MRP1 TR ATPase Assay Protocol

CAT. NO. SBAT02

SIGMA®

SIGMA-ALDRICH®

Determination of the interaction of drugs with the human MRP1 (ABCC1) transporter using the ATPase Assay

For the following membrane products:
SB-MRP1-Sf9-ATPase

Version Number:

1.1

Effective date:

02. February 2007

Replaces:

MDR1-MRP1-2 ATPase Assay Protocol v4.0 – 16.December 2005

Related Procedures:

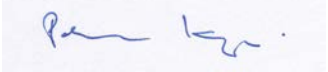
SOP 201: Fee-for Service Screening

Signatures:

Author(s):

Date (dd/mm/yyyy)	Name	Initials	Signature
02. February 2007	Hristos Glavinas, R&D Manager	HG	

Approved:

Date (dd/mm/yyyy)	Name	Initials	Signature
02. February 2007	Peter Krajcsi, PhD, CSO	PK	

1. Introduction **3**
2. Assay principle **3**
3. Deliverables **4**
4. Equipment needed **4**
5. Materials **5**
6. Solutions **6**
6.1. Stock solutions 6
6.2. Assay-mix 7
7. Main steps of the assay **8**
8. What is the signal composed of? **8**
9. Note for MRP1 studies **9**
10. Solvents **10**
11. Controls **10**
12. Suggested assay layout **12**
12.1. Activation studies 12
12.2. Inhibition studies 12
Assay steps **13**
13. Representing the data **14**
13.1. Calculation of the specific (Na_3VO_4 sensitive) transporter ATPase activities 15
13.2. Calculation of the percent transporter ATPase activities 17

1. Introduction

SOLVO Biotechnology has developed a number of assay systems based on the detection of the ATPase activity of ABC transporter proteins. These assays are performed using purified membrane vesicles from Sf9 *(Spodoptera frugiperda)* or mammalian cells, expressing high levels of a selected human ABC transporter protein. The following protocol describes the ATPase assay for measuring human MRP1 (ABCC1).

2. Assay principle

ABC transporters pump substrates out of the cell by using ATP hydrolysis as an energy source. ATP hydrolysis yields inorganic phosphate (Pi), which can be detected by a simple colorimetric reaction. The amount of Pi liberated by the transporter is proportional to the activity of the transporter.

Membrane preparations containing ABC transporters show a baseline ATPase activity that varies for different transporters. Transported substrates increase this baseline ATPase activity, while inhibitors or slowly transported compounds inhibit the baseline ATPase activity and/or the ATPase activity measured in the presence of a stimulating agent. Both activation and inhibition studies can be performed.

This assay is a modification of the method of *Sarkadi et al., J. Biol. Chem. 267:4854 (1992).*

3. Deliverables

- Frozen membrane vesicles, containing 5 mg/ml membrane protein in Eppendorf tubes, labeled with volume, catalog number (transporter) and date of production.
- Data sheet indicating protein content, volume, basal and maximal vanadate sensitive ATPase activities and date of expiry of frozen membrane stocks.
- Assay protocol.

4. Equipment needed

- Water bath/incubator, 37 ^{o}C
- Automatic pipettes, multichannel-pipette with corresponding tips
- 96 well microtiter plates
- Microplate reader with absorbance filter suitable for measuring OD between 630 and 850 nm

