Vesicular Transport Assay Protocol

For ABC Transporter Vesicle Product
with RI-labeled Substrates

This protocol describes the vesicular transport assay for the following ABC Transporter Vesicle Products and the corresponding Reagent kit.

- ABC Transporter Vesicle Products

Human MRP1	(GenoMembrane, Cat. No. GM0010)
Rat Mrp1	(GenoMembrane, Cat. No. GM0011)
Dog Mrp1	(GenoMembrane, Cat. No. GM0017)
Human MRP2	(GenoMembrane, Cat. No. GM0001)
Rat Mrp2	(GenoMembrane, Cat. No. GM0002)
Mouse Mrp2	(GenoMembrane, Cat. No. GM0022)
Dog Mrp2	(GenoMembrane, Cat. No. GM0014)
Monkey Mrp2	(GenoMembrane, Cat. No. GM0018)
Human MRP3	(GenoMembrane, Cat. No. GM0021)
Human MRP4	(GenoMembrane, Cat. No. GM0012)
Rat Mrp4	(GenoMembrane, Cat. No. GM0020)
Human MRP8	(GenoMembrane, Cat. No. GM0013)
Human BCRP	(GenoMembrane, Cat. No. GM0008)
Rat Bcrp	(GenoMembrane, Cat. No. GM0007)
Human BSEP	(GenoMembrane, Cat. No. GM0005)
Rat Bsep	(GenoMembrane, Cat. No. GM0006)
Dog Bsep	(GenoMembrane, Cat. No. GM0019)

- ABC Transporter Vesicle Product for Negative Control

Control	(GenoMembrane, Cat. No. GM0003)

- Vesicular Transport Assay Reagent kit

For MRPs and BCRP	(GenoMembrane, Cat. No. GM3010)
For BSEP	(GenoMembrane, Cat. No. GM3001)

When you prepare the reagents for vesicular transport assay by yourself, please refer to "**Buffer Preparation Protocol**" available at the following homepage.
(http://www.genomembrane.com/E_Technical_Information.html).

Contents

1. Vesicular transport assay

Vesicular transport assay using ABC transporter Vesicle Products allows to evaluate the interaction of compounds with the transporter of interest. ABC Transporter Vesicle Products of GenoMembrane are prepared from purified plasma membranes isolated from an insect cell system (Sf9 cells infected with baculovirus) expressing ABC transporters. These Vesicle Products have inside-out vesicle structure.

Since ABC transporters transport their substrates from inside to outside of the cell across the cell membrane using ATP hydrolysis energy as the driving force, inside-out vesicles transport substrates from the reaction medium into the vesicles.

The amount of a 1) radioisotope-labeled compound, 2) fluorescence-labeled compound or 3) non-labeled compound transported into the vesicles can be measured directly by means of 1) liquid scintillation counter, 2) fluorescence plate reader, 3) LC/MS^{ref}, thereby allowing direct evaluation of the ABC transporter activity.

The protocol described here is designed for vesicular transport assay using radioisotope -labeled compounds. In the following example, Estradiol-17β-D-glucuronide ($E_2$17βG) is used as a standard transport substrate for MRP1, MRP2, MRP3, MRP4 and MRP8, Methotrexate (MTX) is used for BCRP and Taurocholic acid (TCA) is used for BSEP. Substrate transported into vesicles can be determined by counting radioactivity with liquid scintillation counter after the removal of substrate remaining in the reaction medium by filtration.

It is well known that MRP1, MRP2, MRP3 and MRP4 transport some drugs in the **presence of glutathione** (co-transport). Glutathione (2 mM) is, therefore, added for your purpose.

We recommend to use the control product (Cat. No. GM0003) as a negative control to know intrinsic transport activity of Sf9 cell membranes if necessary.

2. Kit Contents

2.1. ABC transporter Vesicle Products

◆ Frozen ABC Transporter Vesicle Product (5 mg/mL, 500 μL)

> One vial of ABC Transporter Vesicle Product contains 2.5 mg protein.
> For vesicular transport assay, 50 μg of protein is needed for one assay. Therefore, 50 assays can be performed with one vial of the Vesicle product based on this protocol.

