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ProductInformation

Mdr1b, rat
recombinant, expressed in *Sf*9 cells,
membrane preparation, for ATPase

Product Number **M9319**
Storage Temperature –70 °C

TECHNICAL BULLETIN

Product Description

Multi-drug resistance (MDR) is a major factor in the failure of many forms of chemotherapy; one that affects patients with a variety of hematological malignancies and solid tumors, including different types of leukemia, breast, ovarian, lung, and lower gastrointestinal tract cancers. In the past few years it has become accepted that the resistance to chemotherapy correlates with the overexpression of at least two ATP-dependent drug-efflux pumps. These human cell membrane proteins, called P-glycoprotein (MDR1, PgP, ABCB1) and multidrug-resistance-associated protein (MRP1), are members of the ATP-binding-cassette (ABC) transporter family. The human MDR1 protein is involved in cancer drug resistance and in the transport of hydrophobic drugs and xenobiotics in the bowel, kidney, liver, and the blood-brain barrier.[1-5]

In humans there is single *MDR1* gene, while in rodents there are two genes, *mdr1a* and *mdr1b*. Based on function and tissue distribution in rodents, the equivalent of the human *MDR1* gene product (PgP) is the product of the rodent *mdr1b* gene. So far no significant differences in function, substrate specificity, or substrate affinity have been reported between these two proteins.

The ABC transporters pump substrates out of the cell by using hydrolysis of ATP as an energy source. Detection of the ATPase activity of the Mdr1b protein is a measure of transporter activity. The assay is performed using purified membrane vesicles from *Sf*9 *(Spodoptera frugiperda)* cells, expressing high levels of Mdr1b protein.

This kit uses an ATPase assay procedure for measuring rat Mdr1b activity. ATP hydrolysis yields inorganic phosphate (P_i), which can be detected by a simple colorimetric reaction. The amount of P_i liberated is proportional to the activity of the transporter.[6]

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.

Reagent

The membrane vesicles are suspended in TMEP solution (50 mM Tris-HCl, 50 mM mannitol, 2 mM EGTA, 8 µg/ml aprotinin, 10 µg/ml leupeptin, 50 µg/ml PMSF, and 2 mM DTT, pH 7.0)

Equipment and Reagents Required But Not Provided

- Trizma® base [Tris(hydroxymethyl)aminomethane, Tris-base], Product Code T1503
- MOPS [3-(N-Morpholino)propanesulfonic acid], Product Code M1254
- Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), Product Code E3889
- Potassium chloride (KCl), Product Code P9333
- Sodium azide (NaN_3), Product Code S8032
- DL-Dithiothreitol (DTT), Product Code D5545
- Sodium dodecyl sulfate (SDS), Product Code L6026
- Potassium phosphate monobasic (KH_2PO_4), Product Code P5379
- (±)-Verapamil hydrochloride, Product Code V4629
- Sodium orthovanadate (Na_3VO_4), Product Code S6508
- Adenosine 5′-triphosphate, disodium salt (ATP), Product Code A2383
- Magnesium chloride hexahydrate, Product Code M2670

- Zinc acetate dihydrate, Product Code 383058
- Ammonium molybdate tetrahydrate, Product Code A7302
- Ascorbic acid
- Dimethyl sulfoxide (DMSO), Product Code D2650
- Ultrapure water (17 MΩ·cm or equivalent)
- 37 °C water bath/incubator
- Multichannel pipettes with corresponding tips
- 96 well plates and plate reader with absorbance filter suitable for measuring absorbance between 630–850 nm

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Use ultrapure water (17 MΩ·cm or equivalent) for preparation of reagents.

1.7 M Tris Solution – Dissolve 20.587 g of Tris-base in 100 ml of water. The solution may be stored at 2–8 °C for at least 1 year.

0.1 M MOPS-Tris Solution – Dissolve 2.09 g of MOPS in 90 ml of water and adjust pH to 7.0 with 1.7 M Tris Solution (~2 ml). Bring the final volume to 100 ml with water. The solution may be stored at 2–8 °C for at least 1 year.

100 mM EGTA-Tris, pH 7.0 Solution – Dissolve 3.804 g of EGTA in ~10 ml of 1.7 M Tris Solution. Add 80 ml of water and adjust pH to 7.0 with 1.7 M Tris Solution. Bring the final volume to 100 ml with water. The solution may be stored at 2–8 °C for at least 1 year.

1 M KCl Solution – Dissolve 7.46 g of KCl in 100 ml of water. The solution may be stored at 2–8 °C for at least 1 year.

0.1 M Sodium Azide Solution – Dissolve 0.65 g of sodium azide in 100 ml of water. The solution may be stored at 2–8 °C for at least 1 year.

