



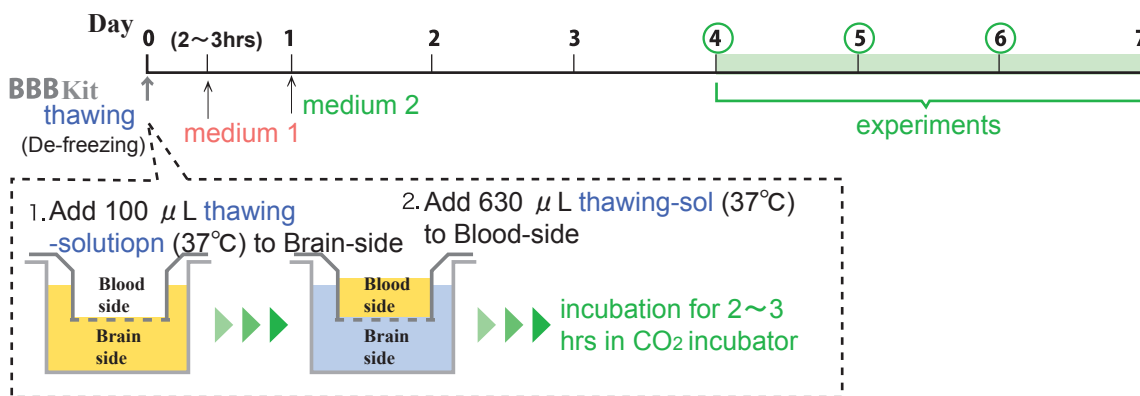
Package

- ©BBB Kit
- ©thawing-solution
- ©medium 1
- ©medium 2
- ©Protocol
- ©Quality Assurance

*Please check the contents.

Protocol [Thawing (De-freezing) and Activation of BBB Kit (PET-12)]

1. Schedule after thawing BBB Kit



Amounts of solutions (to be added)

	Thawing-sol	medium 1	medium 2
Blood-side	630 μ L	500 μ L	500 μ L
Brain-side	1000 μ L	1500 μ L	1500 μ L

※Recipe: medium 2 10%PDS / DMEM F-12:

- Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 Ham (DMEM F-12)
- Fetal Bovine Plasma Derived Serum (PDS) 10% (v/v)
- Heparin 100 μ g/mL
- basic Fibroblast growth factor (bFGF) 1.5 ng/mL
- Insulin-Transferrin-sodium Selenite (ITS) insulin 5 μ g/mL, transferrin 5 μ g/mL, sodium selenite 5 ng/500 nM
- Hydrocortisone 50 μ g/mL
- Gentamicin

2. Storing of BBB Kit, thawing-sol and incubation medium

BBB Kit is stored at -80 °C, and can be used within one month.
Thawing-sol and incubation medium are stored at below -20°C.

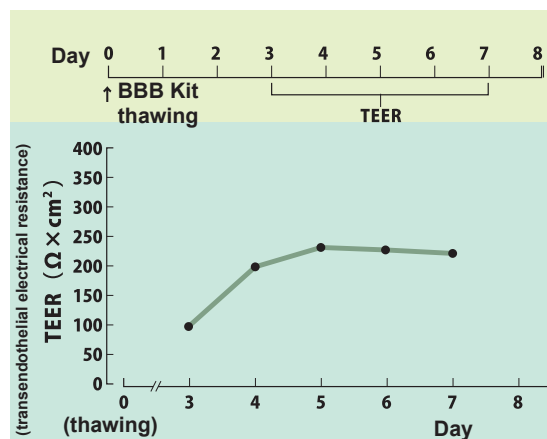
3. Protocol (procedure of activating BBB Kit) (p2)

thawing^{*1~8} ⇒ medium 1^{*9~10} ⇒ incubation^{*11} ⇒ medium 2^{*12~14} ⇒ incubation^{*15} ⇒ experiment^{*16}
(* protocol # see (p2))

4. TEER(trans endothelial electrical resistance) in BBB Kit (PET-12)

TEER in BBB Kit (PET-12) reached more than 150 $\Omega \times \text{cm}^2$, and maintained a plateau up to 7 days. (Thawing-sol and medium 1, 2 do not contain cAMP and its analogs.)

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



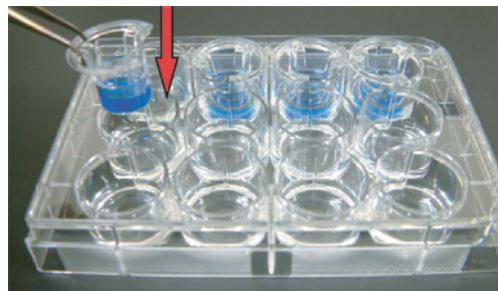
※ BBB Kit is not available for clinical diagnosis, examinations, and treatments.

