

ratMdr1b PE ATPase Kit Assay Protocol

CAT. NO. SBPE06

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**Determination of the interaction of drugs
with the human ratMdr1b transporter using the
SB-ratMdr1b PREDEASY™ ATPase Kit**

For the following membrane product:
SB-ratMdr1b-ATPase

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Related Procedures:

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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 insect cells or mammalian cells, expressing high levels of a selected ABC transporter protein. The following protocol describes the ATPase assay for measuring ratMdr1b.

2. Assay principle

ABC transporters pump substrates out of the cell 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.

As membrane preparations contain other ATPases as well, it is important to distinguish the specific ABC transporter related ATPase activity and the background ATPase activity. ABC transporters are effectively inhibited by Na₃VO₄ therefore the activity of the transporters is measured as the vanadate sensitive portion of the total ATPase activity.

The assay is composed of two different tests which are performed on the same plate. In the activation test transported substrates may stimulate baseline vanadate sensitive ATPase activity. In the inhibition test, which is carried out in the presence of a known activator of the transporter, inhibitors or slowly transported compounds may inhibit the maximal vanadate sensitive ATPase activity. In some cases inhibitors or slowly transported compounds may inhibit the baseline transporter ATPase activity as well. The activation and inhibition tests are complementary assays. Stimulation detected in the activation assay indicate that the compound is a transported substrate of the transporter, while interactions detected in the inhibition test indicate interaction of the test compounds with the transporter, but do not give information on the nature



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(substrate or inhibitor) of the interaction. The assay is a modification of the method of Sarkadi *et al.*, *J. Biol. Chem.* 267:4854 (1992).

3. Deliverables

- SOLVO Biotechnology's SB-ratMdr1b **PREDEASY™** ATPase Assay Kit for ratMDR-1b transporter sufficient for 1, 3 or 10 complete activation and inhibition studies.
- Data sheet indicating protein content, volume, basal and maximal vanadate sensitive ATPase activities and expiration date of frozen membrane stocks.
- Data CD containing assay protocol, MS Excel file for calculations and data representation, and material safety data sheets.

4. Preferred equipment and materials

- Water bath / heating block / thermoshaker (BIOSAN PST-60HL or equivalent), 37 °C (Do not use CO₂ incubator because of insufficient heat conductivity!)
- Automatic pipettes, multichannel-pipette with corresponding tips (Eppendorf Research, Multipette Plus or equivalent, Eppendorf pipette tips or equivalent quality tips.) Electronic pipettes are not recommended, as they require additional amount of membrane preparation. Please note that the volume of membrane preparation provided in the vial A is the double of the amount actually needed for a 96-well plate (that still might not be sufficient for an electronic pipette).
- 96 well microtiter plates (COSTAR 3585 or equivalent)
- Microplate reader with absorbance filter suitable for measuring OD between 590 and 630 nm
- 2 ml, 5ml, 10 ml tubes
- MilliQ water (phosphate free water)



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- Dimethyl sulfoxide (DMSO, A.C.S reagent spectrophotometric grade, $\geq 99,9\%$, Sigma 154938)

For further useful information please review section 12. Troubleshooting.



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5. Materials

Your kit contains the following materials in amounts depending on the requested Kit size:

Kit size (number of plates)		1	3	10	Storage	Assay conditions
Vial	Substance	Amount				
A	Membrane stock (5 mg/ml)	180 µl	3x180 µl	10x180 µl	-80 °C	on ice
B	10x Medium	1 ml	3 ml	10 ml	-80 °C	on ice
C	Phosphate solution (0.4 mM)	100 µl	300 µl	1000 µl	≤-20 °C	on ice
D	Phosphate solution (0.8 mM)	100 µl	300 µl	1000 µl	≤-20 °C	on ice
E	Na-Orthovanadate solution (600 mM)	50 µl	150 µl	500 µl	≤-20 °C	on ice
F	Verapamil solution (100 mM in DMSO)	50 µl	150 µl	500 µl	≤-20 °C	room temp.
G	3x Developer (3x conc.)	4.5 ml	13.5 ml	3x15 ml	≤-20 °C	room temp.
H	10x Blocker (10x conc.)	1.5 ml	3x1.5 ml	15 ml	≤-20 °C	room temp.
I	Inhibitor solution (Cyclosporin A, 2 mM)	50 µl	150 µl	500 µl	≤-20 °C	room temp.
J	MgATP solution (0.2 M)	100 µl	300 µl	1000 µl	≤-20 °C	on ice

Keep the kit compounds during the assay procedure at the temperature specified in this table. Material safety data sheets of the compounds in your Vials are available as pdf files in the MSDS folder on the CD-ROM attached to the KIT box.

