Protocol for RPTEC/TERT1-OCT2 Uptake of 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+) or N,N,N-Trimethyl-2[(7-nitro-1,2,3-benzoxadiazol-4-yl)amino] ethanaminium iodide(EAM-1)

There is an abundance of OCT2 transporter in kidney tissue. However, primary cells lose OCT2 expression after just a few days in culture. To facilitate hOCT2 *in vitro* toxicity studies we have generated RPTEC/TERT1- OCT2 (ATCC® CRL-4031-OCT2™) cell line by stably expressing hOCT2 into the RPTEC/TERT1 (ATCC® CRL-4031™) cell line.

Functionality of the RPTEC/TERT1- OCT2 was demonstrated by uptake of 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP+) or N,N,N-Trimethyl-2[(7-nitro-1,2,3-benzoxadiazol-4-yl)amino] ethanaminium iodide (EAM-1). ASP+ is fluorescent in the RED channel and EAM-1 is fluorescent in the GREEN channel; both are readably been taken up by the OCT2 transporter, after uptake, the cells can be visualized under a fluorescent microscope or lysed and read on a fluorescent plate reader.

Materials:

Material	Company	Catalogue Number
RPTEC/TERT1-OCT2	ATCC	CRL-4031-OCT2
RPTEC/TERT1	ATCC	CRL-4031
Trypsin-EDTA for Primary Cells	ATCC	PCS-999-003
HBSS	Corning	21-023-CV
ASP^+	Thermo Fisher	D288
EAM-1	Macrocyclics	D-100
Black 96-well plates	Corning	354649
M-Per lysis buffer	Thermo Fisher	78501
RPTEC media		
Basal media, DMEM:F12	ATCC	30-2006
Growth kit	ATCC	ACS-4007

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Protocol:

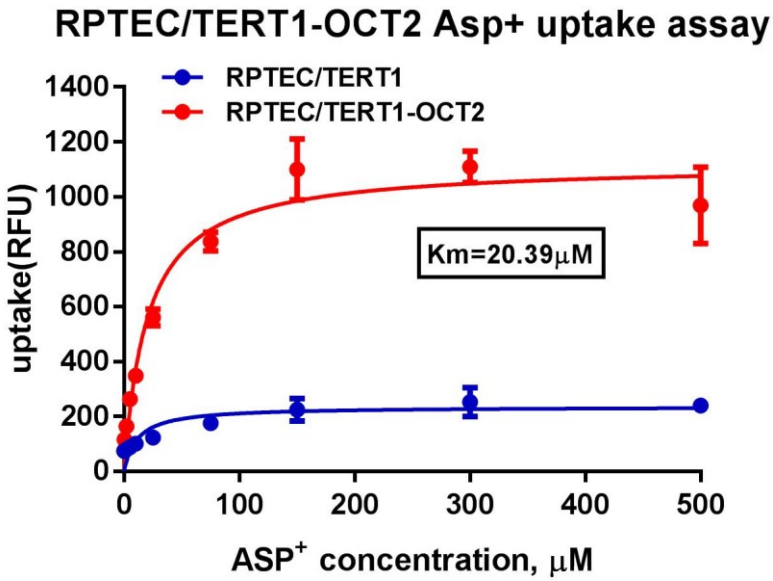
A. Seeding cells

1. Make complete RPTEC media by adding 5 mL of component A and 8mL of component B to the basal DMEM:F12 media, mix well.
2. Thaw both RPTEC/TERT1 and RPTEC/TERT1-OCT2 cells and seed at 1.5-2.0 cells/cm^2 in T75cm tissue culture flasks in RPTEC media (for RPTEC/TERT1-OCT2, need to add 0.3 µg/mL puromycin for long term culturing). Passage cells at least one time prior to seeding cells in 96-well plates for the uptake assay.
3. Trypsinize both RPTEC/TERT1 and RPTEC/TERT1-OCT2 cells and re-suspend each cell line at a concentration of $5x10^5$ cells/mL in RPTEC media without antibiotic.
4. Plate 200 µL of cells into each well of a 96-well collagen I coated plate ensuring that both cells have a minimum of three replicates each.
5. Incubate the cells for 24–36 hours at 37°C and 5% CO_2 incubator.

