

3D Human Blood Brain Barrier

Catalog #: 3D45002

Storage: Store at -80° C until ready for use (for up to one month). After thawing, keep in an incubator at 37°C

Format: 6, 12 or 24 well

Storing of BBB model thawing-sol and incubation medium

BBB is stored at **-80 °C** and should be used within one month.

Media is stored at below **-20°C**.

General Information

The blood brain barrier (BBB) specifically regulates molecular and cellular flux between the blood and the nervous tissue. We develop and characterize a highly reproducible Human in vitro model of the BBB using co-cultures of primary Human brain endothelial cells (HBEC), Human brain pericytes, and Human brain astrocytes to study receptors involved in transcytosis across the endothelial cell monolayer. Many drugs developed to treat Central Nervous System (CNS) disorders are unable to reach the brain parenchyma in therapeutically relevant concentrations. The BBB protects brain nervous tissue from the fluctuation of plasma composition, from pathogenic agents, and maintains homeostasis of the brain parenchyma by restricting non-specific flux of ions, peptides, proteins and even cells into and out the brain.

Model Components:

- 3 Culture Medium, 100 mL each
- Frozen Model
- Suture Removal Kits

Astrocytes and brain pericytes help to develop and maintain specific BBB characteristics in brain capillary endothelial cells. Co-culture of the three cell types in our 3D Human BBB model led to the enhancement of barrier properties; an increase in expressions of tight junction proteins of occludin, claudin-5 and ZO-1 and continuous localizations of ZO-1 and claudin-5.

Our model mimic transport properties of the BBB due to the formation of tight junctions, higher expression of specific carriers, or great cell viability. We developed a 3D in vitro model of the BBB by culturing brain endothelial cells with pericytes and astrocytes layered in an insert. **This model improves endothelial cell polarization and enhance the formation of tight junctions, provide better endothelial cell-to-cell contact that is important for barrier development, and prevent the dilution of secreted neurotrophic factors, and these conditions collectively led to the development of an in vitro model that can truly mimic the BBB.**

Advantages:

- 1) Cells used in the 3D model are all human cells; results obtained are more relevant to human situations rather than those data from animal models, i.e. CAM et al.
- 2) The whole process can be monitored (from cell inoculation to the end of experiment), therefore, more crucial information can be acquired at multiple time points from a single experiment.
- 3) No need to perform post-experimental staining for endothelial markers, this is particularly important, if those markers are changed in experimental conditions involved in the studies.

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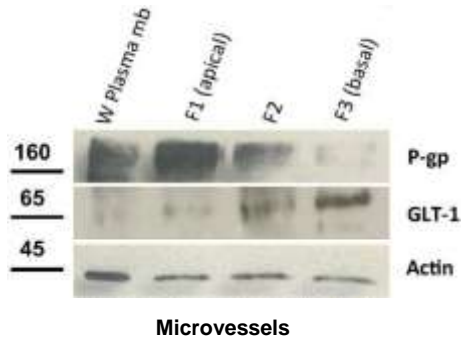
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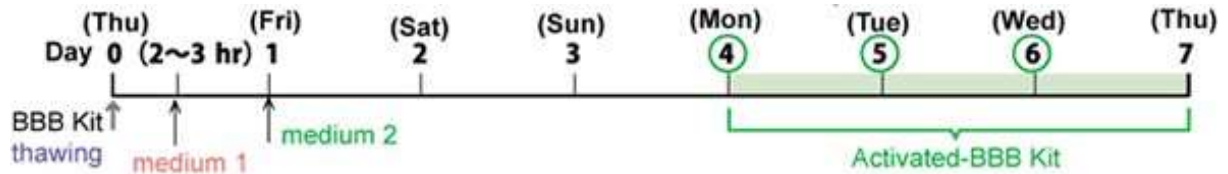
The 3D Human BBB model can be use, but not limited to:

- Drug BBB permeability assay
- Research on BBB physiology
- Cell-cell interactions
- Transport pathway modulations
- Research on BBB toxicology
- Brain endothelial toxicity assays
- Research on BBB pathology
- Disease modeling
- Transport and permeability studies from ions to macromolecules: effect of physiological or pathogenetic factors
- Paracellular barrier and cell polarity studies: TJ protein expression, distribution, polarized distribution of transport proteins, receptors, enzymes etc
- Studies on endo- and transcytosis, receptor-ligand interactions
- Drug transport, drug effect on permeability, localization of receptors, polarity of drug responses
- Co-culture studies: cell-cell and cell-matrix interactions
- Microbial pathogenesis: virus, bacteria, parasite attachment, invasion and penetration
- Compounds screening neuroimmune targets



Protocol

We deliver the 3D Human BBB model in frozen packaged with dry ice. The 3D Human BBB model can be frozen as a whole and stored at -80°C. 4 days prior to your experiment, you just thaw your 3D Human BBB model stored at -80°C. Please read the protocol carefully before thawing the model.



