Vesicular Transport Assay Protocol

For ABC Transporter Vesicle Product (HEK)
with non RI-labeled Substrates

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

- ABC Transporter Vesicle Products (HEK)
  - Human MRP1 (GenoMembrane, Cat. No. GM0110)
  - Human MRP2 (GenoMembrane, Cat. No. GM0101)
  - Human MRP3 (GenoMembrane, Cat. No. GM0121)
  - Human MRP4 (GenoMembrane, Cat. No. GM0112)
  - Human BSEP (GenoMembrane, Cat. No. GM0105)

- ABC Transporter Vesicle Product (HEK) for Negative Control
  - Mock (GenoMembrane, Cat. No. GM0103)

- Vesicular Transport Assay Reagent kit for HEK (GenoMembrane, Cat. No. GM3100)

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

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1. Vesicular transport assay

Vesicular transport assay using ABC transporter Vesicle Products (HEK) allows evaluation of compound interactions with the transporter of interest. ABC Transporter Vesicle Products (HEK) of GenoMembrane are prepared from purified plasma membranes isolated from a mammalian cell system (HEK293F™ cells) expressing ABC transporters. These Vesicle Products (HEK) 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 RI-labeled compound transported into the vesicles can be measured directly by means of 1) liquid scintillation counter, 2) fluorescence plate reader, 3) LC/MS/MS^{ref}, thereby allowing direct evaluation of the ABC transporter activity.

The protocol described here is designed for vesicular transport assay using non RI-labeled compound. In the following example, Estradiol-17β-D-glucuronide ($E_2$17βG) is used as a standard transport substrate for MRP1, MRP2, MRP3, and MRP4 and Taurocholic acid (TCA) is used for BSEP. Substrate transported into vesicles can be quantified by LC/MS/MS 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 Mock product (Cat. No. GM0103) as a negative control to know intrinsic transport activity of HEK293F™ cell membranes if necessary.

2. Kit Contents

2.1. ABC Transporter Vesicle Product (HEK)

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

One vial of ABC Transporter Vesicle Product (HEK) 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 HEK

◆ Buffer A: Reaction Buffer (6 mL ×1 vial)

[Components] 50 mM MOPS-Tris, 70 mM KCl, 7.5 mM $MgCl_2$

- ◆ 10 × Buffer B: 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 C: 10 mM MgATP solution (1.3 mL ×2 vials)
- ◆ Reagent D: 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

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 (HEK).

3. Apparatuses, Materials and Substrates

3.1. Apparatuses

- Water bath or incubator with temperature control at 37 ºC
- Multichannel Pipettes
- Micropipettes
- Suction filtration device
- Centrifuge for 96-well plate
- LC/MS/MS

3.2. Materials

- Pipettes, tips
- Containers for dilution and preparation (tubes, etc.)
- 96-well glass-fiber filter plate
- 96-well plate (for water removal)
- 96-well deep well plate
- Cap mat for micro plate

3.3. Substrates

Non RI-labeled Substrate

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

4. Preparation of solutions

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

Transporter	Non RI-Substrate Solution	Preparation	Final concentration
MRP1 MRP3	0.2 mM $E_2$17βG in DMSO	Dissolve $E_2$17βG in DMSO to make 0.2 mM solution.	1 µM
MRP2	10 mM $E_2$17βG in DMSO	Dissolve $E_2$17βG in DMSO to make 10 mM solution.	50 µM
MRP4	0.6 mM $E_2$17βG in DMSO	Dissolve $E_2$17βG in DMSO to make 00.6 mM solution.	3 µM
BSEP	0.4 mM TCA in DMSO	Dissolve TCA in DMSO to make 0.4 mM solution.	2 µM

5. Vesicular transport assay

The following is the standard procedure for determination of the ATP-dependent transport activity of positive control substrates. The final concentrations of each substrate are listed above.

The example is conducted in quadruplicate. The experimental conditions, such as volumes, should be appropriately adjusted in each case according to the specific experimental design.

(1) For one assay, dilute 10 µL of ABC Transporter Vesicle Product (HEK) with 10 µL of Buffer A to make 20 µL. Adjust volumes of vesicle preparation if necessary.

(i) <ABC Transporter Vesicle Product (HEK) preparation>	(Per assay)	(For 8 assays)
ABC Transporter Vesicle Product (HEK) (5 mg/mL)	10 µL	80 µL
Buffer A	10 µL	80 µL
	(Total 20 µL/assay)	(Total 160 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
Non RI-labeled substrate solution	5 µL	20 µL
Reagent G or Buffer A	0.5 µL	2 µL
(Add Reagent G depending on the study conditions)		
Buffer A	4.5 µL	18 µL
	(Total 30 µL/assay)	(Total 120 µL)

◆ In the case of inhibitory test, add test compound (inhibitor) in (1). Adjust the final reaction medium to 50 µL, by decreasing the volume of Buffer A accordingly.

