ATPase assay Protocol

For ABC Transporter Vesicles Product

This protocol describes ATPase assay using following ABC Transporter Vesicles Products.

- ABC Transporter Vesicles Products

Human MDR1	(GenoMembrane, Cat. No. GM0015)
Mouse Mdr1a	(GenoMembrane, Cat. No. GM0004)
Mouse Mdr1b	(GenoMembrane, Cat. No. GM0016)
Human MRP1	(GenoMembrane, Cat. No. GM0010)
Rat Mrp1	(GenoMembrane, Cat. No. GM0011)
Dog Mrp1	(GenoMembrane, Cat. No. GM0017)
Human MRP2	(GenoMembrane, Cat. No. GM0001)
Rat Mrp2	(GenoMembrane, Cat. No. GM0002)
Mouse Mrp2	(GenoMembrane, Cat. No. GM0022)
Dog Mrp2	(GenoMembrane, Cat. No. GM0014)
Monkey Mrp2	(GenoMembrane, Cat. No. GM0018)

- ABC Transporter Vesicles Products for Negative Control

Control	(GenoMembrane, Cat. No. GM0003)

- ATPase Assay Reagent Kit (GM 3050).

When you prepare the reagents for ATPase assay by yourself, please refer to "**Buffer Preparation Protocol**" available at the following homepage (http://www.genomembrane.com/E_Technical_Information.html).

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1. ATPase assay

The ATPase assay using ABC transporter Vesicles Product allows to evaluate the interaction of compounds with the transporter of interest. ABC Transporter Vesicles Products released by GenoMembrane are purified plasma membranes isolated from an insect cell system (Sf9 cells infected with baculovirus) expressing ABC transporter.

1.1. Principle

ABC transporter-mediated drug transport across membranes is driven by energy derived from hydrolysis of ATP. It is, therefore, possible to evaluate drug interaction with the transporter by determining the ATPase activity. In the ATPase assay, inorganic phosphate generated by ATP hydrolysis, which is linked with transport by ABC transporters, is determined by colorimetry. The method described in this protocol is modified from the procedure reported by Sarkade *et al*. *(J. Biol. Chem*. **267**:4845, 1992).

1.2. Outline of procedure

MgATP is added to assay buffer containing ABC Transporter Vesicles Product and a test compound, and the mixture is incubated for an appropriate time. The reaction is stopped by adding SDS, and starts chromogenic reaction of inorganic phosphate by means of reduction reaction. The ATPase activity due to the ABC transporter is calculated from the amount of inorganic phosphate determined by colorimetry.

1.3. Factors influencing the ATPase assay

Inorganic phosphate measured in the ATPase assay does not accurately reflect the generation of inorganic phosphate by the ABC transporter. The following possibilities should be considered.

- ✓ Contaminant in reaction medium

  Inorganic phosphate might be contaminated in the MgATP and/or Vesicles Product preparations.

- ✓ Orthovanadate-insensitive ATPase

  It is known that the ATPase activity of ABC transporters is inhibited by orthovanadate. However, ABC transporter Vesicles also contain orthovanadate-insensitive ATPase activity (insensitive ATPase) that is not inhibited by orthovanadate. Orthovanadate-sensitive ATPase activity (sensitive ATPase) due to ABC transporters, therefore, can be obtained from the following equation.

  Sensitive ATPase activity = Total ATPase activity – Insensitive ATPase activity

- ✓ Endogenous Orthovanadate-sensitive ATPase activity

  ABC Transporter Vesicles Products may contain a trace amount of endogenous sensitive ATPase originating from the Sf9 cells. It is, therefore, recommended to conduct an ATPase assay with control product (GenoMembrane, Cat. No. GM0003) in addition to the vesicles containing the transporter of interest.

1.4. Controls

It is necessary to use appropriate controls during ATPase assay, as follows:

1) Control without test compound

This is a control assay to measure the basal ATPase activity of the ABC transporter in the absence of substrate.

2) Positive control

This is a control assay to measure the ATPase activity of the ABC transporter in the presence of a typical substrate. **The final concentrations of each substrate are shown in section 5.2.**

3) Background control

This is a control assay to measure inorganic phosphate derived from the reaction buffer, that is, under conditions such that the ATPase activity of the ABC transporter is blocked by adding stop solution prior to the ATPase reaction with MgATP.