***Volume of media to use when feeding models after thawing:**

Plates and Inserts	Blood-side Volume (µL)	Brain-side Volume (µL)
6 well plate	2000	1500
12 Well Plate	500	1500
24 well plate	200	500

3. Protocol (procedure of activating BBB model)

thawing*^{1~8} → medium 1*^{9~10} → incubation*¹¹ → medium 2*^{12~14} → incubation*¹⁵ → experiment*¹⁶ Medium 3

medium 1 Cat.# BBB-GM001; Blood Brain Barrier Growth Media 100mL. – For use when thawing the model.

medium 2 Cat.# NMBBB001; Endo-Neuro-Pharmaceuticals Media 100mL - For use while culturing the model

Medium 3 Cat.# TMBBB001; Blood Brain Barrier Transportation Media 100mL – For use during permeability assays

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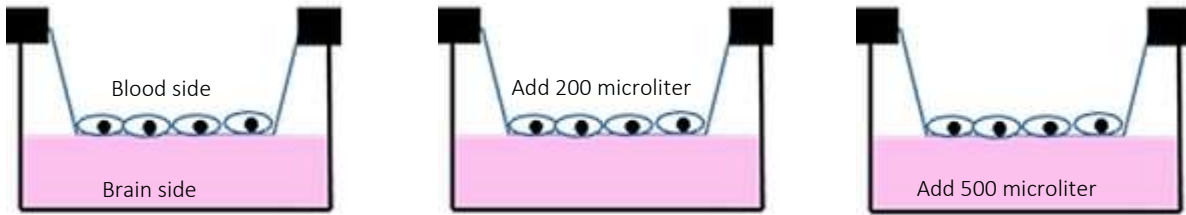
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Thawing the 3D Model:

Image: Volumes of media for a 24 well model (refer protocol for other model sizes):



1. Warm **medium 1 (Blood Brain Barrier Growth Media)** to 37 °C, prior to De-freezing and warm up BBB model. (Move frozen medium 1 to 37 °C water-bath.)
2. Once at 37 °C move **medium 1** to clean bench.
3. Move frozen BBB model to clean-bench. Take off seals. (Do not take a minute.)
4. Wipe up water drops (humidity) on BBB model with clean papers.

[On thawing (day 0)]



Do not touch membrane of insert with pipette, and do not move insert, during procedures of #1 to #9.

5. Under a biosafety cabinet Add 1,000 μL **medium 1** for 6 and 12 well models. (Add 500 μL for 24 well models) to Brain-side, through an opening between Inserts.
6. Add 630 μL thawing-sol (**medium 1**) for 6 and 12 well models (add 200 μL for 24 well models) to Blood-side (inside of Insert)
7. Place model in a 37° C incubator with 5% CO₂. **Note: DO NOT MOVE THE INSERTS UNTIL THE MODEL IS FULLY THAWED.**
8. Once thaw removes old medium on both sides by gently pipetting.

Plates and Inserts	Blood-side Volume (μL)	Brain-side Volume (μL)
6 well plate	2000	1500
12 Well Plate	500	1500
24 well plate	200	500

* Please see above chart: **Volume of media to use when feeding models after thawing** for correct working volume.

9. Incubate for 2-3 hours in a 5% CO₂ incubator.
10. After 2-3 hours, remove the media and repeat step 3 and 4 using 37° C **Endo-Neuro-Pharmaceuticals Media**
11. Incubate overnight in a 37° C incubator with 5% CO₂ until the BBB kit is activated on day 4,5,6, and 7. Use **Medium 3** (Cat.#TMBBB001); Blood Brain Barrier Transportation Media when testing the penetration of a molecule through the brain endothelial and pericytes layer of BBB.
12. You can perform your experiment in days, 4, 5, 6, and 7 after thawing.
13. See cells with inverted microscope.

Endothelial cells on Polycarbonate membrane of BBB model cannot be seen by microscope, therefore

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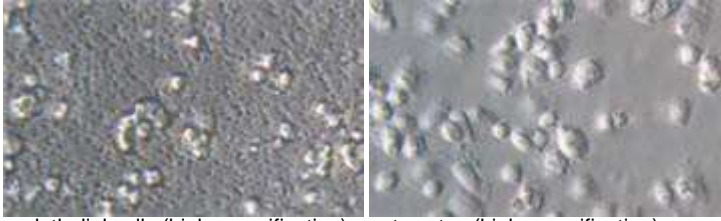
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microscopic examination for astrocytes on bottom side of lower compartment must be done to check cell-proliferation.

BBB model



endothelial cells (high magnification) astrocytes (high magnification)

18. See astrocytes through polycarbonate membranes with inverted microscope.

BBB model

TEER (trans endothelial electrical resistance) in BBB model

TEER in BBB model reaches more than $150 \Omega \times \text{cm}^2$, and maintains a plateau up to 7 days. (Thawing-sol/ medium 1, and medium 2 do not contain cAMP and its analogs.) *BBB model can be used from Day 4 to Day 6 after thawing.

