READY-TO-USE BBB Kit™ Protocol

Protocol: Permeability assay with BBB Kit™ (RBT-24H)

(Ver. 1.0)

When testing the penetration of a molecule through the brain endothelial and pericytes layer of BBB Kit™ 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 (⊿T) by detecting the amount of compound from the lower (basal, brain-side) compartment.

1. Equipments & Reagents

- **Equipment & Reagents required for TEER measurement**
  Please refer to the Protocol for TEER measurement of BBB Kit™
- **Activated BBB Kit™ (RBT-24H, TEER > 150 Ω x cm²)**
- **Micropipette & Sterilized Tips (not provided)**
- **Tweezers (not provided)**
- **24 well Millipore Plate (#PIMW S24 50, Millipore) x 2 plates (not provided)**
- **Assay Buffer (DPBS-H): 10xD-PBS(Ca+/Mg+), 1M HEPES, D-glucose (not provided)**
- **Test compounds (not provided)**
- **Stop watch (not provided)**
- **Orbital Shaker (100 rpm) in Incubator (37℃) (not provided)**

2. Summary

Check TEER of activated BBB Kit™ (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 peameability coefficient

Important Note :

TEER measurement:
BBB Kit™ (RBT-24H) TEER (Ω x cm²) = (Total R - Blank R) x 0.33.
Blank insert should be soaked with medium (DMEM) prior to its use for accurate reading.
Only use BBB Kit™ 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.

※ BBB Kit™ is not available for clinical diagnosis, examinations, and treatments.

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3. Check TEER of activated BBB Kit™

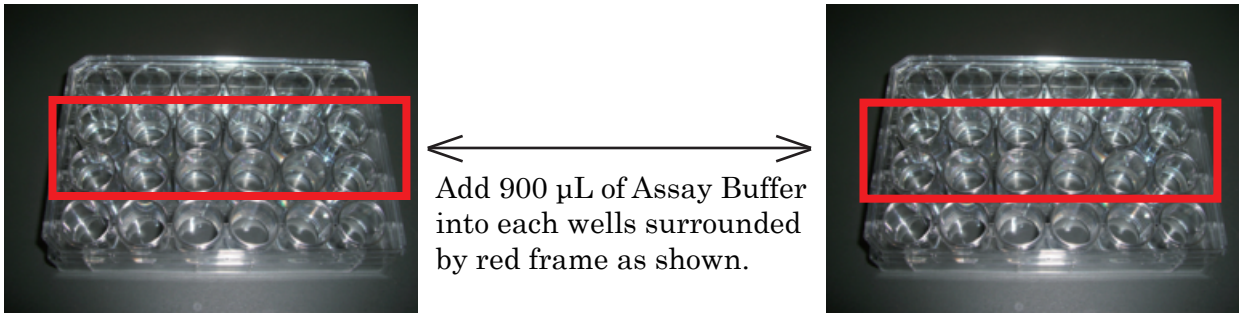
Measure TEER of activated BBB Kit™. Make sure TEER > 150 Ωx cm^2 before assay. Please refer to the protocol for TEER measurement of BBB Kit™.

4. 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
distilled water	89 mL
total	100 mL

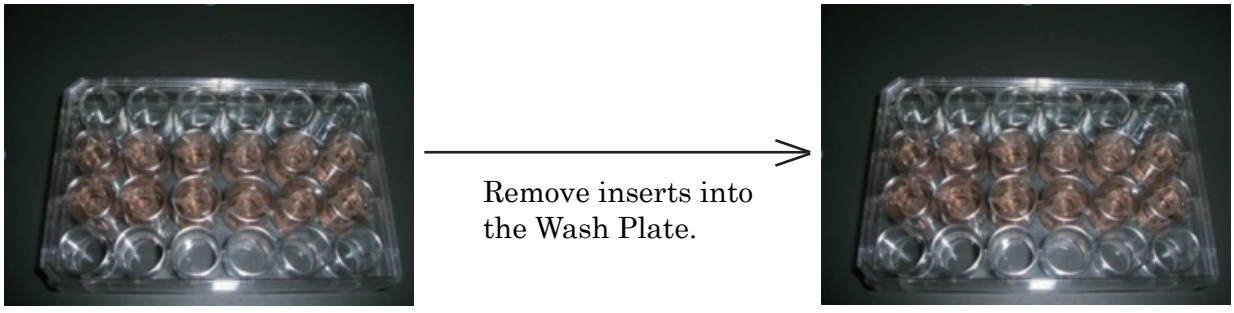
2. Prepare test compounds in Assay Buffer to appropriate concentration, then keep them at 37 °C.
3. Add 900 µL of Assay Buffer into 12 wells of wash and assay plate, then keep them at 37 °C.



Wash Plate　　Assay Plate

5. Permeability Assay

1. Remove all 12 inserts from BBB Kit™ into wells of the Wash Plate containing assay buffer with clean tweezer.



activated BBB Kit™　　Wash Plate

2. One insert at a time,
   ① Pick it up with tweezer and remove culture media from luminal side, then return the insert into Wash Plate.
   ② Add 200 µL of Assay Buffer containing test compound which is kept at 37 °C.
   Note: Do not wash inside of the insert with Assay Buffer.
   ③ Quickly transfer the insert from Wash Plate into Assay Plate.
   ④ As you transfer the first insert into the Assay Plate start stopwatch.
   ⑤ Place on a shaker inside an incubator when all 12 inserts are transferred to Assay Plate.
   ⑥ Incubate at 37 °C, 100 rpm, for <30 minutes.

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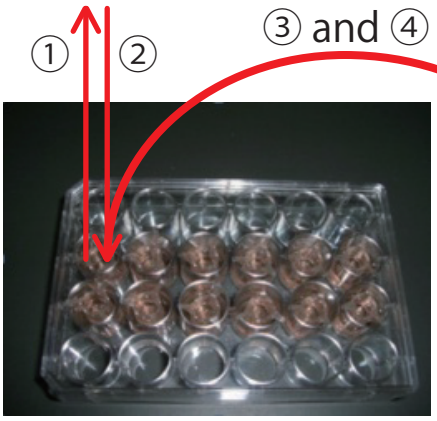

Wash Plate with inserts

Assay Plate with inserts containing Test Compound

Stop Watch

3. 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.

4. Mix collected sample with Vortex. Measure concentration of Test Compound and determine permeability coefficient using Excel form provided.

6. Note

1. We recommend to use Millipore Plate (Millipore corporation #PIMW S24 50).

2. The volume of Assay Buffer and Test Compound dissolved-Assay Buffer varies if other 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 to complete the assay within 30 minutes for accurate evaluation.

	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 Kit activation.

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