Determination of the interaction of drugs with MRP2/MRP3/MRP5 transporter using the fluorescent 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 (MRP2, MRP3 or MRP5) using the vesicular transport assay. The interaction is detected as the modulation of the initial rate of 5(6)-Carboxy-2',7'-dichlorofluorescein (CDCF) transport by the given transporter into membrane vesicles purified from Sf9 or HEK293 cells expressing the transporter.

2. Deliverables

SOLVO Biotechnology's PREDIVEZ Reagent Kit for MRP2, MRP3 and MRP5 transporters 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	10x Assay Mix	3.0 ml	2-8°C	on ice
C	CDCF (1 mM)	330 µl	≤-15 °C	RT
D	MgATP solution (0.2 M)	360 µl	≤-15 °C	on ice
E	Inhibitor drug stock (15 mM Benzbromarone)	150 µl	≤-15 °C	RT
F	CDCF for calibration (80 µM)	150 µl	≤-15 °C	RT
G	10x Washing Mix	3x14.5 ml	2-8 °C	on ice
H	10x Detector Solution	5.25 ml	≤RT	RT
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.

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. CDCF can be detected using Ex: 485 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. (Absolute Transport values)

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

	Calibration curve				Compound 1				Compound 2			
	CDCF				ATP		AMP		ATP		AMP	
	1	2	3	4	5	6	7	8	9	10	11	12
A	20 pmol/80 pmol				300 µM		300 µM		300 µM		300 µM	
B	10 pmol/40 pmol				100 µM		100 µM		100 µM		100 µM	
C	5 pmol/20 pmol				33.3 µM		33.3 µM		33.3 µM		33.3 µM	
D	2.5 pmol/10 pmol				11.1 µM		11.1 µM		11.1 µM		11.1 µM	
E	0 pmol				3.7 µM		3.7 µM		3.7 µM		3.7 µM	
F	ATP		AMP		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					DMSO		DMSO		DMSO		DMSO	

Dark grey wells represent measurement with negative control membrane

5. Assay steps

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 250 µl 10× Assay Mix (Vial **B**) to 2.5 ml with 2.25 ml purified water. (Store 1× Assay 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 400 µl 10× Detector Solution (Vial **H**) to 4 ml with 3.6 ml purified water (Keep this solution at room temperature).
3. Prepare the MgATP solution
   - Dilute 30 µl 0.2 M MgATP solution (Vial **D**) to 500 µl with 470 µl 1× Assay 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× Assay Mix. (Keep the AMP solution on ice).
5. Prepare the Membrane Suspension in 1× Assay Mix following the table below. Homogenize your Membrane stock with gentle pipetting! Keep the suspensions on ice.

	MRP2-Sf9	MRP2-HEK293	MRP3-Sf9 MRP3-HEK293	MRP5-HEK293
1× Assay Mix	1498.5 µl	1426.5 µl	1426.5 µl	1413 µl
Membrane stock	288 µl	360 µl	360 µl	360 µl
1 mM CDCF (Vial C)	13.5 µl	13.5 µl	13.5 µl	27 µl

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 5 minutes (in case of MRP2-HEK293 and MRP5-HEK293), 20 minutes (in case of MRP3-HEK293), 10 minutes (in case of MRP3-Sf9) or 30 minutes (in case of MRP2-Sf9).
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. Dry the filters of the filter plate (a hairdryer can be used to speed up the process).
16. Add 100 µl 1× Detector Solution to every well and incubate for 10 minutes at room temperature.
17. Transfer the liquid under vacuum to a clear, flat-bottom 96 well plate (a black-walled, clear-bottom plate for fluorescence can be used as well).
18. Measure fluorescence at Ex: 485 nm Em: 538 nm.
19. Analyze your data.

Optional assay steps:

Preparation of CDCF calibration curve

With the help of the calibration curve, the interaction of the test drug and the reporter substrate can be presented in absolute transport values (pmol/mg protein/min). The measurement is optional and can be performed on a separate plate as well. However, we suggest preparing a calibration curve before the first experiment is done.

