Human iPS cell training program

Protocols for human iPS cell generation from PBMCs under feeder-free culture conditions

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Protocols for human iPS cell generation from PBMCs under feeder-free culture conditions

This protocol introduces generation of iPS cells without the insertion to a genome into human peripheral blood mononuclear cells (PBMCs) using episopmal vectors containing reprogramming factors. To handle human cells, follow the associated laws and carry out measures against infectious disease.

1.1. MATERIALS

1.1.1. Cells and vectors

- Human peripheral blood mononuclear cells (PBMCs; CTL, CTL-UP1) or fresh blood
- Episomal vectors Set2 or Human iPS Cell Generation™ Episomal Vector Mix(TaKaRa, 3673)

  Set2 (pCE-hOCT3/4, pCE-hSK, pCE-hUL, pCE-mp53DD, pCXB-EBNA1), available from Addgene; http://www.addgene.org/Shinya_Yamanaka

  Episomal expression vectors containing OCT3/4, SOX2, KLF4, L-MYC, LIN28, p53DD, EBNA1 (Fig. 1).

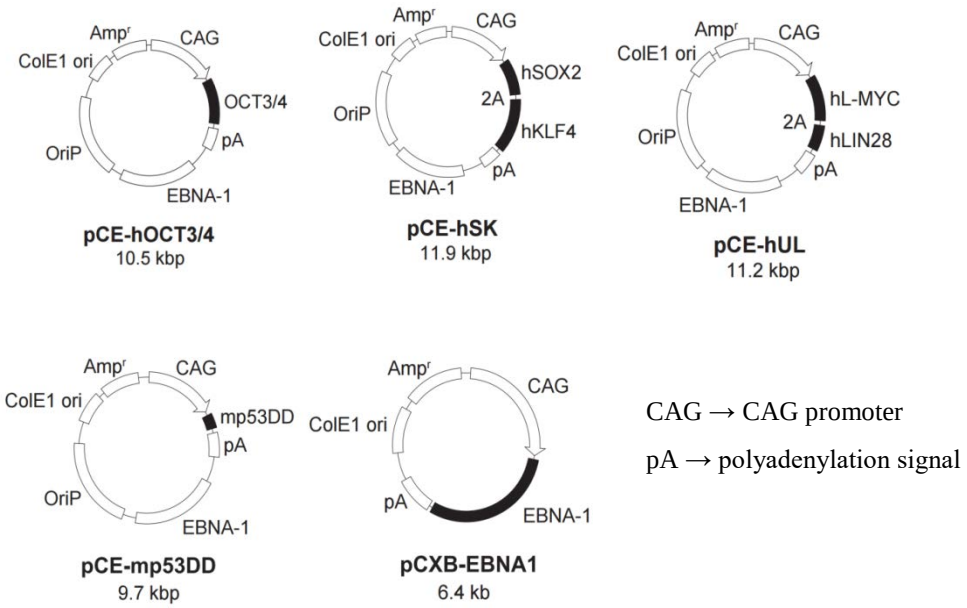


Fig.1 Map of Episomal vectors Set2

1.1.3. Reagents

- BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube - Sodium Citrate (16x125 mm / 8mL) (BD, 362761)
- Phosphate buffered saline Ca, Mg free (PBS; Nacalai tesque, 14249-95)
- StemSpan™-ACF (STEMCELL Technologies, 09805/09855)
- 0.4%Trypan blue stain (BIO RAD, 1450021)
- iMatrix-511（Nippi；892011/892012）
- Bovine serum albumin (BSA; ICN, 810-661)
- Recombinant human IL-3 (R&D, 203-IL-050, 50 µg)
- Recombinant human IL-6 (R&D, 206-IL-050, 50 µg)
- Recombinant Human Thrombopoietin (NS0-expressed) Protein (R&D, 288-TPN-025, 25 µg)
- Recombinant Human Flt-3 Ligand Protein (R&D, 308-FK-025, 25 µg)
- Recombinant Human SCF Protein (R&D, 255-SC-050, 50 µg)
- STEM-CELLBANKER® GMP grade (Conventional product name is STEM-CELLBANKER 3; ZENOAQ, CB045)
- Amaxa® Human CD34 Cell Nucleofector® Kit (Lonza, VAPA- 1003)
- StemFit® AK02N (AJINOMOTO, AK02N)
- 5 mmol/l Y-27632 Solution (Wako, 253-00591)

1.1.4. Reagent setup

- **Plasmid mixture Set2**

  Mix the indicated plasmids.