0.1 M DTT Solution – Dissolve 0.3086 g of DTT in 20 ml of water. Store the solution in aliquots at –20 °C.

5% SDS Solution – Dissolve 5 g of SDS in 100 ml of water. The solution may be stored at room temperature for at least 1 year.

60 mM Sodium Orthovanadate Solution – prepared in water. Store the solution in aliquots at –20 °C.

100 mM KH_2PO_4 Solution – Dissolve 0.1361 g of KH_2PO_4 in 10 ml of water. Store the solution in aliquots at –20 °C.

Phosphate Standard Solutions - 0.5 mM, 1 mM, 2 mM, 4 mM, and 8 mM KH_2PO_4 solutions prepared with water from 100 mM KH_2PO_4 Solution. Store the solutions in aliquots at –20 °C.

0.2 M Mg-ATP Solution – Dissolve 2.2 g of ATP and 0.813 g of $MgCl_2$ in 10 ml of water and adjust pH to 7.0 with 1.7 M Tris Solution. Bring final volume to 20 ml with water. Store the solution in aliquots at –20 °C.

10 mM Verapamil Solution – Prepare in DMSO. Store the solution in aliquots at –20 °C.

15 mM Zinc Acetate Solution – Dissolve 330 mg of Zinc Acetate in 100 ml of water. The solution may be stored at 2–8 °C for at least 2 weeks.

35 mM Ammonium Molybdate and 15 mM Zinc Acetate, pH 5.0 Solution – Dissolve 0.432 g of Ammonium Molybdate in 10 ml of 15 mM Zinc Acetate Solution. Store in a container wrapped with aluminum foil in the dark. Freshly prepare the solution.

10% Ascorbic Acid, pH 5.0 Solution – Dissolve 3 g of Ascorbic Acid in 30 ml of water and adjust pH to 5.0 with NaOH. Freshly prepare the solution.

Assay Mix – Combine the following:

0.1 M MOPS-Tris Solution	5,000 µl
1 M KCl Solution	500 µl
0.1 M Sodium Azide Solution	500 µl
0.1 M DTT Solution	200 µl
100 mM EGTA-Tris, pH 7.0 Solution	10 µl
water	3,790 µl
Total volume	10,000 µl

Add the 0.1 M DTT Solution just prior to use. The rest of the solutions can be pre-mixed and stored at 2–8 °C.

Storage/Stability
The product ships on dry ice and storage at –70 °C is recommended. The membrane activity is not significantly lost after one freeze-thaw cycle. For long term storage, freeze in working aliquots.

Procedure

A – Assays for Activation Studies

Some test compounds may have absorbance between 630–850 nm or interfere with the assay in other ways. In order to separate any nonspecific effects, test compounds should be assayed with and without the presence of 1.2 mM Na_3VO_4. See Table 1 for suggested layout of activation assays.

See method B for information on control assays.

1. Assemble the 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 P_i calibration curve. Add 10 µl of the Phosphate Standard Solutions to the appropriate wells (to obtain a range of 5–80 nmoles of P_i per well). Add 40 µl of 5% SDS Solution to these wells. Perform in duplicates.
3. For Mdr1b assays, use the predetermined membrane protein concentration (5 mg/ml) to prepare a 0.5 mg/ml suspension by diluting the membranes with Assay Mix (mix well).
4. Add 40 µl of the prepared membrane suspension to the appropriate wells of the 96 well plate.
5. Add test compounds in 1 µl of DMSO. Throughout this procedure DMSO is used as a general solvent of test compounds; 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, and acetonitrile). If you are using a different solvent replace DMSO with that solvent in all controls.
6. Transfer plates to 37 °C and preincubate for 5 minutes.
7. Prepare a 25 mM Mg-ATP Solution by diluting 0.2 M Mg-ATP Solution with Assay Mix.
8. Start the ATPase reaction by the addition of 10 µl of 25 mM Mg-ATP Solution.
9. For the Mdr1b assay, incubate the plates at 37 °C for 20 minutes.
10. Stop the ATPase reaction by adding 40 µl of 5% SDS Solution to each well.
11. Prepare the Detection Reagent: Add 5 ml of 35 mM Ammonium Molybdate and 15 mM Zinc Acetate, pH 5.0 Solution to 15 ml of the freshly prepared 10% Ascorbic Acid, pH 5.0 Solution. Invert to mix.
12. Add 200 µl of Detection Reagent to each well.
13. Incubate the plates at 37 °C for 25 minutes and read the absorbance between 630–850 nm. Note: A slow increase in absorbance will continue in the ATP-containing wells, due to ATP cleavage caused by the Detection Reagent.