1. Dilute CDCF solutions for the calibration curve as follows (please note that 800 nM is the starting concentration for MRP2 transporter and 200 nM for MRP3 and MRP5 transporter):
   - Dilute 250 µl 10× Detector Solution (Vial **H**) to 2.5 ml with 2.25 ml purified water.
   - Prepare 800 nM CDCF solution by adding 10 µl of Vial **F** to 990 µl 1× Detector Solution.
   - Prepare 400 nM CDCF solution by mixing 500 µl 800 nM CDCF solution with 500 µl 1× Detector Solution.
   - Prepare 200 nM CDCF solution by mixing 500 µl 400 nM CDCF solution with 500 µl 1× Detector Solution.
   - Prepare 100 nM CDCF solution by mixing 500 µl 200 nM CDCF solution with 500 µl 1× Detector Solution.
   - Prepare 50 nM CDCF solution by mixing 500 µl 100 nM CDCF solution with 500 µl 1× Detector Solution.
   - Prepare 25 nM CDCF solution by mixing 500 µl 50 nM CDCF solution with 500 µl 1× Detector Solution.
2. 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.
3. Add 100 µl of these solutions to the wells of the 96-well filter plate (see Assay Layout 2. on page 5)

4. Filter the solutions to a clear, flat-bottom 96-well plate using the plate-to-plate filtration system.
5. Measure fluorescence at Ex: 485 nm, Em: 538 nm
6. Analyze data using raw data template

CDCF transport by betagal-Sf9 or HEK293-Mock-Ctrl (negative control)

These Sf9 vesicles express the beta-galactosidase enzyme and the HEK293-Mock-Ctrl vesicles show minimal accumulation of CDCF. 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 120 µl 10× Assay Mix (Vial **B**) to 1200 µl with 1080 µl purified water. (Store 1× Assay 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 150 µl 10× Detector Solution (Vial **H**) to 1.5 ml with 1.35 ml purified water. (Keep this solution at room temperature).
2. Prepare the MgATP solution
   - Dilute 15 µl 0.2 M MgATP solution (Vial **D**) to 250 µl with 235 µl 1× Assay 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× Assay Mix. (Keep the AMP solution on ice).
4. Prepare the Membrane Suspension in 1× Assay Mix following the table below. Homogenize your Membrane stock with gentle pipetting! Keep the suspensions on ice.

	MRP2-Sf9	MRP2-HEK293	MRP3-Sf9 MRP3-HEK293	MRP5-HEK293
1× Assay Mix	666 µl	634 µl	634 µl	628 µl
Membrane stock	128 µl	160 µl	160 µl	160 µl
1 mM CDCF (Vial C)	6 µl	6 µl	6 µl	12 µl

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 5 minutes (in case of MRP2-HEK293 and MRP5-HEK293), 20 minutes (in case of MRP3-HEK293), 10 minutes (in case of MRP3-Sf9) or 30 minutes (in case of MRP2-Sf9)
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. Dry the filters of the filter plate (a hairdryer can be used to speed up the process).
15. Add 100 µl 1× Detector to every well and incubate for 10 minutes at room temperature.
16. Transfer the liquid under vacuum to a clear, flat-bottom 96 well plate (a black-walled, clear-bottom plate for fluorescence can be used as well).
17. Measure fluorescence at Ex: 485 nm, Em: 538 nm.
18. Analyze your data.

6. Calculations

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

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:

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

ATP dependent transport (pmol/mg protein/min): For this calculation use Assay Layout 2 on page 5! Set up a calibration curve with the help of the measured fluorescence values and the CDCF concentrations used. Substitute the fluorescence values into the equation of the calibration curve and calculate the amount of CDCF / well (pmol). Divide this value by the amount of protein per well (0.04 mg for MRP2 or 0.05 mg for MRP3 and MRP5) and by the time (5 minutes for MRP2-HEK293 and MRP5-HEK293, 20 minutes for MRP3-HEK293, 10 minutes for MRP3-Sf9 or 30 minutes for MRP2-Sf9).

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 5.). 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.). 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.

7. Expected Results

Relative transport values (%)

This curve shows the effect of the test drug on CDCF transport by the given transporter in percentages. 100% represent CDCF transport by the given transporter 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 CDCF). This representation is commonly used if the affinities of multiple test drugs are compared.

If the test drug interacts with the CDCF transport, then a dose-dependent decrease in transport is observed. The IC_{50} value for the test drug is the concentration where the CDCF 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 CDCF 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.

8. Troubleshooting

In case of a fluorescent test drug, the analysis of the results might be difficult, especially if the excitation and emission spectra of the compounds overlap. In cases like this, we recommend that the fluorescent test compound should be tested using a Vesicular Transport assay utilizing a radioactive reporter substrate.

The sensitivity and scaling of fluorimeters change from instrument to instrument. Even if you prefer to analyze your data using relative transport values, we recommend the preparation of a calibration curve, in order to see the fluorescence values your instrument produces. Differences among fluorimeters may account for higher background fluorescence values. It is also important that the fluorescence values obtained from the measurement falls into the linear phase of the calibration curve.

Some test compounds that are not highly soluble in aqueous solutions may precipitate in high concentrations, which might not be visible. In cases like this an increase in fluorescence in both ATP and AMP wells is observed, due to co-precipitation and incomplete filtration. We recommend the use of lower test drug concentrations, in order to get valid results.