1mg/mL pCE-hOCT3	0.63 μ L
1mg/mL pCE-hSK	0.63 μ L
1mg/mL pCE-hUL	0.63 μ L
1mg/mL pCE-mp53DD	0.63 μ L
1mg/mL pCXB-EBNA1	0.50 μ L
Total	3.02 μ L(3.02 μ g)

- **Gene transfer reagents mixture**

  Prepare the gene transfer reagents as shown below.

Nucleofector solution	81.8 μL
Supplement solution	18.2 μL
Plasmid mixture Set2	3.02 μL
Total	103.02 μL

- **10% bovine serum albumin (BSA; 10%)**

  To make PBS containing 10% bovine serum albumin (BSA), put 5.0 g BSA in 50 ml of PBS. Store 2 to 8 °C under sterile conditions after reconstitution.

- **10 μg/mL Recombinant human Interleukin-3 (IL-3; 10 μg/mL)**

  Dissolve recombinant human IL-3(50 μg) at 10μg/mL in 5mL of sterile PBS containing 0.1% bovine serum albumin. After reconstitution, divide suitable tube and store -20 to -70 °C under sterile conditions. Use within 3 months and avoid repeated freeze-thaw cycles.

- **100 μg/mL Recombinant human Interleukin-6(IL-6; 100 μg/mL)**

  Dissolve recombinant human IL-6(50 μg) at 100μg/mL in 0.5mL of sterile PBS containing 0.1% bovine serum albumin. After reconstitution, divide suitable tube and store -20 to -70 °C under sterile conditions. Use within 3 months and avoid repeated freeze-thaw cycles.

- **300 μg/mL Recombinant Human Thrombopoietin(TPO; 300 μg/mL)**

  Dissolve recombinant human TPO (25 μg) at 300μg/mL in 83.3 μL of sterile PBS containing 0.1% bovine serum albumin. After reconstitution, divide suitable tube and store -20 to -70 °C under sterile conditions. Use within 3 months and avoid repeated freeze-thaw cycles.

- **300 μg/mL Recombinant Human Flt-3 Ligand Protein (Flt-3 Ligand; 300 μg/mL)**

  Dissolve recombinant human Flt-3 Ligand Protein (25 μg) at 300μg/mL in 83.3 μL of sterile PBS containing 0.1% bovine serum albumin. After reconstitution, divide suitable tube and store -20 to -70 °C under sterile conditions. Use within 3 months and avoid repeated freeze-thaw cycles.

- **300 μ g/mL Recombinant Human SCF Protein (SCF; 300 μ g/mL)**
  Dissolve recombinant human Flt-3 Ligand Protein (50 μ g) at 300μg/mL in 166.6 μ L of sterile PBS containing 0.1% bovine serum albumin. After reconstitution, divide suitable tube and store -20 to -70 ° C under sterile conditions. Use within 3 months and avoid repeated freeze-thaw cycles.

- **Medium for human PBMCs culture with Cytokines**
  Mix the 10 μ L of indicated cytokines shown below into 10mL of StemSpan™-ACF medium. The final concentration was shown in the parenthesis.
  ·10μg/mL Recombinant human Interleukin-3(final conc. 10ng/mL)
  ·100μg/mL Recombinant human Interleukin-6(final conc. 100ng/mL)
  ·300μg/mL Recombinant Human TPO(final conc. 300ng/mL)
  ·300μg/mL Recombinant Human Flt-3 Ligand Protein(final conc. 300ng/mL)
  ·300μg/mL Recombinant Human SCF Protein(final conc. 300ng/mL)

- **StemFit® AK02N**
  This medium is comprised of three components, and the safekeeping temperature zone is divided into "refrigeration" and "the freezing".
  ・A solution 400mL (refrigeration, 2-8 degrees)
  ・B solution 100mL (freezing, -20 degrees or less)
  ・C solution 2mL (freezing, -20 degrees or less)
  To thaw, place a solution under room temperature for several hours or under 2 to 8 °C for a night. Thaw a solution C under room temperature, add into solution B. Mix well and transfer bottle of solution A. After mix well, divide suitable tube or bottle and store -20 to -70 ° C. After preparation, use within 2weeks.