Calculating TEER

TEER calculation is dependent on the model size. See below for the different well formats:

TEER measurement :

12 well TEER ($\Omega \times \text{cm}^2$) = (Total R - Blank R) x 0.33.

6 well TEER ($\Omega \times \text{cm}^2$) = (Total R - Blank R) x 1.72

24 well TEER ($\Omega \times \text{cm}^2$) = (Total R - Blank R) x 1.57

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Sample TEER Calculation – 12 Well Plate

TEER measurement : $12 \text{ well TEER } (\Omega \times \text{cm}^2) = (\text{Total R} - \text{Blank R}) \times 0.33$

1. Calculate Blank R = (Probe reading in 1xPBS) – (Probe and blank insert reading)



Probe reading in 1XPBS

$807 - 247 = 560$

560 = Blank R



Probe and blank insert reading.

2. Measure insert:

Total R read out: 1238

TEER: $12 \text{ well TEER } (\Omega \times \text{cm}^2) = (\text{Total R} - \text{Blank R}) \times 0.33$

$= 1238 - 560 = 678 \times 0.33 = 223.74 \Omega \times \text{cm}^2$



3. Minimum TEER value for activated BBB should be than $150 \Omega \times \text{cm}^2$. This insert is activated and ready to use.

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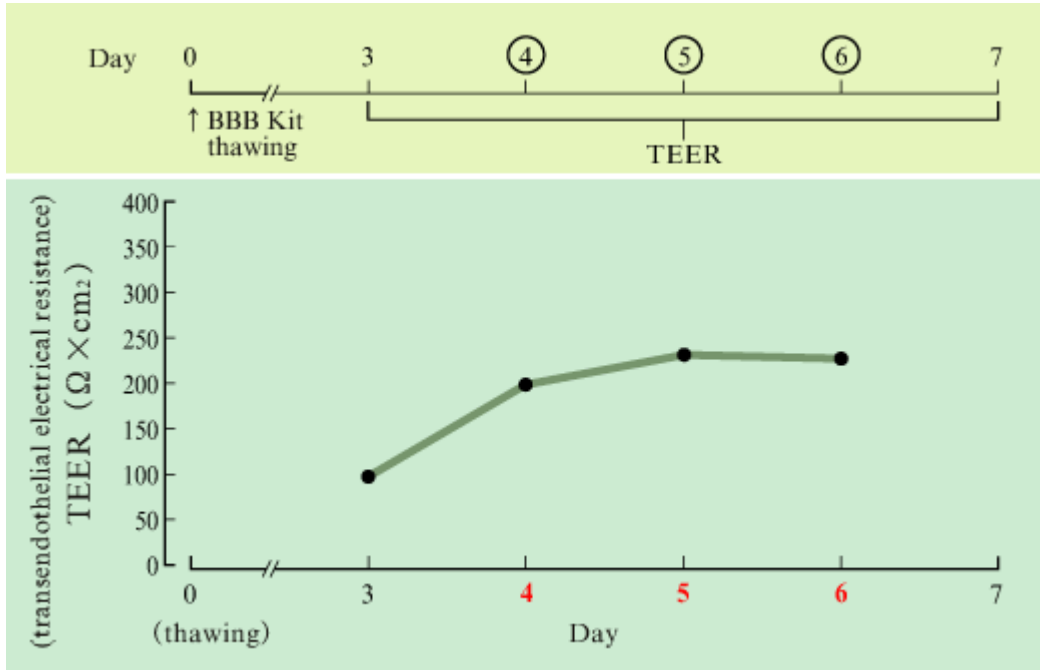
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BBB model



3D Human Blood Brain Barrier Permeability assay

When testing the penetration of a molecule through the brain endothelial and pericytes layer of BBB representing the BBB, in a blood-to-brain direction, the molecule is applied to the upper (luminal, blood-side) compartment of the insert. Transport is measured after a given time (ΔT) by detecting the amount of compound from the lower (basal, brain-side) compartment.

Test compound



Δt

Test compounds (not provided)

- Stopwatch (not provided)

Collect sample Measure Conc. Calculate Papp

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- Orbital Shaker (100 rpm) in Incubator 37°C (not provided)

Summary:

Check TEER of activated BBB Make sure **TEER >150Ω x cm²**

→ Prepare Assay buffer, test compounds, wash plate and assay plate → Permeability Assay

→ Measure concentration of test compound in lower compartment → Calculate permeability coefficient

TEER measurement :

12 well TEER (Ω x cm²) = (Total R - Blank R) x 0.33.

6 well TEER (Ω x cm²) = (Total R - Blank R) x 1.72

24 well TEER (Ω x cm²) = (Total R - Blank R) x 1.57

Blank insert should be soaked with medium (DMEM) prior to its use for accurate reading Only use inserts with TEER value of 150 Ω x cm² or more for assay.

