ratMrp2
PREDIVEZ Protocol

CAT. NO. SBPV03

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Determination of the interaction of drugs with rat Mrp2 using the rat Mrp2 Fluorescent PREDIVEZ Kit

For the following membrane product:
SB-ratMrp2-HEK293-VT

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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 rat Mrp2 transporter 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 rat Mrp2 into membrane vesicles purified from mammalian cells expressing the transporter.

2. Principle

The rat Mrp2 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 CDCF, 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 fluorescent drug surrogate, CDCF, as a probe substrate in a competition type assay.

Rat Mrp2 mediates the transport of CDCF efficiently. Drugs that interact with the transporter modulate the initial rate of CDCF transport measured without any other compounds added. If a test drug is a transported substrate of the transporter it might

compete with CDCF thus reducing the rate of CDCF transport. If a compound is an inhibitor of the transporter, it will block the transport of CDCF into the membrane vesicles.

3. Deliverables

- SOLVO Biotechnology's SB-ratMrp2 Fluorescent PREDIVEZ Vesicular Transport Assay Kit for rat Mrp2 transporter sufficient for the analysis of 3, 6 or 9 test compounds. The contents of the kit are listed on the next page.
- Data sheet indicating protein content, volume, ATP dependent transport at 5 μM CDCF concentration and date of expiry of frozen membrane stocks.
- Data CD containing assay protocol, Microsoft 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]
- 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 Tip-Tubs (Eppendorf, Cat. No. 0030 058.607)
- MilliQ water

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

5. Kit contents

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

Kit size (number of cpds)		3	6	9	Storage	Storage during the assay
Vial	**Substance**	**Amount**				
A	Membrane stock (5 mg/ml)	3x420 µl	6x420 µl	9x420 µl	-80 °C	on ice
B	10x Assay Mix	1.0 ml	2.0 ml	3.0 ml	≤+4 °C	on ice
C	CDCF (500 µM)	110 µl	220 µl	330 µl	≤-20 °C	RT
D	MgATP solution (0.2 M)	120 µl	240 µl	360 µl	≤-20 °C	on ice
E	Inhibitor drug stock (15 mM Benzbromarone)	50 µl	100 µl	150 µl	≤-20 °C	RT
F	CDCF for calibration (20 µM)	50 µl	100 µl	150 µl	≤-20 °C	RT
G	10x Washing Mix	14.5 ml	2x14.5 ml	3x14.5 ml	≤+4 °C	on ice
H	10x Detector Solution	1.75 ml	3.5 ml	5.25 ml	≤RT	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.

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.

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
	Calibration curve				Compound 1				Compound 2			
	CDCF				+ ATP		-ATP (AMP)		+ ATP		-ATP (AMP)	
A	20 pmol				300 µM		300 µM		300 µM		300 µM	
B	10 pmol				100 µM		100 µM		100 µM		100 µM	
C	5 pmol				33.3 µM		33.3 µM		33.3 µM		33.3 µM	
D	2.5 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		-ATP		1.23 µM		1.23 µM		1.23 µM		1.23 µM	
G	+ATP		-ATP		0.41 µM		0.41 µM		0.41 µM		0.41 µM	
H	+ATP		-ATP		DMSO		DMSO		DMSO		DMSO	

Dark grey wells represent measurement with negative control membrane

7. Assay steps

Prepare your solutions fresh before use. Always use MilliQ water as distilled water to prepare the solutions. The steps are for assaying **1 compound** (see Assay Layout on page 6)!

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 10x Assay Mix (Vial **B**) to 2.5 ml with 2.25 ml distilled water. (Store 1x Assay 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)

   Dilute 400 µl 10x Detector Solution (Vial **H**) to 4 ml with 3.6 ml distilled 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 1x Assay 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 1x Assay Mix.

   Keep the AMP solution on ice.
4. Prepare the Membrane Suspension in 1x Assay Mix.

   Homogenize your Membrane stock with gentle pipeting. Add 360 µl Membrane stock (Vial **A**) and 27 µl CDCF (Vial **C**) to 1413 µl 1x Assay Mix. (Mix well, gently!)

   Keep your suspensions on ice.
5. Place a 96 well plate on ice.

6. Add 50 µl Membrane Suspension to each well 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 (see Assay Layout on page 6)
8. Preincubate your plate, MgATP and AMP solution at 37°C for 10 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 30 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.
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 Millipore filter plate.
14. Under vacuum, remove the liquid from the wells and wash them 5 times with 200 µl 1x 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 1x 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 probe 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 250 µl 10x Detector Solution (Vial **H**) to 2.5 ml with 2.25 ml distilled water.
2. Prepare 200 nM CDCF solution by adding 10 µl of Vial **F** to 990 µl 1x Detector Solution.
3. Prepare 100 nM CDCF solution by mixing 500 µl 200 nM CDCF solution with 500 µl 1x Detector Solution.
4. Prepare 50 nM CDCF solution by mixing 500 µl 100 nM CDCF solution with 500 µl 1x Detector Solution.
5. Prepare 25 nM CDCF solution by mixing 500 µl 50 nM CDCF solution with 500 µl 1x Detector Solution.
6. 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.
7. Add 100 µl of these solutions to the wells of the 96-well filter plate (see Assay Layout 2. on page 6)
8. Filter the solutions to a clear, flat-bottom 96-well plate using the plate-to-plate filtration system.
9. Measure fluorescence at Ex: 485 nm, Em: 538 nm
10. Analyze data using raw data template

CDCF transport by SB-HEK293-CTRL (negative control)

The kit contains a vial of SB-HEK293-CTRL membrane (Vial **I**), that serves as a negative control. These vesicles are derived from the parental cell line in which rat Mrp2 was expressed and show minimal accumulation of CDCF. 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 120 µl 10x Assay Mix (Vial **B**) to 1200 µl with 1080 µl distilled water. (Store 1x Assay 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)

   Dilute 150 µl 10x Detector Solution (Vial **H**) to 1.5 ml with 1.35 ml distilled 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 1x Assay 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 1x Assay Mix.

   Keep the AMP solution on ice.

3. Prepare the Membrane Suspension in 1x Assay Mix.

   Homogenize your Membrane stock with gentle pipeting. Add 160 µl Membrane stock (Vial **I**) and 12 µl CDCF (Vial **C**) to 628 µl 1x Assay Mix. (Mix well, gently!)

   Keep your suspensions on ice.

4. Place a 96 well plate on ice.
5. Add 50 µl Membrane Suspension to each well indicated on the Assay Layout 2. (page 6). This way one well will contain 50 µg total membrane protein.
6. Add 0.75 µl of DMSO/ test drug (in DMSO or in your solvent) to every well
7. Preincubate your plate, MgATP and AMP solution at 37°C for 10 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 30 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 1x Washing Mix to each well.
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 1x Washing Mix.
14. Dry the filters of the filter plate (a hairdryer can be used to speed up the process).
15. Add 100 µl 1xDetector 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.

8. 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/min): For this calculation use Assay Layout 2! 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 (0.05 mg) and by the time (30 min).

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.). 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 CDCF transport by rat Mrp2 in percentages. 100% represent CDCF transport by rat Mrp2 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 probe 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 rat Mrp2 in absolute transport values. This representation is important to monitor the performance of the transporter or for other purposes, e.g. publications.

10. 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 probe 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 ATP – 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.