Determination of the interaction of drugs with MDR1 transporter using the PREDIVEZ Reagent Kit

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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 given transporter (MDR1) 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.

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 can be used both for radiolabeled form and unlabeled form of NMQ.

2. Deliverables

SOLVO Biotechnology's PREDIVEZ Reagent Kit for MDR1 transporter sufficient for the analysis of 9 test compounds. The kit does not contain the membrane vesicles! The contents of the kit are listed in the table below.

Vial	Substance	Amount	Storage	Storage during the assay
B	3x Start Mix	12.0 ml	2-8°C	on ice
C	NMQ (500 µM)	210 µl	≤-15 °C	RT
D	MgATP solution (0.2 M)	360 µl	≤-15 °C	on ice
E	Inhibitor drug stock (20 mM Verapamil)	150 µl	≤-15 °C	RT
F*	10x QND (2500 ng / ml – internal standard)	900 µl	≤-15 °C	RT
G	10x Washing Mix	3x14.5 ml	2-8 °C	on ice
J	AMP solution (0.2 M)	360 µl	≤-15 °C	on ice

Keep the kit compounds during the assay procedure at the temperature specified in this table.
*-use only in case of LC/MS analysis

3. 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 [Merck Millipore Multiscreen HTS 96 well filter plates with FB filters (Cat. No. MSFBN6B10) or equivalent]
- Rapid filtration apparatus [Multiscreen™ HTS Vacuum Manifold from Merck Millipore (Cat. No MSVMHTS00) or equivalent]
- Fluorimeter suitable for the 96-well format. Lucifer yellow can be detected using Ex: 430 nm, Em: 538 nm wavelengths.
- 2 ml, 5 ml tubes
- 150 ml cylinder and Reagent Reservoir (Eppendorf, Cat. No. 0030 058.607)
- Purified water
- Dimethyl sulfoxide (Sigma-Aldrich 34869)
- Membrane vesicles

4. Suggested assay layouts

Assay Layout 1. (Relative Transport values)

Assay layout for presenting results in percentages:

	Compound 1				Compound 2				Compound 3			
	ATP		AMP		ATP		AMP		ATP		AMP	
	1	2	3	4	5	6	7	8	9	10	11	12
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.

Assay Layout 2.a (Absolute Transport values for radiolabeled NMQ)

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

					Compound 1				Compound 2			
					ATP		AMP		ATP		AMP	
	1	2	3	4	5	6	7	8	9	10	11	12
A					300 µM		300 µM		300 µM		300 µM	
B					100 µM		100 µM		100 µM		100 µM	
C					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	
E					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	
G	ATP		AMP		0.41 µM		0.41 µM		0.41 µM		0.41 µM	
H	ATP		AMP		DMSO		DMSO		DMSO		DMSO	

Dark grey wells represent measurement with negative control membrane

Assay Layout 2.b. (Absolute Transport values for unlabelled NMQ for LC/MS analysis)

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

					Compound 1				Compound 2			
	Calibration		Controls		ATP		AMP		ATP		AMP	
	1	2	3	4	5	6	7	8	9	10	11	12
A	200 ng/ml				300 µM		300 µM		300 µM		300 µM	
B	150 ng/ml				100 µM		100 µM		100 µM		100 µM	
C	100 ng/ml				33.3 µM		33.3 µM		33.3 µM		33.3 µM	
D	25 ng/ml				11.1 µM		11.1 µM		11.1 µM		11.1 µM	
E	2 ng/ml		**ATP**		3.7 µM		3.7 µM		3.7 µM		3.7 µM	
F	1 ng/ml		**ATP**		1.23 µM		1.23 µM		1.23 µM		1.23 µM	
G	0.5 ng/ml		**AMP**		0.41 µM		0.41 µM		0.41 µM		0.41 µM	
H	0		**AMP**		DMSO		DMSO		DMSO		DMSO	

Dark grey wells represent measurement with negative control membrane

5. Assay steps for measuring radiolabelled NMQ transport

Prepare your solutions fresh before use. Always use purified water to prepare the solutions. The steps are for assaying **1 compound** (see Assay Layout on page 5)!

