Protocol for

TRANSiPORT (G) Cells

This protocol contains necessary reagents and the procedures for thawing, seeding and cell-based assay of HEK293 cell lines transiently expressing the following SLC transporter.

TRANSiPORT (G) Cells

Human OATP1B1 (G)	(GenoMembrane, Cat. No. GM1102G)
Human OATP1B3 (G)	(GenoMembrane, Cat. No. GM1106G)
Human OAT1 (G)	(GenoMembrane, Cat. No. GM1103G)
Human OAT3 (G)	(GenoMembrane, Cat. No. GM1104G)
Human OCT1 (G)	(GenoMembrane, Cat. No. GM1108G)
Human OCT2 (G)	(GenoMembrane, Cat. No. GM1105G)
Human MATE1 (G)	(GenoMembrane, Cat. No. GM1114G)
Human MATE2K (G)	(GenoMembrane, Cat. No. GM1115G)
Human NTCP (G)	(GenoMembrane, Cat. No. GM1113G)
Human OATP2B1 (G)	(GenoMembrane, Cat. No. GM1107G)
Human OATP1A2 (G)	(GenoMembrane, Cat. No. GM1116G)
Human OCT3 (G)	(GenoMembrane, Cat. No. GM1118G)
Rat Ntcp (G)	(GenoMembrane, Cat. No. GM1120G)
Rat Oatp1a4 (G)	(GenoMembrane, Cat. No. GM1121G)
Rat Oatp1b2 (G)	(GenoMembrane, Cat. No. GM1122G)
Mock (G)	(GenoMembrane, Cat. No. GM1101G)

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1. Introduction

Cell-based assay using Transporter Transiently Expressing Cell lines allows evaluating the interaction of compounds with the transporter of interest. TRANS*i* PORT (G) Cells are HEK293 cells transiently overexpressing a SLC transporter protein. One vial contains 8-12 million cells designed for one 24-well plate for single use.

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

We recommend to use the Mock (G) (Cat. No. GM1101G) as a negative control depending on your needs since high background is observed in some compounds due to endogenous transporter of HEK293 cells and/or non-specific binding.

2. Kit Contents and Storage

◆ TRANSi PORT (G) Cells (9.6-14.4 million cells in 1 mL freezing medium)

For cell-based assay, one 24-well plate assay or 96-well plate assay can be performed with one vial of the TRANSi PORT (G) Cells based on this protocol.

- Stored at liquid nitrogen container
- Transport activity for each lot; written in the Data Sheet available at the following homepage
  https://www.genomembrane.com/product-1

3. Reagents, Equipment and Materials Requirements

3.1 Reagents

- DMEM (SIGMA, D6046, etc.)
- FBS* (Biowest, S1810, etc.)
  ***Incubate at 55 °C for 30 minutes to inactivate prior to use**
- 10x HBSS (Gibco, 14065056, etc.)
- HEPES (nacalai tesque, 02443-34, etc.)
- Tricine (nacalai tesque, 02437-24, etc.)
- MES (nacalai tesque, 02442-44, etc.)
- 1M NaOH (nacalai tesque, 37421-05, etc.)
- 1M HCl (nacalai tesque, 37314-15, etc.)
- Total protein assay reagents (PIERCE, 23225, etc.)

3.2 Equipment

- CO_2 incubator
- Tissue culture hood

- Thermostatic water bath
- Vacuum pump
- Inverted microscope
- Low-speed centrifuge
- Hot Plate
- Pipet-Aid, micropipette, etc.

3.3 Materials

- Poly-Lysine coat 24 well plate (Corning, 356414, etc.)
- Poly-Lysine coat 96 well plate (Corning, 356461, etc.)

  Note; *Using a plate other than a poly-Lysine coated plate may cause a cell detachment when the assay is performed. We recommend using a Poly-Lysine coated plate.*
- Disposable pipette, disposable tips, conical tube, etc.

4. Preparation of solution

Buffer

a) Mix all reagents as shown in the table below. Adjust pH with 1M NaOH and make the solution to the designated volume.

[pH 7.4]

Reagent	Final Concentration	Amount (g or mL)
10x HBSS	1x	50 mL
HEPES	25 mM	2.979 g
Milli-Q Water		Appropriate volume
Total Volume		500 mL

[pH 8.0 or 8.4]

Reagent	Final Concentration	Amount (g or mL)
10x HBSS	1x	50 mL
Tricine	25 mM	2.240 g
Milli-Q Water		Appropriate volume
Total Volume		500 mL

[pH 6.0]

Reagent	Final Concentration	Amount (g or mL)
10x HBSS	1x	50 mL
MES	25 mM	2.666 g
Milli-Q Water		Appropriate volume
Total Volume		500 mL

b) Store in a refrigerator.

