Universal Sulfotransferase Activity Kit

Catalog Number EA003

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

SECTION PAGE

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PRINCIPLE OF THE ASSAY

The Universal Sulfotransferase Activity Kit provides a simple, non-radioactive high-throughput compatible format for assaying the enzyme activity of all sulfotransferases that use 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as the donor substrate (1). This kit takes advantage of a coupling phosphatase to remove inorganic phosphate quantitatively from the leaving nucleotide 3'-phosphoadenosine- 5'-phosphate (PAP; Figure 1) (1). The released inorganic phosphate is then detected by malachite green phosphate detection reagents. The amount of inorganic phosphate released by the coupling phosphatase is proportional to PAP consumed or product generated; therefore, the rate of inorganic phosphate production reflects the kinetics of a sulfotransferase reaction.

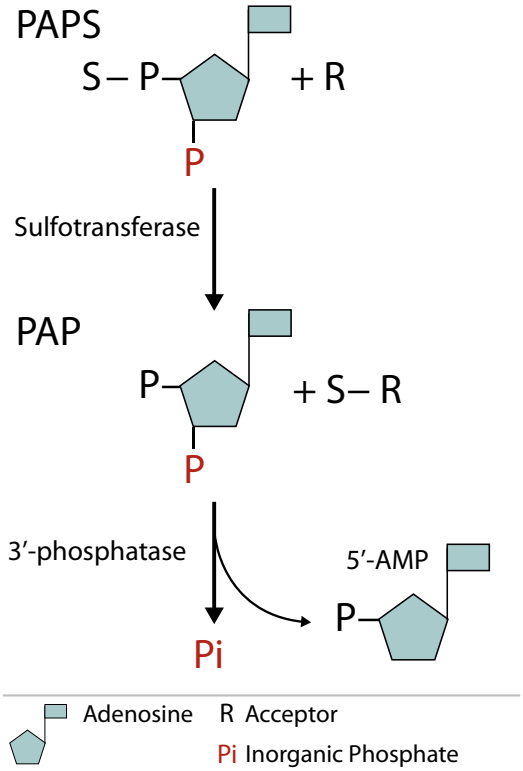

Figure 1: Sulfotransferase activity assay principle. The sulfotransferase reaction converts PAPS to PAP. The coupling 3'-phosphatase releases one inorganic phosphate from PAP. The inorganic phosphate is detected using malachite green reagents as described by Prather et al. (1).

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 PAP and Coupling Phosphatase 3 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 3	894233	200 µL of 0.5 µg/µL of Coupling Phosphatase 3 in 20 mM Tris, 120 mM NaCl, 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.
PAP	894235	100 µL of 5 mM PAP in 10 mM sodium borate, pH 9.0.	
Phosphatase Buffer 3	894234	2 vials (1.5 mL/vial) of a 10X solution of 500 mM Tris, 150 mM $MgCl_2$, pH 7.5	May be stored at room temperature.*
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

- Sulfotransferase
- PAPS (R&D Systems®, Catalog # ES019; PAPS from other commercial sources should be purified before use. In particular, PAP, Na^+, and Li^+ should be removed.[†])
- Acceptor substrate for sulfotransferase
- 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)

[†]For more information, see the Technical Hints and Limitations section.

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 3 to 4.5 mL of deionized water in a tube. Mix well. **Make fresh and discard after use.**

Enzymes and Substrates - As a starting point, prepare the following working solutions:
- Sulfotransferase (1 ng/mL-100 ng/µL)
- Coupling Phosphatase 3 (100 ng/µL)
- Acceptor substrates (0.5-5 mM)
- PAPS (1 mM)

All solutions should be prepared in 1X Assay Buffer. Optimal conditions may vary and need to be experimentally determined. **Make fresh and discard after use.**

0.1 mM PAP - Add 5 µL of 5 mM PAP 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 microplate wells in duplicate.
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 OD. 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	1.504	1.498	1.501	1.412
50	2500	0.871	0.852	0.861	0.773
25	1250	0.489	0.490	0.489	0.400
12.5	625	0.290	0.287	0.288	0.199
6.25	313	0.185	0.188	0.186	0.097
3.13	156	0.135	0.136	0.135	0.046
1.56	78	0.111	0.113	0.112	0.023
0	0	0.089	0.090	0.089	———

