Procedures for Thawing, Seeding, and Cell-based Assay of Cryopreserved Transporter Transiently Expressing HEK293 Cell Lines

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.

Transporter Transiently Expressing Cell Lines

Human OATP1B1	(GenoMembrane, Cat. No. GM1002)
Human OATP1B3	(GenoMembrane, Cat. No. GM1006)
Human OAT1	(GenoMembrane, Cat. No. GM1003)
Human OAT3	(GenoMembrane, Cat. No. GM1004)
Human OCT1	(GenoMembrane, Cat. No. GM1008)
Human OCT2	(GenoMembrane, Cat. No. GM1005)
Human MATE1	(GenoMembrane, Cat. No. GM1014)
Human MATE2K	(GenoMembrane, Cat. No. GM1015)
Human NTCP	(GenoMembrane, Cat. No. GM1013)
Mock	(GenoMembrane, Cat. No. GM1001)

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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. Transporter Transiently Expressing Cell Lines products 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 (Cat. No. GM1001) 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

◆ Transporter Transiently Expressing Cell Lines products (8-12 million cells in 1 mL freezing medium)

For cell-based assay, 24 assays (1 plate) can be performed with one vial of the Transporter Transiently Expressing Cell Lines products 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.lifetechnologies.com/

3. Reagents, Equipment and Materials Requirements

3.1 Reagents

- DMEM (SIGMA, D6046, etc.)
- FBS* (Biowest, S10581S1820, etc.)

  ***Incubate at 55 °C for 30 minutes to inactivate prior to use**
- 10x HBSS (Gibco, 14065056, etc.)
- Sodium butyrate (Wako, 193-01522, etc.)
- HEPES (nacalai tesque, 02443-34, etc.)
- Tricine (nacalai tesque, 02437-24, 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.)
- 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

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 15 mL conical tube.
(3) Incubate the vial in water bath until the vial is ~90% 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 15 mL conical tube.
(5) Rinse the vial once by adding 1 mL culture medium and bring back into the 15 mL conical tube.
(6) Gently invert 15 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 12.5 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 11.5 mL of culture medium in a 15 mL conical tube.
    ii Incubate the vial in water bath until the vial is ~90% thawed. There should still be a small, visibly frozen portion remaining in the vial.
    iii Decant the vial into 11.5 mL culture medium in a 15 mL conical tube.
    iv Rinse the vial once by adding 1 mL culture medium and bring back into the 15 mL conical tube.
    v Mix thoroughly using pipette.
(10) Plate 500 μL cell suspension into each well of 24 well poly-Lysine coat 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.
    ◆ Instead you can use culture medium supplemented with 5 mM sodium butyrate in order to induce the expression of some transporters, however, it is known to have cytotoxicity. Refer to the datasheet for more information.
(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.*

* ***pH gradient****;*

i) ***MATE1:*** *Use pre-warmed buffer (pH 7.4) for cell washing and pre-incubation (#4 and #5), change to the buffer* ***(pH 8.0)*** *pre-warmed at 37 ºC from addition of test compound (#3 and #6), followed by washing with chilled buffer* ***(pH 8.0)*** *(#8).*

ii) ***MATE2K:*** *Use pre-warmed buffer (pH 7.4) for cell washing and pre-incubation (#4 and #5), change to the buffer* ***(pH 8.4)*** *pre-warmed at 37 ºC from addition of test compound (#3 and #6), followed by washing with chilled buffer* ***(pH 8.4)*** *(#8).*

(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 500 µL pre-warmed buffer.

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

(6) Remove the buffer, add pre-warmed 250 µL **Dosing Solution** 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 500 µL chilled buffer. 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) Add 150 µL 1M NaOH, allow to stand for more than 15 min.

* Confirm the cells being lysed with microscope.

(10) Add 150 µL 1M HCl to the lysate.

* Use a portion of the lysate for protein assay, in which a range of 20-2,000 µg/ml is recommended for measurement.

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

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}.

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