Assay :

- Make sure final concentration of DMSO is equal to or less than 0.2% (v/v) when test compound is dissolved in DMSO. Concentration of test compound used should not be at the concentration which cause any cytotoxic effects. For unknown compounds start with 1μM and adjust concentration as required.
- Use orbital shaker (100 rpm) during incubation period for obtaining accurate result.

Check TEER of activated 3D BBB Model

Measure TEER of activated BBB. Make sure TEER > 150 Ω x cm² before assay. Please refer to the protocol for TEER measurement of BBB.

Prepare assay buffer (DPBS-H), test compounds, wash & assay plate

1. Preparation of assay buffer (DPBS-H); Mix as follows.

10 x Dulbecco's PBS (Ca+/Mg+)	10 mL
1M HEPES (pH 7.0 - 7.6)	1 mL
D-glucose	0.45 g
<u>distilled water</u>	<u>89 mL</u>
total	100 mL

2. Prepare test compounds in Assay Buffer to appropriate concentration, then keep them at 37 °C.
3. Add 900 μL of Assay Buffer into 12 wells of wash and assay plate, then keep them at 37 °C.

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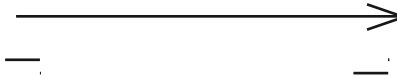
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Wash plate



Add 900 μ L of assay buffer into each well surrounded by red frame as shown.



Assay plate

Permeability Assay

1. Remove all 12 inserts from BBB into wells of the wash plate containing assay buffer with clean tweezers.



Activated BBB Model

Remove inserts into the wash plate.



Wash plate

2. **One insert at a time:**

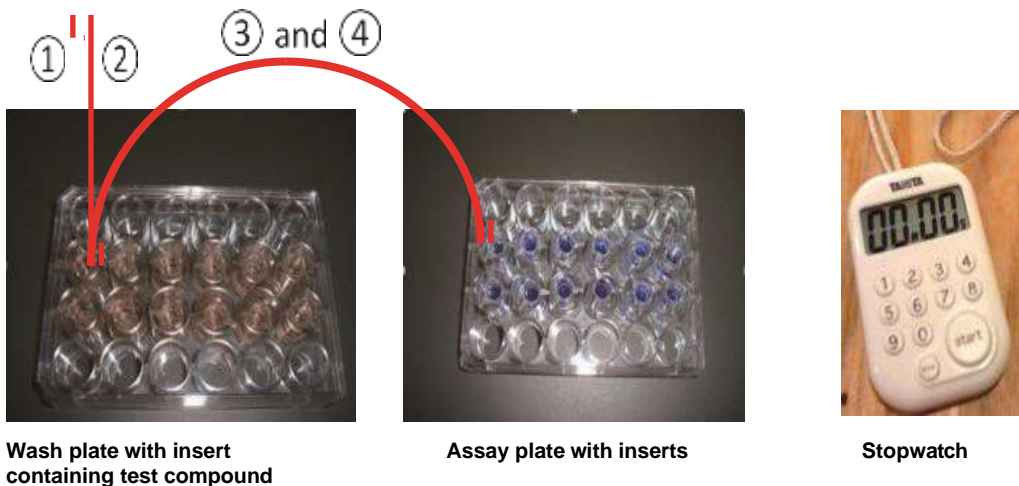
- Pick it up with tweezers and remove culture media from luminal side, then return the insert into wash plate.
- Add 200 μ L of assay buffer containing test compound which is kept at 37 °C.
 - o Note: Do not wash inside of the insert with assay buffer.
- Quickly transfer the insert from wash plate into assay plate.
- As you transfer the first insert into the assay plate start stopwatch.
- Place on a shaker inside an incubator when all 12 inserts are transferred to assay plate.
- Incubate at 37 °C, 100 rpm, for <30 minutes.

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Collect assay buffer from inserts and Assay Plate for measuring concentration of test compound (Apical and Basal concentration). Make sure you perform pipetting action x10 times to have no test compounds remaining at the bottom of the well.

Mix collected sample with Vortex. Measure concentration of test compound and determine permeability coefficient using Excel form provided.

1. We recommend using Millipore plate (Millipore corporation #PIMW S24 50).
2. The volume of assay buffer and test compound dissolved-assay buffer varies if another assay plate is used. Please refer below.
3. Assay time:

The amount of test compound which penetrate through to the brain-side will be greater when assay time is increased. Although concentration measurement will be easier this way especially when detection-limit is low, the barrier-function (tight junction-function) of BBB Kit will deteriorate with time and hence increase paracellular transport of test compound. For this we recommend completing the assay within 30 minutes for accurate evaluation.

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	Blood-side Volume (μL)	Brain-side Volume (μL)
Millipore Plates	100	600
	200*	900*
	300**	1,200**
Corning Plates	100	1,000
	200	1,300
	300	1,600
BD Plates	100	1,000
	200	1,300
	300	1,600

*Recommendation to use for permeability assay. **Volume used for BBB model activation.