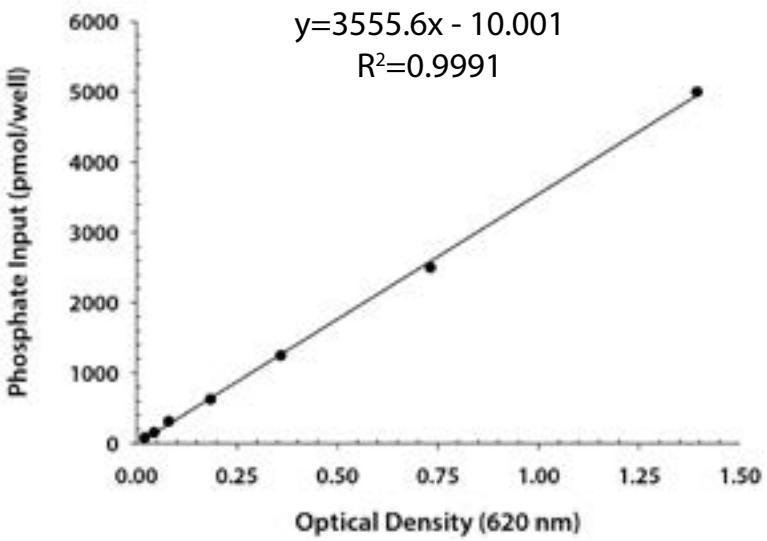

Figure 2: A phosphate standard curve determined using 1X Assay Buffer. The slope of the linear regression line, 3555.6 pmol/OD, represents the amount of phosphate corresponding to a unit of absorbance at 620 nm. It is referred to as the phosphate conversion factor (CF) in subsequent calculations. This standard curve, prepared using the data in Table 1, is provided for demonstration only.

SULFOTRANSFERASE ASSAY PROTOCOL

This is a standard protocol for sulfotransferase assays using a 50 µL reaction volume.* The reaction may be scaled up proportionally. 1X Assay Buffer can be used in most sulfotransferase reactions. Individual sulfotransferase reactions can also be optimized by adjusting the pH, incubation time, and the concentrations of sulfotransferase, donor and acceptor substrates, and salt and metal ions. 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 PAPS, acceptor substrate, and Coupling Phosphatase 3 in a final volume of 25 µL/well immediately prior to the experiment.

	Volume/well
PAPs	10 µL
Acceptor Substrate	10 µL
Coupling Phosphatase 3	5 µL
Total Volume	25 µL

2. Initiate the reaction by adding 25 µL/well of the working sulfotransferase solution.
3. For a negative control, use 1X Assay Buffer in place of the sulfotransferase solution.
4. For the Coupling Phosphatase 3 control, add 20 µL of 1X Assay Buffer, 5 µL of Coupling Phosphatase 3 (100 ng/µL), and 25 µL 0.1 mM PAP 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 for 20 minutes.
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 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 SIALYLTRANSFERASE SPECIFIC ACTIVITY

Varying amounts of recombinant mouse CHST7 (R&D Systems®, Catalog # 5108-ST) were incubated with 15 mM N-acetyl glucosamine, 200 µM PAPS, and 500 ng of Coupling Phosphatase 3 in 50 µL of 1X Assay Buffer for 20 minutes at 37 °C. The corrected ODs were plotted versus the amount of the enzyme (Figure 3).