4) Orthovanadate control

Every assay, including control assays, should be conducted with and without orthovanadate to measure sensitive ATPase activity.

1.5. Solvent for test compounds

Dimethyl sulfoxide (DMSO) is employed to dissolve test compounds, and is present at the final concentration of 2 % in the assay mixture in this protocol; 2 % DMSO is also added to every well of each plate to obtain a uniform reaction condition. It has been confirmed that 2 % DMSO, or 1 % ethanol, methanol or acetonitrile, does not affect the ATPase activity of ABC transporters.

2. Kit Contents

2.1. ABC transporter Vesicles Product

◆ Frozen ABC Transporter Vesicles Product (5 mg/mL, 500 μL)

One vial of ABC Transporter Vesicles Product contains 2.5 mg protein. In ATPase assay 20 μg of protein is needed for one assay, this means 125 assays can be performed with one vial of the Vesicles product based on this protocol.

Stored at –80 ˚C.

Expiration date; written in the data sheet

◆ Product data sheet

2.2. ATPase Assay Reagent Kit

• Reaction Buffer	MOPS, Tris, DTT, EGTA, ouabain, KCl, NaN_3	(30 mL × 2)
• Orthovanadate Solution (100 mM)	Sodium Orthovanadate	(0.5 mL × 2)
• MgATP Solution (200 mM)	Magnesium Chloride, ATP2Na	(0.5 mL × 2)

• 10 mM-Phosphate Standard Solution	Sodium Dihydrogen Phosphate	(1 mL × 2)
• Stop Solution (10 w/v % SDS)	Sodium Dodecyl Sulfate	(5 mL × 2)
• Reducing Agent	Ascorbic Acid	(2 g × 2)
• Coloring Solution A (35 mM)	Hexaammonium Heptamolybdate Tetrahydrate	(2.5 mL × 2)
• Coloring Solution B (15 mM)	Zinc Acetate Dihydrate	(2.5 mL × 2)
• pH-adjusting Agent (10 N NaOH)	Sodium Hydroxide	(1 mL × 2)

Two sets of bottles (1 kit) contain sufficient volume of reagents to perform 80 ATPase assays and 16 phosphate standards (one full 96-well plate) based on this protocol

3. Apparatuses and other products required

3.1. Apparatuses

- Water bath or incubator
- Micropipettes (20, 200, 1000 µL), multichannel pipette (20 ~ 200 µL)
- Microplate reader covering the wavelength range of 630 ~ 850 nm

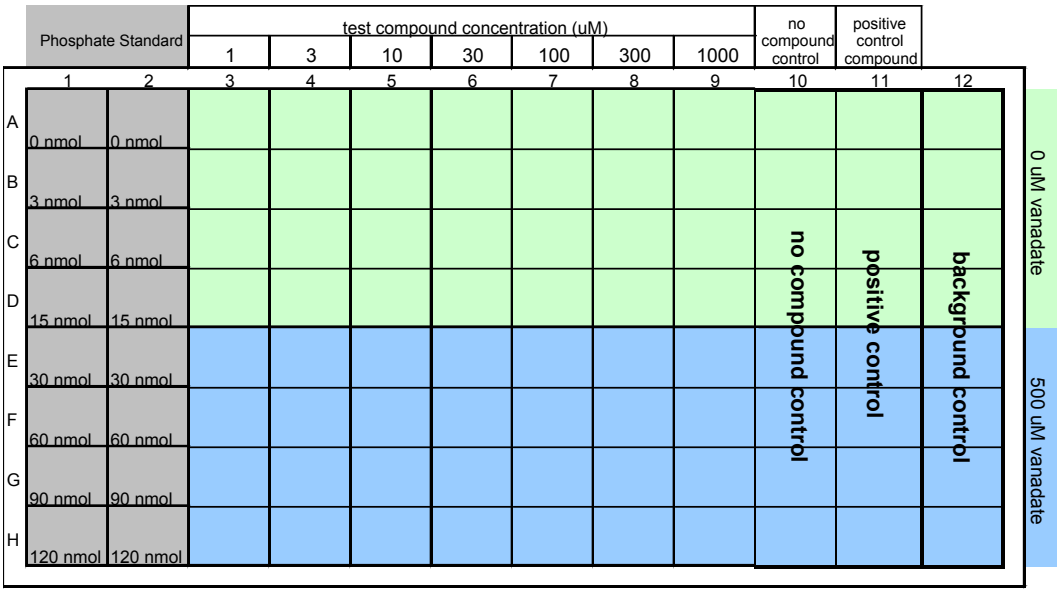
3.2. Materials

- Pipettes, Chips
- Tubes for dilution and preparation
- 96-well microtiter plate

4. Preparation of ATPase assay

This is a standard procedure to determine the concentration-dependent reaction of test compounds.