5. Materials

Substance	*Cat. Number*	*Storage*
Tris-Base (Tris[hydroxymethyl]aminomethane)	Sigma T-1503	RT, >1year
MOPS (3-[N-Morpholino]propanesulfonic acid)	Sigma M-1254	RT, >1year
EGTA	Sigma E-3889	RT, >1year
KCl	Sigma P-9333	RT, >1year
Na-azide	Sigma S-8032	RT, >1year
DTT	Sigma D-5545	4 °C, >1 year
SDS	Sigma L-6026	RT, >1year
KH_2PO_4	Sigma P-5379	RT, >1year
NEM	Sigma E-1271	4 °C, >1 year
Glutathione	Sigma G-4251	4 °C, >1 year
Na-Orthovanadate (Na_3VO_4)	Sigma S-6508	RT, >1year
ATP (disodium salt)	Sigma A-2383	-20 °C, >1 year
$MgCl_2$ (hexahydrate)	Sigma M-2670	RT, >1year
Zinc acetate (dihydrate)	Sigma Z-4540	RT, >1year
Ammonium-molybdate (tetrahydrate)	Sigma A-7302	RT, >1year
Ascorbic acid	Pharmacy grade	4 °C, >1 year
DMSO	Sigma D-2650	RT, >1year
MilliQ water MilliQ water is made by filtering distilled water through a Millipore Ultra-Pure Water System Purification Pak. Use MilliQ water to make all solutions.	Millipore 67733	RT, >1year

6. Solutions

6.1. Stock solutions

Solution	*Storage*
1.7 M Tris Dissolve 20.587 g of Tris in 100 ml distilled water.	4 °C, >1 year
0.1 M MOPS-Tris Dissolve 2.09 g of MOPS in 90 ml distilled water, adjust pH to 7.0 with 1.7 M Tris (about 2 ml). Bring solution to 100 ml with distilled water.	4 °C, >1 year
100 mM EGTA-Tris pH 7.0 Dissolve 3.804 g of EGTA in about 10 ml of 1.7 M Tris. Add 80 ml of distilled water and adjust pH to 7.0 with 1.7 M Tris. Bring solution to 100 ml with distilled water.	4 °C, >1 year
1 M KCl Dissolve 7.46 g of KCl in 100 ml distilled water.	4 °C, >1 year
0.1 M Na-azide Dissolve 0.65 g of Na-azide in 100 ml distilled water.	4 °C, >1 year
0.1 M DTT Dissolve 0.3086 g of DTT in 20 ml distilled water.	-20 °C in small aliquots
5% SDS Dissolve 5 g of SDS in 100 ml distilled water.	RT, > 1 year
100 mM KH_2PO_4 Dissolve 0.1361 g of KH_2PO_4 in 10 ml distilled water.	-20 °C in small aliquots
Phosphate standard solutions 0.5 mM, 1 mM, 2 mM, 4 mM and 8 mM KH_2PO_4 prepared from 100 mM stock in distilled water.	-20 °C in small aliquots
60 mM Na-Orthovanadate 60 mM Na-Orthovanadate in distilled water.	-20 °C in small aliquots
0.2 M MgATP Dissolve 2.2 g of ATP and 0.8 g $MgCl_2$ in 10 ml of distilled water and adjust pH to 7.0 with 1.7 M Tris. Bring solution to 20 ml with distilled water.	-20 °C in small aliquots

Solution	*Storage*
300 mM NEM Dissolve 0.375 g of NEM in 10 ml of 0.1 M MOPS-Tris.	-20 °C in small aliquots
300 mM Glutathione Dissolve 0.922 g Glutathione in 8 ml distilled water and adjust pH to 6.8 with 10 M NaOH. Bring solution to 10 ml with distilled water.	-20 °C in small aliquots
150 mM NEM-GS Mix 300 mM NEM with 300 mM glutathione in 1:1 ratio.	Prepare fresh
15mM Zinc Acetate Dissolve 330 mg of Zinc Acetate in 100 ml of distilled water.	4 °C, 2 weeks
35 mM Ammonium Molybdate in 15 mM Zinc Acetate pH 5.0 Dissolve 0.432 g of Ammonium Molybdate in 10 ml of Zn Acetate Store in a container wrapped with aluminum foil in the dark.	Prepare fresh
10% Ascorbic Acid pH 5.0 Dissolve 3 g Ascorbic Acid in 30 ml distilled water and adjust pH to 5.0 with NaOH (prepared freshly).	Prepare fresh

6.2. Assay-mix

Ingredient	*Volume (µl)*	*Final concentration*
0.1 M MOPS-TRIS, pH 7.0	5,000	50 mM
1 M KCl	500	50 mM
0.1 M Na-azide	500	5 mM
0.1 M DTT	200	2 mM
100 mM EGTA-TRIS, pH 7.0	10	0.1 mM
Distilled Water	3,790	
Total volume	10,000	

Add DTT freshly before use. The rest of the solutions can be pre-mixed and stored at 4 °C.

