FIBROBLAST CULTURES FROM SKIN BIOPSY

Materials and media needed:

-glass petridish
-forceps
-scalpel
-RPMI-1640 (Gibco) supplemented with antibiotics and 20% FCS
-12 well plates (Corning)
-freezing medium (10% DMSO in culture medium)
-freezing tubes

Procedure:

1. Cut the tissue into small pieces with a scalpel in a glass petridish with a small amount of medium to avoid drying
2. Add 250 ul medium per well into 12 well plate and tap the plate until the medium is evenly distributed
3. Carefully transfer 1-2 tissue pieces with forceps in the middle of the well
4. Day 3 and 5: carefully add 3 drops of the medium with 1 ml pipette
5. Day 7 and 10: change the medium (0.5 ml)
6. Day 14: remove the rest of the attached tissues from the wells and change the medium (1 ml)
7. Day 18: split the cells into 4 x 10 cm dish
8. Change the medium twice a week
9. Freeze the cells at -150°C when almost confluent; 2 vials from one 10 cm dish

Characterization

1) Immunostainings

Materials:

-PBS
-4% PFA
-0.5% Tx-100 in PBS
-0.1% Tween-20 in PBS
-Ultra V Block, Thermo Scientific
-primary antibodies (Rb-IgG anti-Oct4, Santa Cruz Biotechnology, 1:500; Mo-IgM anti-TRA160, Millipore, 1:200 and Rat- IgM anti- SSEA3, Millipore, 1:70 or Mo-IgG anti-SSEA4, Millipore, 1:200)
-fluorescent secondary antibodies (Alexa Fluor A21206, A21042 and A21212 or A21202, Invitrogen, all 1:500)
-Vectashield mounting medium with Dapi, Vector Laboratories
-round coverslips (13 mm), VWR International

Methods:

-Remove culture medium from the 24 well plates
-Wash wells 2 times with PBS
-Fix cells with 4% PFA for 15 min
-Wash
-Permeabilize cells with 0.5% Tx-100/PBS for 15 min (Oct4)
-Wash
-Add blocking reagent and incubate max 10 min at RT
-Incubate with primary antibodies diluted in 0.1% Tween/PBS overnight at +4°C
-Wash
-Incubate with fluorescent secondary antibodies diluted in 0.1% Tween/PBS for 30 min at RT
-Wash
-Add mounting medium with Dapi and cover with coverslip

2) qPCR/PCR

A) RNA isolation and cDNA synthesis

Sample collection
-Aspirate culture medium from the 3.5 cm dish
-Wash 2 times with PBS
-Scrape cells from the plates and centrifuge them down in eppendorf tubes
-Remove washing buffer and add 350ul RA1 buffer (Macherey-Nagel NucleoSpin® RNA II) +/- 3.5 ul beta mercaptoethanol
-Vortex vigorously to lyse the cells and freeze immediately at -75°C

RNA isolation
-Add 3.5 ul beta mercaptoethanol (if not already added) and vortex
-Isolate RNA according to Macherey-Nagel NucleoSpin® RNA II (without DNAse treatment)
-Elute RNA in 40 ul DEPC H_2O

DNaseI treatment
-50 ul reaction (40 µl of tot. RNA after column purification)
-5 ul RQ1 DNase 10x buffer (Promega)
-2 ul RiboLock Rnase Inhibitor 40U/ul stock (Fisher Scientific)
-3 ul RQ1 RNase-Free DNase 1U/uL stock (Promega)
-Wash the walls of the eppendorf with the RNA-DNase mix
-Incubate at 37°C for 20 min.

RNA purification
-Clean-up the RNA with another round of RNA purification according to Macherey Nagel NucleoSpin® RNA Clean-up
-Elute RNA in 40 ul DEPC H_2O
-Measure the RNA concentration

RT reaction
-20 ul reaction (2 ug total RNA)
-4 ul RT buffer 5x (Promega)
-2.5 ul dNuTPs-mix 2,5 mM stock (á 2,5 mM/dNuTP)
-1 ul oligoT 500 ug/mL stock (Promega)
-0.2 ul Random Hexamers 500 ug/mL stock (Promega)
-0.5 ul RiboLock Rnase Inhibitor 40U/ul stock (Fisher Scientific)
-0.5 ul MMLV RTase 200U/ul stock (Promega)
-11.3 ul DEPC H_2O contains 2 ug RNA

1. Denaturate RNA for 1 min at 65°C, put into ice

2. Make master mix from the reaction components and add to the RNA, mix by pipetting and spin down
3. Incubation at 37°C for 90 min
4. Inactivation at 95°C for 5 min, put into ice, spin down
5. Dilute the 20 ul RT reaction 2x by adding 20 ul DEPC H_2O to make the final reaction as 1ug RNA/20ul RT reaction

B) qPCR for pluripotency genes

Materials:

-Endogenous primers: Oct4 F/ TTGGGCTCGAGAAGGATGTG
R/ TCCTCTCGTTGTGCATAGTCG,
Sox2 F/ GCCCTGCAGTACAACTCCAT
R/ TGCCCTGCTGCGAGTAGGA,
KLF4 F/ CCGCTCCATTACCAAG
R/ CACGATCGTCTTCCCCTCTT,
c-Myc F/ AGCGACTCTGAGGAGGAACA
R/ CTCTGACCTTTTGCCAGGAG,
Nanog F/ CTCAGCCTCCAGCAGATGC
R/ TAGATTTCATTCTCTGGTTCTGG,
TDGF1 F/ TCAGAGATGACAGCATTTGGC
R/ TTCAGGCAGCAGGTTCTGTTTA
GAPDH F/ GGTCATCCATGACAACTTTGG
R/ TGAGCTTCCCGTTCAGCTC

-5 x HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne)
-RT samples
-DEPC H_2O
-Corbett pipetting robot CAS-1200
-Corbett qPCR-machine Rotor-Gene 6000

Methods:

<u>Pipetting robot CAS-1200 mixing procedure for 20 ul</u>
Master-mix/RT (15 ul) and primer mix (5 ul) separately:
-4 ul of 5 x HOT FIREPol EvaGreen + 11 ul RT/DEPC H_2O
-5 ul primer mix F/R 2 uM + 20 ul extra

<u>Corbett qPCR-machine:</u>

95°C 15min/
40 cycles 95°C 20sec
60°C 20sec
72°C 20sec/
dissociation step from 75°C

C) PCR for Sendai virus

Materials:

-Sendai virus specific primers: SeV F/ GGATCACTAGGTGATATCGAGC,
R/ACCAGACAAGAGTTTAAGAGATATGTATC
-REDTaq DNA Polymerase (Sigma)
-RT samples
-BioRad PCR-machine

Methods:

-25 ul reaction
-13.38 ul DEPC H_2O
-2.5 ul 10x PCR buffer (includes 11 mM $MgCl_2$)
-0.125 ul dNuTP mix, 25 mM stock each
-5 ul F+R primer mix, 2 uM stock
-2 ul DMSO, 50% stock
-1 ul RT template
-1 ul REDTaq DNA polymerase, 1 U/ul stock

	95°C 2 min/
32 cycles	95°C 30 sec
	56°C 30 sec
	72°C 30 sec/
	72°C 7 min
	16°C ∞

-Electrophoresis in 2% agarose gel, take about 2-7 ul of multiplication reaction.