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ProductInformation

MRP2, human
recombinant, expressed in *Sf*9 cells,
membrane preparation, for Vesicular Transport

Product Code **M9069**
Storage Temperature –70 °C

TECHNICAL BULLETIN

Product Description

ATP Binding Cassette (ABC) transporters are membrane proteins characterized by homologous ATP-binding and large, multispanning transmembrane domains. Three major groups of ABC transporters are involved in cancer multidrug resistance (MDR). These are the classical P-glycoprotein (MDR1, ABCB1), the multidrug resistance associated proteins (MRPs, in the ABCC subfamily), and the ABCG2 protein, an ABC half-transporter. All these proteins catalyze an ATP-dependent active transport of chemically unrelated compounds, including anticancer drugs.[1]

MRP2 (ABCC2) is an organic anion transporter found in the liver, kidney, and gut epithelium apical membranes. Its transport of glucuronate conjugates plays a role in the detoxification of endogenous and xenobiotic substances, and may cause multidrug resistance (MDR) in tumor cells.

One of the simplest methods invented for measuring transport is the vesicular transport assay. The human MRP2 transporter is expressed in *Sf*9 insect cells using the baculoviral expression system. Membrane preparations from infected cells always contain some closed membrane vesicles that have an inside-out orientation (5-10% of total lipid). In the case of these inside-out vesicles, transport of substrates across the membrane takes molecules from the surrounding buffer and transports them into the vesicles. The rate of this transport is temperature and ATP dependent.

The procedure in this kit determines the interaction of compounds with the MRP2 transporter using the vesicular transport assay. The interaction is detected by changes in the initial rate of ^{3}H-β-estradiol 17-(β-D-glucuronide) transport by MRP2 into membrane vesicles purified from *Sf*9 cells expressing the transporters.

Rapid filtration of the membrane suspension through a filter that retains membrane vesicles allows removal of the substrate molecules that are “outside” leaving the membrane vesicles with transported molecules trapped “inside” on the filter.

The quantity of transported molecules can be determined by any adequate method like HPLC, LC/MS/MS separation and detection. Also, the transported molecules can be labeled with fluorescent or radioactive tags. This protocol utilizes ^{3}H-β-estradiol 17-(β-D-glucuronide) for the detection of the transported substrate in a competition type assay.

MRP2 mediates the transport of β-estradiol 17-(β-D-glucuronide) ($E_2$17βG) very efficiently. Compounds that interact with the transporter modulate the initial rate of $E_2$17βG transport measured without any other compounds added. If a substance is a transported substrate of the transporter it might compete with $E_2$17βG, thus reducing the rate of $E_2$17βG transport. If a compound is an inhibitor of the transporter, it will block the transport of $E_2$17βG into the membrane vesicles. Some compounds can be co-transported with $E_2$17βG. These substances will increase the rate of $E_2$17βG transport compared to the control level.

The MRP2 transporter is known to transport some small, positively charged and hydrophobic molecules only in the presence of L-glutathione (co-transport), which is readily available in living cells. Also, in the vesicular transport some compounds only modulate the rate of $E_2$17ßG transport of the MRP2 transporter in the presence of L-glutathione. Therefore, it is suggested to determine the interaction of test compounds in the presence of 2 mM L-glutathione.

Reagent
The membrane vesicles are suspended in TMEP solution (50 mM Tris-HCl, 50 mM mannitol, 2 mM EGTA, 8 µg/ml aprotinin, 10 µg/ml leupeptin, 50 µg/ml PMSF, and 2 mM DTT, pH 7.0)