Do not use Substances from any other type of ATPase assay Kit.

6. 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.



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7. 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.



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- 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 Na₃VO₄.

8. Controls

We suggest the following controls to be set up for the measurement of the different components of the signal.

Ctrl 1: Baseline ATPase activity: This control shows the basic level of Pi liberation in the presence of a given membrane, transporter and solvent for the drugs. The Pi liberated in this well is the result of Pi contamination, non-transporter related ATP cleavage and the baseline ATPase activity of the transporter. For obtaining the vanadate-sensitive baseline ATPase activity subtract Ctrl 2.

Ctrl 2: Na₃VO₄ insensitive ATPase activity: Pi liberated in this well is the result of Pi contamination and non-transporter related ATP cleavage.

Ctrl 3: ATPase activity of fully activated membranes: It is the positive control for activation studies. The Pi liberated in this well is the result of Pi contamination, non-transporter related ATP cleavage and maximal transporter ATPase activity. For obtaining the full vanadate-sensitive ATPase activity, subtract Ctrl 4.

Ctrl 4: Na₃VO₄ insensitive ATPase activity of fully activated membranes: It is used in calculations for inhibition studies. The Pi liberated in this well is the result of Pi contamination and non-transporter related ATP cleavage in the presence of a strong activator and vanadate.

Ctrl 5: Inhibited ATPase activity of fully activated membranes: It is the positive control for inhibition studies. This control is designed to show if a known inhibitor of the transporter inhibits the ATPase activity of the fully activated membranes. For obtaining the inhibited vanadate-sensitive ATPase activity, subtract Ctrl 4.



9. Suggested assay layout

We suggest using the following assay layout. It is designed for activation and inhibition measurements of a drug at 8 concentrations (3-fold serial dilution starting from 300 μ M) performed in duplicates. Note that in case of inhibition studies, test drugs are assayed in the presence of a strong activator. . The plate map below indicates final concentrations!

9.1. Assay plate map for an activation and inhibition study

Plate Map (for two parallels)	Controls				test drug (μ M)		test drug (μ M) + vanadate		test drug (μ M) + activator		test drug (μ M) + activator + vanadate	
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank				300.00	300.00	300.00	300.00	300.00	300.00	300.00	300.00
B	4 nmol Pi				100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
C	8 nmol Pi				33.33	33.33	33.33	33.33	33.33	33.33	33.33	33.33
D	Ctrl1				11.11	11.11	11.11	11.11	11.11	11.11	11.11	11.11
E	Ctrl2				3.70	3.70	3.70	3.70	3.70	3.70	3.70	3.70
F	Ctrl3				1.23	1.23	1.23	1.23	1.23	1.23	1.23	1.23
G	Ctrl4				0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41
H	Ctrl5				0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14

Legend

Blank	Assay mix + 10 μ l distilled water
4 nmol Pi	Assay mix + 10 μ l 0.4 mM Pi
8 nmol Pi	Assay mix + 10 μ l 0.8 mM Pi
Ctrl1	Membrane suspension + 1 μ l solvent
Ctrl2	Membrane suspension + vanadate (1.2 mM) + 1 μ l solvent
Ctrl3	Membrane suspension + activator + 1 μ l solvent
Ctrl4	Activated membrane suspension + vanadate (1.2 mM) + 1 μ l solvent
Ctrl5	Activated membrane suspension + 1 μ l inhibitor
	Membrane suspension + 1 μ l test drug from drug plate
	Membrane suspension + vanadate + 1 μ l test drug from drug plate
	Activated membrane suspension + 1 μ l test drug from drug plate
	Activated membrane suspension + vanadate + 1 μ l test drug from drug plate

Figure 1: Assay plate map for an activation and inhibition study

Template file on your CD utilizes the same plate map for analyzing your data.



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10. Assay steps

NOTICE!!!

Please note that the 3- and 10- plate size PREDEASY ATPase Kits contain 3 and 10 separate membrane preparation vials (colored cap). Please thaw only the number that you are going to use in an hour.

Prepare your solutions freshly before use. Always use MilliQ (phosphate free) water as distilled water to prepare the solutions.

