MDR1
PREDIVEZ Protocol

CAT. NO. SBPV02

SIGMA

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Determination of the interaction of drugs with the MDR1 transporter using the SB-MDR1-K PREDIVEZ Kit

For the following membrane product:
SB-MDR1-K-VT

Version Number:

1.3

Effective date:

21.Feb.2011

Replaces:

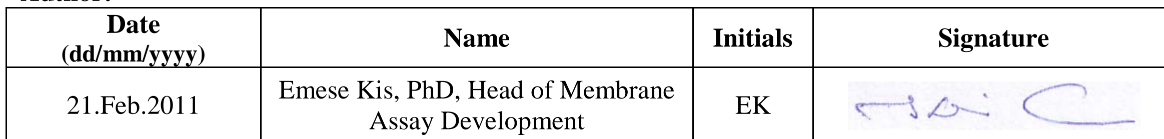
1.2

Related Procedures:

SOP FFSS01

Signatures:

Author:



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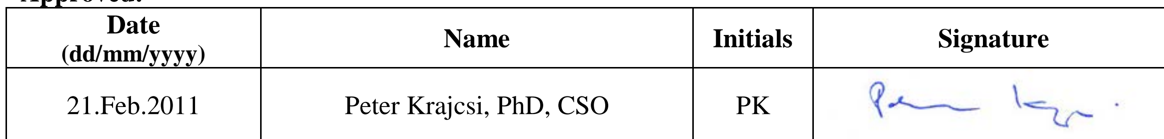

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1. Introduction

Most ABC transporters transport substrates across the cell membrane using ATP as an energy source. One of the simplest methods invented for measuring this transport is the vesicular transport assay. This assay protocol describes the determination of the interaction of test drugs with the MDR1 transporter using the vesicular transport assay. The interaction is detected as the modulation of the initial rate of N-methyl-quinidine (NMQ) transport of MDR1 into membrane vesicles purified from mammalian cells expressing the transporter.

2. Principle

The human MDR1 transporter can be stably expressed in mammalian cells. Membrane preparations prepared from these cells always contain some closed membrane vesicles that are in inside-out orientation. Due to the orientation of the transporter, the transported substrates accumulate inside the vesicle. In case of low permeability substrates, such as NMQ, the molecules get trapped inside the vesicle. The rate of this transport is temperature and ATP dependent.

Rapid filtration of the membrane suspension through a filter that retains membrane vesicles allows us to separate the transported molecules trapped from the rest of the buffer.

The quantity of transported molecules can be determined by any adequate method like HPLC, LC/MS/MS separation and detection. Also, the transported molecule can be labeled by fluorescent or radioactive tags. This protocol utilizes a drug surrogate, NMQ, available in radiolabeled form, for the determination of the transported substrate amount in a competition type assay.

NMQ is a transported substrate of MDR1. Drugs that interact with the transporter modulate the initial rate of NMQ transport measured without any other compounds

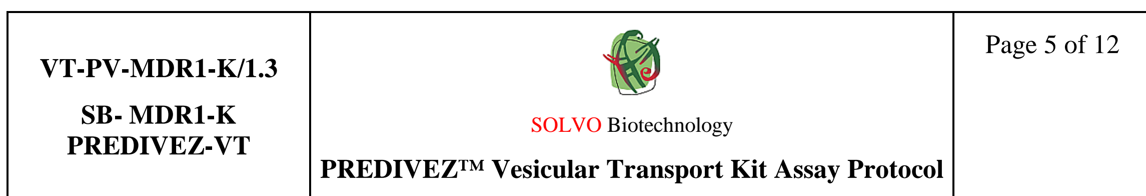
added. If a test drug is a transported substrate of the transporter it might compete with NMQ thus reducing the rate of NMQ transport. If a compound is an inhibitor of the transporter, it will block the transport of NMQ into the membrane vesicles.

3. Deliverables

- SOLVO Biotechnology's SB-MDR1-K PREDIVEZ Vesicular Transport Assay Kit for MDR1 transporter sufficient for the analysis of 3, 6 or 9 test compounds.
- Data sheet indicating protein content, volume, ATP dependent transport at 2 μM NMQ concentration and date of expiry of frozen membrane stocks.
- Data CD containing assay protocol, MS Excel file for calculations and data representation, and material safety data sheets.

