Infection of mHAT9d with Retrovirus

◯Materials

Serum-free medium of mHAT9d：

DMEM+ham /F-12, HEPES, no phenol red (Gibco) 500ml

50×B27 Serum- free Supplement 10ml

Mouse Recombinant FGF2(R&D): 12.5μg(final conc.25ng/ml) :Add 250μl of 0.1%BSA sterile PBS into the bottle and dissolve the powder and divide the solution into two siliconized eppendorf each 125μl(12.5μg).

Mouse Recombinant EGF(R&D): 50μg(final conc. 100ng/ml):Add 1ml of sterile PBS into the bottle and dissolve the powder and divide the solution into four siliconized eppendorf each 250μl(50μg).

Primaria 100×20mm dish (BD Falcon)

Primaria 24 well plate (BD Falcon)

Retro-X Concentrator (Takara)

5 μg/μl polybrene

◯First culture of mHAT9d:

1. Thaw serum tube with mHAT9d quickly at 37℃.
2. Place them in 10ml medium to dilute them
3. Centrifuge them at 4℃, 1500rpm for 3min and aspirated the supernatant.
4. Add 1ml medium to the cells and suspend them, and count the cells.
5. Place 1ml cell suspension into 9ml medium in 10cm dish.
6. Place the dish in CO2 incubator.

mHAT9d Medium usage:

For first dilution:10ml

For suspension :1ml

As a medium :9ml

◯How to maintain the cells

Maintain one 10cm culture dish (grow up to 70〜90% confluent).

For subculturing, transfer 1/10 of these cells in 10 cm dish (1×107cells, 70〜90%) into 10 cm dish containing 10ml serum-free medium.

For infection, transfer 2×105 cells of 10 cm dish(1×107cells, 70〜90%) into each well of 24 well plate containing 1ml of serum-free medium.

Store the remains with cell banker at −80℃.

◯Splitting the Cells

Rate:1:10

Medium replacement:2～3 days intervals

Yield:Typically 1×107cells can be harvested from one 10 cm culture dish(70〜90% confluent).

1. Wash cells two times with PBS(−) (Ca2+, Mg2+-free).
2. Add 2mL of 0.25%tripsin +0.02% EDTA to a 10cm dish.
3. Incubate these cells at 37℃ for 20 min. Remove the cells from the dish surface by tapping the rim of the culture dish.
4. Add 5ml of Serum-free medium to a 10cm dish. Gently pipette the cell suspension up and down 10 times.
5. Transfer the cell suspension into the 50mL tube.
6. Centrifuge the cell suspension for 5 min at 1300-1500 rpm.
7. Aspirate the supernatant and add 1ml of serum-free medium to the pellet.
8. Add 1/10 of the suspension into a 10 cm dish containing 10ml of serum-free medium.
9. Incubate the dish at 37℃, 5% CO2 for a few days.

mHAT9d Medium usage:

For first dilution:5ml

For suspension :1ml

As a medium :10ml

〇Cryopreservation

1. Check if the cells are 70〜90% confluent.
2. Aspirate the Serum-free medium.
3. Wash cells two times with PBS(−).
4. Add 2mL of 0.25%tripsin +0.02% EDTA to a 10cm dish.
5. Incubate these cells at 37℃ for 20 min. Remove the cells from the dish surface by tapping the rim of the culture dish.
6. Add 5ml of Serum-free medium to a 10cm dish. Gently pipette the cell suspension up and down 10 times.
7. Transfer the cell suspension into the 50mL tube.
8. Centrifuge the cell suspension for 5 min at 1300-1500 rpm.
9. Aspirate the supernatant.
10. Add 1mL of Cell Banker into the cell pellet and transfer the cell suspension into the serum tube.
11. Store it at −80℃ and after a few days, store it in the nitrogen tank.

 mHAT9d Medium usage:

 For first dilution:5ml

Retrovirus: (transduction efficiency of Plat-e)

pGC YFP XN(43.5%)

MSCV GFP(68.31%)

MSCV LMP(91.02%)

MSCV LMP C/EBPβ sh1(5.92%)

MSCV LMP C/EBPβ sh3(73.66%)

MSCV GFP Runx2 WT(45.89%)

MSCV LMP Runx2 sh1351#94(68.66%)

Titer of Plat-e (conditions:60 mm dish 2×106cells 70〜90% confluent medium 4ml) :1×107units/ml ⇒In the case of 10cm dish(1×107cells), we can get 5×107units/ml

Individual titer estimated :

pGC YFP XN(43.5%)：2.2×107units/ml

MSCV GFP(68.31%)：3.4×107units/ml

MSCV LMP(91.02%) : 4.5×107units/ml

MSCV LMP C/EBPβ sh1(5.92%) : 2.9×106units/ml

MSCV LMP C/EBPβ sh3(73.66%) : 3.6×107units/ml

MSCV GFP Runx2 WT(45.89%)：2.3×107units/ml

MSCV LMP Runx2 sh1351#94(68.66%)：3.4×107units/ml

1. Take Retrovirus stocks from No.101Box(−130℃) and unfreeze them.
2. Combine 1 volume of 4×Retro-X Concentrator with 3 volumes of clarified supernatant.

 Retrovirus 1000μl

 Retro-X Concentrator 330μl

 Total 1330μl

1. Mix by gentle inversion.
2. Incubate mixture overnight at 4℃.
3. Centrifuge sample at 1,500×g for 45 min.
4. Remove supernatant.
5. Add 1ml of the medium into each pellet.
6. Store them at －70℃.

Retrovirus infection of mHAT9d:

1. Seed 2×105 mHAT9d cells in each well of a 24 well plate (BD Falcon).
2. After 4 hours, 1ml of medium containing virus concentration solution and 1.6μl of 5 μg/μl polybrene (final conc.8μg/ml)
3. The next day, add 1 ml of the medium into the well and divide the cells into two wells (yield: 6×105cells)
4. Check if cells are 70〜90%. Split these cells of into 4 wells (yield:1.2×106cells)
5. Check if cells are 70〜90%. Split these cells into 10cm dish.
6. Subculture them with a 10cm dish and cryopreserve the remains. In the case of MSCV-LMP retrovirus vector, Add 6μl of 5mg/ml puromycin into 10ml of the serum-free medium(final conc. 3μg/ml).
7. The day after, check the efficiency of infection.



mHAT9d Medium usage :

For first dilution of mHAT9d : 5 ml

For suspension : 1 ml

As a medium : 1 ml (per one well)