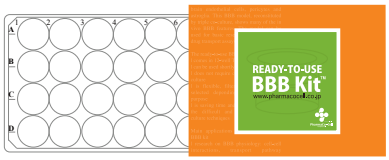
READY-TO-USE BBB Kit™ (RBT-24) Protocol

(Ver. 2.0)

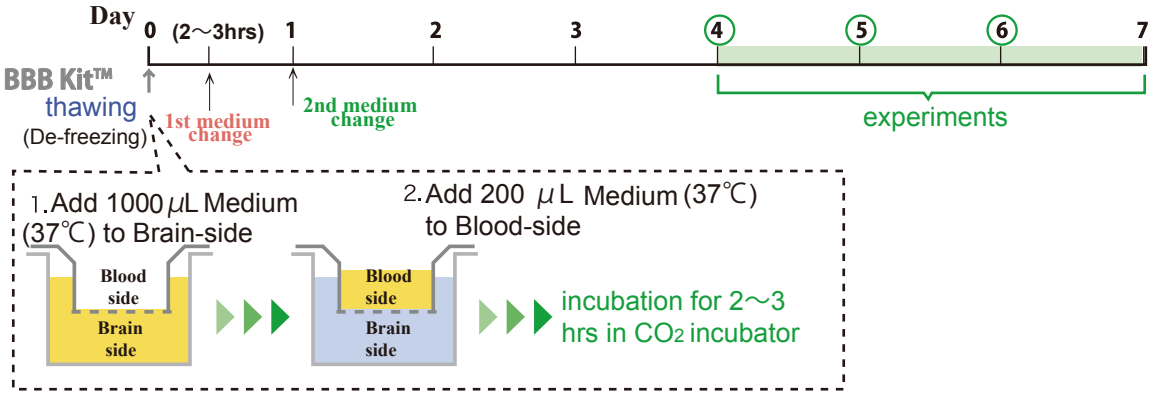
Package
- BBB Kit™ (RBT-24)
- Medium
- Blank insert (Millicell®)
- Protocol
- Quality Assurance



*Please check the contents.

Protocol: Thawing and Activation of BBB Kit™ (RBT-24)

1. Schedule after thawing BBB Kit™



Amounts of Medium (to be added)

	Thawing (De-freezing)	1st medium change	2nd medium change
Blood-side	200 μL	300 μL	300 μL
Brain-side	1000 μL	1200 μL	1200 μL

※ Medium Composition

10%PDS / DMEM F-12:

DMEM / Nutrient Mixture F-12 Ham (DMEM F-12) supplemented with

Fetal Bovine Plasma Derived Serum (PDS)	10% (v/v)
Heparin	1850 U/mL
basic Fibroblast growth factor (bFGF)	1.5 ng/mL
Insulin-Transferrin-sodium Selenite (ITS)	insulin 5 μg/mL; transferrin 5 μg/mL; sodium selenite 5 ng/mL
Hydrocortisone	500 nM
Gentamicin	50 μg/mL

2. Storing of BBB Kit™

BBB Kit™ (RBT-24) is sotred at -80℃, and can be used within one month.
Medium is stored at below -20℃.

3. Summary: Thawing and activating of BBB Kit™ (p2)

thawing*1~8 ⇒ 1st medium change *9~10 ⇒ incubation*11 ⇒ 2nd medium change*12~14 ⇒ incubation*15 ⇒ experiment*16

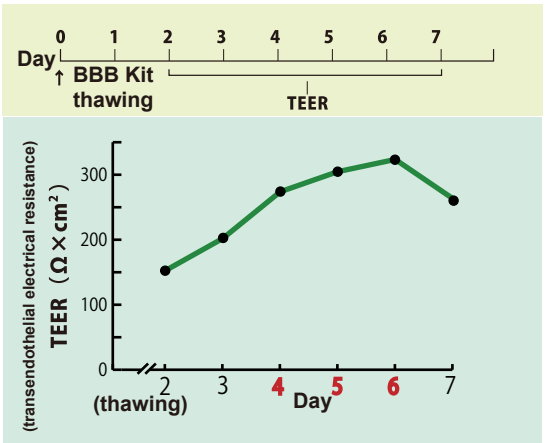
(*protocol # see p2)

4. TEER (trans endothelial electrical resistance) in BBB Kit™

TEER in BBB Kit™ (RBT-24) reached more than 150 Ω×cm², and maintained a plateau up to 7 days from thawing.

* Medium does not contain cAMP and its analogs.

* BBB Kit™ can be used from Day 4 to Day 6 after thawing.



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

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3. Procedures (from thawing to activation of BBB Kit™)

[On thawing (day 0)]

1. Warm frozen Medium to 37 ℃ in water-bath, prior to De-freezing a BBB Kit™.

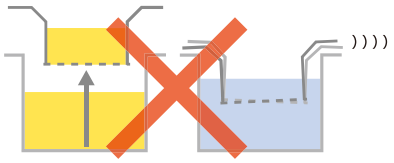
2. Prepare clean bench before taking out BBB Kit™ from freezer.
   - Have ~200 μL and ~1,000 μL pipettors (P200 and P1000) ready to use with sterilized pipette tips attached.
   - Have pair of scissors ready to use as BBB Kit™ is contained in sealed plastic bag.
   - Move Medium from water-bath to clean bench.