Equipment and Reagents Required But Not Provided
- MOPS [3-(N-Morpholino)propanesulfonic acid], Product Code M1254
- Trizma® base [Tris(hydroxymethyl)aminomethane, Tris-base], Product Code T1503
- Potassium chloride (KCl), Product Code P9333
- Magnesium chloride hexahydrate, Product Code M2670
- Adenosine 5′-triphosphate, disodium salt (ATP), Product Code A2383
- L-Glutathione reduced (GSH), Product Code G4251
- Benzbromarone, Product Code B5774
- ^{3}H-β-estradiol 17-(β-D-glucuronide), ~1 mCi/ml
- β-estradiol 17-(β-D-glucuronide) ($E_2$17βG) Product Code E1127
- Dimethyl sulfoxide (DMSO), Product Code D2650
- OptiPhase SuperMix scintillation cocktail, PerkinElmer Product Code 1200-439
- Ultrapure water (17 MΩ·cm or equivalent)
- Filter plates (Millipore MultiScreen®$_{HTS}$ 96 well Filter Plates with glass fiber filter or equivalent, Millipore Product Code MSFB N6B 10)
- Rapid filtration apparatus (Millipore 96 well plate filtration system or equivalent)
- Plate incubator/shaker
- Multichannel pipettes with corresponding tips
- 96 well liquid scintillation system

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Use ultrapure water (17 MΩ·cm or equivalent) for preparation of reagents.

1.7 M Tris Solution - Dissolve 20.587 g of Tris-base in 100 ml of water. The solution may be stored at 2–8 °C for at least 1 year.

0.1 M $MgCl_2$ Solution – Prepare in water. The solution may be stored at 2–8 °C for at least 1 year.

0.1 M MOPS-Tris Solution – Dissolve 2.09 g of MOPS in 90 ml of water and adjust pH to 7.0 with 1.7 M Tris Solution (~2 ml). Bring the final volume to 100 ml with water. The solution may be stored at 2–8 °C for at least 1 year.

1 M KCl Solution – Prepare in water. The solution may be stored at 2–8 °C for at least 1 year.

0.14 M KCl Solution – Prepare in water. The solution may be stored at 2–8 °C for at least 1 year.

7.5 mM Benzbromarone Solution – Prepare in DMSO. The solution may be stored at –20 °C for at least 1 year.

0.2 M Mg-ATP Solution - Dissolve 2.2 g of ATP and 0.813 g of $MgCl_2$ in 10 ml of water and adjust pH to 7.0 with 1.7 M Tris Solution. Bring final volume to 20 ml with water. The solution may be stored at –20 °C for at least 1 year.

7.5 mM $E_2$17βG Solution – Prepare in DMSO. The solution may be stored at –20 °C for at least 1 year.

300 mM GSH Solution – Dissolve 0.922 g of GSH in 8 ml of water and adjust pH to 6.8 with 10 M NaOH. Bring final volume to 10 ml with water. The solution may be stored at –20 °C in small aliquots.

Assay Mix – Combine the following:

0.1 M MOPS-Tris Solution	5 ml
0.14 M KCl Solution	5 ml
0.1 M $MgCl_2$ Solution	0.75 ml

The solution can be prepared and stored at 2–8 °C.

Washing Mix – Combine the following:

0.1 M MOPS-Tris Solution	200 ml
1 M KCl Solution	35 ml
water	265 ml

The solution can be prepared and stored at 2–8 °C.

Storage/Stability
Store the product at –70 °C.

Procedure

This procedure is for the determination of the interaction of compounds with the MRP2 transporter using the ^{3}H-β-estradiol 17-(β-D-glucuronide) vesicular transport assay in a 96 well format.

Positive control – Benzbromarone inhibits the ^{3}H-$E_2$17βG transport of the MRP2 transporter. Due to this inhibition, it can be used as a positive control by replacing the test compound with 1 µl of 7.5 mM Benzbromarone Solution giving a final concentration 100 µM. Controls should be run in duplicate with both Assay Mix and Assay Mix with ATP.

Membrane negative control – There is a low endogenous ^{3}H-$E_2$17βG transport detected in membranes expressing a mutant (defective) variant of the MRP1 transporter (Product Code M9819; MDR1, MRP, and BSEP Control). However, for the study of transport of cold compounds, use of this control as a negative control is suggested.

96 Well Assay

1. Prepare the Reaction Suspension – Combine the following:

Membrane Suspension	1,000 µl
^{3}H-$E_2$17βG, ~1 mCi/ml	10 µl
7.5 mM $E_2$17βG Solution	50 µl
300 mM GSH Solution	50 µl
Assay Mixture	3,890 µl

2. Add 50 µl of the Reaction Suspension to the wells of a standard 96 well plate, **not** the filter plate.

3. Add test compound solutions to give the final reaction concentrations indicated in Table 1. The volume of the test compound solution should not exceed 1 µl. Add DMSO to the solvent only assays (row H).
   Note: If the test compound is not dissolved in DMSO, substitute the solvent used in the wells marked for DMSO.