7. Main steps of the assay

1. Preparation of the assay-mix
2. Preparation of membrane suspension in assay-mix, in case of inhibition studies addition of a known substrate, distribution onto microplates
3. Serial dilution of the drugs to be assayed, addition of reagents to the plate wells
4. Preincubation at 37 ^{o}C, starting the assay by the addition of MgATP
5. Incubation at 37 ^{o}C, termination of the ATPase activity by SDS
6. Measurement of the Pi liberation by spectrophotometry
7. Calculation of the results by using the OD values, compared to standard phosphate concentrations

8. What is the signal composed of?

As a result of the relatively complex composition of the assay, transporter activities from OD values can only be calculated with adequate controls. Therefore it is important to understand how the different experimental conditions modify the readout (OD, reflecting Pi concentration). The Pi measured is composed of the following elements:

1. There is some Pi present in the final assay suspension (containing the membranes and ATP). This is mainly due to the contamination of the membranes and ATP with Pi, and some non-enzymatic ATP hydrolysis.

2. Some of the ATPase activities in the membranes are Na_3VO_4 insensitive. This is important to determine since the transporter-specific Na_3VO_4 sensitive activity can only be calculated by subtracting this background Na_3VO_4 insensitive activity. This activity may somewhat vary with membrane stocks, thus including this control in the calculations also decreases inter-assay variability.

3. Membranes always contain a small amount of Na_3VO_4 sensitive ATPase activity not related to transporter ATPase. This can be assayed by measuring the Na_3VO_4 sensitive ATPase activity of a control membrane (purified from cells infected with a virus expressing e.g. beta-galactosidase). This experiment is routinely performed in our laboratory to validate membrane production and purification. If you wish to set up this control experiment, control membranes are available from SOLVO Biotechnology.

4. The rest of the signal is the transporter specific ATPase activity of the membrane suspension, which is Na_3VO_4 sensitive. Transporters show some baseline ATPase activity that varies for different transporters and different membrane preparations. Substrates of the transporter stimulate the baseline ATPase activity, while inhibitors or slowly transported compounds inhibit the baseline ATPase activity.

5. Some drugs are colorful or might interfere with the assay in any other nonspecific way. In order to separate any nonspecific effect test drugs should be assayed with and without the presence of 1.2 mM Na_3VO_4.

9. Note for MRP1 studies

The MRP1 transporter is known to transport some small, positively charged molecules only in the presence of glutathione (co-transport), which is readily available in living cells. Also, in the ATPase assay some drugs only modulate the ATPase activity of the MRP1 transporter in the presence of glutathione. Therefore, we suggest assaying the interaction of the test drugs with the MRP1 transporter with and without the presence of 2 mM glutathione (added to the assay mix) both in inhibition and activation studies.

10. Solvents

Throughout this protocol DMSO is used as a general solvent of test drugs; the final concentration of DMSO is 2% in the assay during the incubation period. Controls are also designed according to this. However, the assay tolerates up to 2 % of a wide range of organic solvents (DMSO, ethanol, methanol, acetonitrile). If you are using a different solvent replace DMSO with that solvent in all controls.

11. Controls

We suggest the following controls to set up for measuring the different elements of the signal. Perform all controls in duplicates.