- Store at –80˚C.
- Expiration date; written in the Data Sheet
- Transport activity for each lot; written in the Data Sheet attached with the product

It is recommended for reliable and reproducible data to minimize freeze/thaw cycle by dividing aliquot to small portions.

2.2. ABC Transporter Vesicle Reagent kit (for MRPs and BCRP)

◆ Buffer A2: Reaction Buffer (6 mL ×1 vial)
[Components] 50 mM MOPS-Tris, 70 mM KCl, 7.5 mM $MgCl_2$

◆ 10 × Buffer B2: 10 × Stopping and Washing Buffer (20 mL ×1 vial)

[Components] 400 mM MOPS-Tris, 700 mM KCl

***Note**; Dilute the "× 10 Buffer B" to "× 1" with cold ultra pure water and cool on ice before the assay.*

- ◆ Reagent C2: 10 mM MgATP solution (1.3 mL ×2 vials)
- ◆ Reagent D2: 10 mM MgAMP solution (1.3 mL ×2 vials)
- ◆ Reagent G: 200 mM Glutathione solution (0.1 mL ×1 vial)

All Reagents must be stored at –20˚C until use.
Expiration date; written in the box

2.3. ABC Transporter Vesicle Reagent Kit (for BSEP)

- ◆ Buffer A1: Reaction Buffer (6 mL ×1 vial)

  [Components] 10 mM Hepes-Tris, 100 mM KNO_3, 10 mM $Mg(NO_3)_2$, 50 mM Sucrose
- ◆ 10 × Buffer B1: 10 × Stopping and Washing Buffer (20 mL ×1 vial)

  [Components] 100 mM Hepes-Tris, 1000 mM KNO_3, 500 mM Sucrose

  ***Note**; Dilute the "10 × Buffer B" to" ×1" with cold ultra pure water and cool on ice before the assay.*
- ◆ Reagent C1: 10 mM MgATP solution (1.3 mL ×2 vials)
- ◆ Reagent D1: 10 mM MgAMP solution (1.3 mL ×2 vials)

All Reagents must be stored at –20˚C until use.
Expiration date; written in the box

One Regent Kit contains sufficient volume of reagents to perform 100 assays based on this protocol, which is equivalent to two vials of ABC Transporter Vesicle products.

3. Apparatuses, Materials and Substrates

3.1. Apparatuses

- Water bath or incubator with temperature control at 37 ºC
- Micropipettes
- Suction filtration device
- Liquid scintillation counter

3.2. Materials

- Pipettes, tips
- Containers for dilution and preparation (tubes, etc.)
- 96 well glass-fiber filter plate
- Liquid scintillation cocktail and vials for measurement

3.3. Substrates

Non-RI-labeled Substrate

Transporter	Non-RI-labeled Substrate
MRP1, MRP2, MRP3, MRP4, MRP8	Estradiol-17β-D-glucuronide Sodium salt ($E_2$17βG)
BCRP	Methotrexate (MTX)
BSEP	Taurocholic acid (TCA)

RI-labeled Substrate

Transporter	RI-labeled Substrate
MRP1, MRP2, MRP3, MRP4, MRP8	[^{3}H] Estradiol-17β-D-glucuronide (^{3}H-$E_2$17βG)
BCRP	[^{3}H] Methotrexate (^{3}H-MTX)
BSEP	[^{3}H] Taurocholic acid (^{3}H-TCA)

4. Preparation of solutions

4.1. Preparation of Non RI-labeled substrate solutions (positive control)

Transporter	Non-RI Substrate Solution	Preparation
MRP1 MRP4 MRP8	1 mM $E_2$17βG in DMSO	Dissolve $E_2$17βG in DMSO to make 1 mM solution.
MRP2	5 mM $E_2$17βG in DMSO	Dissolve $E_2$17βG in DMSO to make 5 mM solution.
MRP3	0.1 mM $E_2$17βG in DMSO	Dissolve $E_2$17βG in DMSO to make 0.1 mM solution.
BCRP	10 mM MTX	Dissolve MTX in DMSO to make 10 mM solution.

