


<p>VT-PV-MRP3-HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT</p>	<div style="text-align: center;">  SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol </div>	<p>Page 1 of 14</p>
--	---	---------------------

Determination of the interaction of drugs using the MRP3-HEK293 Fluorescent PREDIVEZ Kit

For the following membrane products:
SB-MRP3-HEK293

Version Number:

1.1

Effective date:

16. Mar. 2016

Replaces:

1.0

Written by:

2016.03.16.

X 

Zsolt Safar
 Scientist
 Signed by: Zsolt Sáfár


Approved by:

2016. 03. 16.

X 


Emese Kis PhD
 Head of Production Laboratory
 Signed by: Emese Kis



<p>VT-PV-MRP3- HEK293/1.1</p> <p>SB-MRP3-HEK293 PREDIVEZ-VT</p>	 <p>SOLVO Biotechnology</p> <p>PREDIVEZ™ Vesicular Transport Kit Assay Protocol</p>	<p>Page 2 of 14</p>
---	--	---------------------

1.	Introduction.....	3
2.	Principle.....	3
3.	Deliverables	4
4.	Equipment and Materials needed.....	4
5.	Materials	5
6.	Suggested assay layouts	6
7.	Assay steps	7
8.	Calculations	12
9.	Expected Results	13
10.	Troubleshooting	13



<p>VT-PV-MRP3-HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT</p>	<div style="text-align: center;">  SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol </div>	<p>Page 3 of 14</p>
--	---	---------------------

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 MRP3 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 of MRP3 into membrane vesicles purified from mammalian cells expressing the transporter.

2. Principle


The human MRP3 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, for the detection of the transported substrate in a competition type assay.

MRP3 mediates the transport of CDCF efficiently. Drugs that interact with the transporter modulate the initial rate of CDCF transport measured without any other



<p>VT-PV-MRP3-HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT</p>	 <p>SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol</p>	<p>Page 4 of 14</p>
---	---	---------------------

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-MRP3-HEK293 Fluorescent PREDIVEZ Vesicular Transport Assay Kit for MRP3 transporter sufficient for the analysis of 3, 6 or 9 test compounds.
- Data sheet indicating protein content, volume 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]
- Fluorimeter suitable for the 96-well format (Ex: 485 nm Em: 538 nm)
- 2 ml, 5 ml tubes
- 150 ml cylinder and Reagent Reservoir (Eppendorf, Cat. No. 0030 058.607)
- MilliQ water
- Dimethyl sulfoxide (DMSO, A.C.S reagent spectrophotometric grade, ≥99,9%, Sigma 154938)



VT-PV-MRP3-HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT	 SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol	Page 5 of 14
---	--	--------------

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)	3x500 µl	5x500 µl	7x500 µl	-80 °C	on ice
B	10x Assay Mix	1.0 ml	2.0 ml	3.0 ml	≤+4 °C	on ice
C	CDCF (0.5 mM)	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)	250 µl	2x250 µl	3x250 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 μ M		300 μ M		300 μ M		300 μ M		300 μ M		300 μ M	
B	100 μ M		100 μ M		100 μ M		100 μ M		100 μ M		100 μ M	
C	33.3 μ M		33.3 μ M		33.3 μ M		33.3 μ M		33.3 μ M		33.3 μ M	
D	11.1 μ M		11.1 μ M		11.1 μ M		11.1 μ M		11.1 μ M		11.1 μ M	
E	3.7 μ M		3.7 μ M		3.7 μ M		3.7 μ M		3.7 μ M		3.7 μ M	
F	1.23 μ M		1.23 μ M		1.23 μ M		1.23 μ M		1.23 μ M		1.23 μ M	
G	0.41 μ M		0.41 μ M		0.41 μ M		0.41 μ M		0.41 μ M		0.41 μ M	
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:

	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 μ M		300 μ M		300 μ M		300 μ M	
B	10 pmol				100 μ M		100 μ M		100 μ M		100 μ M	
C	5 pmol				33.3 μ M		33.3 μ M		33.3 μ M		33.3 μ M	
D	2.5 pmol				11.1 μ M		11.1 μ M		11.1 μ M		11.1 μ M	
E	0 pmol				3.7 μ M		3.7 μ M		3.7 μ M		3.7 μ M	
F	+ATP		-ATP		1.23 μ M		1.23 μ M		1.23 μ M		1.23 μ M	
G	+ATP		-ATP		0.41 μ M		0.41 μ M		0.41 μ M		0.41 μ M	
H	+ATP		-ATP		DMSO		DMSO		DMSO		DMSO	

Dark grey wells represent measurement with negative control membrane




<p>VT-PV-MRP3-HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT</p>	 <p>SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol</p>	<p>Page 7 of 14</p>
--	--	---------------------

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** (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 μ l 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 μ l 10x Detector (Vial **H**) to 4 ml with 3.6 ml distilled water (Keep this solution at room temperature).
3. Prepare the MgATP solution
 - Dilute 30 μ l 0.2 M MgATP solution (Vial **D**) to 500 μ l with 470 μ l Assay Mix.
 - Keep the MgATP solution on ice.
4. Prepare the AMP solution
 - Dilute 30 μ l 0.2 M AMP solution (Vial **J**) to 500 μ l with 470 μ l Assay Mix.
 - Keep the AMP solution on ice.
5. Prepare the Membrane Suspension in Assay Mix.
 - Homogenize your Membrane stock with gentle pipetting. Add 360 μ l Membrane stock (Vial **A**) and 27 μ l CDCF (Vial **C**) to 1413 μ l Assay Mix. (Mix well, gently!)
 - Keep your suspensions on ice.
6. Place a 96 well plate on ice.



