[TS culture protocol]

➢ **Materials**

- DMEM/F12 (Wako #048-29785)
- 30% BSA (Wako #017-22231)
- ITS-X (Wako #094-06761)
- L-Ascorbic acid (Wako #013-12061) : 0.03 g/ml Stock (in H_2O)
- VPA (Wako #227-01071)
- 55mM 2-Me (GIBCO #21985-023)
- FBS (GIBCO #16141-079) : Heat inactivation (56°C, 30 min)
- Penicillin-Streptomycin (GIBCO #15140122)
- PBS (Wako #166-23555)
- TrypLE (GIBCO #12604-021)
- Col IV (Corning #354233)
- EGF (Wako #053-07871) : 100 μg/ml Stock (in 0.2% BSA/PBS)
- A83-01 (Wako #035-24113) : 5 mM Stock (in DMSO)
- CHIR99021 (Wako #038-23101) : 3 mM Stock (in DMSO)
- Y27632 (Wako #036-24023) : 10 mM Stock (in H_2O)
- SB431542 (Wako #031-24291) : 10 mM Stock (in DMSO)
- Cell Banker 1 (Nippon Zenyaku Kogyo #CB011)

➢ **Media preparation**

- Inhibitor Cocktail: 1200 μl

3 mM CHIR99021	800 μl
5 mM A83-01	120 μl
10 mM SB431542	120 μl
DMSO	160 μl

Store at -20˚C

▫ Basal medium: 500 ml

DMEM/F12	486 ml
BSA	5 ml
ITS-X	5 ml
Penicillin-Streptomycin	2.5 ml
55mM 2-Me	900 µl
100 µg/ml EGF	250 µl
FBS	1000 µl
L-Ascorbic acid	25 µl

Store at 4˚C

▫ TS medium: 10 ml

Basal medium	10 ml
10 mM Y27632	5 µl
VPA	1.25 µl
Inhibitor Cocktail	10 µl

Store at 4˚C, Use within one week

➢ **Thawing cells** (@10 cm dish)

1. Coat a dish with 5 ml PBS containing 10 µg/ml Col IV for >1.5 hours at 37°C
2. Aspirate PBS/Col IV
3. Wash the dish with 10 ml PBS
4. Add 10 ml TS medium and keep the dish at 37°C for at least 10 minutes
5. Thaw TS cells in a 37°C water bath
6. Transfer the cells to a centrifuge tube containing 2 volumes of Basal medium
7. Centrifuge the cells
8. Suspend ~1 x 10^6 cells in the pre-warmed TS medium

➢ **Passaging cells** (@10 cm dish)

1. When the cells reach 70-90 confluency (it takes 2-3 days), passage the cells as follows
2. Coat a dish with Col IV and pre-warm TS medium (see Steps 1-4 of “Thawing cells”)
3. Aspirate TS medium
4. Add 5 ml TrypLE and keep the dish at 37°C for ~15 minutes
5. Add 5 ml Basal medium and transfer the cells to a centrifuge tube
6. Centrifuge the cells
7. Suspend ~1 x 10^6 cells in the pre-warmed TS medium (the split ratio is about 1:4)