4.2. Preparation of RI-labeled substrate solution (positive control)

Transporter	RI-Substrate Solution	Preparation
MRP1	[^{3}H] $E_2$17βG; 40 μCi/mL (Final conc.: 8 μCi/mL)	Dilute 1 mCi/mL of [^{3}H] $E_2$17βG to 25-fold with Buffer A2
MRP2 MRP3 MRP4 MRP8	[^{3}H] $E_2$17βG; 20 μCi/mL (Final conc.: 4 μCi/mL)	Dilute 1 mCi/mL of [^{3}H] $E_2$17βG to 50-fold with Buffer A2
BCRP	[^{3}H] MTX; 100 μCi/mL (Final conc.: 20 μCi/mL)	Dilute 1 mCi/mL of [^{3}H] MTX to 10-fold with Buffer A2
BSEP	[^{3}H] TCA; 10 μCi/mL (Final conc.: 2 μCi/mL)	Dilute 1 mCi/mL of [^{3}H] TCA to 100-fold with Buffer A1

5. Vesicular transport assay

The following is the standard procedure for determination of the ATP-dependent transport activity of positive references. The final concentrations of each substrate are shown in section 6.

The example is conducted in quadruplicate. The experimental condition such as volumes etc. should be appropriately adjusted in each case according to the experimental design to be used.

(1) For one assay, dilute 10 µL of ABC Transporter Vesicle Product with 9 µL of Buffer A to make 19 µL. Adjust volumes of vesicle preparation if necessary.

(i) <ABC Transporter Vesicle Product preparation>	(Per assay)	(For 8 assays)
ABC Transporter Vesicle Product (5 mg/mL)	10 µL	80 µL
Buffer A	9 µL	72 µL
	(Total 19 µL/assay)	(Total 152 µL)

(2) Prepare the Assay mixture on ice as follows.

(ii) <Assay mixture>	(Per assay)	(4 assays for ATP/AMP)
Reagent C or D	20 µL	80 µL
RI-labeled substrate solution	10 µL	40 µL
Non RI-labeled substrate solution	0.5 µL	2 µL
(In the case of BSEP, use Buffer A1)		
Reagent G or Buffer A	0.5 µL	2 µL
(Add Regent G depending on study condition)		
	(Total 31 µL/assay)	(Total 124 µL)

(3) Pre-incubate the assay tube containing the vesicle product preparation (i) at 37˚C for 5 min. At the same time, incubate each assay mixture containing Reagent C or D (ii) as well.

(4) Add 31 µL of pre-incubated each assay mixture containing Reagent C or D (ii) to assay tubes containing the vesicle product (i) and mix by pipetting several times to start reaction.

(5) Incubate the assay tube at 37˚C for appropriate time (Adjust reaction time depending on the study design).

(6) Add 200 µL of chilled 1×Buffer B to stop reaction. Then, place the assay tube on ice until filtration.

(7) Filter the reaction medium with 96-well glass-fiber filter plate as follows:

7-1) Add 1×Buffer B to all wells of 96-well glass fiber filter plate including unused wells, and filter by suctioning to make the filter wet. Pre-wet step is important for increasing suction efficiency and decreasing the probe adsorption to glass fiber filter as well.

7-2) Transfer the reaction medium onto pre-wet filters using a multi-channel pipette, and filter by suctioning.

7-3) Wash the filter 5 times with chilled 1×Buffer B (200 µL per well).

7-4) Determine 10 µL of RI-labeled substrate solution (total radioactivity)

7-5) Place the filter in a vial, and add liquid scintillation cocktail.

7-6) Measure the radioactivity (cpm) of the filter with a liquid scintillation counter.

In order to obtain fine results, perform the step from (6) to (7) immediately.

Note; **When you use glass-fiber filter instead of 96-well filter plate, conduct the following procedure from step (7).**

Confirm necessary quantity of Buffer B, because quantity of the Buffer B in Reagent Kit is not enough for following procedure.

7-1) Filter the reaction medium through glass-fiber filter by suctioning.

7-2) Wash the filter sufficiently* with chilled 1× Buffer B.