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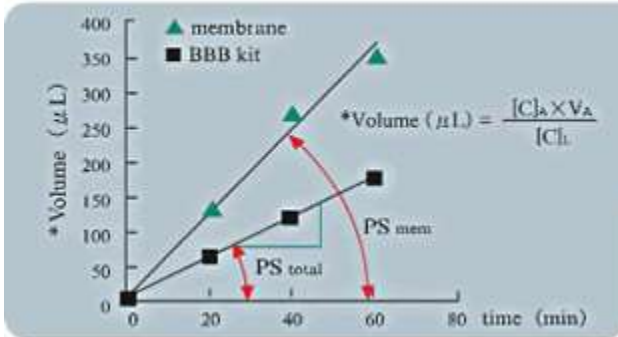
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Analysis:

1. Calculation of apparent permeability



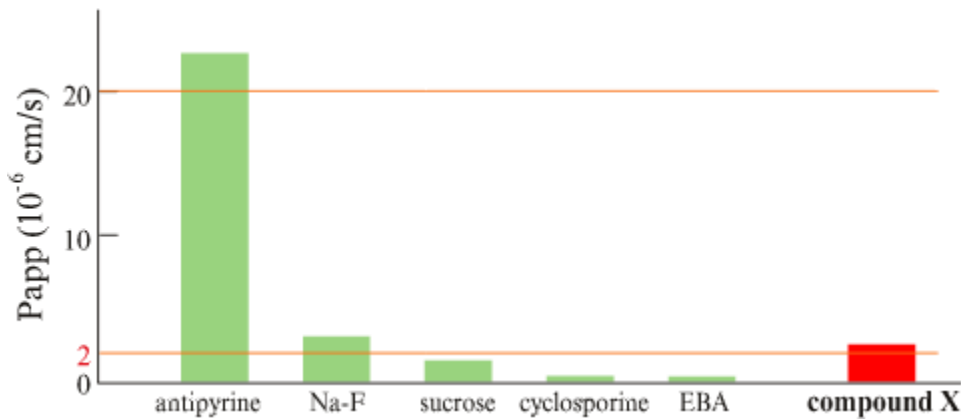
Calculation of apparent permeability

$$\frac{1}{P_{Se}} = \frac{1}{P_{S_{total}}} - \frac{1}{P_{S_{mem}}}$$

$$P_e (\text{cm/min}) = \frac{P_{S_e} (\mu L/\text{min})}{A (\text{cm}^2)}$$

$$P_{app} (\text{cm/s}) = \frac{V_A}{A \times [C]_{luminal}} \times \frac{\Delta [C]_{Abuminal}}{\Delta T}$$

