions of the sialyltransferase (1-100 ng/µL), Coupling Phosphatase 2 at 10 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. **Make fresh and discard after use.**

0.1 mM CMP - Add 5 µL of 5 mM CMP to 245 µL of 1X Assay Buffer and mix well. **Make fresh and discard after use.**

PHOSPHATE STANDARD CURVE DETERMINATION

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 (O.D.) 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 O.D. (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	1.646	1.676	1.661	1.572
50	2500	0.895	0.892	0.894	0.805
25	1250	0.487	0.492	0.490	0.401
12.5	625	0.292	0.285	0.289	0.200
6.25	313	0.187	0.184	0.186	0.097
3.13	156	0.134	0.138	0.136	0.047
1.56	78	0.113	0.109	0.111	0.022
0	0	0.088	0.090	0.089	-

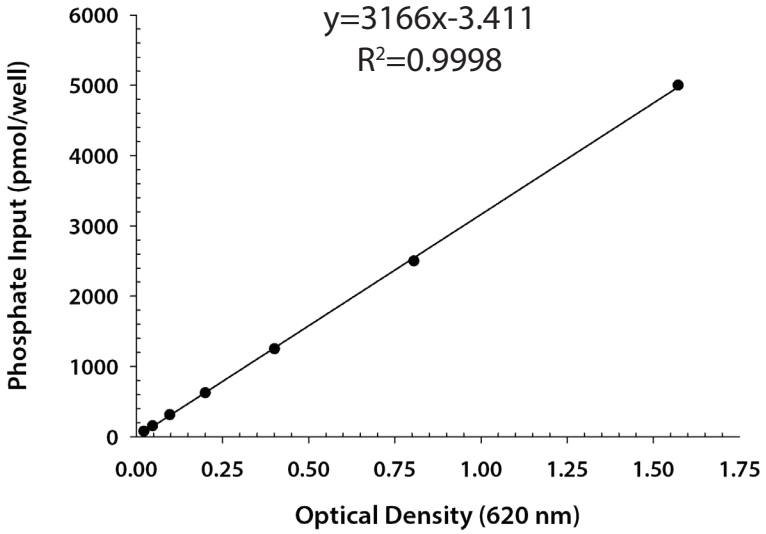

Figure 2: A phosphate standard curve determined using 1X Assay Buffer. The slope of the linear regression line, 3166 pmol/OD, represents the amount of phosphate corresponding to a unit of absorbance at 620 nm. It is referred to as the phosphate conversion factor (CF) in subsequent calculations. This standard curve is provided for demonstration only.

SIALYLTRANSFERASE ASSAY PROTOCOL

1X Assay Buffer can be used in most sialyltransferase reactions. Individual sialyltransferase reactions can also be optimized by adjusting the pH, the concentrations of sialyltransferase, 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 2 in a final volume of 25 µL/well.

	Volume/well
Donor Substrate	10 µL
Acceptor Substrate	10 µL
Coupling Phosphatase 2	5 µL
Total Volume	25 µL

2. Initiate the reaction by adding 25 µL/well of the working sialyltransferase solution.
3. For a negative control, use 25 µL/well of 1X Assay Buffer in place of the working sialyltransferase solution.
4. For the Coupling Phosphatase 2 control, add 20 µL of 1X Assay Buffer, 5 µL of Coupling Phosphatase 2 (10 ng/µL), and 25 µL 0.1 mM CMP 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 on the bench for 20 minutes to stabilize the color development.
11. Determine the optical density of each well using a microplate reader set to 620 nm, and adjust the O.D. by subtracting the reading of the negative control.
12. Calculate product formation using the conversion factor determined from the phosphate standard curve.