*When you use a filter of 25 mm in diameter (Such as Millipore #APFF02500, Whatman #1825-025), washing with 10 mL of the Washing Buffer B twice is appropriate.

7-3) Determine 10 µL of RI-labeled substrate solution (total radioactivity).

7-4) Place the filter in a vial, and add liquid scintillation cocktail.

7-5) Measure the radioactivity (cpm) of the filter with a liquid scintillation counter.

6. Data analysis

(1) The amount of substrate trapped by one filter can be calculated using the following formula.

$$\text{Amount of substrate on one filter (pmol)} = \frac{\text{Radioactivity of filter (cpm)}}{\text{Total radioactivity in assay tube (cpm) }*1} \times \text{Amount of substrate in one reaction medium (pmol) }*2$$

*****1; refer to the step 7-4) above

*****2; Amount of substrates in one reaction medium are as follows

	Substrate	Final Conc.	Amount of substrate in a reaction medium
MRP1	$E_2$17βG	10 µM	500 pmol (= 1000 µM × 0.5 µL)
MRP2	$E_2$17βG	50 µM	2500 pmol (= 5000 µM × 0.5 µL)
MRP3	$E_2$17βG	1 µM	50 pmol (= 100 µM × 0.5 µL)
MRP4	$E_2$17βG	10 µM	500 pmol (= 1000 µM × 0.5 µL)
MRP8	$E_2$17βG	10 µM	500 pmol (= 1000 µM × 0.5 µL)
BCRP	MTX	100 µM	5000 pmol (=10000 µM × 0.5 µL)
BSEP	TCA	figure out from radioactivity	

(2) Subtract the radioactivity of one reaction medium with MgAMP from that with MgATP. This is the amount of ATP-dependent substrate transport (pmol).

(3) Divide the amount of ATP-dependent substrate transport (pmol) by the amount of protein used (0.05 mg) and the reaction time (min), in order to obtain the amount of ATP-dependent substrate transport per unit amount of protein and per unit time (pmol/mg protein/min).

(4) Dividing the amount of substrate transport (pmol/mg protein/min) by concentrations (µM) gives the volume of substrate solution transported (µL/mg protein/min).

7. Recommended equipments

7.1. Apparatuses

	Maker	Bland Name
96 well glass-fiber filter plate	Millipore	MultiScreenHTS+ Hi Flow FB Plate MultiScreenHTS-FB Plate
Suction Filtration device	Millipore	MultiScreenHTS Vacuum Manifold
96 well separation machine	Millipore	Multiple Punch
96 well separation equipment	Millipore	Disposable Punch Tips
Liquid scintillation cocktail	PerkinElmer	UltimaGold

7.2. RI-labeled compounds (Substrates)

MRP1, MRP2, MRP3 MRP4, MRP8	**1 mCi/mL [^{3}H] $E_2$17βG** (PerkinElmer # NET-1106, American Radiolabeled Chemicals # ART1320, etc.)
BCRP	**1 mCi/mL [^{3}H] MTX** (Moravek # MT701, etc.)
BSEP	**1 mCi/mL [^{3}H] TCA** (PerkinElmer # NET-322, etc.)

8. References

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- Kato Y, Takahara S, Kato S, Kubo Y, Sai Y, Tamai I, Yabuuchi H, and Tsuji A. Involvement of multidrug resistance-associated protein 2 (Abcc2) in molecular weight-dependent biliary excretion of beta-lactam antibiotics. *Drug Metab Dispos*. **36** (6): 1088-96 (2008). ⇒**LC-MS/MS analysis**

- Yamaguchi K, Murai T, Yabuuchi H, and Kurosawa T. Measurement of transport activities of bile acids in human multidrug resistance-associated protein 3 using liquid chromatography-tandem mass spectrometry. *Anal Sci*.; **26** (3): 317-23 (2010). ⇒**LC-MS/MS analysis**

GenoMembrane

2-3-18 Namamugi, Tsurumi-ku
Yokohama, Kanagawa 230-0046 JAPAN
TEL: +81-45-508-2326
FAX: +81-45-716-8884
URL: http://www.genomembrane.com/
E-mail: info@genomembrane.com