4. Equipment and Materials needed

- Plate incubator/shaker.
- Automatic pipettes and multichannel pipettes with corresponding tips
- 96-well plates (Costar, Cat. No. 3585, or equivalent)
- Filterplates [Millipore multiscreen HTS 96 well filter plates with FB filters (Cat. No. MSFBN6B10) or equivalent]
- Rapid filtration apparatus [Multiscreen™ HTS Vacuum Manifold from Millipore (Cat. No MSVMHTS00) or equivalent]
- 96 well Liquid scintillation system
- 2 ml, 5 ml tubes
- 150 ml cylinder and Tip-Tubs (Eppendorf, Cat. No. 0030 058.607)
- MilliQ water



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

5. Materials

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

Kit size (number of cpds)		**3**	**6**	**9**	**Storage**	**Assay conditions**
Vial	**Substance**	**Amount**				
A	Membrane stock (5 mg/ml)	3x420 µl	6x420 µl	9x420 µl	-80 °C	on ice
B	3x Start Mix	4.0 ml	8.0 ml	12.0 ml	≤+4 °C	on ice
C	NMQ (500 µM)	70 µl	140 µl	210 µl	≤-20 °C	RT
D	MgATP solution (0.2 M)	120 µl	240 µl	360 µl	≤-20 °C	on ice
E	Inhibitor drug stock (20 mM Verapamil)	50 µl	100 µl	150 µl	≤-20 °C	RT
F*	^{3}H-NMQ	250 µl			≤-20 °C	RT
G	10x Washing Mix	14.5 ml	2x14.5 ml	3x14.5 ml	≤+4 °C	on ice
H	Inhibitor drug stock (20 mM Digoxin)	50 µl	100 µl	150 µl	≤-20 °C	RT
I	Negative control membrane stock (5 mg/ml)	200 µl	2x200 µl	3x200 µl	-80 °C	on ice
J	AMP solution (0.2 M)	120 µl	240 µl	360 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.

* - supplied in a separate container in the same shipment

Do not use substances from any other type of PREDIVEZ Kit.

6. Suggested assay layouts

Assay Layout 1. (Relative Transport values)

Assay layout for presenting results in percentages:

	1	2	3	4	5	6	7	8	9	10	11	12
	Compound 1				Compound 2				Compound 3			
	+ ATP		-ATP (AMP)		+ ATP		-ATP (AMP)		+ ATP		-ATP (AMP)	
A	300 µM		300 µM		300 µM		300 µM		300 µM		300 µM	
B	100 µM		100 µM		100 µM		100 µM		100 µM		100 µM	
C	33.3 µM		33.3 µM		33.3 µM		33.3 µM		33.3 µM		33.3 µM	
D	11.1 µM		11.1 µM		11.1 µM		11.1 µM		11.1 µM		11.1 µM	
E	3.7 µM		3.7 µM		3.7 µM		3.7 µM		3.7 µM		3.7 µM	
F	1.23 µM		1.23 µM		1.23 µM		1.23 µM		1.23 µM		1.23 µM	
G	0.41 µM		0.41 µM		0.41 µM		0.41 µM		0.41 µM		0.41 µM	
H	DMSO		DMSO		DMSO		DMSO		DMSO		DMSO	

Note: If your test drug is not dissolved in DMSO replace DMSO with that solvent. The numbers indicate final concentrations

Assay Layout 2. (Absolute Transport values)

Assay layout for calculating ATP dependent transport (pmol/mg/min) transport values:

	1	2	3	4	5	6	7	8	9	10	11	12
	Totals and control				Compound 1				Compound 2			
	+ ATP		-ATP (AMP)		+ ATP		-ATP (AMP)		+ ATP		-ATP (AMP)	
A	Total C1*		Total C2*		300 µM		300 µM		300 µM		300 µM	
B	Total C3*		Total control*		100 µM		100 µM		100 µM		100 µM	
C	Verapamil		Verapamil		33.3 µM		33.3 µM		33.3 µM		33.3 µM	
D	Digoxin		Digoxin		11.1 µM		11.1 µM		11.1 µM		11.1 µM	
E	C1		C1		3.7 µM		3.7 µM		3.7 µM		3.7 µM	
F	C2		C2		1.23 µM		1.23 µM		1.23 µM		1.23 µM	
G	C3		C3		0.41 µM		0.41 µM		0.41 µM		0.41 µM	
H	DMSO		DMSO		DMSO		DMSO		DMSO		DMSO	

Dark grey wells represent measurement with negative control membrane
* - these wells contain 2.5 µl of the original membrane suspension for the determination of absolute transport. There is no need for the addition of ATP to these wells.