DETERMINATION OF SIALYLTRANSFERASE SPECIFIC ACTIVITY

In this example, varying amounts of recombinant mouse ST6GALNAC2 (R&D Systems®, Catalog # 6468-GT) were incubated with 25 nmol of CMP-Neu5Ac (Sigma, Catalog # C8271), 1 mg of asialofetuin (Sigma, Catalog # A4781), and 50 ng of Coupling Phosphatase 2 in 1X Assay Buffer at 37 °C for 20 minutes using the protocol provided. The corrected ODs were plotted against the amounts of the sialyltransferase (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)

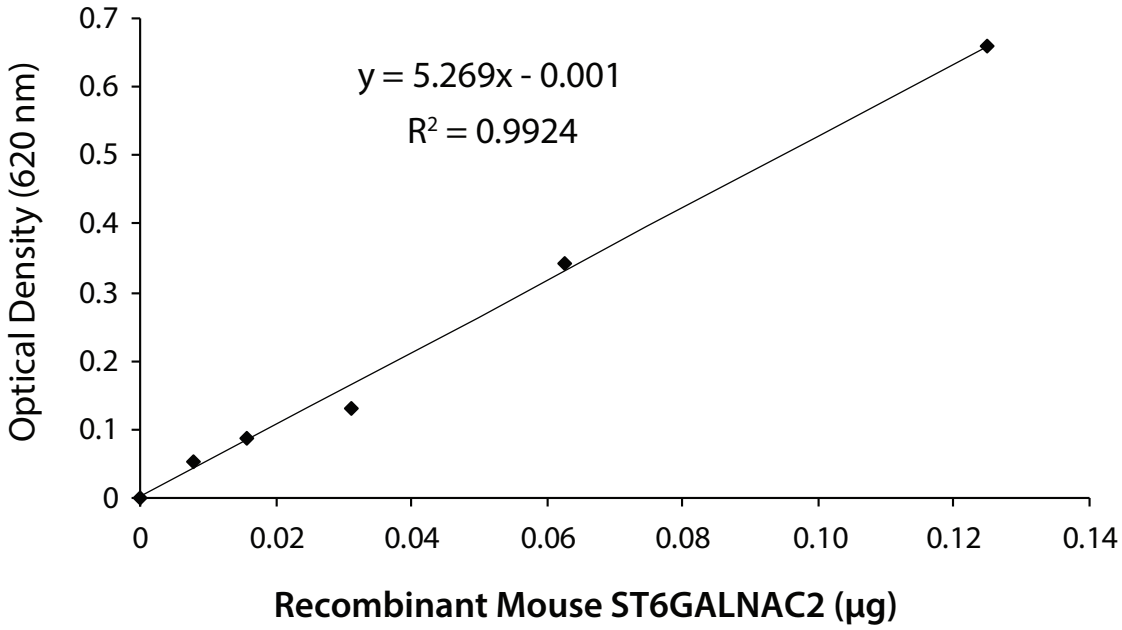

Figure 3: Recombinant mouse ST6GALNAC2. Using the conversion factor of 3166 pmol/OD, the specific activity was calculated to be 834 pmol/min/µg ((5.269 x 3166) ÷ 20).

TECHNICAL HINTS & LIMITATIONS

- Malachite Green Reagents are highly sensitive to phosphate. All reagents must be phosphate-free. In particular, phosphate-based 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 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 sialyltransferase reactions may be used for detection. Alternatively, the reactions can be diluted prior to detection.
- The provided Coupling Phosphatase 2 at 50 ng/well is sufficient to release phosphate from 10 nmol/well of CMP in 20 minutes at 37 °C using 1X Assay Buffer. Should different assay conditions be used, the amount of Coupling Phosphatase 2 may need adjustment.
- Coupling Phosphatase 2 shows the optimal activity from pH 6.0-8.0 and less than 30% variation from pH 5.5-8.5. The enzyme loses ~70% of its activity in the presence of 0.6 M NaCl as compared to that in the absence of salt. Metal ions including Mg^{2+}, Mn^{2+}, and Ca^{2+} at a concentration of 10 mM have no effect on the enzyme activity. The enzyme is stable at room temperature and loses ~50% of its activity at 37 °C after overnight incubation.
- If the reaction conditions for a sialyltransferase and Coupling Phosphatase 2 are not compatible, a decoupled assay may be performed in which the phosphatase reaction can be carried out after the sialyltransferase reaction. In this case, the strength of the phosphatase buffer is recommended to be 4X higher than that of the sialyltransferase buffer.
- Pipetting concentrated proteins or polypeptides can cause foaming. Any foam which has formed should be eliminated.
- Always perform an assay blank using the assay buffer and substrates to monitor for 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).

NOTES

NOTES