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

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

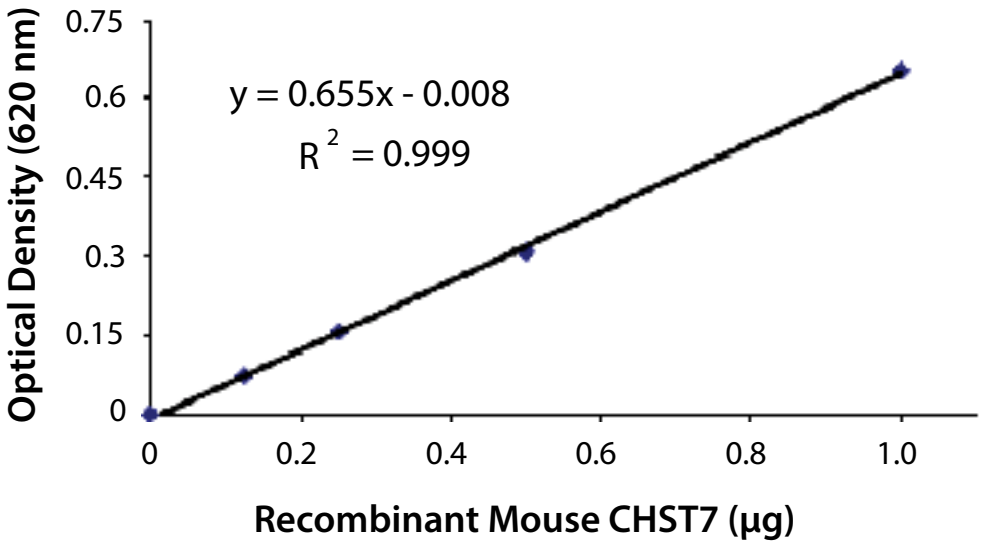

Figure 3: Recombinant mouse CHST7 assay. Using the conversion factor of 3555.6 pmol/OD determined in Figure 2, the specific activity was calculated to be 116.4 pmol/min/mg ((0.655 x 3555.6) ÷ 20).

Table 2: Coupling rates for the Coupling Phosphatase 3 coupled sulfotransferase reactions. The coupling rates listed are for 20 minute reactions in 50 µL of 1X Assay Buffer 3 at 37 °C and were calculated based on the specific rate constant of 117 nmol/min/mg/mM. For rate constant determination and calculation of the coupling rate, see references 1 and 2.

NaCl (mM)	Concentration of Coupling Phosphatase 3 (µg/mL))					
	2	6	10	14	18	22
0	0.79	0.93	0.96	0.97	0.98	0.98
5	0.73	0.91	0.94	0.96	0.97	0.97
10	0.68	0.89	0.93	0.95	0.96	0.97
20	0.59	0.85	0.91	0.93	0.95	0.96
50	0.42	0.73	0.83	0.88	0.91	0.92
75	0.33	0.65	0.77	0.84	0.87	0.89
100	0.28	0.58	0.72	0.79	0.84	0.86
150	0.21	0.47	0.62	0.71	0.77	0.80
200	0.16	0.40	0.55	0.64	0.71	0.75
300	0.12	0.30	0.43	0.53	0.61	0.65
500	0.07	0.20	0.31	0.39	0.46	0.51

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 linear response region for phosphate detection is between 100-4000 pmol/well. Higher levels of phosphate may cause precipitation of the phosphate-malachite complex. To ensure that the phosphate content falls into this range, the sulfotransferase reaction volume can be scaled up or a portion of the sulfotransferase reaction may be used for detection.
- The exact relationship between inorganic phosphate produced and PAPS consumed is reflected in the coupling rate (Table 2), a parameter that defines the product-signal conversion (1). For accurate enzyme kinetic determination, the final assay data may be adjusted using the determined coupling rate. The provided Coupling Phosphatase 3 at 10 µg/mL will achieve a coupling rate of approximately 0.96 at 37 °C using 1X Assay Buffer.
- Coupling Phosphatase 3 is slightly active with PAPS. The ratio of activity toward PAPS and PAP is about 1:350 (1). Using excessive amounts of coupling phosphatase can increase the coupling rate but will also increase the hydrolysis of PAPS, resulting in elevated background. Although this background can be measured using a negative control (without sulfotransferase), achieving a coupling rate > 98% is not recommended.
- Coupling Phosphatase 3 shows activity across a wide pH range with the optimal activity around pH 7.5 and requires Mg^{2+} for activity. Mn^{2+} is not suggested in all buffers as it causes high background due to elevated activity on the donor PAPS. Na^{+} and Li^{+} are inhibitors of the enzyme, with a Ki of 17.2 mM and 0.122 mM, respectively (1). The coupling rates at different concentrations of Na^{+} are listed in Table 2. The enzyme loses ~ 20% of its activity at 37 °C after overnight incubation.
- If the reaction conditions for a sulfotransferase and Coupling Phosphatase 3 are not compatible, a decoupled assay may be performed in which the phosphatase reaction can be carried out after the sulfotransferase reaction. In this case, the strength of the phosphatase buffer is recommended to be 4X higher than that of the sulfotransferase buffer.
- Pipetting concentrated proteins or polypeptides can cause foaming. Any foam which has formed should be eliminated.

REFERENCES

1. Prather, B. *et al.* (2012) Anal. Biochem. **423(1)**:86.
2. Wu, Z.L. (2011) PLoS ONE **6(8)**:e23172.

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