4.1. Plate layout



4.2. Dilution of phosphate standard

Dilute 10 mM standard phosphate to 0, 0.05, 0.1, 0.25, 0.5, 1.0, 1.5 and 2.0 mM with reaction buffer according to the following dilution schedule:

	Dilution mixture							
10 mM NaH_2PO_4 (µL)	0	5	10	25	50	100	150	200
Reaction buffer (µL)	1000	995	990	975	950	900	850	800
Final concentration of phosphate (mM)	0	0.05	0.1	0.25	0.5	1.0	1.5	2.0
Phosphate amount in 60 µL (nmol)	0	3	6	15	30	60	90	120

4.3. 3 mM Orthovanadate Solution

Add 120 µL of 100 mM Orthovanadate to 3880 µL of reaction buffer to make 3 mM Orthovanadate and place on ice.

4.4. 12 mM MgATP Solution

Add 300 µL of 200 mM MgATP to 4700 µL of reaction buffer to make 12 mM MgATP solution and place on ice.

4.5. Test compound Solution

Prepare test compound solutions in DMSO at 50 times the final concentration. For example, with the plate layout shown in section 4.1., prepare 50-fold concentrations (0.05, 0.15, 0.5, 1.5, 5, 15 and 50 mM) first. Add 12 µL of each solution to 188 µL of reaction buffer to make 3-fold concentrations (percentage of DMSO is 6%).

The solutions at 3-fold concentration are diluted in the assay process to final concentrations of 1, 3, 10, 30, 100, 300 and 1000 µM, respectively, (final percentage of DMSO becomes 2 %).

Mix the prepared solutions well and then place them on ice. Prepare materials for positive controls in the same manner.

4.6. 6 % DMSO solution

Add 24 µL of DMSO to 376 µL of reaction buffer to make 6 % DMSO solution, to be used for the no compound control and background control.

4.7. 2 mg/mL ABC Transporter Vesicles Products

Add 350 µL of ABC Transporter Vesicles Products (5 mg/mL) to 525 µL of reaction buffer to make a 2 mg/mL dilution of product, and place on ice. Mix well by using a pipette or by gentle inversion-shaking.

Do not use a Vortex mixer.

4.8. Detection Reagent Solution 1

Dissolve **Reducing Agent** (2 g) in 15 mL ultra pure water, adjust with **pH-adjusting Agent** (ca. 1 mL) to

pH 5.0, and then dilute to 20 mL with ultra pure water.

Note; Detection Reagent Solution 1 should be prepared upon each assay performance.

4.9. Detection Reagent Solution 2

Equivalent volume of **Coloring Solution A** (2.5 mL) and **Coloring Solution B** (2.5 mL) are mixed to obtain a volume of 5 mL.

Note; Detection Reagent Solution 2 should be prepared upon each assay performance. The mixed solution cannot be stored.

5. ATPase Assay procedure

5.1. Assay Steps

(1) Place a 96-well plate on ice.

(2) Add 60 µL each of 0, 0.05, 0.1, 0.25, 0.5, 1.0, 1.5 and 2.0 mM standard phosphate (prepared as described in section 4.2.) to wells A ~ H of the first and second lines of the plate. Add the other solutions to the plate as well according to the layout shown in section 4.1.

(3) Add 10 µL each of 2 mg/ml ABC Transporter Vesicles Products (prepared as described in section 4.7.) to wells A ~ H of the third to twelfth lines of the plate.

(4) Add 20 µL each of the 3-fold concentrations of test compound solution (prepared as described in section 4.5.) to wells A ~ H of the third to ninth lines of the plate.