1. Prepare serial dilution of the drug to be assayed. Use a column of a separate 96 well microtiter plate and DMSO as solvent, Note that test drug solutions will be diluted 50-times when added to the reaction mixture in step 12.
2. Prepare 1xBlocker solution and 1xDeveloper solution
Dilute 1.2 ml 10xBlocker (Vial H) to 12 ml with 10.8 ml distilled water.
Dilute 4 ml 3xDeveloper (Vial G) to 12 ml with 8 ml distilled water.
(Shake well 3xDeveloper before use.)
Keep these solutions at room temperature.
3. Prepare the Assay Mix
Dilute 870 µl 10xMedium (Vial B) to 8700 µl with 7830 µl distilled water. (Mix well!)
Keep the Assay Mix on ice.
4. Prepare the MgATP solution
Dilute 50 µl 0.2 M MgATP solution (Vial J) to 1000 µl with 950 µl Assay Mix in a 1.5-2 ml tube.
Keep the MgATP solution on ice.
5. Prepare the Membrane Suspension in Assay Mix (Follow flow chart in Fig2).
Thaw the Membrane stock in 25 °C waterbath, and then homogenize it with gentle pipetting (2-3 times). Dilute 120 µl Membrane stock (Vial A) to 6000 µl with 5880 µl Assay Mix. (Mix well, by gentle vortexing for 1-2 seconds and/or pipetting!)



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Prepare activated membrane suspension for the inhibition study by removing 3000 µl from the original membrane suspension and adding 7.5 µl 100 mM Verapamil (Vial F) to it. Mark this solution as “activated” membrane suspension.

Prepare membrane suspension for the activation study by pipetting 2600 µl original membrane suspension. Add 6.5 µl DMSO. Mark this solution as “basic” membrane suspension.

Label four 5 ml tubes as follows CTRL1, CTRL2, CTRL3, CTRL4.

Pipet 1200 µl from the “basic” membrane suspension to the tube CTRL1. Add 3 µl distilled water.

Pipet another 1200 µl from the “basic” membrane suspension to the tube CTRL2. Add 3 µl 600 mM Na-orthovanadate solution (Vial E).

Pipet 1600 µl suspension from the “activated” suspension to the tube CTRL3. Add 4 µl distilled water.

Pipet 1200 µl from the “activated” membrane suspension to the tube CTRL4. Add 3 µl 600 mM Na-orthovanadate solution (Vial E).

Keep your suspensions on ice.

6. Place a 96 well plate on ice.
7. Add 40 µl Assay Mix to the wells of phosphate calibration.
8. Dispense your suspensions according to the suggested plate setup (Fig1) adding 40 µl of the appropriate membrane suspension to each well. This way one well will contain 4 µg membrane (total) protein. Change pipette/dispenser tips always between different suspensions!
9. Add 10 µl distilled water to the appropriate wells of phosphate calibration. Add 10 µl 0.4 mM, and 10 µl 0.8 mM KH₂PO₄ solution (Vial C, VialD) to the appropriate wells of phosphate calibration.
10. Add 1µl DMSO (or your solvent) to the control wells (Ctrl1, Ctrl2, Ctrl3, Ctrl4).
11. Add 1µl Inhibitor to the wells of Ctrl5.



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12. Add 1 µl of serial dilution of your test drug (in DMSO or in your solvent) to the appropriate wells. (Use 8-channel pipette to minimize time of pipetting.)
Change pipette tips after each addition to a column.
13. Preincubate your plate and MgATP solution at 37°C for 10 minutes. (If the bottom of the wells do not touch directly the heated surface of your incubator, you may need a longer incubation time.)
14. Start reaction by adding 10 µl MgATP solution to each well except of the wells of phosphate calibration. (Use a repeater pipette to minimize time of pipetting.)
15. Incubate your plate at 37°C for 10 minutes.
16. Stop the ATPase reaction by adding 100 µl of 1xDeveloper solution to the wells at room temperature. (Use 8-channel pipette or repeater pipette to minimize time of pipetting.)
17. After two minutes add 100 µl of 1x Blocker solution to the wells at room temperature.
18. Incubate your plate for 30 min at 37 °C.
19. Read the OD between 590 and 630 nm. The color is stable for at least 3 hours.
20. Analyze your data.



