Echelon Biosciences Inc.

Malachite Green Phosphatase Assay Kit

K-1500 (192 tests)

Support: echelon@echelon-inc.com

Description: Assay for the in vitro measurement of lipid and protein phosphatase activity.

Materials Provided

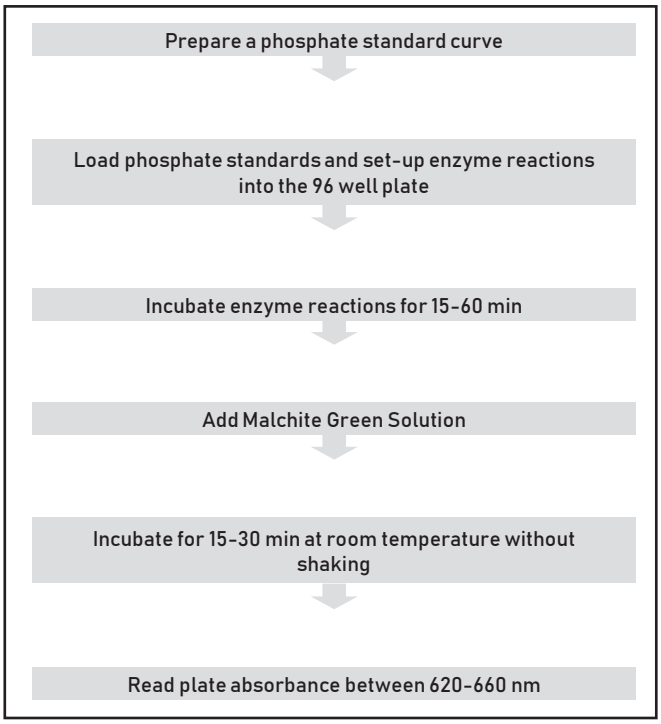
Catalog #	Description	Amount
K-1501	Malachite Green Solution	20 mL
K-1502	1 mM Phosphate Standard	1 mL
---	96 well plate	2 plates

Additional Materials Provided by User:

- Microplate Reader with capability to read absorbance between 620 and 660 nm
- Source of Phosphatase Enzyme and Substrate

Storage: Store kit components at 4 °C for up to 3 months. Store long term at –20 °C.

Quick Protocol



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Background

Echelon's Malachite Green Phosphatase Assay kits are designed for the in vitro measurement of lipid phosphatase activity. The liberated phosphate forms a colored complex with molybdate/malachite green, which is quantified by reading the absorbance at 620 nm. The Echelon assay uses water-soluble lipid substrates so there is no need to make phospholipid vesicles. The assay is fast, convenient and offers a flexible non-radioactive alternative for measuring specific phosphatase activities. The Malachite Green phosphatase assays can measure as little as 50 pmoles of free phosphate and has a high level of accuracy in the range of 200 to 2,000 pmol of phosphate per assay point (see figure 1).

Assay Notes

Warm Malachite Green Solution to room temperature before use. Before proceeding, check that your enzyme preparations and reaction solutions are free from contaminating phosphate by adding 100 µL Malachite Green Solution to 25 µL sample in a well of the 96-well polystyrene microplate.

- Phosphate contamination can be detected visually as a change from yellow to green or by reading between 620 and 660 nm in a microplate reader. There is phosphate contamination if the solution turns green.
- Phosphate buffers such as PBS are incompatible in this assay.
- Detergents used to clean labware may contain high levels of phosphate. Use caution either by using phosphate-free detergent for dish washing or by using disposable plasticware.
- Do not shake the plate during incubation as the malachite green may precipitate.

Protocol for the Detection of Phosphatase Activity

Please read this entire section and the assay notes before beginning the assay

1. Prepare a set of phosphate standards by diluting the phosphate standard solution provided in this kit. See Table 1.
   - Diluted standards should range between 200 and 2000 pmoles phosphate per well in a volume of 25 µL (or a similar volume as the enzyme reaction).
   - It is advisable to make up phosphate standards in buffers consistent with those to be used in the enzyme assay.
2. Detection of free phosphate
   a. Pipet 25 µL phosphate standards, enzyme reactions, and blanks (reaction buffer used as diluent) into the wells of the microplate.
   b. Add 100 µL of Malachite Green Solution. Add to the wells carefully without creating bubbles. (Bubbles interfere with absorbance measurement).
   c. Allow color development to proceed for 15 to 30 minutes at room temperature. DO NOT SHAKE.
   d. Mix briefly by gently tapping the plate before reading. Measure absorbance at a wavelength between 620 and 660 nm in a microplate reader.
   e. Plot absorbance vs. pmoles phosphate / well for the standard curve. Use computer analysis software to generate a non-linear regression curve. Using this one can interpolate the amount of free phosphate in the enzyme reactions. (See Figure 1.) Alternatively, use linear regression analysis. This method is sufficient for analysis but provides a less precise fit.