(5) Add 20 µL of 6% DMSO (prepared as described in section 4.6.) to A ~ H of the tenth and twelfth lines of the plate.

(6) Add 20 µL of positive control at 3-fold concentration to wells A ~ H of the eleventh line of the plate.

(7) Add 30 µL of Stop Solution to wells A ~ H of the twelfth line of the plate.

(8) Add 10 µL of reaction buffer to wells A ~ D of the third to twelfth lines of the plate.

(9) Add 10 µL of 3 mM orthovanadate solution (prepared as described in section 4.3.) to wells E ~ H of the third to twelfth lines of the plate.

(10) Shake gently to mix solutions inside of wells.

(11) Shield the plate with a cover and incubate at 37 °C for 3 min.

(12) Add 20 µL of 12 mM MgATP solution (prepared as described in section 4.4.) with a multi-channel pipette to wells A ~ H of the third to twelfth lines, and start the reaction by shaking the plate.

(13) Shield the plate with a cover and incubate at 37 °C for appropriate time. Reaction times for each positive control are shown in section 5.2.

(14) Stop the reaction by adding 30 µL of Stop Solution with the multi-channel pipette to all wells except for the background control (wells A ~ H of the the first to eleventh lines).

(15) Mix 20 mL of Detection Reagent Solution 1 (prepared as described in section 4.8.) with 5 ml of Detection Reagent Solution 2 (prepared as described in section 4.9.), and add 200 µL of the mixed solution to all wells (wells A ~ H of the first to twelfth lines) with the multi-channel pipette.

(16) Shield the plate with a cover and incubate at 37 °C for 20 min to develop color, then determine the absorbance in a range of 630 ~ 850 nm.

5.2. Positive control substrate

		Positive control	Reaction time
MDR1	Human MDR1	50 μM Verapamil	30 min
	Mouse Mdr1a		30 min
	Mouse Mdr1b		60 min
MRP1	Human MRP1	10 mM NEM-GS	60 min
	Rat Mrp1		
	Dog Mrp1		
MRP2	Human MRP2	1 mM Probenecid	60 min
	Rat Mrp2		
	Mouse Mrp2		
	Dog Mrp2		
	Monkey Mrp2		

6. Data analysis

(1) Make a calibration plot for phosphate from the absorbance data obtained with the standard phosphate solutions, and then calculate the correlation between phosphate amount and absorbance.

(2) Determine the amount of inorganic phosphate generated in each well from the correlation equation.

(3) Calculate the average value of inorganic phosphate.

(4) Subtract [average of data with orthovanadate] from [average of data without orthovanadate] to obtain inorganic phosphate generated by sensitive ATPase activity.

(5) Calculate sensitive ATPase activity from the amount of generated inorganic phosphate as follows,

sensitive ATPase activity (nmol Pi /min/mg protein)
= [generated inorganic phosphate (nmol)] ÷ [reaction time (min)] ÷ [protein amount (mg)]

(Ex.) Protein 2 mg/mL[1)] × 10 µL[2)] = 20 µg (0.02 mg), reaction time 60 min, generated inorganic phosphate 15 nmol

ATPase activity: 15 ÷ 60 ÷ 0.02 = 12.5 nmol Pi/min/mg protein

[1)] Protein concentration of Vesicles Products dilution prepared as described in section 4.7.