Table 1.

Activation Assay Layout Guidelines – It is designed for a duplicate measurement of 2 different compounds at 8 concentrations (3-fold serial dilution starting from 100 µM).

	1	2	3	4	5	6	7	8	9	10	11	12
	Calibration (P_i/well)		**Controls**		**Compound A**		**Compound A + Na_3VO_4**		**Compound B**		**Compound B + Na_3VO_4**	
A	0		Control 1		100 µM		100 µM		100 µM		100 µM	
B	5 nmol		Control 2		33.3 µM		33.3 µM		33.3 µM		33.3 µM	
C	10 nmol		Control 3		11.1 µM		11.1 µM		11.1 µM		11.1 µM	
D	20 nmol		Control 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	

B – Control Assays

As a result of the multiple reactions occurring during the assay, transporter activities from absorbance values can only be calculated with adequate controls. Therefore, it is important to understand how the different experimental conditions modify the measured absorbance values, reflecting P_i concentration. The P_i measured is composed of the following elements:

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

Some of the ATPase activity in the membranes is Na_3VO_4 insensitive. It is important to determine this activity, 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 vary with membrane stocks, thus including this control in the calculations also decreases inter-assay variability.

Membranes always contain a small amount of Na_3VO_4 sensitive ATPase activity not related to transporter ATPase. This activity is low and does not significantly impact the results of the assay. To confirm this, an optional fifth control may be run. The Na_3VO_4 sensitive ATPase activity may be measured using control membranes expressing a mutant (defective) variant of the MRP1 transporter (Product Code M9819; MDR1, MRP, and BSEP Control). The Na_3VO_4 sensitive ATPase activity may also be measured using control membranes expressing β-galactosidase. This assay is routinely performed to validate membrane production and purification. Control membranes expressing β-galactosidase are available from SOLVO Biotechnology.

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.

It is suggested the following controls be set up for measuring the different elements of the signal. Perform all controls in duplicates.

If you are using a solvent other than DMSO, replace DMSO with that solvent in all controls.

Control 1 – Assay Background Control: Add 40 µl of 0.5 mg/ml prepared membrane suspension (step A,3) and 40 µl of 5% SDS Solution to the wells. Add 1 µl of DMSO. Preincubate at 37 °C for 5 minutes. Add 10 µl of 25 mM Mg-ATP Solution (step A,7) to the wells and incubate at 37 °C. Develop color reaction for inorganic phosphate. This will show the P_i present in the well without any enzymatic ATP hydrolysis. Control 1 serves as a quality check of the components of the assay mix, solvents, ATP, and membrane used.

Control 2 – Na_3VO_4 Insensitive ATPase Activity: Add 40 µl of 0.5 mg/ml prepared membrane suspension (step A,3) and 1 µl of 60 mM Sodium Orthovanadate Solution to the wells (final Na_3VO_4 concentration is 1.2 mM). Add 1 µl of DMSO. Preincubate at 37 °C for 5 minutes. Start the reaction by adding 10 µl of 25 mM Mg-ATP Solution (step A,7) to the wells and incubate at 37 °C. Stop the reaction by adding 40 µl of 5% SDS Solution per well at the end of the incubation period and develop color reaction for inorganic phosphate. This will show the ATPase activity insensitive to Na_3VO_4.

Control 3 – Baseline ATPase Activity: Add 40 µl of 0.5 mg/ml prepared membrane suspension (step A,3) and 1 µl of DMSO to the wells. Preincubate at 37 °C for 5 minutes. Start the reaction by adding 10 µl of 25 mM Mg-ATP Solution (step A,7) to the wells and incubate at 37 °C. Stop the reaction by adding 40 µl of 5% SDS Solution to each well at the end of the incubation period and develop color reaction for inorganic phosphate. This will show the basic level of P_i liberation in the presence of a given membrane, transporter, and solvent for the test compounds. For obtaining the vanadate-sensitive baseline ATPase activity subtract Control 2.

Control 4 – Fully Activated (Maximal) ATPase Activity: Add 40 µl of 0.5 mg/ml prepared membrane suspension (step A,3). For Mdr1b ATPase activity, add 1 µl of 10 mM Verapamil Solution (final concentration of verapamil is 200 µM). Preincubate at 37 °C for 5 minutes. Start the reaction by adding 10 µl of 25 mM Mg-ATP Solution (step A,7) to the wells and incubate at 37 °C. Stop the reaction by adding 40 µl of 5% SDS Solution to each well at the end of the incubation period and develop color reaction for inorganic phosphate. This will show the maximum level of P_i liberation in the presence of a given membrane and transporter. For obtaining the full vanadate-sensitive ATPase activity subtract Control 2.