2. Evaluation of Papp



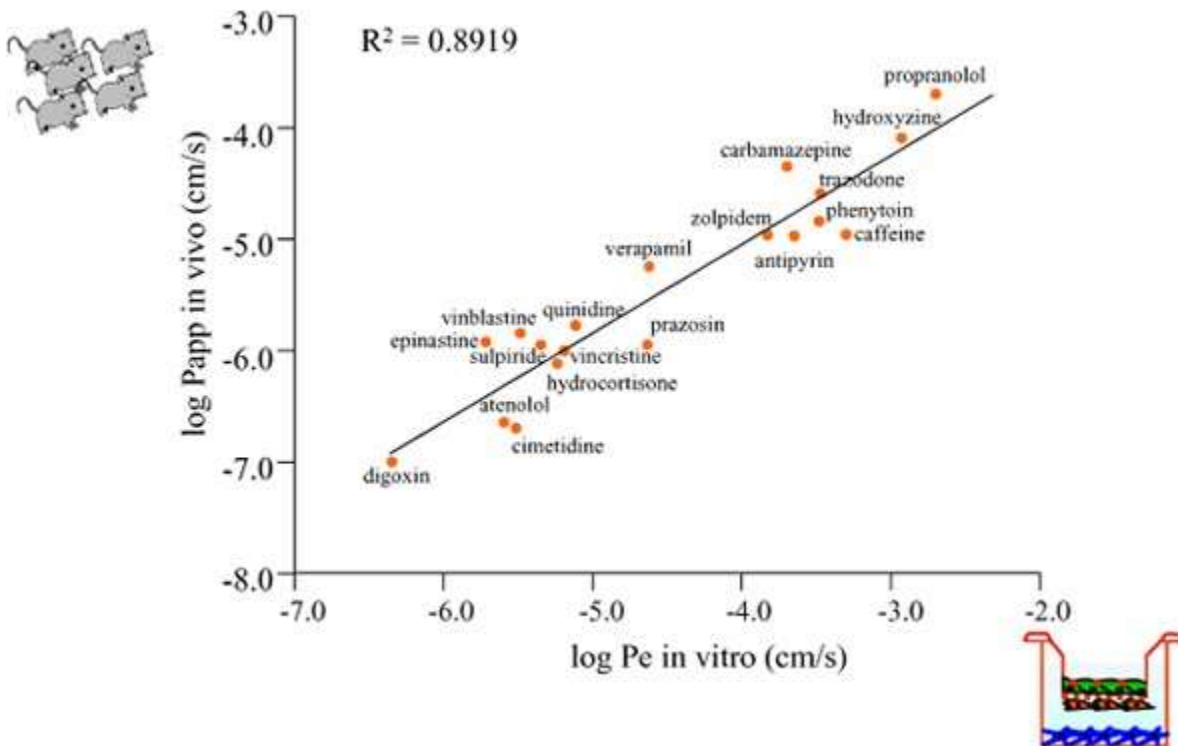
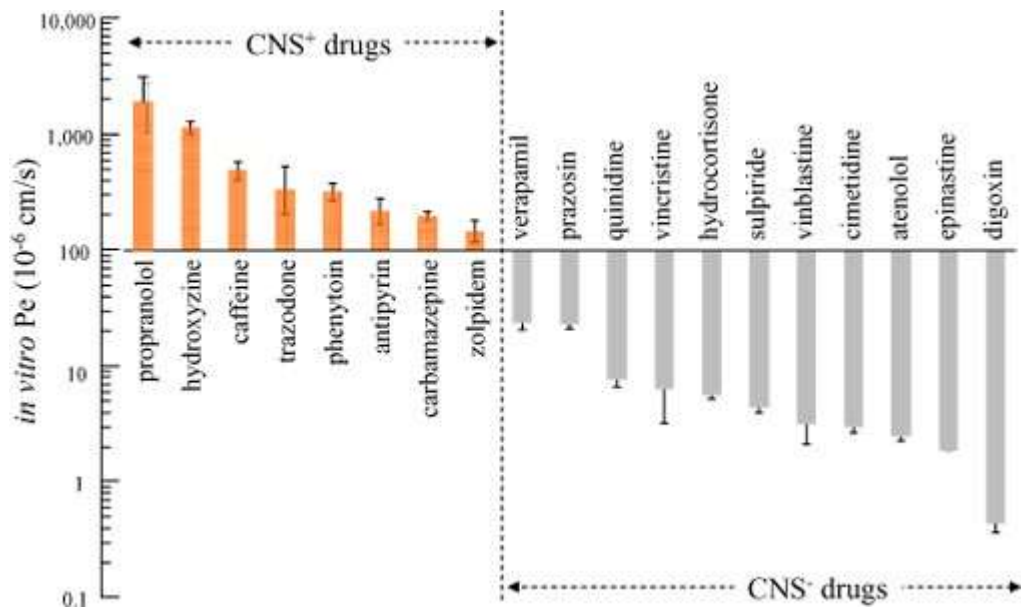
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We obtained a very good correlation between the BBB and in vivo permeabilities of drugs. You can evaluate the BBB-permeability by our BBB model, quantitatively. When you design molecular modifications of your compound or vectors carrying your compound into the brain, you can easily evaluate the BBB-permeability, quantitatively.

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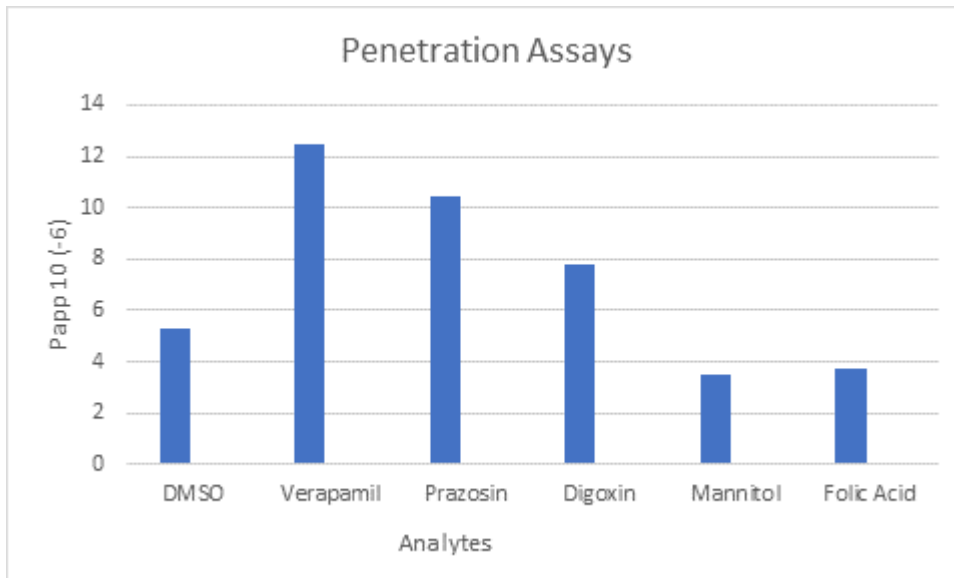
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	Papp ($\times 10^{-6}$ cm/s)	Permeability	ex)
Permeability	>20	very good	Antipyrine
	10~20	good	-
	2~10	low	Na-F
	<2	very low	ESA, sucrose

*Transwell® is trademark of Corning, Incorporated, Corning, NY, USA.