B. ASP^+/EAM-1 uptake assay

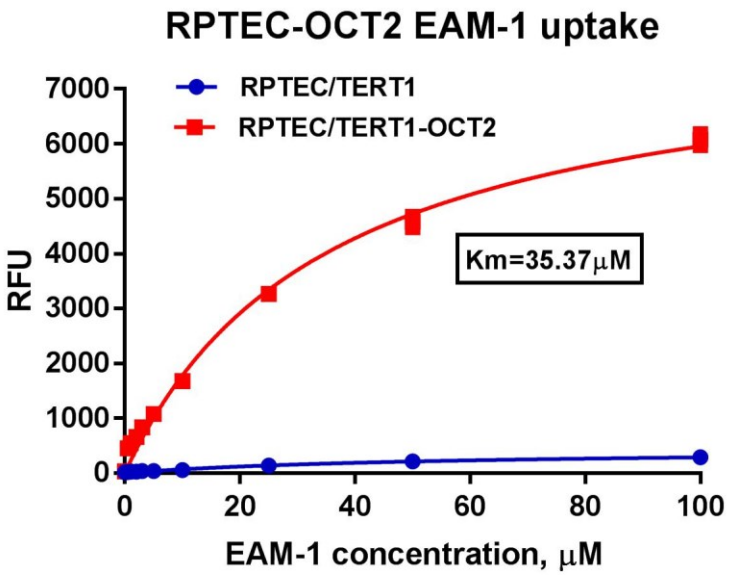
1. Make sure the cells are more than 90% confluent before you start the assay.
2. Wash the cells two times with 200 µL warm (37°C) HBSS in the 96-well plate.
3. Incubate the 2nd wash at 37°C and 5% CO_2 for 10 minutes; it is important to remove all remaining wash liquid to the last drop using a pipet.
4. Add 100 µL of 100 µM of ASP+ or 5 µM of EAM-1 in warm HBSS and incubate at 37°C and 5% CO_2 for 20 minutes.
5. Remove all remaining ASP+ or EAM-1 to the last drop by using pipet tips.
6. Wash 4 times with cold (4°C) HBSS.
7. (Optional) The last wash can remain on the cells for up to 10 minutes to visualize the cells in the Red (for ASP+) or GREEN (for EAM-1) channel.
8. Remove the wash to the last drop using pipet tips and add 100 µL of M-Per lysis buffer.
9. Incubate for 10 minutes at room temperature protected from light.
10. Read on a fluorescent plate reader at 525_{ex}/$580\text{-}640_{em}$ (for ASP+) or 490_{ex}/$510\text{-}580_{em}$ (for EMA-1).
11. Calculate the fluorence intensity ratio of ASP+ or EAM-1 over parental.

Uptake ratio= RFU of (RPTEC/TERT1-OCT2-Blank) / RFU of (RPTEC/TERT1-Blank)



Asp+ concentration	0μM	0.5μM	2μM	5μM	10μM	25μM	75μM	150μM	300μM	500μM
uptake ratio	1.017543	1.523934	2.033221	2.989123	3.455444	4.540586	4.776906	4.883032	4.38785	4.050386

Fig. 1. RPTEC/TERT1-OCT2 ASP+ uptake assays: cells were seeded on 96 well plate, 24 hours later, different concentration of Asp+ were added in the HBSS buffer and incubate at 37°C for 20 minutes; uptake was stopped by washing the wells with ice-cold HBSS, after 4 wash of the HBSS, a 100 µL of lysate buffer was added to each well, fluorescence intensity is measured using the Promega GloMax®-Multi Detection System



EAM-1 concentration	0μM	0.5μM	1μM	2μM	3μM	5μM	10μM	25μM	50μM	100μM
uptake ratio	2.267334	21.56116	23.0057	22.65206	22.73024	28.13118	29.92067	23.72269	21.58114	21.21723

Fig. 2. RPTEC/TERT1-OCT2 EAM-1 uptake assays: cells were seeded on 96 well plate, 24 hours later, different concentration of EAM-1 were added in the HBSS buffer and incubate at 37°C for 20 minutes; uptake was stopped by washing the wells with ice-cold HBSS, after 4 wash of the HBSS, a 100 µL of lysate buffer was added to each well, fluorescence intensity is measured using the Promega GloMax®-Multi Detection System