Ctrl 1: **Assay background control:** add 40 µl membrane in assay mix and 40 µl 5% SDS to the wells. Add 1 µl DMSO. Preincubate at 37 ^{o}C for 5 minutes. Add 10 µl of 25 mM MgATP to the wells and incubate at 37 ^{o}C. Develop color reaction for inorganic phosphate. This will show the Pi present in the well without any enzymatic ATP hydrolysis. Ctrl 1 serves as a quality check of the components of the assay mix, solvents, and the ATP and membrane used.

Ctrl 2: Na_3VO_4 insensitive ATPase activity: add 40 µl membrane in assay mix and 1 µl of 60 mM Na_3VO_4 to the wells (final Na_3VO_4 concentration is 1.2 mM). Add 1 µl DMSO. Preincubate at 37 ^{o}C for 5 min. Start the reaction by adding 10 µl of 25 mM MgATP to the wells and incubate at 37 ^{o}C. Stop the reaction by adding 40 µl 5% SDS at the end of the incubation period and develop color reaction for inorganic phosphate. This will show the ATPase activity not sensitive to Na_3VO_4.

Ctrl 3: Baseline ATPase activity: add 40 µl membrane in assay mix. Add 1 µl DMSO. Preincubate at 37°C for 5 min. Start the reaction by adding 10 µl of 25 mM MgATP to the wells and incubate at 37 °C. Stop the reaction by adding 40 µl 5% SDS at the end of the incubation period and develop color reaction for inorganic phosphate. This will show the basic level of Pi liberation in the presence of a given membrane, transporter and solvent for the drugs. For obtaining the vanadate-sensitive baseline ATPase activity subtract Ctrl 2.

Ctrl 4: Fully activated (maximal) ATPase activity: add 40 µl membrane in assay mix. For MRP1 transporter add 3.3 µl of 150 mM NEM-GS (final concentration 10 mM) and 1 µl DMSO. Preincubate at 37 °C for 5 min. Start the reaction by adding 10 µl of 25 mM MgATP to the wells and incubate at 37 °C. Stop the reaction by adding 40 µl 5% SDS/well at the end of the incubation period and develop color reaction for inorganic phosphate. This will show the maximum level of Pi liberation in the presence of a given membrane and transporter. For obtaining the full vanadate-sensitive ATPase activity subtract Ctrl 2.

12. Suggested assay layout

We suggest using the following assay layouts. It is designed for a duplicate measurement of 2 different drugs at 8 concentrations (3-fold serial dilution starting from 100 µM). Note that in case of inhibition studies test drugs are assayed in the presence of a strong activator.

12.1. Activation studies

	1	2	3	4	5	6	7	8	9	10	11	12
	Calibration (Pi/well)		**Controls**		**Test drug A**		**Test drug A + Na_3VO_4**		**Test drug B**		**Test drug B + Na_3VO_4**	
A	0		Ctrl 1		100 µM		100 µM		100 µM		100 µM	
B	5 nmol		Ctrl 2		33.3 µM		33.3 µM		33.3 µM		33.3 µM	
C	10 nmol		Ctrl 3		11.1 µM		11.1 µM		11.1 µM		11.1 µM	
D	20 nmol		Ctrl 4		3.7 µM		3.7 µM		3.7 µM		3.7 µM	
E	40 nmol				1.23 µM		1.23 µM		1.23 µM		1.23 µM	
F	80 nmol				0.41 µM		0.41 µM		0.41 µM		0.41 µM	
G					0.137 µM		0.137 µM		0.137 µM		0.137 µM	
H					0.046 µM		0.046 µM		0.046 µM		0.046 µM	