7. Assay steps

Prepare your solutions freshly before use. Always use MilliQ water as distilled water to prepare the solutions. The steps are for assaying **1 compound**!

1. Prepare serial dilution of the drug to be assayed. (Use DMSO as solvent).
2. Dilute reagents as follows:

    Dilute 1 ml 3x Start Mix (Vial **B**) to 3 ml with 2 ml distilled water. (Store 1x Start Mix on ice)

    Dilute 4.25 ml 10x Washing Mix (Vial **G**) to 42.5 ml with 38.25 ml distilled water. (Store 1x Washing Mix on ice or in the fridge)

3. Prepare the MgATP solution

    Dilute 30 µl 0.2 M MgATP solution (Vial **D**) to 500 µl with 470 µl Start Mix.

    Keep the MgATP solution on ice.

    Prepare the AMP solution

    Dilute 30 µl 0.2 M AMP solution (Vial **J**) to 500 µl with 470 µl Start Mix.

    Keep the AMP solution on ice.

4. Prepare the Membrane Suspension in Start Mix.

    Homogenize your Membrane stock. Add 360 µl Membrane stock (Vial **A**) and 11 µl NMQ (Vial **C**) to 1423 µl Start Mix. Add 6 µl ^{3}H-NMQ (Vial F). (Mix well, gently!)

    Keep your suspensions on ice.

5. Place a 96 well plate on ice.
6. Add 50 µl Membrane Suspension to the wells of the first 4 columns. This way one well will contain 50 µg total membrane protein.
7. Add 0.75 µl of serial dilution of your test drug (in DMSO or in your solvent) to the appropriate wells
8. Preincubate your plate, MgATP and AMP solution at 37°C for 15 minutes.

9. Start reaction by adding 25 µl MgATP or AMP solution to the appropriate wells (see Assay Layout).
10. Incubate your plate at 37 °C for 3 minutes
11. Wet the first four columns of the Millipore filter plate with 100 µl distilled water per well and set up the filtering apparatus. Use a plate sealer on the remaining wells to ensure adequate vacuum and remove the liquid. This step can be done during preincubation.
12. Stop the reaction by adding 200 µl of ice cold 1xWashing Mix to the wells.
13. Transfer all the solution from the 96 well plate to the Millipore filter plate.
14. Under vacuum, remove the liquid from the wells and wash them 5 times with 200 µl Washing Mix.
15. Pipette 2.5 µl the membrane suspension (prepared in step 1.) into one well of a filterplate. The radioactivity (cpm) measured on this filter will be used to calculate *total activity* in one well (see Calculations).
16. Dry filter plates (you can use a hair drier to speed up the process.) till completely dry.
17. Add 100 µl of scintillation cocktail and after 30 min measure radioactivity in each well. Record cpm values.

Optional assay steps:

NMQ transport by SB-K-CTRL (negative control)

The Kit contains a vial of SB-K-CTRL membrane as well (Vial **I**), that serves as a negative control. These vesicles show minimal accumulation of NMQ. Transport in the absence (DMSO – recommended) or in the presence of a test drug (one concentration – highest is recommended) can be tested. The measurement is optional and can be performed on a separate plate as well.

1. Dilute reagents as follows:

Dilute 400 µl 3x Start Mix (Vial **B**) to 1200 µl with 800 µl distilled water. (Store 1x Start Mix on ice)

Dilute 1.6 ml 10x Washing Mix (Vial **G**) to 16 ml with 14.4 ml distilled water. (Store 1x Washing Mix on ice or in the fridge)

2. Prepare the MgATP solution

    Dilute 15 µl 0.2 M MgATP solution (Vial **D**) to 250 µl with 235 µl Start Mix.

    Keep the MgATP solution on ice.

    Prepare the AMP solution

    Dilute 15 µl 0.2 M AMP solution (Vial **J**) to 250 µl with 235 µl Start Mix.

    Keep the AMP solution on ice.

3. Prepare the Membrane Suspension in Start Mix.

    Homogenize your Membrane stock. Add 160 µl Membrane stock (Vial **I**) and 4.8 µl NMQ (Vial **C**) to 632.6 µl Start Mix. Add 2.6 µl ^{3}H-NMQ. (Mix well, gently!)