3. Move a BBB Kit™ in a frozen state to clean-bench quickly. And then take off the seal.

4. Wipe up the waterdrops (humidity) on a BBB Kit™ with clean papers.

During steps from #5 to #6 on this manual,
- Do not touch membrane of insert with pipette tip.
- Do not move inserts.
- Do not perform pipetting action neither in Blood nor in Brain-side.

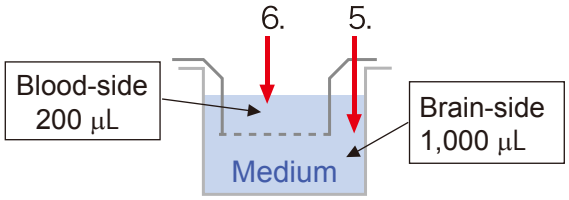
Otherwise, TEER in each well may not achieve more than 150Ω×cm².

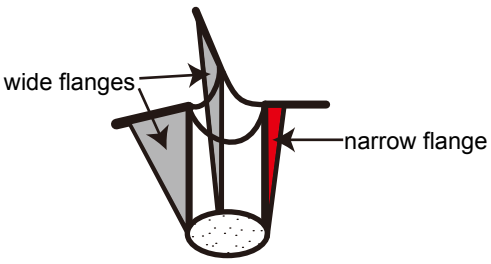


5. Add 1,000 μL Medium to Brain-side (to all 12-wells) through a space between insert and well.

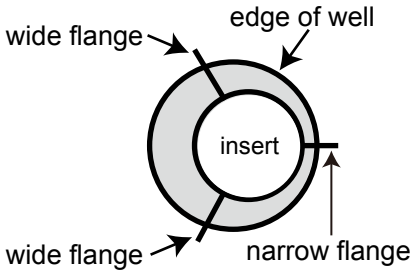
Note: As BBB Kit™ insert is not symmetrical in shape* it will not be located at the center of each well. Add Medium from the largest space between insert and well. You may attach the end of the pipette tip on the wall of well as you add Medium. It is important that you make sure you add 1 mL to each well correctly.

* Each BBB Kit™ insert has three flanges extending on to the plate. Two of them are wider than the other one.





Structure of BBB Kit™ insert



Top view of BBB Kit™ insert in culture well

6. Add 200 μL Medium to Blood-side (inside of the insert) (to all 12-wells).

7. Wipe up humidity on the surface and bottom of the BBB Kit™ with clean papers.

8. Incubate the BBB Kit™ for 2 to 3 hours in CO_2 incubator. Anything less or longer duration could adversely affect the function of BBB Kit™.
   Warm Medium to 37℃ in water-bath or CO_2 incubator during this step.

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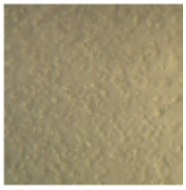
9. Look at astrocytes through a inverted microscope to confirm the attachment of cells to the bottom of well.

When you are observing astrocytes, you must adjust the focus point at the space between the well and insert. Once you focused, move the field of vision toward the center of insert. You should be able to observe astrocytes attached around center of well. Gently shake the plate to confirm astrocytes are attached.

astrocytes



endothelial cells

Note: Endothelial cells on Polyethylene terephthalate (PET) membrane of the insert can not be seen by microscope, therefore microscopic examination for astrocytes on the bottom side of lower compartment has to be done to check cell-proliferation.

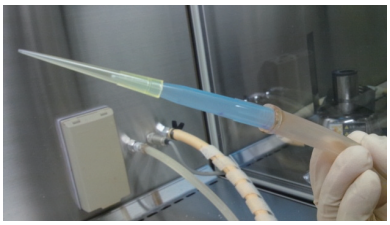
10. Prior to changing Medium, prepare followings in clean bench.

•Micropipette :

P1000 and P5000, or two P1000 attached with sterilized tips.

•Aspirator :

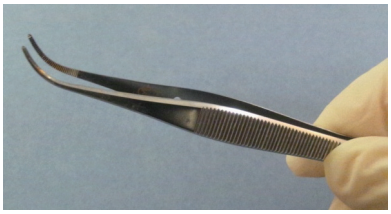
For ease of handling we recommend to attach P1000 tip (without filter) at end of the aspirator hose, and attach sterilized P200 tip (without filter) at end of this P1000 tip. It is probably not easy to handle if you use Pasteur pipette at the end of the aspirator hose.



Aspirator attached with P200 & P1000 tip

•Tweezers:

We recommend No.7 with curved end. Dry-heat sterilized tweezers are ideal, but if you do not have this, dip the tweezers in 70% EtOH and dry alcohol in burning flame should be more than adequate.



Tweezers with curved end

•Medium :

Move Medium from water bath to clean bench.