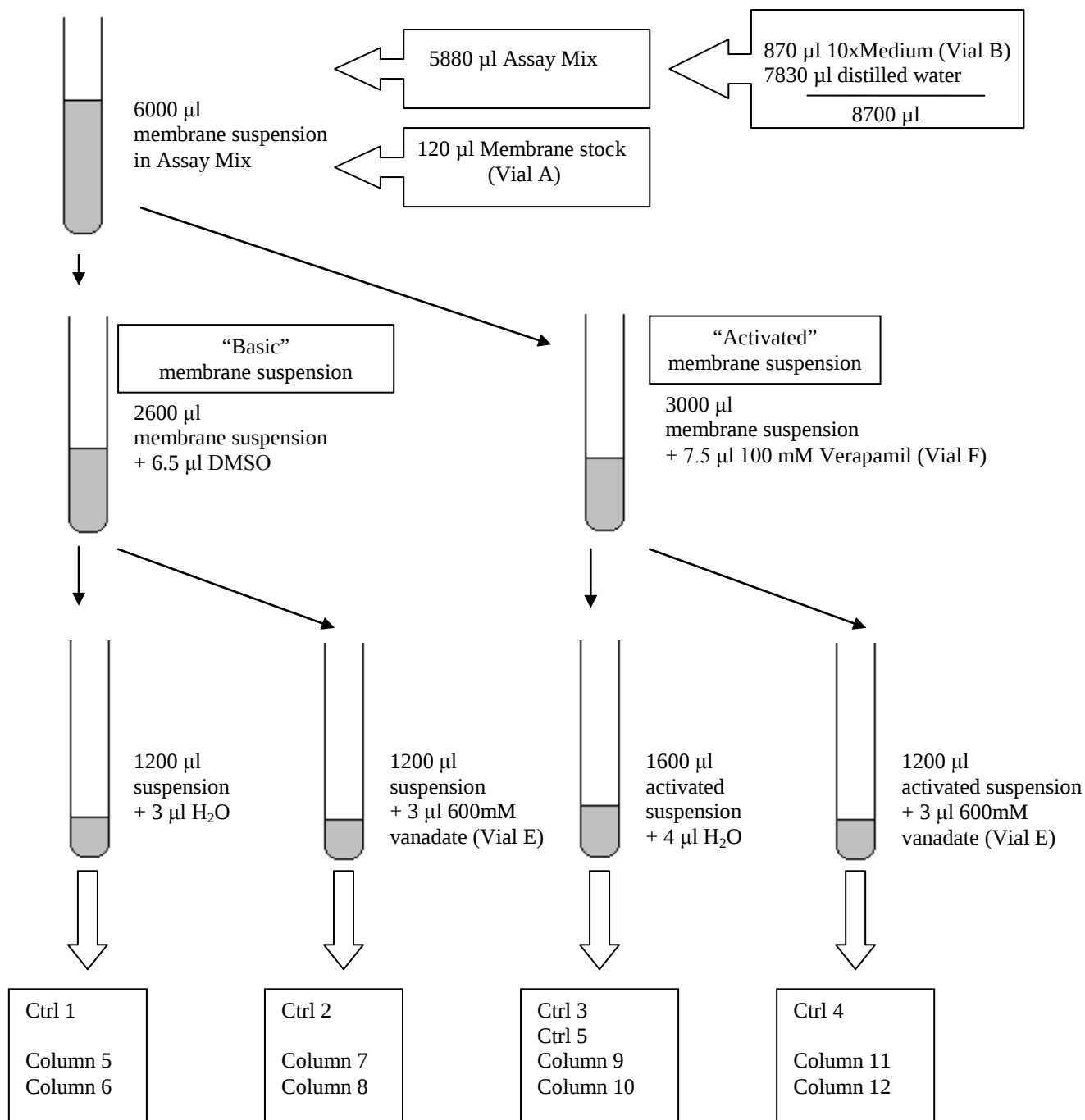


Figure 2: Flow chart for the preparation of membrane suspensions

Add 1 µl DMSO (or your solvent) to the wells of Ctrl1-4, add 1 µl Inhibitor to the wells of Ctrl 5 and add 1 µl of serial dilution of your test drug (in DMSO or in your solvent) to the appropriate wells



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11. Representing the data

There are two main representations of the data:

1. *Calculating the absolute value 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. *Calculating the transporter ATPase activities in percentages*

In this representation the vanadate sensitive baseline ATPase activity and the maximal vanadate sensitive ATPase activity are taken as 0% and 100% ATPase activity of the transporter, respectively. Vanadate sensitive activity 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.



