

DATA SHEET AND PROTOCOL for hMRP4 (ABCC4) ProATPase Kit

Human MRP4 Sf9-membranes for ATPase activity determination

Membranes Cat. #PM21004; Lot #PN-M-1001

ProATPase Kit Cat. #PB21006; Lot#PB-ATP-1001

Contents: 2 x 500 μ L @ 5 mg/mL total protein (determined by BCA protein assay) in MRP Resuspension Buffer*

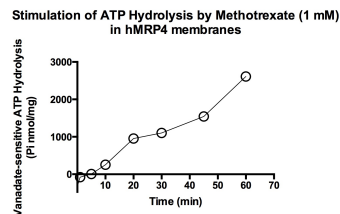
*MRP Resuspension Buffer: 50 mM Tris-HCl, pH 7.0; 50 mM mannitol; 2 mM EGTA; 2 mM DTT; 8 μ g/mL aprotinin and 10 μ g/mL leupeptin.

Store at -70 to -80C upon receipt. Aliquot to smaller working volumes to minimize freeze-thawing cycles.

Human multidrug resistance protein 4 (hMRP4) membranes are prepared from Sf9 insect cells infected with baculovirus to overexpress MRP4. ProNovus hMRP4 ProMembranes should be used to investigate drug interactions with hMRP4 *in vitro*.

Representative data showing time dependent ATP hydrolysis by hMRP4 when stimulated by methotrexate:

Vanadate-sensitive ATP-hydrolysis stimulated by Methotrexate (60 min): 20 nmol Pi/mg/min



This product is strictly for laboratory research use only.

ProATPase Kit contents for 100 sample wells (store at -20C):

Part 21003-A: 5 mL of 15 mM zinc acetate solution

Part 21003-B: 216 mg of ammonium molybdate

Part 21003-C: 1.5 g of ascorbic acid, containing sodium hydroxide pellets

Part 21003-D: 1 mL of 5 mM sodium phosphate (standard)

Part 21003-E: 7 mL ProATPase Assay Buffer

Part 21003-F: 0.5 mL of 0.2 M MgATP

Part 21003-G: 300 μ L of 10 mM orthovanadate

Chemical reagents you will need, NOT supplied in the ProATPase kit:

- Glutathione (GSH): 300 mM stock, prepared fresh.
- 5 mL of 5% SDS solution used as Stopping Solution
- Ultra-pure water
- Appropriate substrate or inhibitor for transporter

Prior to initiating ProATPase Assay Protocol, do the following:

1. Rapidly thaw out membranes in a 37C water bath. Place on ice once thawed.
2. Next, thaw out remaining kit components at room temperature. Place on ice once thawed.
3. Prepare the following solutions:
 - a. 35 mM ammonium molybdate/15 mM zinc acetate solution:
 - i. Dissolve the 216 mg of ammonium molybdate with 5 mL of zinc acetate solution.

- ii. The solution will be slightly opaque. In our experience, we recommend using a sonicating bath for 10 – 30 min to dissolve major clumps of ammonium molybdate.
 - iii. Keep solution in the dark.
 - b. Add 15 mL of ultra-pure water to the 1.5 g of ascorbic acid, containing sodium hydroxide pellets.
 - i. The included sodium hydroxide pellets bring the pH of the solution to approximately 5.0, resulting in an acidic solution necessary for color development.
4. **NOTE: These reagents must be made fresh, on the day of the assay. Solutions may not be stored once mixed and dissolved in water.**
5. Prepare phosphate standards as shown below:

	Phosphate Standards							
	A	B	C	D	E	F	G	H
5 mM sodium phosphate (μL)	250	250	250 μL of B	250 μL of C	250 μL of D	250 μL of E	250 μL of F	0
ProATPase Assay Buffer (μL)	0	250	250	250	250	250	250	250
Final concentration phosphate (mM)	5	2.5	1.3	0.63	0.31	0.16	0.08	0
nmol Phosphate in 50 μL reaction	250	125	63	31	16	8	4	250

6. Distribute 50 μL of standards in triplicate (or duplicate, if desired) to Columns 10-12 and A through H of 96-well assay plate.

Initiating ProATPase Assay Protocol:

- 7. We recommend performing the ProATPase assay at least in duplicates. Z-factor (Z') for ProATPase Assay performed in duplicates is in the range of 0.78 – 0.90.
- 8. Final reaction volume per well is 50 μL. For each 50 μL reaction, prepare the following in a 96-well UV/optical plate, placed on an ice block:
 - a. 4 μL membranes (final of 20 μg protein)
 - b. 36.5 μL ProATPase Assay Buffer
 - c. 1 μL of 300 mM GSH stock (6 mM final GSH)
 - d. 5 μL 10 mM orthovanadate **or** ProATPase Assay Buffer, where appropriate
 - e. 2 μL of test compound (final solvent concentration is 4%)
 - i. Note: The ProATPase Assay can handle 4% solvent concentration for DMSO, water or methanol. Always include proper solvent blank to ensure there is no interference with assay.
- 9. Pre-incubate these components (with no Mg-ATP) for 3 min at 37C with gentle shaking in assay plate.
- 10. Start reaction by adding 1.5 μL Mg-ATP (final concentration of 5 mM) to all wells (except phosphate standards).
- 11. Cover plate and incubate for 60 min at 37C with gentle shaking.
- 12. Terminate ATP-hydrolysis by adding 40 μL of 5% SDS Stopping Solution to each well, including phosphate standards.
- 13. To initiate measurement of inorganic phosphate:
 - a. Mix 5 mL of ammonium molybdate/zinc acetate solution with 15 mL of 10% ascorbic acid at pH 5.0. This makes the Developing Solution.
 - i. Solution will be orange-yellow in color.
 - b. Add 200 μL of above Developing Solution to each well, including standards. Tap plate gently to mix.
 - c. Cover plate and incubate at 37C for 10 min with gentle shaking.
 - i. Color development occurs anywhere from 10 min to 40 min, depending on how well solution is mixed during addition and stimulation of ATP hydrolysis by test compound.
 - ii. As a rule of thumb, we generally follow phosphate standards and incubate according to good color development in the phosphate standards.
 - iii. Highest phosphate standard concentration is a deep blue color.
 - d. After desired color is reached, measure absorbance at, or around 720 nm.
 - i. Some test compounds may interfere with absorbance reading at 720 nm, so absorbance may be read anywhere from 630 nm to 850 nm. In our experience, 720 nm works consistently well.

Data Analysis: Calculating Vanadate-sensitive ATP Hydrolysis:

1. Using the absorbance measurement for free phosphate standard curve and make a calibration plot (X-Y scatter, linear regression and obtain equation of line).
2. Using the standard curve, convert absorbance measurements for samples to “nmol of Pi.”
3. Calculate the average value of “nmol of Pi” for samples with and without orthovanadate. Following this conversion, calculate as follows:
 - a. Vanadate-sensitive ATP Hydrolysis = nmol Pi without orthovanadate – nmol Pi with orthovanadate
4. Finally, to obtain “nmol Pi/mg of protein/min”, divide values in 3a by “mg of protein” and “assay min”, for example:
 - a. Vanadate-sensitive ATP Hydrolysis ÷ 0.020 mg of protein ÷ 20 min

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