4. Prepare Assay Mix with ATP – Add 90 µl of the 0.2 M Mg-ATP Solution to 1,410 µl of Assay Mix.

5. Preincubate the plate, 1,500 µl of the Assay Mix, and 1,500 µl of the Assay Mix with ATP at 37 °C for 5 minutes.

6. Wet the filter plate and set up the filtration apparatus.

7. Add 25 µl of Assay Mix with ATP or Assay Mix to the wells as indicated in Table 1. Shake plate with the shaker. Incubate the plate at 37 °C for 8 minutes.
   Note: Depending on the equipment available, the assays can be run one row at a time or in blocks. The general consideration is that the filtration should take place within 2 minutes after stopping the assay with cold Assay Mix (step 8).

8. Stop the reaction by adding 200 µl of ice cold Washing Mix to each well. Transfer the reaction mixtures to the filter plate and filter.

9. Wash each well five times, each time with 200 µl of ice cold Washing Mix.

10. Pipette 2.5 µl the Reaction Suspension (prepared in step 1) into one well of the filter plate. The radioactivity (cpm) measured on this filter in this well is the total activity per well/20 for each assay.

11. Dry the filter plate. A heat gun (hair drier) may be used to speed up the process.

12. Add 50 µl of scintillation cocktail and measure radioactivity in each well. Record cpm values.

Table 1.
Assay Layout Guidelines - Preparation of reaction mixtures

	1	2	3	4	5	6	7	8	9	10	11	12
	Compound 1				**Compound 2**				**Compound 3**			
	Assay Mix with ATP		**Assay Mix (without ATP)**		**Assay Mix with ATP**		**Assay Mix (without ATP)**		**Assay Mix with ATP**		**Assay Mix (without ATP)**	
A	100 µM		100 µM		100 µM		100 µM		100 µM		100 µM	
B	33.3 µM		33.3 µM		33.3 µM		33.3 µM		33.3 µM		33.3 µM	
C	11.1 µM		11.1 µM		11.1 µM		11.1 µM		11.1 µM		11.1 µM	
D	3.7 µM		3.7 µM		3.7 µM		3.7 µM		3.7 µM		3.7 µM	
E	1.23 µM		1.23 µM		1.23 µM		1.23 µM		1.23 µM		1.23 µM	
F	0.41 µM		0.41 µM		0.41 µM		0.41 µM		0.41 µM		0.41 µM	
G	0.137 µM		0.137 µM		0.137 µM		0.137 µM		0.137 µM		0.137 µM	
H	DMSO		DMSO		DMSO		DMSO		DMSO		DMSO	

Calculations

ATP dependent transport (cpm) – For each tested compound, subtract cpm values measured without the presence of ATP (Assay Mix) from the cpm values measured in the presence of ATP (Assay Mix with ATP) for controls and samples. Take the average of the duplicates.

ATP dependent transport (pmol/mg/min) – Multiply the total activity per well/20 by 20 to get Total activity (cpm). Calculate the rate of transport in pmol/mg membrane protein/min using the following:

$$\frac{\text{ATP dependent transport (cpm)}}{\text{Total activity (cpm)}} \times \frac{E_2 17\beta G \text{ concentration (nM)} \times \text{Volume (ml)}}{\text{membrane protein (mg)} \times \text{time (minutes)}}$$

For assays performed according to the described procedure, the value of the right part of the equation is 4,687.5

ATP dependent transport (%) – Calculate the percent activation or inhibition of the test compound. In this representation the ATP dependent transport determined in the compound free (solvent only) control is taken as 100% and all other values are represented on this relative scale. Use the following formula:

$$\frac{\text{ATP dependent transport in the presence of test compound (cpm)}}{\text{ATP dependent transport in compound free control (cpm)}} \times 100$$

References

1. Glavinas, H., *et al*., The role of ABC transporters in drug resistance, metabolism, and toxicity. Curr. Drug Deliv., **1**, 27-42 (2004).

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