Vesicular Transport Assay Protocol

For ABC Transporter Vesicle Product
with Fluorescent 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 MDR1	(GenoMembrane, Cat. No. GM0015)
Mouse Mdr1a	(GenoMembrane, Cat. No. GM0004)
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 BCRP	(GenoMembrane, Cat. No. GM0008)
Rat Bcrp	(GenoMembrane, Cat. No. GM0007)

- ABC Transporter Vesicle Product for Negative Control

Control	(GenoMembrane, Cat. No. GM0003)

- Vesicular Transport Assay Reagent Kit

For MDR1	(GenoMembrane, Cat. No. GM3030)
For MRPs and BCRP	(GenoMembrane, Cat. No. GM3010)

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 with fluorescent substrate, *N*-Methylquinidine (NMQ) for MDR1, 5(6)-Carboxy-2', 7'-dichlorofluorescein (CDCF) for MRP2 and MRP3, and Lucifer yellow (LFY) for BCRP.

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 Products (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.

- Stored 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 Set (for MDR1)

◆ 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 E: 10% SDS (13 mL ×1 vial)

  Check "Regent E" whether SDS precipitates in the solution due to low storage temperature, and if necessary, warm to 37°C to dissolve the precipitates.
- ◆ Detection Reagent (Reagent F): 0.1N H_2SO_4 (11 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 Set (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)
- ◆ Raegent 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

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 37ºC
- Micropipettes
- Suction filtration device
- Centrifuge for 96-well plate
- Fluorescence plate reader

3.2. Materials

- Pipettes, tips
- Containers for dilution and preparation (tubes, etc.)
- 96-well glass-fiber filter plate
- 96-well plate for fluorometer (black plate)

3.3. Fluorescent substrates

Transporter	Fluorescent Substrate
MDR1	*N*-Methylquinidine Iodide (NMQ)
MRP2, MRP3	5(6)-Carboxy-2', 7'-dichlorofluorescein (CDCF)
BCRP	Lucifer yellow (LFY)

4. Preparation of fluorescent substrate and Reagent

4.1. Fluorescent substrate

50 μM NMQ	Dissolve NMQ with Buffer A2 and make 1 mM NMQ solution. Then, store the solution at -20℃ in the dark until use. Subsequently dilute the NMQ solution with Buffer A2 to 50 μM before use.
50 μM CDCF	Dissolve CDCF in organic solvent (preferably DMF), and prepare the stock solution of 100 mM. Subsequently dilute the stock solution with buffer A2 to prepare 50 μM CDCF solution.
100 μM LFY	Dissolve LFY in ultra pure water, and prepare the stock solution of 10 mM. Subsequently dilute the stock solution with buffer A2 to prepare 100 μM LFY solution.

◆ *Before conducting the assay, it is recommended to adjust excitation energy and detection time of the fluorescence plate reader to gain more than 40,000/ 5 μL fluorescence intensity per one-well using the prepared the fluorescent substrate solutions.*

4.2. Reagent

Please prepare Reagent E and Detection Reagent as described below by yourself as necessary.

Reagent	Use in Procedure step	Purpose
Reagent E (10% SDS)	5(10) (12)	To lyse and elute the ABC Transporter Vesicle Products from a filter plate.
Reagent F (0.1 N H_2SO_4)	5 (13)	Detection Reagent for NMQ
0.1 N NaOH	5 (13)	Detection Reagent for CDCF
DMSO	5 (13)	Detection Reagent for LFY

***Note**; Some compounds may have the same fluorescent wavelength as the above fluorescent substrate. Thus, it is recommended to confirm the fluorescence intensity of the test compound of interest before conducting inhibition assay to avoid overwrapping of the fluorescent wavelength of the test compound with one of the fluorescent substrate.*

5. Vesicular transport assay

The following is the standard procedure for determination of the inhibitory activity of test compounds. The final concentrations of each fluorescent 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) Dilute ABC Transporter Vesicle Products on ice with Buffer A as follows to prepare 24.5 -x µL.