1. Prepare serial dilution of the drug to be assayed or of the Inhibitor (Vial **E**). (Use DMSO as solvent).
2. Dilute reagents as follows:
   - Dilute 1 ml 3× Start Mix (Vial **B**) to 3 ml with 2 ml purified water. (Store 1× Start Mix on ice)
   - Dilute 4.25 ml 10× Washing Mix (Vial **G**) to 42.5 ml with 38.25 ml purified water. (Store 1× 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 1× Start Mix. (Keep the MgATP solution on ice).

4. Prepare the AMP solution
   - Dilute 30 µl 0.2 M AMP solution (Vial **J**) to 500 µl with 470 µl 1× Start Mix. (Keep the AMP solution on ice).
5. Prepare the Membrane Suspension in 1× Start Mix. Homogenize your Membrane stock with gentle pipetting. Add 360 µl Membrane stock and 11 µl NMQ (Vial **C**) to 1423 µl Start Mix. Add 6 µl ^{3}H-NMQ. Keep the suspension on ice.
6. Place a 96 well plate on ice and add 50 µl Membrane Suspension to each well of the first 4 columns.
7. Add 0.75 µl of serial dilution of your test drug (in DMSO or in your solvent) to the appropriate wells (see Assay Layout on page 5)
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 on page 4).
10. Incubate your plate at 37 °C for 3 minutes.
11. Wet the first four columns of the Merck Millipore filter plate with 100 µl purified water per well and set up the filtering apparatus. Use a plate sealer on the remaining wells to ensure adequate vacuum.
12. Stop the reaction by adding 200 µl of ice cold 1x Washing Mix to every well.
13. Transfer all the solution from the 96 well plate to the Merck Millipore filter plate.
14. Under vacuum, remove the liquid from the wells and wash them 5 times with 200 µl 1× Washing Mix per well.
15. Pipette 10 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 K-Ctrl (negative control)

The K-Ctrl vesicles show minimal accumulation of NMQ. Transport in the presence of DMSO (or solvent) 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 3× Start Mix (Vial **B**) to 1200 µl with 800 µl purified water. (Store 1× Start Mix on ice)
   - Dilute 1.6 ml 10× Washing Mix (Vial **G**) to 16 ml with 14.4 ml purified water. (Store 1× 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 1× Start Mix. (Keep the MgATP solution on ice).
3. Prepare the AMP solution
   - Dilute 15 µl 0.2 M AMP solution (Vial **J**) to 250 µl with 235 µl 1× Start Mix. (Keep the AMP solution on ice).
4. Prepare the Membrane Suspension in 1× Start Mix. Homogenize your Membrane stock. Add 160 µl Membrane stock and 4.8 µl NMQ (Vial **C**) to 632.6 µl Start Mix. Add 2.6 µl ^{3}H-NMQ. Keep the suspensions on ice.
5. Place a 96 well plate on ice and add 50 µl Membrane Suspension to each well indicated on the Assay Layout 2. page 5.
6. Add 0.75 µl of DMSO/ test drug (in DMSO or in your solvent) to each well.
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. on page 5).
9. Incubate your plate at 37 °C for 3 minutes.

10. Wet the appropriate wells of the Merck Millipore filter plate with 100 µl purified 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 1× Washing Mix to each well.
12. Transfer all the solution from the 96 well plate to the Merck Millipore filter plate.
13. Under vacuum, remove the liquid from the wells and wash them 5 times with 200 µl 1× Washing Mix.
14. Pipette 10 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.

6. Calculations

ATP dependent transport (cpm): Take the average of the duplicates. Subtract cpm values measured in the absence of ATP from the cpm values measured in the presence of ATP for control and samples.

ATP dependent transport (pmol/mg protein/min): Calculate *Total activity (cpm)* by multiplying the cpms measured in the designated well prepared in step 15 by 5. Calculate the rate of transport in pmol/mg membrane protein/min using the following formula.