You will use many types of equipment described above during this step. We recommend removing all unneccesary equipments out from the clean bench to have plenty of working space available.

11. Changing Medium

The order of changing Medium is designed as such that the time of endothelial cells which is attached to the membrane (blood-side) being exposed without Medium is kept to minimum.

① Hold wide flange of the insert with tweezers and pick up it carefully without dropping the insert.

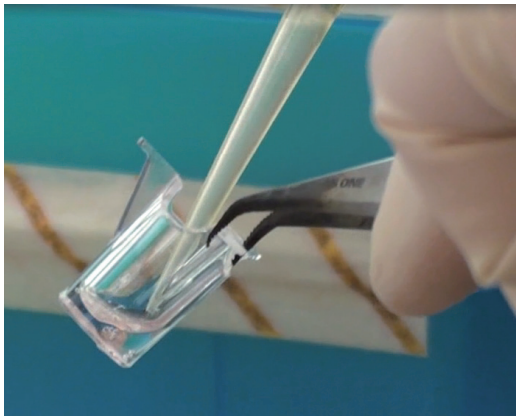
Note: Avoid membrane part of the insert from contacting any part of the plate or any equipment.

② Remove the present Medium of the **brain-side** with aspirator.

Note: Freezing Medium contain DMSO, so it is important to remove Medium completely at this stage. There are not many astrocytes at periphery of the well, you may attach end of the aspirator at the peripery of the well.

③ Remove the present Medium of **blood-side** with aspirator.

Note: Tip up the insert so that membrane of the insert is perpendicular to the line of your sight.
Move end of the aspirator from top to bottom of the inside wall of the insert slowly.
Although it is important to remove Medium cotaining DMSO completely from the insert, you must never contact end of the aspirator with the membrane of the insert.
Doing this you may adversely affect the function of the BBB Kit™.

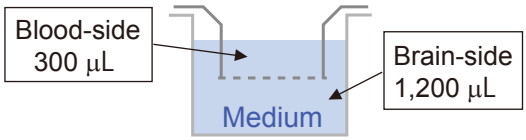


④ Return the insert back to its well once Medium has been aspirated.

Note: Some users often hit the bottom of the insert against plate during this handling, and had poor TEER reading from the respective insert so please be careful.

⑤ Add 300 µL new Medium to the blood-side very gently with P1000 preset as 300 µL, and then add 1,200 µL new Medium to the brain-side with P5000 preset as 1,200 µL (or P1000 preset as 600 µL).

Note: Make sure you add specified volume of Medium to all wells and inserts.
If you are using two P1000s, one preset as 300 µL and the other as 600 µL, you may like to mark them so that you do not use them wrong way around.



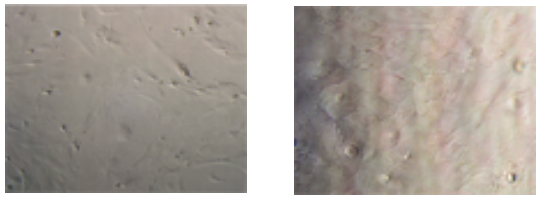
⑥ Repeat ①～⑤ in each well.

Note: Please be careful to remember which inserts you have changed Medium and which ones are not. It is easy to lose track of which ones you have and which ones you have not.

12. Incubate the BBB Kit™ in CO_2 incubator, overnight. Store remains of medium in the refrigerator.

[On day1 (the next day after thawing of BBB Kit™)]

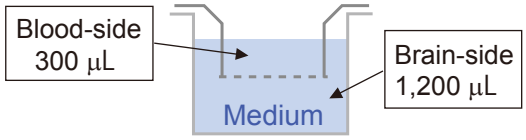
13. Look at astrocytes just below the insert through a inverted microscope.



astrocytes

14. Warm Medium to 37 ℃ in water-bath.

15. Change medium in exactly the same way described at step 11.

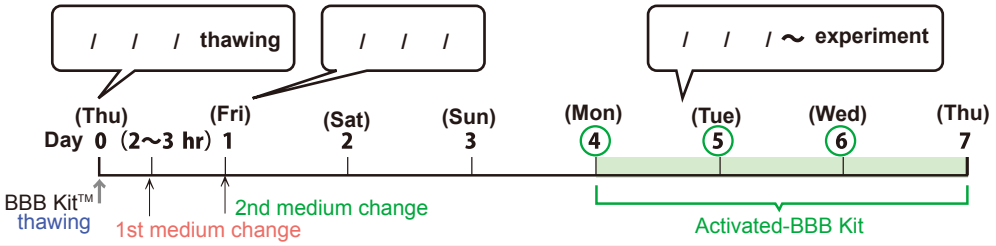


16. Incubate the BBB Kit™ in CO_2 incubator for 3 days *.

17. On Day 4, BBB Kit™ is activated functionally (TEER > 150 Ω×cm²), and maintains BBB functions until Day 7.

Note: Be sure to measure TEER of all inserts of activated BBB Kit™ you use before your experiments. Please refer to the protocol for TEER measurement of BBB Kit™.

Schedule (example)



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