    Keep your suspensions on ice.

4. Place a 96 well plate on ice.
5. Add 50 µl Membrane Suspension to the wells indicated on the Assay Layout 2. This way one well will contain 50 µg total membrane protein.
6. Add 0.75 µl of DMSO/ test drug / positive control drug (in DMSO or in your solvent) to the wells
7. Preincubate your plate, MgATP and AMP solution at 37°C for 15 minutes.
8. Start reaction by adding 25 µl MgATP or AMP solution to the appropriate wells (see Assay Layout 2.).
9. Incubate your plate at 37 °C for 3 minutes
10. Wet the appropriate wells of the Millipore filter plate with 100 µl distilled water per well and set up the filtering apparatus. Use a plate sealer on the remaining wells to ensure adequate vacuum.

11. Stop the reaction by adding 200 µl of ice cold 1xWashing Mix to the wells.
12. Transfer all the solution from the 96 well plate to the Millipore filter plate.
13. Under vacuum, remove the liquid from the wells and wash them 5 times with 200 µl Washing Mix.
14. Pipette 2.5 µl the membrane suspension (prepared in step 1.) into one well of a filterplate. The radioactivity (cpm) measured on this filter will be used to calculate *total activity* in one well (see Calculations).

15. Dry filter plates (you can use a hair drier to speed up the process.) till completely dry.

16. Add 100 µl of scintillation cocktail and after 30 min measure radioactivity in each well. Record cpm values.

8. Calculations

ATP dependent transport (cpm): Subtract cpm values measured without the presence of ATP from the cpm values measured in the presence of ATP for control and samples. Take the average of the duplicates.

ATP dependent transport (pmol/mg/min): Calculate *Total activity (cpm)* by multiplying the cpms measured in the designated well prepared in step 15 by 20. Calculate the rate of transport in pmol/mg membrane protein/min using the following

$$\frac{ATP\,dependent\,transport\,(cpm)}{Total\,activity\,(cpm)} * \frac{NMQ\,concentration\,(nM)*Volume\,(ml)}{membrane\,protein\,(mg)*time\,(min)}$$

formula.

If the assay is performed in the conditions described the value of the second part of the equation is 1000.

Membrane validation: During membrane validation the test done is identical to *drug free control* (samples H1-4). ATP dependent transport measured under these circumstances is indicated on the datasheet.

ATP dependent transport (%): Calculate the percent activation or inhibition of the test drug. In this representation the ATP dependent transport determined in the *drug free control* is taken as 100% and all other values are represented on this relative scale. Use the following formula:

$$\frac{ATP\ dependent\ transport\ in\ the\ presence\ of\ test\ drug\ (cpm)}{ATP\ dependent\ transport\ in\ drug\ \ free\ control\ (cpm)} * 100$$

Positive control: The NMQ transport of MDR1 is fully (under 10% of the drug free control) inhibited by 200 µM Verapamil. You can assay this inhibition by replacing test drug with 0.75 µl of 20 mM Verapamil (final concentration 200 µM). The FDA recommended MDR1 substrate, Digoxin, is also provided in the kit. Using 200 µM Digoxin concentration, the MDR1-mediated NMQ transport can be inhibited by about 50%.

Suggested membrane negative control: As a confirmation of the results and the transporter specificity, we suggest the use of negative control vesicles, in case of MDR1, SB-K-CTRL vesicles, lacking human MDR1 transporter expression.

Calculation of results using the raw data template file

Use your Excel Template file to calculate results in case of applying the suggested Assay Layouts (see page 8.). The Template file is designed to analyze one test drug at a time!

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 amount/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.

The file can be used in both calculation modes: percentages and absolute transport values as well. Analyze your results.

9. Expected Results

Relative transport values (%)

This curve shows the effect of the test drug on NMQ transport by MDR1 in percentages. 100% represent NMQ transport by MDR1 in the absence of test drug (row H in the plate setup), while 0% is the transport in the absence of ATP (non-specific binding of NMQ). This representation is very helpful if the activities of multiple test drugs are compared.

If the test drug interacts with the NMQ transport, then a dose-dependent decrease in transport is observed. The IC_{50} value for the test drug is the concentration where the NMQ transport is inhibited by 50%. In case of a non-interactor, the curve does not fall below 100%.