MRP5 cGMP Ves Tr Assay Protocol

CAT. NO. SBVT08

Determination of the interaction of drugs with the MRP5 transporter using the ^{3}H-cGMP vesicular transport assay (for 96 well filterplates)

For the following membrane product:
SB-MRP5-HEK293-VT

Version Number:

1.2

Effective date:

21.Feb.2011

Replaces:

1.1

Related Procedures:

SOP FFS01: Fee-for Service Screening

Signatures:

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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 MRP5 transporter using the vesicular transport assay. The interaction is detected as the modulation of the initial rate of ^{3}H-Guanosine 3′,5′-cyclic monophosphate (cGMP) transport of MRP5 into membrane vesicles purified from mammalian cells expressing the transporter.

2. Principle

Membrane preparations prepared from mammalian cells overexpressing MRP5 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 cGMP, 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 ^{3}H labeled cGMP for the detection of the transported substrate in a competition type assay.

MRP5 mediates the transport of cGMP efficiently. Drugs that interact with the transporter modulate the initial rate of cGMP transport measured without any other compounds added. If a test drug is a transported substrate of the transporter it might compete with cGMP thus reducing the rate of cGMP transport. If a compound is an inhibitor of the transporter, it will block the transport of cGMP into the membrane vesicles.

3. Deliverables

- Frozen membrane vesicles, containing 5 mg/ml membrane protein, labeled with volume, catalog number (transporter) and date of production.
- Data sheet indicating protein content, volume, ATP dependent cGMP transport at 1 µM in pmol cGMP /mg membrane protein/min and date of expiry of frozen membrane stocks.
- Assay protocol.

4. Equipment

- Plate incubator/shaker.
- Multichannel pipettes with corresponding tips
- Rapid filtration apparatus
  - Millipore 96 well plate filtration system or equivalent.
- 96 well liquid scintillation system

5. Materials

Substance	*Cat. Number*	*Storage*
MOPS (3-[N-Morpholino]propanesulfonic acid)	Sigma M-1254	RT, >1year
KCl	Sigma P-9333	RT, >1year
Sucrose	Sigma S-0389	RT, >1year
Tris-Base (Tris[hydroxymethyl]aminomethane)	Sigma T-1503	RT, >1year
$MgCl_2$	Sigma M-8266	RT, >1year
ATP (disodium salt)	Sigma A-2383	-20 °C, >1 year
AMP (disodium salt)	Fluka 01930	-20 °C, >1 year
Benzbromarone	Sigma B-5774	4 °C, >1 year
Quercetin dihydrate	Sigma Q-0125	RT, >1year
Guanosine 3',5'-cyclic phosphate	Sigma G-7504	-20 °C, >1 year
Guanosine 3',5'-cyclic phosphate, ammonium salt, [8-^{3}H] 1.0 mCi/ml	Moravek MT-517	store as stated by the supplier
DMSO	Sigma D-2650	RT, >1year
Filterplates (Millipore multiscreen HTS 96 well filter plates with FB filters or equivalent)	Millipore MSFB6B10	RT, >1year
OptiPhase 'supermix' scintillation cocktail	PerkinElmer	RT, > 1 year
MilliQ water Filtering distilled water through a Millipore Ultra-Pure Water System Purification Pak makes MilliQ water. Use MilliQ water to make all solutions.	Millipore 67733	RT, >1year

6. Solutions

Stock solutions

Solution	*Storage*
1.7 M Tris Dissolve 20.587 g of Tris in 100 ml distilled water.	4 °C, >1 year
0.1 M MOPS-Tris Dissolve 2.09 g of MOPS in 90 ml distilled water, adjust pH to 7.0 with 1.7 M Tris (about 2 ml). Bring solution to 100 ml with distilled water.	4 °C, >1 year
1 M $MgCl_2$ Dissolve 95.21 g $MgCl_2$ in distilled water.	4 °C, >1 year
1 M KCl Dissolve 74.56 g KCl in distilled water.	4 °C, >1 year
1 mM cGMP in distilled water	-20 °C, >1 year
20 mM Benzbromarone in DMSO	-20 °C, >1 year
20 mM Quercetin in DMSO	-20 °C, >1 year
0.2 M Mg-ATP Dissolve 2,2 g of ATP and 0,813 g $MgCl_2$ in 10 ml of distilled water and adjust pH to 7.0 with 1.7 M Tris. Bring solution to 20 ml with distilled water.	-20 °C, >1 year
0.2 M AMP Dissolve 1.56 g of AMP in 10 ml of distilled water and adjust pH to 7.0 with HCl. Bring solution to 20 ml with distilled water.	-20 °C, >1 year