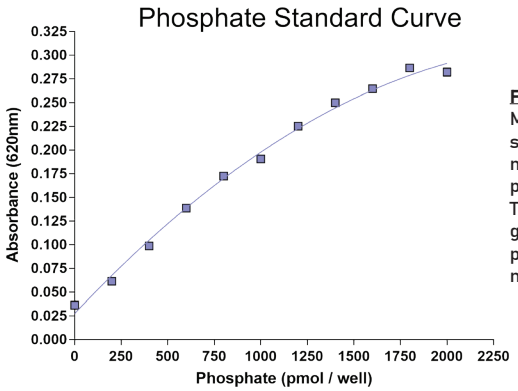

Figure 1: Malachite Green standard curve measuring free phosphate in solution. The standard curve was generated using polynomial second order non-linear regression

Support protocol: Recombinant PTEN Enzyme

The following support protocol has been validated in the Malachite green assay with our recombinant PTEN enzyme (Cat # E-3000). Further optimization of this protocol and/or different enzymes and enzyme buffers can be used depending on the needs and experience of the user.

Buffer and Reagent Preparation

TBS: 25 mM Tris-Cl, pH 7.4, 140 mM NaCl, 2.7 mM KCl. Store at room temperature.

Table 1. Phosphate Standards

Make a 0.1 mM phosphate standard solution by adding 100 µL of 1 mM phosphate solution to 900 µL of distilled water or diluent. Use 0.1 mM phosphate stock to make additional dilutions from chart below.

Phosphate concentration (pmol/25 µL)	Volume of 0.1 mM stock (µL)	Volume of distilled water or diluent (µL)
2,000	100	25
1,800	90	35
1,600	80	45
1,400	70	55
1,200	60	65
1,000	50	75
800	40	85
600	30	95
400	20	105
200	10	115
0 (Blank)	0	125

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PTEN Reaction Buffer: TBS with 10 mM DTT. Make fresh before use and keep on ice. For 5 mL: Add 50 µL of 1 M DTT to 5 mL TBS.

Malachite Green Solution and Phosphate Standards: Malachite Green Solution (K-1501) and 1 mM phosphate standard (K-1502) are included in the K-1500 kit. Warm up Malachite Green Solution to room temperature before use. Make phosphate standard dilutions in PTEN Reaction Buffer following the protocol in the kit. Prepare sufficient volume of each phosphate standards for loading 25 µL per well in triplicate.

PIP3 Substrate: Reconstitute PI(3,4,5)P3 powder (cat # P-3908) in ddH2O to make a 1 mM stock solution. For example, add 510 µL ddH2O to a vial of 0.5 mg PI(3,4,5)P3. Use 3 µL (3,000 pmol) for each assay point. Store the stock solution of reconstituted PI(3,4,5)P3 at -20 °C after use for up to 3 months.

PTEN enzyme reactions

Set up the PTEN assay in the 96-well flat bottom clear plate (provided in K-1500 kit). Set up phosphate standards, assay controls or enzyme reactions 25 µL per well in triplicate. Refer to the following steps and Table 2 below:

1. Add 25 µL of phosphate standard solution to the standard wells.
2. Add corresponding volumes of PTEN reaction buffer to the control and reaction wells.
3. Reconstitute 2.5 µg PTEN Enzyme (E-3000) in 50 µL dH2O for 50 ng/µL solution. Flip vial gently to mix and spin down. Add PTEN enzyme (E-3000) 3 µL/well (150 ng/reaction) to Enzyme-only control and Enzyme Reaction wells.
4. Add 3 µL/well of 1 mM PIP3 substrate to Substrate-only control and Enzyme Reaction wells to start the PTEN reactions. Seal plate and mix gently on plate shaker briefly (if available). Let reactions proceed at 37 °C for desired time, usually 30 to 60 minutes.

Note: PTEN is temperature sensitive, keep it on ice while working on bench.

Determine PTEN enzyme activity

1. Add 100 µL of room temperature Malachite Green solution (K-1501) to each well of the controls, phosphate standards, and PTEN reactions. Seal plate and cover with aluminum foil to protect from light. Incubate without shaking for 20 minutes at room temperature for green color development.
2. Read absorbance at 620 nm in a plate reader.
3. Draw a standard curve with Absorbance (620 nm) on the Y axis and phosphate (pmol) on the X axis. Use polynomial second order non-linear regression analysis to generate a best fit curve (Y=A+B*X+C*X2). A linier curve fit will work well in most regions below 2000 pmol of free phosphate.
4. Determine the free phosphate in pmol from each reaction or control by interpolation from the standard curve.
5. Determine the percent conversion of PIP3 using the formula below:

   % PIP3 conversion = [(Free phosphate in reaction, pmol) – (Background phosphate, pmol)] * 100% ÷ 3000 pmol

"Background phosphate" is the average free phosphate value from the "Substrate-only" controls. The "Enzyme-only" controls usually yield negligible free phosphate values when compared to Buffer controls.