<p>VT-PV-MRP3-HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT</p>	 <p>SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol</p>	<p>Page 8 of 14</p>
--	--	---------------------


7. Add 50 µl Membrane Suspension to the wells of the first 4 columns. This way one well will contain 50 µg total membrane protein.
8. 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).
9. Preincubate your plate, MgATP and AMP solution at 37°C for 10 minutes.
10. Start reaction by adding 25 µl MgATP or AMP solution to the appropriate wells (see Assay Layout).
11. Incubate your plate at 37 °C for 20 minutes
12. 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.
13. Stop the reaction by adding 200 µl of ice cold 1xWashing Mix to every well.
14. Transfer all the solution from the 96 well plate to the Millipore filter plate.
15. Under vacuum, remove the liquid from the wells and wash them 5 times with 200 µl 1x Washing Mix per well.
16. Dry the filters of the filter plate (a hairdryer can be used to speed up the process).
17. Add 100 µl 1xDetector to every well and incubate for 10 minutes at room temperature.
18. Transfer the liquid under vacuum to a clear 96 well plate (a black-walled, clear-bottom plate for fluorescence can be used as well).
19. Measure fluorescence at Ex: 485 nm, Em: 538 nm.
20. 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



<p>VT-PV-MRP3-HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT</p>	 <p>SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol</p>	<p>Page 9 of 14</p>
--	--	---------------------

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 (Vial **H**) to 2.5 ml with 2.25 ml distilled water.
2. Prepare 0.2 µM CDCF solution by adding 10 µl of Vial **F** to 990 µl 1x Detector.
3. Prepare 0.1 µM CDCF solution by mixing 500 µl 0.2 µM CDCF solution with 500 µl 1x Detector solution.
4. Prepare 0.05 µM CDCF solution by mixing 500 µl 0.1 µM CDCF solution with 500 µl 1x Detector solution.
5. Prepare 0.025 µM CDCF solution by mixing 500 µl 0.05 µM 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-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-FT-Mock-CTRL (negative control)

The Kit contains a vial of SB-HEK293-FT-Mock-CTRL membrane as well (Vial **I**), that serves as a negative control. These vesicles 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:



<p>VT-PV-MRP3-HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT</p>	 <p>SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol</p>	<p>Page 10 of 14</p>
--	--	----------------------

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 (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 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 Assay Mix.

Keep the AMP solution on ice.

4. Prepare the Membrane Suspension in Assay Mix.

Homogenize your Membrane stock. Add 160 µl Membrane stock (Vial **I**) and 12 µl CDCF (Vial **C**) to 628 µl 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 the wells indicated on the Assay Layout 2. This way one well will contain 50 µg total membrane protein.

7. Add 0.75 µl of DMSO/ test drug (in DMSO or in your solvent) to the wells


8. Preincubate your plate and MgATP 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 2.).

10. Incubate your plate at 37 °C for 20 minutes


11. 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.



VT-PV-MRP3- HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT	 SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol	Page 11 of 14
--	--	---------------

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. Dry the filters of the filter plate (a hairdryer can be used to speed up the process).
16. Add 100 µl 1xDetector to the wells and incubate for 10 minutes at room temperature.
17. Transfer the liquid under vacuum to a clear 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.



<p>VT-PV-MRP3-HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT</p>	 <p>SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol</p>	<p>Page 12 of 14</p>
---	--	----------------------

8. Calculations

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

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:

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


ATP dependent transport (pmol/mg protein/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 (mg) and by the time (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



<p>VT-PV-MRP3-HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT</p>	 <p>SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol</p>	<p>Page 13 of 14</p>
--	--	----------------------

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 CDCF transport by MRP3 in percentages. 100% represent CDCF transport by MRP3 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 very helpful if the activities 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 curve does not fall below 100%.


Absolute transport values (pmol/mg protein/min)

This curve shows the effect of the test drug on CDCF transport by MRP3 in absolute transport values. This representation is important to check 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



VT-PV-MRP3- HEK293/1.1 SB-MRP3-HEK293 PREDIVEZ-VT	 SOLVO Biotechnology PREDIVEZ™ Vesicular Transport Kit Assay Protocol	Page 14 of 14
--	--	---------------

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.

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 the incomplete filtration. We recommend the use of lower test drug concentrations, in order to get valid results.