Permeability data courtesy of Visikol

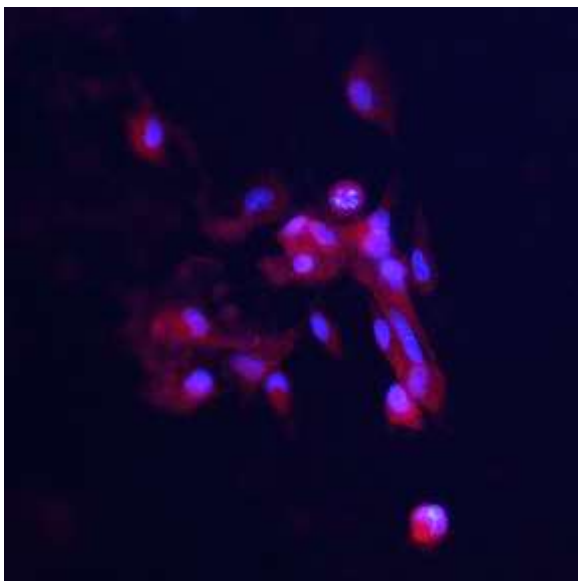
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ACE-2 staining (red) and DAPI nuclear counterstain (blue) of the endothelial cells on the bottom of the inserts. Images collected on a microscope.

ACE- 2 Staining

- a. TEER was checked on the inserts.
 - i. minimum TEER value to activate the BBB should be greater than $150\Omega \times \text{cm}^2$.
 - ii. The lowest number we got when testing all six inserts to be tested was $165.66\Omega \times \text{cm}^2$.
 - iii. The inserts had a range of $165.66\Omega \times \text{cm}^2$ to $270.93\Omega \times \text{cm}^2$
- b. Membranes with fixed cells were removed from the inserts into PBS.
- c. Blocked with antibody blocking solution for 30 min RT followed by incubation with a permeabilization solution for 20 min RT.
- d. inserts were placed in 6-well plate and incubated with primary anti-ACE2 antibody (AF933, R&D Systems) at 5 ug/mL overnight at 4 degrees C.
- e. The membranes were washed 3 times 15 min each in PBS.
- f. Incubated with anti-goat Cy3 secondaries (Jackson ImmunoResearch) for 30 min RT.
- g. Washed 3 times 15 min each in PBS, placed onto histological slides and mounted under iBright with DAPI to counter stain cell nuclei.
- h. Collected images on FL microscope.

Note: Can be tricky to remove and handle the membranes with cells so they do not become crooked.

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Plate and Wells information:

Membrane material	Polyethylene Terephthalate	Polyester
Pore Size (μm)	3.0	0.4
Membrane Diameter (mm)	6.5	12
Membrane Surface Area (cm^2)	0.33	1.12
Apical Volume (μL)	200/300	500
Basolateral Volume (μL)	900/1200	1500
Height of insert (mm)	16	-
Pore Density (pores/cm^2)	2×10^6	4×10^6
Membrane Thickness (μm)	9	10
Optical Property	Translucent	Clear
Cell visibility	Poor	good

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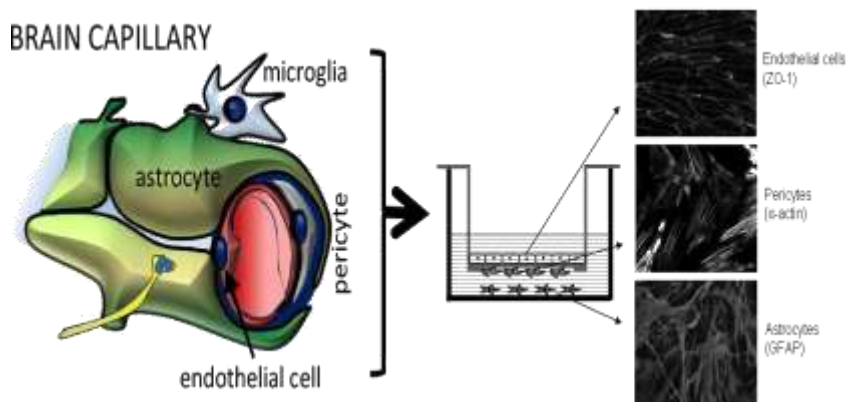
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3D Human Blood Brain Barrier Compound Penetration



1. RESULT

1) Purpose

The objective of this study was to evaluate the BBB permeability of the test articles by 3D Human BBB model

2) Study conditions

This study was performed under GLP conditions. All work was performed with appropriate local health regulations and ethical approval. Two replicates were applied for each test compound.