- **StemFit® AK02N medium containing 10 μ mol/L Y-27632**
  To the required amount of StemFit® AK02N medium, add 1/500 of 5mmol/L Y-27632. Use it after a preparation by the end of the day

1.1.5. Equipment

- Pipetman and tips (Eppendorf)
- 15 ml conical tube (IWAKI, 2325-015)
- 50 ml conical tube (IWAKI, 2345-050)
- 5 ml plastic disposable pipette (IWAKI, 7153-005)
- 10 ml plastic disposable pipette (IWAKI, 7154-010)

- 25 ml plastic disposable pipette (IWAKI, 7155-025)
- 50 ml plastic disposable pipette (Nunc, 170353)
- Portable Pipet-Aid® XP Pipette Controller(Drummond Scientific Company)
- 1.5mL micro tube (WATSON, 131-7155CS)
- 1 ml cryovial (NUNC, 375353)
- 6-well tissue culture plate (Falcon, 353046)
- 24-well tissue culture plate (Falcon, 353047)
- BIOLOGICAL SAFETY CABINET(SANYO, MHE-131AJ)
- Microscope（OLYMPUS, CKX41）
- Centrifuge machine (TOMY, AX-310)
- TC10/20 Automatic cell counter (BIO RAD:145001 /1450101J1)
- Cell Counting Slides for TC10™/TC20™ Cell Counter (BIORAD, 1450011/ 1450015)
- "Mr.Frosty" Cryo 1°C Freezing Container (Nalgene, 5100-0001)
- CO_2 incubator(Thermo Fisher Scientific, Forma)
- Deep freezer (SANYO, MDF-U384)
- Mini Water bath (TITEC e-Thermo Bucket ETB, 0059042-000)
- Nucleofector II b Device (Lonza)
- Stereoscopic microscope（OLYMPUS, SZ61）
- Transmission illumination base set(OLYMPUS, SZ2-ILA-LED-SET)
- One monitor type C-mount camera(MEIJI TECHNO, HD1500MM)

1.2. Methods

1.2.1. Isolation of PBMCs from human blood using the BD Vacutainer® CPT™ Tube with Sodium Citrate

1. The BD Vacutainer® CPT™ Tube with Sodium Citrate and medium (StemSpan ACF, PBS) should be at room temperature (18-25ºC).

2. Collect blood into the tube (8mL).

3. After collection, remix the blood sample immediately prior to centrifugation by gently inverting the tube 8 to 10 times.

4. Centrifuge tube sample at room temperature (18-25ºC) in a horizontal rotor (swing-out head) for 20 minutes at 1800×g (slow acceleration and slow deceleration).

5. After centrifugation, (mononuclear cells and platelets will be in a whitish layer just under the plasma layer) remove with a micropipette (P1000) approximately half of the plasma without disturbing the cell layer.

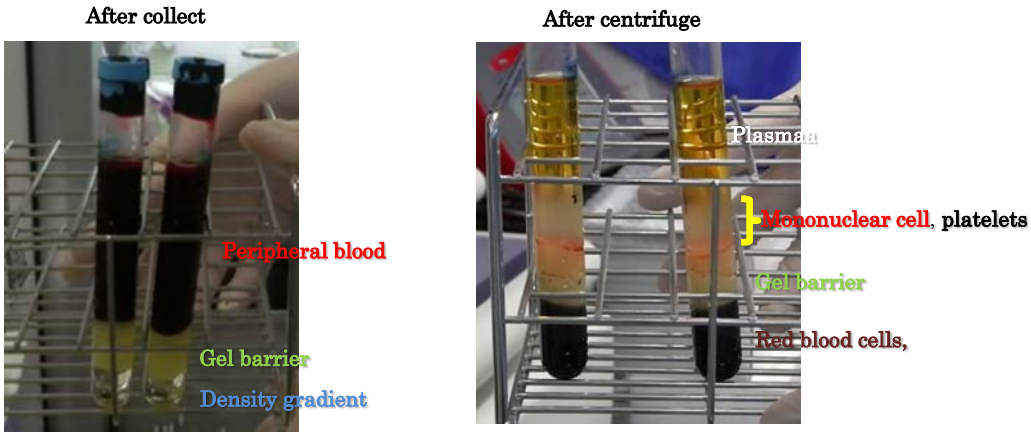


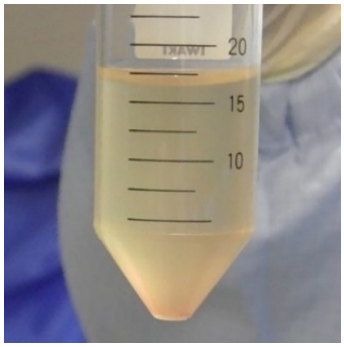
Fig.2 Isolation of PBMCs from human blood using the BD Vacutainer® CPT™ Tube with Sodium Citrate