Assay-mix:

Ingredient	*Volume (ml)*
1.7 M Tris	2.94
Sucrose	
1 M $MgCl_2$	10
Total volume:	100

Dissolve 8.56 g Sucrose in 50 ml distilled water. Add Tris and $MgCl_2$ solutions according to the above table. Adjust pH with 5 M HCl to 7.4. Bring the solution to 100 ml with distilled water. The solution can be pre-mixed and stored at 4 °C.

Washing-mix:

Ingredient	*Volume (ml)*
0.1 M MOPS-Tris	200
1 M KCl	35
MilliQ water	265
Total volume:	500

The solution can be pre-mixed and stored at 4 ^{o}C.

7. Assay steps (for 1 plate)

1. Mix 612 µl of membrane suspension with 4447.2-x* µl of assay-mix. Add x* µl of 1 mM cGMP. Add 40.8 µl of ^{3}H-cGMP. Add 50 µl of this suspension to all wells of a standard 96 well plate (not the filterplate).

2. Add test drugs (in 0.75 µl DMSO) and DMSO as indicated on the plate setup below.

3. Mix 90 µl of Mg-ATP with 1410 µl of assay-mix.

4. Mix 90 µl of AMP with 1410 µl of assay-mix.

5. Preincubate plate, ATP and AMP at 37 ^{o}C for 15 min.

6. Wet the filter plate as recommended by the supplier and set up the filtering apparatus.

7. Add 25 µl of ATP and AMP (prepared in steps 3 and 4) to the wells as indicated on the plate setup below. Shake plate with the shaker. Incubate at 37 ^{o}C for 15 min.

    - Depending on your equipment you can run the assay with one row at a time, or in blocks. The general consideration is that filtration should take place in 2 minutes after stopping the assay with cold washing-mix.

8. Stop the reaction by adding 200 µl ice cold washing-mix. Transfer samples to the filter plate and filter.

9. Wash wells with 5*200 µl of ice cold washing-mix.

10. 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).

11. Dry filters plate (you can use a hair drier to speed up the process.).

12. Add 100 µl of scintillation cocktail and measure radioactivity in each well. Record cpm values.

*The amount of unlabeled cGMP to add depends on the concentration of the labeled cGMP used. This concentration can be calculated from the information available on the datasheet supplied with the labeled cGMP batch. Add the amount of unlabeled cGMP to achieve a final concentration (labeled and unlabeled together) of 1 µM. In order to keep the final assay volume unchanged, subtract the volume of the unlabeled cGMP added to each tube from the amount of assay-mix.

8. Suggested assay layout

Preparation of reaction mixtures for MRP5-mediated cGMP transport inhibition

	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.

9. Calculations

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

ATP dependent transport (pmol/mg/min): Calculate *Total activity (cpm)* by multiplying the cpms measured in the designated well prepared in step 10 by 20. Calculate the rate of transport in pmol/mg membrane protein/min using the following formula.

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

If the assay is performed in the conditions described the value of the second part of the equation is 166.67.

Membrane validation: During membrane validation the test done is identical to samples in wells e.g. H1-4 in the assay layout above. 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 fee 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: Quercetin and Benzbromarone inhibit the cGMP transport of the MRP5 transporter. You can assay this inhibition by replacing the test drug with 0.75 µl 20 mM Quercetin (final concentration 200 µM) or 0.75 µl 20 mM Benzbromarone (final concentration 200 µM).

Suggested membrane negative control: There is a low endogenous cGMP transport detected in membranes prepared from control mammalian cells not expressing the MRP5 transporter. However, if you are studying transport of cold cpds we would advise you to use SB-HEK293-CTRL as negative control.