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

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

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:

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

7. Assay steps for measuring cold NMQ transport using LC/MS analysis

1. Prepare serial dilution of the drug to be assayed or of the Inhibitor (Vial **E**). (Use DMSO as solvent).
2. Dilute reagents as follows:
   - Dilute 1 ml 3× Start Mix (Vial **B**) to 3 ml with 2 ml purified water. (Store 1× Start Mix on ice)
   - Dilute 4.25 ml 10× Washing Mix (Vial **G**) to 42.5 ml with 38.25 ml purified water. (Store 1× Washing Mix on ice or in the fridge)
   - Dilute 72 µl 10x QND internal standard (Vial **F**) to 720 µl with 648 µl MeOH: water (50:50, V/V)
3. Prepare the MgATP solution
   - Dilute 30 µl 0.2 M MgATP solution (Vial **D**) to 500 µl with 470 µl 1× Start Mix. (Keep the MgATP solution on ice).
4. Prepare the AMP solution

- Dilute 30 µl 0.2 M AMP solution (Vial **J**) to 500 µl with 470 µl 1× Start Mix. (Keep the AMP solution on ice).

5. Prepare the Membrane Suspension in 1× Start Mix. Homogenize your Membrane stock with gentle pipetting. Add 360 µl Membrane stock and 11 µl NMQ (Vial **C**) to 1423 µl Start Mix. Keep the suspension on ice.
6. Place a 96 well plate on ice and add 50 µl Membrane Suspension to each well of the first 4 columns.
7. Add 0.75 µl of serial dilution of your test drug (in DMSO or in your solvent) to the appropriate wells (see Assay Layout on page 5)
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 on page 4).
10. Incubate your plate at 37 °C for 3 minutes.
11. Wet the first four columns of the Merck Millipore filter plate with 100 µl purified water per well and set up the filtering apparatus. Use a plate sealer on the remaining wells to ensure adequate vacuum.
12. Stop the reaction by adding 200 µl of ice cold 1x Washing Mix to every well.
13. Transfer all the solution from the 96 well plate to the Merck Millipore filter plate.
14. Under vacuum, remove the liquid from the wells and wash them 5 times with 200 µl 1× Washing Mix per well.
15. Pipette 10 µl of the membrane suspension (prepared in step 1) into 3 wells of the filterplate.
16. MeOH:water (70:30, V/V, 150 µl) is added to the filters and incubated for 10 minutes at RT.
17. Placing a 96-well plate beneath the filter plate, the MeOH:water is eluted (vacuum is applied) and collected in the 96 well plate. The elution process is repeated (two rinses total).

18. After filtration, 200 µl of each sample is transferred to a 96 well plate (for LC/MS analysis), followed by the addition of 20 µl of 1x QND internal standard (250 ng/ml, diluted from Vial **F**).
19. The samples were then analyzed by LC/MS/MS using the following conditions: (If the samples cannot be analyzed within 1 day samples should be dried using a SpeedVac instrument and store refrigerated.)

HPLC Column

Analytical: Waters, Atlantis C18 (100 x 2.1 mm, 5 µm), or equivalent

Chromatographic Conditions

Mobile Phase A: 0.2% Formic Acid in Water

B: 0.2% Formic Acid in Methanol

Flow Rate: 650 µl per minute (total flow)

Run time ~ 3.0 minutes

Typical retention times: N-methyl Quindine ≅ 1.9 min; IS (Quindine) ≅ 2.0 min;

2 µl injection

LC Program

Step No.	Time (min)	%B
1	0.00	15
2	0.10	15
3	2.00	45
4	2.10	98
5	2.50	98
6	2.60	15
7	3.00	Stop

The system is plumbed to divert flow from the analytical column to waste for the first 1.5 minutes after sample injection. During this time, no LC flow is delivered to the

MS. At 2.5 minutes, flow from the LC system is directed to the MS for analysis. The flow is diverted again to waste at 2.5 minutes.