[2)] Added amount of the ABC Transporter Vesicles Products dilution

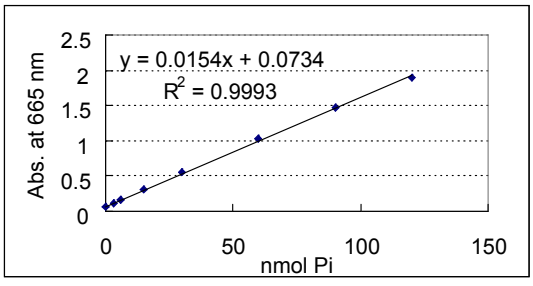
6.1. Example of data analysis

◆ Absorbance data

	Standard phosphate		Test compounds concentration (µM)							No compound control	Positive control	Background control	
			1	3	10	30	100	300	1000				
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.060	0.065	0.525	0.688	0.820	0.868	0.857	0.926	0.928	0.370	0.773	0.163	0 µM Vanadate
B	0.113	0.109	0.521	0.716	0.858	0.895	0.886	0.924	0.909	0.373	0.800	0.163	
C	0.155	0.156	0.558	0.734	0.843	0.896	0.844	0.942	0.915	0.378	0.848	0.155	
D	0.305	0.302	0.563	0.744	0.898	0.889	0.865	0.950	0.900	0.357	0.769	0.160	
E	0.557	0.551	0.307	0.311	0.323	0.305	0.309	0.328	0.318	0.312	0.298	0.163	500 µM Vanadate
F	1.040	1.023	0.301	0.309	0.317	0.309	0.306	0.319	0.320	0.322	0.299	0.156	
G	1.487	1.462	0.307	0.315	0.296	0.312	0.308	0.315	0.317	0.315	0.305	0.158	
H	1.932	1.869	0.308	0.320	0.325	0.313	0.308	0.310	0.324	0.305	0.307	0.155	

◆ Calibration for standard phosphate

nmol Pi (x)	Abs. (y)
0	0.063
3	0.111
6	0.156
15	0.304
30	0.554
60	1.032
90	1.475
120	1.901



◆ Inorganic phosphate (nmol)

Inorganic phosphate is calculated from the correlation equation

	Test compounds concentration (μM)							No compound control	Positive control	Background control	
	1	3	10	30	100	300	1000				
	3	4	5	6	7	8	9	10	11	12	
A	29.325	39.909	48.481	51.597	50.883	55.364	55.494	19.260	45.429	5.818	0 μM Vanadate
B	29.065	41.727	50.948	53.351	52.766	55.234	54.260	19.455	47.182	5.818	
C	31.468	42.896	49.974	53.416	50.039	56.403	54.649	19.779	50.299	5.299	
D	31.792	43.545	53.545	52.961	51.403	56.922	53.675	18.416	45.169	5.623	
E	15.169	15.429	16.208	15.039	15.299	16.532	15.883	15.494	14.584	5.818	500 μM Vanadate
F	14.779	15.299	15.818	15.299	15.104	15.948	16.013	16.143	14.649	5.364	
G	15.169	15.688	14.455	15.494	15.234	15.688	15.818	15.688	15.039	5.494	
H	15.234	16.013	16.338	15.558	15.234	15.364	16.273	15.039	15.169	5.299	

◆ Average of inorganic phosphate (nmol)

Test compounds concentration (μM)							No compound control	Positive control	Background control
1	3	10	30	100	300	1000			
30.412	42.019	50.737	52.831	51.273	55.981	54.519	19.227	47.019	5.640
15.088	15.607	15.705	15.347	15.218	15.883	15.997	15.591	14.860	5.494

◆ Generated inorganic phosphate (nmol)

Test compounds concentration (μM)							No compound control	Positive control	Background control
1	3	10	30	100	300	1000			
15.325	26.412	35.032	37.484	36.055	40.097	38.523	3.636	32.159	0.146

◆ Orthovanadate sensitive ATPase activity (nmol Pi /min/mg protein)

Test compounds concentration (μM)							No compound control	Positive control	Background control
1	3	10	30	100	300	1000			
12.771	22.010	29.194	31.236	30.046	33.415	32.102	3.030	26.799	0.122

7. FAQ

Q1) GenoMembrane recommends storing the ABC Transporter Vesicles Products frozen at -80 °C. Are they inactivated by freeze-thaw cycles?

A1) The ATPase activity of the ABC Transporter Vesicles Products was confirmed to be not decreased significantly after five freeze-thaw cycles. For precision assay, it is recommended to store in aliquots at -80°C to avoid repeated freeze-thaw.

Q2) How do you improve the sensitivity?

A2) Increase the volume of ABC Transporter Vesicles Products or extend the incubation period.

Q3) Does Orthonanadate-sensitive ATPase activity correspond to ATPase activity of ABC transporter?

A3) ABC Transporter Vesicles Products always contain a small amount of Orthonanadate-sensitive ATPase activity not related to ATPase activity of ABC transporter. Test compound may affect the activity as well. It is, therefore, recommended to conduct an ATPase assay with Control Product (GenoMembrane, Cat. No. GM0003).

Q4) Which 96-well plate is recommend for use in the ATPase assay?

A4) Polystyrene plate F96; #269620, Nunc

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