12.2. Inhibition studies

	1	2	3	4	5	6	7	8	9	10	11	12
	Calibration (Pi/well)		**Controls**		**Test drug A + activator at fixed concentration**		**Test drug A + Na_3VO_4 + activator at fixed concentration**		**Test drug B + activator at fixed concentration**		**Test drug B + Na_3VO_4 + activator at fixed concentration**	
A	0		Ctrl 1		100 µM		100 µM		100 µM		100 µM	
B	5 nmol		Ctrl 2		33.3 µM		33.3 µM		33.3 µM		33.3 µM	
C	10 nmol		Ctrl 3		11.1 µM		11.1 µM		11.1 µM		11.1 µM	
D	20 nmol		Ctrl 4		3.7 µM		3.7 µM		3.7 µM		3.7 µM	
E	40 nmol				1.23 µM		1.23 µM		1.23 µM		1.23 µM	
F	80 nmol				0.41 µM		0.41 µM		0.41 µM		0.41 µM	
G					0.137 µM		0.137 µM		0.137 µM		0.137 µM	
H					0.046 µM		0.046 µM		0.046 µM		0.046 µM	

Assay steps

1. Assemble assay mix and keep it on ice. Place a 96 well plate on ice.
2. Add 40 µl of assay mix to the wells used for Pi calibration. Add 10 µl of the phosphate standard solutions to the appropriate wells (to obtain 5-80 nmoles of Pi/well). Add 40 µl 5% SDS to these wells. Perform in duplicates.
3. Use the predetermined membrane protein concentration (5 mg/ml) to prepare a 1 mg/ml suspension by diluting the membranes in assay mix (mix well).
   In case of inhibition studies remove the amount of membrane suspension required for controls (8 wells). Add the strong activator to the rest of the suspension:

MRP1	82.5 µl 150 mM NEM-GS/ml (final concentration 10 mM)

4. Add 40 µl of membrane suspension to the desired wells of a 96-well plate.
5. Add the required reagents to controls. Add test drugs in 1 µl DMSO.
6. Transfer plates to 37 ^{o}C, preincubate for 5 min.
7. Prepare a 25 mM MgATP solution by diluting 0.2 M MgATP solution in assay mix.
8. Start the ATPase reaction by the addition of 10 µl of 25 mM Mg-ATP.
9. Incubate the plates at 37 °C in a water bath, (60 min for MRP1).
10. Stop the ATPase reaction by adding 40 µl 5% SDS to each well.
11. Prepare the detection reagent: Add 10 ml of 35 mM Ammonium Molybdate in 15 mM Zinc Acetate to 30 ml of the freshly prepared 10% ascorbic acid. Invert to mix.
12. Add 200 µl of detection reagent to each well.
13. Incubate the plates at 37 °C for 25 minutes, read the OD between 630 and 850 nm. Note that further slow increase in color occurs in ATP-containing wells, due to ATP cleavage caused by the detection reagent.

13. Representing the data

There are two main representations of the data:

1. Calculation of specific activities

Calculate the Na_3VO_4 sensitive transporter ATPase activities in terms of Pi liberated/mg membrane protein/min. This is obtained by subtracting the Pi liberated in the presence of Na_3VO_4 from the Pi liberated without Na_3VO_4. Pi liberated is determined using a KH_2PO_4 calibration curve and the results are calculated based on the amount of membrane protein/well and incubation time.

2. Calculation of percent transporter ATPase activaties

In this representation the baseline ATPase activity (Ctrl 3) and the maximal ATPase activity (Ctrl 4) are taken as 0% and 100% ATPase activity of the transporter, respectively. OD values determined in the presence of test drugs are represented as percent activity on this relative scale. Note that some drugs are inhibitors of the baseline transporter ATPase activity. These will show negative percent values in this representation.