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11.1. Calculation of the specific (Na₃VO₄ sensitive) transporter ATPase activities

11.1.1. Calculation steps

1. Set up a calibration curve using the calibration well OD values and the amount of KH₂PO₄ used (nmol/well).
2. Calculate the average OD values of the measurements of the calibration points and controls (Ctrl 1, Ctrl 2, Ctrl 3, Ctrl 4, Ctrl5), respectively. Calculate the average OD values of your samples.
3. Determine the amount 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 1. This will give you the baseline Na₃VO₄ sensitive ATPase activity.
5. Subtract Pi liberated in Ctrl 4 from Pi liberated in Ctrl 3. This will give you the fully activated Na₃VO₄ sensitive transporter ATPase activity.
6. Subtract Pi liberated in Ctrl 4 from Pi liberated in Ctrl 5. This will give you the inhibited Na₃VO₄ sensitive transporter ATPase activity.
7. Subtract the Pi values determined in the presence of Na₃VO₄ from the Pi values measured without Na₃VO₄ for each compound examined. This will give you the Na₃VO₄ sensitive transporter ATPase activity for each drug and drug concentration assayed.
8. Calculate the Na₃VO₄ 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.



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11.1.2. Expected results

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.



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11.2. Calculation of the percent transporter ATPase activities

11.2.1. Calculation steps

1. Subtract (Ctrl 1 – Ctrl 2) from (Ctrl 3 – 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 1. 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.

11.2.2. 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. Further insight into the ATPase inhibitory effect of drugs can be gained by performing inhibition studies.



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In case of inhibition studies percent 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 percent inhibition values may turn into negative. 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.

11.3. Calculating results using the template file

Use your Excel Template file to calculate results in case of applying the standard plate map (see Section 11.1). All required fields are highlighted with light green and are editable. Fields that you do not need to change are read only. Charts are editable. Copy your raw data to the RAW DATA field of the template file. Fill header (date, membrane batch, membrane weight/well, incubation time, etc.) carefully. Check your test drug concentrations and change the value of the highest final concentration if it is necessary. Fill the DRUG NAME field. Analyze your results.



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12. Troubleshooting

Problems you can come across while using the **PREDEASY™** kit often originate from the high sensitivity of the phosphate detection method. Any phosphate impurities from distilled water or laboratory vials will interfere with your results. Use phosphate free MilliQ (or equivalent) water for all dilutions to perform the experiment. Detergents used in laboratory wash may contain high amount of phosphate that may contaminate your solutions. Use preferably disposable pipette tips and polymer vials. Change your pipette tips / dispensers always when pipetting / dispensing different solutions!

All vials contain more substance than the amount required for performing the experiment. In case of small volumes, please remove substance from the cap of the vial by shaking or centrifugation before opening the vial. Please consider the included amounts (Table 1.) and do not use large volume digital pipettes for pipetting the membrane suspension and small volumes. Please use tubes with adequate volumes to prepare your suspensions to minimize loss because of non-specific binding to vial surfaces.

Your freshly prepared solutions (Developer, blocker) have to be used within 5 hours from preparation.

Some useful guidelines:

1. Your 1x Developer solution must be yellow after dilution. If it is green, your distilled water or laboratory dishes are contaminated with phosphate.
2. Wells A1-A4 must be yellow even after color development. If they are green, your pipette tips or distilled water are contaminated with phosphate.
3. Wells B1-B4, C1-C4 must be green after color development. If they are yellow, phosphate standard solution (assay step 2) was not added to the wells. If all wells are yellow, probably you added the Blocker solution together or immediately after the Developer to the wells.



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4. If all wells (except A1-A4, B1-B4, C1-C4) are dark green after color development, probably you did not add Blocker solution to the wells.
5. If insufficient activation is detected, check temperature of the reaction mixture after preincubation time. Use a waterbath or a heating block with sufficient heat conductivity to perform the incubations. (96 well plates are floating on the surface of water.) Shaking is not needed, but gentle shaking (up to 300 rpm) may improve homogenization of the reaction mixture. Avoid hard shaking, and shaking during color development!
6. If no or minimal difference is observed between the controls, a possible problem might be the presence of vanadate in all solutions, due to dispensing all your suspensions with the same tip.

Visit our website (www.solvo.com) for further details about PREDEASY™ kits.