Mass Spectrometer Conditions

An LC/MS method employing ESI+ ionization was used with the following (or equivalent) settings:

Source /Gas (ESI+) Settings

IonSpray Voltage	1500 V
Temperature	450°C
Curtain Gas	12
Collision Gas	8

Compound dependent settings	Analyte (NMQ)	IS (QND)
Declustering Potential	25	25
Focusing Potential	400	400
Entrance Potential	10	10
Collision Energy	25 eV	40 eV
Collision Cell Exit Potential	20	20
Mass Transitions	339.1 > 339.2 amu	325.0 > 81.1 amu

Optional assay steps:

NMQ transport by K-Ctrl (negative control)

The K-Ctrl vesicles show minimal accumulation of NMQ. Transport in the presence of DMSO (or solvent) 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 3× Start Mix (Vial **B**) to 1200 µl with 800 µl purified water. (Store 1× Start Mix on ice)
   - Dilute 1.6 ml 10× Washing Mix (Vial **G**) to 16 ml with 14.4 ml purified water. (Store 1× Washing Mix on ice or in the fridge)
   - Dilute 40 µl 10x QND internal standard (Vial **F**) to 400 µl with 360 µl MeOH:water (50:50, V/V).
2. Prepare the MgATP solution
   - Dilute 15 µl 0.2 M MgATP solution (Vial **D**) to 250 µl with 235 µl 1× Start Mix. (Keep the MgATP solution on ice).
3. Prepare the AMP solution
   - Dilute 15 µl 0.2 M AMP solution (Vial **J**) to 250 µl with 235 µl 1× Start Mix. (Keep the AMP solution on ice).
4. Prepare the Membrane Suspension in 1× StartMix. Homogenize your Membrane stock. Add 160 µl Membrane stock and 4.8 µl NMQ (Vial **C**) to 635.2 µl Start Mix. Keep the suspensions on ice.
5. Place a 96 well plate on ice and add 50 µl Membrane Suspension to each well indicated on the Assay Layout 2. page 5.
6. Add 0.75 µl of DMSO/ test drug (in DMSO or in your solvent) to each well.
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. on page 5).
9. Incubate your plate at 37 °C for 3 minutes.
10. Wet the appropriate wells of the Merck Millipore filter plate with 100 µl purified 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 1× Washing Mix to each well.
12. Transfer all the solution from the 96 well plate to the Merck Millipore filter plate.

13. Under vacuum, remove the liquid from the wells and wash them 5 times with 200 µl 1× Washing Mix.
14. Pipette 10 µl of the membrane suspension (prepared in step 1) into 3 wells of the filterplate.
15. MeOH:water (70:30, V/V, 150 µl) is added to the filters and incubated for 10 minutes at RT.
16. Placing a 96-well plate beneath the filter plate, the MeOH:water is eluted (vacuum is applied) and collected in the 96 well plate. The elution process is repeated (two rinses total).
17. After filtration, 200 µl of each sample is transferred to a 96 well plate (for LC/MS analysis), followed by the addition of 20 µl of 1x QND internal standard (250 ng/ml, diluted from Vial **F**).
18. The samples were then analyzed by LC/MS/MS using the conditions described above.

8. Calculations

ATP dependent transport (relative): Subtract area under curve (AUC), or calculated concentration values (ng/ml) measured without the presence of ATP from the AUC or calculated concentration (ng/ml) values measured in the presence of ATP for control and samples. Take the average of the duplicates.

ATP dependent transport (pmol/mg protein/min): Calculate *Total activity (ng/ml)* by dividing the concentration measured in the designated well prepared in step 15 by 0.3 ml (the volume solvent added to filters), then by 0.005 ml (volume of suspension added to filter). Calculate the rate of transport in pmol/mg membrane protein/min using the following formula.

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

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

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:

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

9. Expected Results

Relative transport values (%)

This curve shows the effect of the test drug on NMQ transport by the given transporter in percentages. 100% represent NMQ transport by the given transporter in the absence of test drug, while 0% is the transport in the absence of ATP (non-specific binding of NMQ). This representation is commonly used if the affinities 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 transport of the reporter substrate typically does not change.

Absolute transport values (pmol/mg protein/min)

This curve shows the effect of the test drug on NMQ transport by the given transporter in absolute transport values. This representation is important to monitor the performance of the transporter or for other purposes, e.g. publications.