(3) Prepare LC/MS/MS standard of substrate solution using elution buffer including internal standard.

(4) 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.

(5) Add 30 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.

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

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

(8) Filter the reaction mixture using 96-well glass-fiber filter plate as follows:

8-1) Add 1×Buffer B to all wells of 96-well glass-fiber filter plate including unused wells, and filter by suction 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.

8-2) Add vesicle product of equivalent to 50 µg protein to each well for standard curve on filterplate.

8-3) Transfer the reaction medium onto pre-wet filters using a multi-channel pipette, and filter by suctioning.

8-4) Wash the filter 5 times with chilled 1×Buffer B (200 µL per well).

In order to obtain reliable results, perform the step from (7) to (8) immediately.

(9) Carefully wipe off any remaining liquid on the back of the filter plate.

♦If the bottom of the filter plate is wet, it is impossible to obtain correct results because it will contaminate other wells during the elution performed in (11) and (12).

(10) To remove residual buffer from the wells, place a filter plate on top of a 96-well plate (for centrifugal water removal) and centrifuge at 550 x g for 1 minute.

(11) Place the filter plate directly onto a 96-well deep-well plate, aligning it carefully for LC/MS/MS sample collection.

(12) Add 50 µL of the elution solution containing the internal standard to each well of the filter plate. In parallel, add 50 µL of each concentration from the standard curve (prepared in step 3) into the corresponding wells designated for standards (prepared in step 8-2).

(13) To collect the eluted samples into the deep-well plate, centrifuge the stacked plates (filter plate on top of deep-well plate) at 550 x g for 2 minutes.

(14) Repeat step (12) and step (13).

(15) Dilute the collected samples with the aqueous mobile phase and analyze by LC/MS/MS.

6. Data analysis

(1) The amount of substrate retained on each filter is calculated using the following formula.

$$\text{Amount of substrate per filter (pmol)} = \text{Concentration of substrate in the reaction mixture (pmol/}\mu\text{L)} \times \text{Volume of extract applied (µL)}$$

(2) The amount of ATP-dependent substrate transport (pmol) was determined by subtracting the amount of substrate measured under the MgAMP condition from that measured under the MgATP condition.

(3) The ATP-dependent substrate transport rate (pmol/mg protein/min) was calculated by dividing the ATP-

dependent transport amount (pmol) by the amount of protein used (0.05 mg) and the reaction time (min).

(4) The apparent transport volume (μL/mg protein/min) was calculated by dividing the substrate transport rate (pmol/mg protein/min) by the substrate concentration in the reaction mixture (μM).

7. Recommended equipment

7.1. Apparatuses

	Maker	Bland Name
96-well glass-fiber filter plate	PALL	AcroPrep™ 96-well Filter Plates, 350 μL, 1.0 μm, glass fiber (#8031)
Suction Filtration device	Millipore	MultiScreenHTS Vacuum Manifold
96-well plate	TFS	96-Well Microplate
96-well deep well plate	TFS	96-well deep well plate (2 mL)
Microplate Cap Mat	TFS	96-Well Pre-Slit Microplate Cap Mat

7.2. Non RI-labeled substrate

・ β-Estradiol 17-(β-D-glucuronide) sodium salt

MW	470.49
Molecular formula	$C_{24}H_{31}O_8Na$
Maker	Sigma

・ Taurocholic acid sodium salt hydrate

MW	537.68 (anhydrous basis)
Molecular formula	$C_{26}H_{44}NNaO_7S \cdot xH_2O$
Maker	Sigma

8. References

・ Iwanaga T, Nakakariya M, Yabuuchi H, Maeda T, and Tamai I. Involvement of bile salt export pump in flutamide-induced cholestatic hepatitis. *Biol Pharm Bull*. **30** (4):739-44 (2007).

・ Yabuuchi H, Tanaka K, Maeda M, Takemura M, Oka M, Ohashi R, and Tamai I. Cloning of the dog bile salt export pump (BSEP; ABCB11) and functional comparison with the human and rat proteins. *Biopharm Drug Dispos*. **29** (8): 441-8 (2008).

・ Yamaguchi K, Murai T, Yabuuchi H, and Kurosawa T. Measurement of the transport activities of bile salt export pump using LC-MS. *Anal Sci*. **25** (9): 1155-8 (2009). ⇒**LC-MS analysis**

・ 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