13.1. Calculation of the specific (Na_3VO_4 sensitive) transporter ATPase activities

13.1.1. Calculation steps

1. Set up a calibration curve using the calibration curve OD values and the amount of KH_2PO_4 used (nmol/well).
2. Calculate the average OD values of the duplicate measurements of the calibration curve and controls (Ctrl 1, Ctrl 2, Ctrl 3, Ctrl 4), respectively. Calculate the average OD values of your samples.
3. Determine the nanomoles of Pi liberated in the controls and in the samples by using the calibration curve and the average OD values calculated in step 1 and 2.
4. Subtract Pi liberated in Ctrl 2 from Pi liberated in Ctrl 3. This will give you the baseline Na_3VO_4 sensitive ATPase activity.
5. Subtract Pi liberated in Ctrl 2 from Pi liberated in Ctrl 4. This will give you the fully activated Na_3VO_4 sensitive transporter ATPase activity.
6. Subtract the Pi values determined in the presence of Na_3VO_4 from the Pi values measured without Na_3VO_4 for each compound examined. This will give you the Na_3VO_4 sensitive transporter ATPase activity for each drug and drug concentration assayed.
7. Calculate the Na_3VO_4 sensitive transporter ATPase activity in terms of Pi liberated/mg membrane protein/min. Divide the numbers by the amount of membrane protein added to one well and the time of incubation in minutes.

13.1.2. Expected results

The following table shows the typical baseline and maximal Na_3VO_4 sensitive ATPase activities of MRP1 transporter. All figures are shown in nmol Pi/mg membrane protein/min:

Membrane preparation	Baseline activity	Maximal activity
SB-MRP1-Sf9-ATPase	3-6	9-18

In case of activation studies test drugs that are activators of the baseline ATPase activity of the transporter will increase the rate of ATP cleavage compared to the baseline ATPase activity. The Na_3VO_4 sensitive transporter ATPase activity will fall between the baseline Na_3VO_4 sensitive ATPase activity and the fully activated Na_3VO_4 sensitive ATPase activity. Inhibitors of the baseline ATPase activity will decrease the rate of ATP cleavage compared to the baseline ATPase activity. The Na_3VO_4 sensitive transporter ATPase activity will be between 0 and the baseline Na_3VO_4 sensitive ATPase activity.

In case of inhibition studies test drugs that are inhibitors of the maximal and/or the baseline ATPase activity will decrease the rate of ATP cleavage compared to the fully activated ATPase activity. The Na_3VO_4 sensitive transporter ATPase activity will be between 0 and the maximal Na_3VO_4 sensitive ATPase activity.

13.2. Calculation of the percent transporter ATPase activities

13.2.1. Calculation steps

1. Subtract Ctrl 3 from Ctrl 4. This will give you the OD representing the maximal Na_3VO_4 sensitive drug stimulated transporter ATPase activity.
2. Subtract Ctrl 2 from Ctrl 3. This will give you the OD representing the baseline Na_3VO_4 sensitive transporter ATPase activity.
3. Subtract the OD values measured in the presence of Na_3VO_4 from the OD values measured without Na_3VO_4 for each test drug and subtract the OD representing the baseline Na_3VO_4 sensitive ATPase activity (determined in step 2) from every difference. This will give you the OD representing the drug stimulated transporter ATPase activity for each drug and drug concentration assayed.
4. Calculate the percent transporter ATPase activities. Simply divide each OD value from step 3 by the OD value calculated in step 1. Multiply the result by 100 to get the results in percentage.

13.2.2. Expected results

The substances used to determine the maximal drug stimulated transporter ATPase activities are strong activators of transporters. In activation studies compounds tested are not expected to give significantly higher activation even at the highest concentrations used, so results greater than 100% are not common. Some drugs are inhibitors (or, sometimes in smaller concentrations activators, while in larger concentrations inhibitors) of the baseline transporter activity. In this case the percent stimulation may turn into negative numbers. Further insight into the ATPase inhibitory effect of drugs can be gained by performing inhibition studies.

In case of inhibition studies per cent inhibition values usually fall between 100% and 0%. However, if a certain drug inhibits both the maximal Na_3VO_4 sensitive ATPase

activity and the baseline Na_3VO_4 sensitive ATPase activity per cent inhibition values may turn into negative numbers. Detailed information on whether a certain drug inhibits the baseline ATPase activity and/or the maximal ATPase activity of the transporter can be gained by assaying the test drug for both activation and inhibition.