(i) <ABC Transporter Vesicle Product preparation>	(Per assay)	(For 16 assays)
ABC Transporter Vesicle Products (5 mg/mL)	10 µL	160 µL
Buffer A	14.5 - x µL	232-16x µL
(Total	24.5 - x µL/assay)	(Total 392-16x µL)

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

(ii) <Assay mixture>	(Per assay)	(8 assays for ATP/AMP)
Reagent C or D	20 µL	160 µL
Fluorescent substrate solution	5 µL	40 µL
Test compound	x µL	8x µL
Reagent G or Buffer A	0.5 µL	4 µL
(Add Regent G depending on study condition)		
(Total	25.5 + x µL/assay)	(Total 204+8x µL)

◆ ***Add the test compound (inhibitor) at the step (1) or (2), depending on the purpose of the study. In this protocol, the inhibitor is added at step (2).***

(3) Preincubate 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 preincubated assay mixture containing Reagent C or D (ii) to an assay tube 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).

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

(8) Remove liquid on the bottom of 96 well glass fiber filter plate by wiping thoroughly or shaking out.

◆ *When the bottom of 96 well glass fiber filter plate is wet, the extract solution by 10% SDS in step (11) might be entered to unexpected wells of fluorometer plate, and it causes an odd result.*

(9) Place the fluorometer plate under the glass fiber filter plate.

(10) Add 50 μL of 10% SDS (Reagent E) to each well of the filter plate.

(11) Centrifuge the filter plate with fluorometer plate at 2000 rpm for 1 minute and collect dissolved vesicles to the fluorometer plate.

(12) Repeat step (10) and step (11).

(13) Add 100 μL of Detection Reagent to each well of the fluorometer plate.

(14) Measure the fluorescence intensity of each well with adequate wave length using a fluorescent plate reader.

◆ *To measure the total fluorescence intensity in one reaction solution, add the 5 μL of prepared fluorescent substrate to empty wells of same glass fiber filter plate which were pre-wetted at the step (7-1). And conduct step from (9) to (14). If necessary, measure background of empty wells as blank.*

6. Data analysis

(1) The amount (pmol) of Fluorescent substrate per one well can be calculated using the following formula.

$$\text{Amount of substrate per one well (pmol)} = \frac{\text{Fluorescence intensity of each well}}{\text{Total fluorescence intensity}^{*1}} \times \text{Amount of substrate in a reaction medium (pmol)}^{*2}$$

*1; Refer to the #5 (14) above

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

	Substrate	Final Conc.	Amount of substrate in a reaction medium
MDR1	NMQ	5 μM	250 pmol (= 50 μM × 5 μL)
MRP2	CDCF	5 μM	250 pmol (= 50 μM × 5 μL)
MRP3	CDCF	5 μM	250 pmol (= 50 μM × 5 μL)
BCRP	LFY	10 μM	500 pmol (= 100 μM × 5 μL)

(2) Subtract the amount of substrate (pmol) with MgAMP from that with MgATP, and thereby obtain the amount of ATP-dependent fluorescent 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) 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	Pall	AcroPrep™ 96-well Filter Plates, 350 µL, 1.0 µm, glass fiber (#8031)
Suction Filtration device	Millipore	MultiScreenHTS Vacuum Manifold
Fluorometer plate	Nunc	U96 Microwell plate black (#267342, etc)

7.2. Fluorescence substrate

・ *N*-Methylquinidine (NMQ)

MW	374.90
Molecular formula	$C_{21}H_{27}ClN_2O_2$
Ex	355 nm (use a 355 nm filter)
Em	448 nm (use a 460 nm filter)
Maker	Sigma #SBNMQ

・ 5(6)-Carboxy-2', 7'-dichlorofluorescein (CDCF)

MW	445.21
Molecular formula	$C_{21}H_{10}Cl_2O_7$
Ex	495 nm (use a 485 nm filter)
Em	529 nm (use a 535 nm filter)
Maker	Life Technologies #C368

・ Lucifer yellow (LFY)

MW	521.56
Molecular formula	$C_{13}H_9K_2N_5O_9S_2$
Ex	427 nm (use a 405 nm filter)
Em	535 nm (use a 535 nm filter)
Maker	Life Technologies #L1177

8. References

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