- ◆ Expiration of buffers is 3 months after preparation.

5. Methods

5.1 Thawing and seeding Cells

All cell culture procedures will be performed in a sterile tissue culture hood

(1) Warm DMEM containing 10 % heat inactivated FBS (culture medium) at 37 °C.
(2) Add 9 mL of culture medium in a 50 mL conical tube.
(3) Incubate the vial in water bath until the vial is ~50% thawed. There should still be a small, visibly frozen portion remaining in the vial.
(4) Decant the vial into 9 mL culture medium in a 50 mL conical tube.
(5) Rinse the vial once by adding 1 mL culture medium and bring back into the 50 mL conical tube.
(6) Gently invert 50 mL conical tube a few times to mix.
(7) Centrifuge at 220 × g for 5 minutes.
(8) Remove the supernatant.
(9) Suspend cell pellet with 15 mL fresh culture medium.
    * It is possible to thaw and seed the cells without centrifugation. All you need is to perform the following steps instead of (2)-(9).
        i Add 14 mL of culture medium in a 50 mL conical tube.
        ii Incubate the vial in water bath until the vial is ~50% thawed. There should still be a small, visibly frozen portion remaining in the vial.
        iii Decant the vial into 14 mL culture medium in a 50 mL conical tube.
        iv Rinse the vial once by adding 1 mL culture medium and bring back into the 50 mL conical tube.
        v Mix thoroughly using pipette.
(10) Plate 500 μL (for 24-well plate) or 125 μL (for 96-well plate) cell suspension into each well.
    Note; *Using a plate other than a poly-Lysine coated plate may cause a cell detachment when the assay is performed. We recommend using a Poly-Lysine coated plate.*
(11) Gently rock the plate to spread cells evenly.
(12) Incubate the plate at 37 °C with 5 % CO_2.
(13) After 3-4 hours, remove the medium and feed with warm culture medium.
(14) Return plates to CO_2 incubator and incubate until the transport assay 14-27 h after.

5.2 Cell-based assay

Note;

** Conduct the transport assay on a hot plate with appropriate device to keep the cells at 37 ºC.*

** HEK293 cells are easily detached, thus it is recommended to add any solution gently along the inside wall of plate well. Avoid pouring directly on the cells.*

**** Please note the pH of Transport Buffer***

* Pre-incubation Buffer (#1, #4, #5): pH7.4 for all transporters.

* Dosing Solution (#3, #6): pH8.0 for MATE1, pH8.4 for MATE2K, pH6.0 for OATP1A2 and pH7.4 for the other transporters.

(1) Warm adequate amount of buffer at 37 ºC.

(2) Chill adequate amount of buffer until use at #8.

(3) Preparation of **Dosing Solution**.

i) Uptake assay

Prepare **Test Compound Solution (Substrate)** at the designated concentration with the buffer, warm at 37 ºC.

ii) Inhibition assay

A) **Probe Substrate Solution**

Prepare the solution at the double (×2) of the final concentration with the buffer.

B) **Test Compound Solution (Inhibitor)**

Prepare the solution at the double (×2) of the designated concentration with the buffer.

C) **Mixture Solution**

Mix equal volume of "**Probe Substrate Solution**" and "**Test Compound Solution (Inhibitor)**" to 1:1, warm at 37 ºC.

Note; *Use organic solvent (DMSO is mostly appropriate) to dissolve the test compound, if necessary, note that the final concentration of DMSO must be in less than 0.5 % in the assay.*

(4) Remove the medium from the wells, wash the cells with the pre-warmed buffer (500 µL for 24-well plate, 200 µL for 96-well plate).

(5) Remove the buffer from the wells, add pre-warmed buffer (500 µL for 24-well plate, 200 µL for 96-well plate), and incubate for 10 min.

(6) Remove the buffer, add pre-warmed **Dosing Solution** (250 µL for 24-well plate, 100 µL for 96-well plate) prepared at #3 to start the transport assay.

(7) Remove the buffer from an each well immediately after appropriate time point to stop the assay.

(8) Wash the cells by quick adding/aspirating procedure with chilled buffer (500 µL for 24-well plate, 200 µL for 96-well plate). Repeat washing procedure 3 times quickly.

* It is recommended to conduct this procedure (#7 and #8) for each well one by one to obtain stable result due to the transport assay being performed in a short time.

* It is important to conduct this procedure (#7 and #8) as quickly as possible.

(9) Measure the amount of the compound transported into the cell by appropriate method.