3) Data Summary

Table 1. BBB Test Result

Compound	Test Conc. (μM)	Incubation Time (hour)	Mean P_e (nm/s)	Mean %Recovery	Permeability
Atenolol	10	4	<0.184*	<90.4	Low
Propranolol	10	4	52.1	98.5	High

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Sulpiride	10	4	<0.184	<93.5	Low
Hydroxyzine Dihydrochloride	10	4	29.5	52.4	High
Hydrocortisone	10	4	9.17	87.6	Moderate
Cimetidine	10	4	<0.101	<93.4	Low
Propranolol hydrochloride	10	4	99.3	41.7	High
Atenolol	10	4	<0.184	<95.7	Low
<p>Classification: Low permeability: $P_e < 1.00$ nm/s Moderate permeability: $1.00 < P_e < 10.0$ nm/s High permeability: $P_e > 10.0$ nm/s</p> <p><i>* The signal responses of Atenolol, Sulpiride, and Cimetidine in receiver samples were undetectable. For the convenience of calculation, 1/300 of the measured peak area ratio (PAR) of the relevant AD (A>B Donor) samples were used as the PAR values of Atenolol, Sulpiride, and Cimetidine in receiver samples.</i></p>					

2. MATERIALS

1) Preparation of PBS (100 mM phosphate, pH = 7.4 ± 0.05)

2.6 g KH₂PO₄ and 18.5 g K₂HPO₄·3H₂O were dissolved in 1000 mL of ultra-pure water, mixed thoroughly. The pH was adjusted to 7.40 ± 0.05, using either 1 M sodium hydroxide or 1M hydrochloric acid.

Table 2. Test Item Information

Compound ID	Lot#	M.W.	F.W.	Formula	Purity%
Sulpiride	BCCC9209	341.43	341.43	C ₁₅ H ₂₃ N ₃ O ₄ S	98
Hydroxyzine Dihydrochloride	BCBT3136	374.91	447.83	C ₂₁ H ₂₇ ClN ₂ O ₂ ·2HCl	98
Hydrocortisone	SLCB9138	362.5	362.5	C ₂₁ H ₃₀ O ₅	98.0
Cimetidine	BCCC3082	252.34	252.34	C ₁₀ H ₁₆ N ₆ S	98
Propranolol hydrochloride	BCCB3791	259.35	295.8	C ₁₆ H ₂₁ NO ₂ ·HCl	98

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Atenolol	LRAC4829	266.34	266.34	C14H22N2O3	98
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3. METHODS

1) Preparation of Donor Solution

- a) For Sulpiride, 0.200 mM working solution was prepared by diluting 10.0 mM stock solution with Ethanol. For Hydrocortisone, 0.200 mM working solution was prepared by diluting 10.0 mM stock solution with DMSO. For others test compounds, 0.200 mM working solution was prepared by diluting 10.0 mM stock solution with H₂O. For control compounds, 0.200 mM working solution was prepared by diluting 10.0 mM stock solution with DMSO.
 - b) 10 μM donor solution (5% DMSO) was prepared by diluting 20 μL of working solution with 380 μL PBS.
- 2) 150 μL of 10 μM donor solutions to each well of the donor plate. Duplicates were prepared.
 - 3) 300 μL of PBS was added to each well of the plate.
 - 4) The donor plate and acceptor plate were combined and incubated for 4h at room temperature with shaking at 300 rpm.
 - 5) Preparation of T0 sample: 20 μL donor solution was transferred to new well followed by the addition of 250 μL PBS (DF: 13.5), 130 μL ACN (containing internal standard) as T0 sample.
 - 6) Preparation of acceptor sample: The plate was removed from incubator. 270 μL solution was transferred from each acceptor well and mixed with 130 μL ACN (containing internal standard) as acceptor sample.
 - 7) Preparation of donor sample: 20 μL solution was transferred from each donor well and mixed with 250 μL PBS (DF: 13.5), 130 μL ACN (containing internal standard) as donor sample.
 - 8) Acceptor samples and donor samples were all analyzed by LC/MS/MS.
 - 9) The equation used to determine permeability rates (P_e) was displayed as follow.

$$P_e = C \times \left(-\ln\left(1 - \frac{[drug]_{acceptor}}{[drug]_{equilibrium}}\right) \right) \times 10^7, \text{ where } C = \left(\frac{V_D \times V_A}{(V_D + V_A) \times Area \times time} \right)$$

$$[drug]_{equilibrium} = ([drug]_{donor} \times V_D + [drug]_{acceptor} \times V_A) / (V_D + V_A)$$

$$V_D = 0.15 \text{ mL}; V_A = 0.30 \text{ mL}; Area = 0.28 \text{ cm}^2; time = 14400 \text{ s.}$$

$$[drug]_{acceptor} = (Aa/Ai \times DF)_{acceptor}; [drug]_{donor} = (Aa/Ai \times DF)_{donor};$$

$$Aa/Ai: \text{ Peak area ratio of analyte and internal standard; } DF: \text{ Dilution factor.}$$

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