C – Assays for Inhibtion Studies

Note that in case of inhibition studies test compounds are assayed in the presence of a strong activator.

Some compounds may have absorbance between 630–850 nm or interfere with the assay in other ways. In order to separate any nonspecific effects, test compounds should be assayed with and without the presence of 1.2 mM Na_3VO_4. See Table 2 for suggested layout of inhibition assays.

See method B for information on control assays.

1. Perform steps A1 and A2.
2. For Mdr1b assays, use the predetermined membrane protein concentration (5 mg/ml) to prepare a 0.5 mg/ml suspension by diluting the membranes with Assay Mix (mix well). In inhibition studies, remove the amount of prepared membrane suspension required for controls (8 wells). Add the strong activator to the rest of the suspension: 25 µl of 10 mM Verapamil Solution per ml of membrane suspension (final concentration of verapamil is 200 µM).
3. Perform steps A4 – A13

Table 2.

Inhibition Assay Layout Guidelines – It is designed for a duplicate measurement of 2 different compounds at 8 concentrations (3-fold serial dilution starting from 100 µM). Note that in case of inhibition studies test compounds are assayed in the presence of a strong activator.

	1	2	3	4	5	6	7	8	9	10	11	12
	Calibration (P_i/well)		**Controls**		**Compound A + activator at fixed concentration**		**Compound A + Na_3VO_4 + activator at fixed concentration**		**Compound B + activator at fixed concentration**		**Compound B + Na_3VO_4 + activator at fixed concentration**	
A	0		Control 1		100 µM		100 µM		100 µM		100 µM	
B	5 nmol		Control 2		33.3 µM		33.3 µM		33.3 µM		33.3 µM	
C	10 nmol		Control 3		11.1 µM		11.1 µM		11.1 µM		11.1 µM	
D	20 nmol		Control 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	

Results

There are two presentations of the data:

A. Calculation of Specific Activities:

Calculate the Na_3VO_4 sensitive transporter ATPase activities in terms of P_i liberated/mg membrane protein/min. This is obtained by subtracting the P_i liberated in the presence of Na_3VO_4 from the P_i liberated without Na_3VO_4. P_i 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.

Calculation of the specific Na_3VO_4 sensitive transporter ATPase activities:

1. Calculate the average absorbance values of the duplicate measurements of the Phosphate Standard Solutions, controls (Control 1, 2, 3, 4), and test samples.
2. Set up a calibration curve using the absorbance values of the Phosphate Standard Solutions and the amount of KH_2PO_4 used (nmol/well).
3. Determine the nanomoles of P_i liberated in the controls and in the samples by using the calibration curve and the average absorbance values calculated in steps 1 and 2.
4. Subtract P_i liberated in Control 2 from P_i liberated in Control 3. This will give you the baseline Na_3VO_4 sensitive ATPase activity.
5. Subtract P_i liberated in Control 2 from P_i liberated in Control 4. This will give you the fully activated Na_3VO_4 sensitive transporter ATPase activity.
6. Subtract the P_i values determined in the presence of Na_3VO_4 from the P_i values measured without Na_3VO_4 for each compound examined. This will give you the Na_3VO_4 sensitive transporter ATPase activity for each test compound and test compound concentration assayed.

7. Calculate the Na_3VO_4 sensitive transporter ATPase activity in terms of P_i 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.

Expected results:
In activation studies, test compounds that are activators of the baseline transporter ATPase activity 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 inhibition studies, test compounds 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.

B. Calculation of Percent Transporter ATPase Activities:

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

Calculation of the percent transporter ATPase activities:

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

Expected results:
The maximal compound stimulated transporter ATPase activities are determined in the presence of a strong activator of the transporter. 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 compounds are inhibitors (or in some cases, activators at lower concentrations and inhibitors at higher concentrations) of the baseline transporter activity. In this case the percent stimulation may turn into negative numbers. Further insight into the ATPase inhibitory effect of compounds can be gained by performing inhibition studies.

In inhibition studies, percent inhibition values usually fall between 0–100%. However, if a certain compound inhibits both the maximal Na_3VO_4 sensitive ATPase activity and the baseline Na_3VO_4 sensitive ATPase activity, percent inhibition values may turn into negative numbers. Detailed information on whether a certain compound inhibits the baseline ATPase activity and/or the maximal ATPase activity of the transporter can be gained by assaying the test compound for both activation and inhibition.

References

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