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

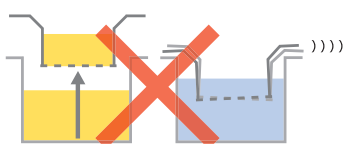
[On thawing (day 0)]

1. Warm thawing-sol to 37 °C, prior to de-frozen and warm up BBB Kit. (Move frozen thawing-sol to 37 °C water-bath.)
2. Move thawing-sol to clean-bench.
3. Move a BBB Kit in frozen to clean-bench. Take off seals. (Do not take a minute.)
4. Wipe up waterdrops (humidity) on BBB Kit with clean papers.

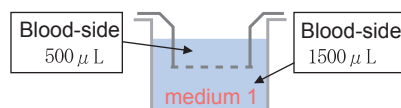
10. Remove thawing-sol from Brain-side, and add 1,500 μL medium 1 (red arrow), then remove thawing-sol from Blood-side, and add 500 μL medium 1. (Do not touch cells, carelessly. Add medium 1, very gently.)



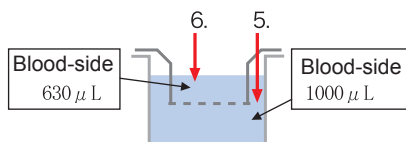
5. Add 1,000 μL thawing-sol to Brain-side (to all 12-wells), through an opening between Inserts.



Do not touch membrane of insert with pipette, and do not move insert, during procedures of #1 to #5.



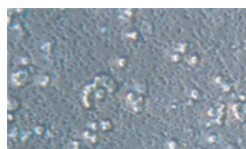
6. Add 630 μL thawing-sol to Blood-side (inside of Insert) (to all 12-wells).



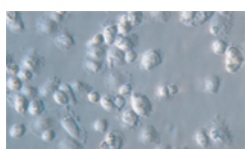
7. Stir up gently Blood-side (inside of Insert) with a pipette, 5 to 10 times. Wipe up humidity on surface and bottom of BBB Kit. (Do not stir up Brain-side.)

8. Incubate BBB Kit for 2 to 3 hrs in CO₂ incubator. During this incubation, warm medium 1 to 37 °C

9. Check cells with inverted microscope.



endothelial cells (high magnification)

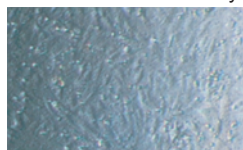


astrocytes (high magni)

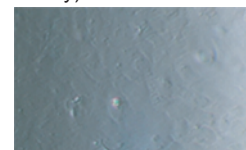
11. Incubate BBB Kit with medium 1 in CO₂ incubator, overnight.

[On day 1 (the next day after thawing of BBB kit)]

12. Check cells to be monolayer (confluency) with inverted microscope.



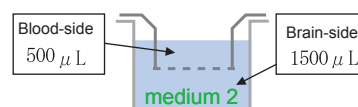
endothelial cells (low magnification)



astrocytes (low magni)

13. Warm medium 2 to 37 °C in water-bath. (Move frozen medium 2 to 37 °C water-bath.)

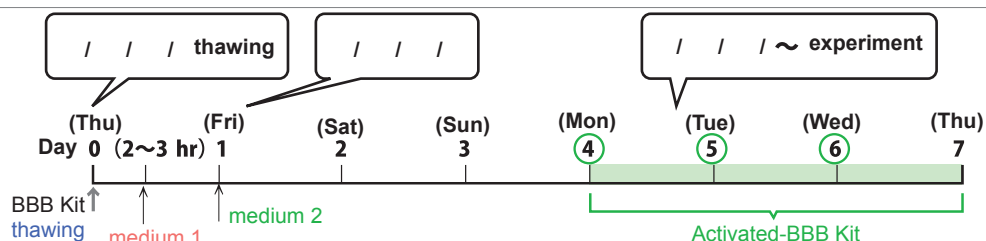
14. Remove medium 1 from Brain-side, and add 1,500 μL medium 2 (red arrow), then remove medium 1 from Blood-side, and add 500 μL medium 2. (Do not touch cells, carelessly. Add medium 2, very gently.)



15. Incubate BBB Kit with medium 2 in CO₂ incubator for 3 days. (from thawing day (Day 0) to Day 4)

16. On Day 4, BBB Kit is activated functionally, and maintains BBB function until day 7. Use activated-BBB Kit on Day 4. (You can store activated-BBB Kit in CO₂ incubator at 37 °C. We recommend you to use the BBB Kit until Day 6.)

Schedule (example)



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