(Reference)

【For Liquid scintillation counter】

a. Add 1M NaOH (150 µL for 24-well plate, 75 µL for 96-well plate), allow to stand for more than 15 min.
   - Examine if the cells are lysed under a microscope.

b. Add 1M HCl (150 µL for 24-well plate, 75 µL for 96-well plate) to the lysate.

* Use a portion of the lysate for protein determination; 50-200 µg/well for 24-well plate and 10-50 µg/well for 96-well plate.

【For LC-MS/MS】

24-well

a. Add 100-200 µL of ultra-pure water into each well.
b. Scrap the cells with a cell-scraper.
c. Collect the cell suspension in a tube
d. Apply ultrasonic wave to each tube.
e. Transfer a portion of the cell suspension to new tube. Then, add the equal volume of acetonitrile.
   * Use a portion of the lysate for protein determination; 50-200 µg/well for 24-well plate.
f. Centrifuge for 10 minutes at 20,000× g under 4 ˚C.
g. Analyze the supernatant.

96-well

a. Add 150 µL of 50% acetonitrile into each well.
b. Stand for 20 minutes at room temperature.
c. Centrifuge for 10 minutes at 550 × g under 4 ˚C.
d. Analyze the supernatant.
   * Prepare wells for protein determination or perform protein determination after diluting the supernatant until acetonitrile is no longer affected; 10-50 µg/well for 96-well plate.

5.3 Data analysis

5.3.1. Uptake assay

Divide the amount of the compound transported into the cell (pmol/well) by the reaction time (min) and the amount of protein in the well (mg protein/well) to obtain the uptake velocity per unit amount of protein (pmol/min/mg protein). Dividing the uptake velocity (pmol/min/mg protein) by concentrations (µM) gives the uptake clearance (CL_{uptake}: µL/min/mg protein).

K_m and **V_{max}** are calculated by using the following equations.

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

***V_{max}** represents the maximum uptake rate, **S** is the substrate concentration in the medium, and **K_m** is the Michaelis constant.

5.3.2. Inhibition assay

i) **IC_{50}** is calculated by using the following equations.

$$CL_{uptake\ (+Inhibitor)} = \frac{CL_{uptake\ (control)}}{1 + [I]/IC_{50}}$$

***I** represents the concentration of the inhibitor, and **IC_{50}** is the half maximal inhibitory concentration.

ii) Calculate inhibition constant (**K_i**) as follows.

$$K_i = \frac{IC_{50}}{1 + [S]/K_m}$$

*When the substrate concentration is markedly lower than K_m, K_i can be approximated to IC_{50}.

6. Recommended Substrate

Transporter	Substrate	Cat. No.
hOATP1B1 hOATP1B3	Estradiol-17β-D-glucronide	・Estradiol-17β-D-glucuronide, [Estradiol-6,7-3H(N)]- (American Radiolabeled Chemicals Inc., #ART1320), etc. ・β-Estradiol 17-(β-D-glucuronide) sodium salt (Sigma, # E1127), etc.
hOAT3 hOAT4 hOATP2B1 hOATP1A2	Estrone-3-sulfate	・Estrone Sulfate, Ammonium Salt, [6, 7-^{3}H(N)]- (PerkinElmer, #NET-203), etc. ・Estrone 3-sulfate potassium salt (Sigma, E9145), etc.
hOAT1	*p*-aminohippuric acid	・Aminohippuric acid, *p*-[Glycyl-2-^{3}H]- (PerkinElmer, #NET-053), etc. ・p-Amino hippuric acid (Sigma, A1422), etc.
hOCT2 hMATE1 hMATE2K	Metformin	・Metformin hydrochloride [biguanido-^{14}C] (1,1-Dimethyliguanide hydrochloride ^{14}C) (American Radiolabeled Chemicals Inc., #ARC1738) , etc. ・Metformin hydrochloride (Sigma, D5035), etc.
hOCT1	Tetraethylammonium	・Tetraethylammonium Bromide, [ethyl 1-^{14}C]- (American Radiolabeled Chemicals Inc., #ARC0577), etc. ・Tetraethylammonium Chloride (Sigma, T2265), etc.
hNTCP rNtcp rOatp1a4 rOatp1b2	Taurocholic acid	・Taurocholic acid, [^{3}H(G)] (PerkinElmer, #NET-322), etc. ・Taurocholic acid sodium salt hydrate (Sigma, T4009), etc.
hOCT3	ASP (4-(4-(dimethylamino)styryl)-N-methylpyridinium)	・4-(4-(dimethylamino)styryl)-N-methylpyridinium) (Sigma,#D3418), etc.

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