Support Protocol: Immunoprecipitation of PTEN From Cells

The following support protocol for immunoprecipitation of PTEN from cells has been validated for the PTEN enzyme. Further optimization of this protocol and/or different IP protocols can be used to IP PTEN or other enzymes from cells depending on the needs and experience of the user. See Table 3.

Buffer and Reagent Preparation

Lysis Buffer: Prepare the Lysis Buffer according to the recipe listed in the Table 3 on the following page. Add the protease inhibitor cocktail fresh at a 1:100 dilution. Place buffer on ice and chill until ice cold.

PTEN Reaction Buffer: TBS with 10 mM DTT. Make fresh before use and keep on ice. For 5 mL: Add 50 µL of 1 M DTT to 5 mL TBS.

Cell Lysis

1. Place cell culture dish on ice and wash cells twice with ice cold PBS.
2. Add 0.5 mL of ice cold Lysis Buffer to cells. Scrape cells and transfer mixture into a cooled 1.5 mL centrifuge tube.
3. Incubate cells for 15 minutes with constant agitation at 4 °C.
4. Centrifuge cells for 10 minutes at 14,000 x g to pellet cells.
5. Transfer supernatant to a fresh, cooled 1.5 mL centrifuge tube and place on ice. Discard pellet.

Immunoprecipitation

1. Transfer 400 µL of the cell lysate to a fresh, cold, 1.5 mL centrifuge tube.
2. Add 8 µL of the anti-PTEN antibody (Cell Signaling) to the lysate.
3. Incubate 3 hours to overnight at 4 °C with agitation.
   Add 60 µL of the 50% Protein A agarose beads to the mixture and

Table 2. PTEN Assay Setup

	Step 1	Step 2	Step 3	Step 4
Sample	Standard, µL	PTEN Rxn Buffer, µL	PTEN Enzyme, µL	PIP_3 Substrate, µL
Phosphate Standard	25	-	-	-
Buffer Control	-	25	-	-
Substrate-only control (PIP_3)	-	22	-	3
Enzyme-only control (PTEN)	-	25 - volume of PTEN	3	-
Enzyme Reaction (PTEN + PIP_3)	-	22 - volume of PTEN	3	3

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Table 3, PTEN Immunoprecipitation Materials

Materials Needed:	Company	Catalog Number
1.5 mL Centifuge Tubes	N/A	N/A
Lysis Buffer (25 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 1mM EDTA, 5% Glycerol)	N/A	N/A
Protease Inhibitor Cocktail	Sigma	P8340
Protein A Agarose Beads	Pierce	20333
PTEN (D4.3) XP™ Rabbit mAb	Cell Signaling	9188
PBS	N/A	N/A

This protocol is written for a 100 mm dish of NIH 3T3 cells (90% confluent) for about 15-20 mg/mL of cellular protein. To maximize PTEN activity; keep all solutions ice cold, carry out all reactions on ice or at 4°C, and use a centrifuge that is equilibrated at 4°C.

incubate 2-3 hours at 4 °C.

4. Briefly centrifuge to pellet beads. Discard the supernatant.
5. Wash the bead complex three times with PTEN Reaction Buffer. Centrifuge and discard solution after each wash.
6. Resuspend bead complex in 30 µL of PTEN Reaction buffer. This is enough for one PTEN enzyme reaction.

Start PTEN enzyme reactions

Proceed immediately with the PTEN reactions by adding PI(3,4,5)P3 Substrate to the bead complex. Run enzyme reaction 1-4 hours at 37 °C. Follow "Detection of Phosphatase Activity (Malachite Green Assay Protocol)".

References:

1. B. Martin et al. J. Biol. Chem. 1985 260 14932
2. K.W. Harder et al. Biochem. J. 1994 298 395
3. R.B. Campbell et al. J. Biol. Chem. 2003 278 36

Related Products

Products	Catalog Number
Assays	
PIP3 Mass ELISA	K-2500s
PI3-Kinase Activity ELISA: Pico	K-1000s
PTEN Activity ELISA	K-4700
Malachite green solution	K-1501
Malachite green kit + 2 substrates	K-1520
Malachite green kit + 3 substrates	K-1530
Malachite green kit + 4 substrates	K-1540
Enzymes	
PTEN	E-3000
SHIP2	E-1000

Products	Catalog Number
Substrates	
PIP3	P-3904, P-3908, P-3916
PI(4,5)P2	P-4504, P-4508, P-4516
PI(3,4)P2	P-3404, P-3408, P-3416
PI(3,5)P2	P-3504, P-3508, P-3516
PI(3)P	P-3004, P-3008, P-3016
PI(4)P	P-4004, P-4008, P-4016
PI(5)P	P-5004, P-5008, P-5016
Inhibitors (PTEN)	
VO-Ohpic	B-0351
SF1670	B-0350

Please visit our website at www.echelon-inc.com for more phospholipid products.

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