6. Collect cell layer with a micropipette (P1000) and transfer to a 50 mL conical tube.

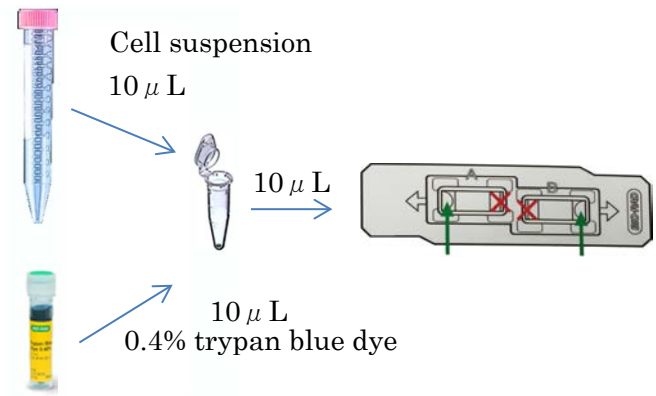
7. Add 12mL of PBS to the 50mL conical tube.

8. Centrifuge the 50mL conical tube at 160×*g* for 5 minutes (slow acceleration and slow deceleration).

9. Remove the supernatant and resuspend 6mL of StemSpan™-ACF.



10. Count the cells; Mix 10µL of the cell suspension with 10µL of 0.4% trypan blue dye in a 1.5mL micro tube. Gently pipet up and down ten times to mix. Pipet 10µL of the cell suspension into the either chamber on the counting slide. After loading a sample, insert a counting slide into the slide slot of the TC20 cell counter.



Use the displayed size gate positions (in microns) as a guide for subsequent count on the TC20 cell counter. To select a population on the histogram displayed at the beginning of cell count. When the histogram is displayed in the low size gate is flashing and positions of gates (in microns) are displayed in the lower right corner. Use the **up** or **down** arrow keys to move the low gate to the desired position (low gate: 4, high gate: 30). Press **Enter** to select the high gate. Use the **up** or **down** arrow keys to move the high gate to the desired position. Press **Enter** to confirm position of both gates. Press Enter to proceed with the count.

1.2.2. Stock of PBMCs (from 1-2-1. step No.10)

If stock the isolated cells, please perform the following operation.

1. Transfer the separated sample (at least 3×10^6 cells/tube) into a 15mL conical tube.

2. Centrifuge the tube at 200×g for 10 minutes (slow acceleration and slow deceleration).

3. Remove the supernatant and resuspend 1mL of STEM-CELLBANKER® GMP grade.

4. Transfer the resuspension to a 1mL of size cryovial and place the cryovials into holes in tube holder of "Mr. Frosty".

5. Place unit in bottom of -70°C to -80°C mechanical freezer. Leave undisturbed for a minimum of 4 hours.

6. Remove frozen tubes from unit and place in a permanent, long-term storage freezer (e.g. -130°C or below).

1.2.3. Preculture for PBMCs to transfect reprograming –related genes

1.2.3.1. Preculture for PBMCs to transfect reprograming –related genes (use fresh pBMCs; from 1-2-1. step No.10)

If seed the isolated cells, please perform the following operation.

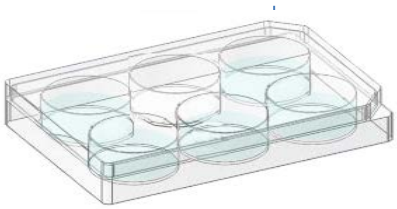
1. To prevent the evaporation of the medium, add each 2mL of PBS into a 6well-plate avoid central well and put the plate on 37°C/5%CO_2 incubator.

2. (From 1-2-1. step No. 10) Resuspend the cells to become 3×10^6 cells/well with 1.5mL of medium for human PBMCs culture with Cytokines and seed into the central well.

3. Culture for 7 days on 37°C/5%CO_2 incubator.

1.2.3.2. Preculture for PBMCs to transfect reprograming –related gene*s(use the frozen cells;From 1-2-2. step No.6)*

1. To prevent the evaporation of the medium, add each 2mL of PBS into 6well-plate avoid central well and put the plate on 37°C/5%CO_2 incubator.



2. Prepare 5mL of StemSpan-ACF (-Cytokine) in a 15 mL conical tube.

3. Take a cryovial of frozen cells out of the liquid nitrogen tank, open the cap once in hood so as to let out liquid nitrogen invaded in the tube, and incubate the vial in a 37℃ water bath until almost cells thawed.
   NOTE) In case that liquid nitrogen invades into frozen tubes, the tube will be burst out along with rapid thawing. To avoid above situation, it is necessary to loosen the cap once or to avoid warming before complete thaw.

4. Transfer the cells to the 15mL conical tube.

5. Centrifuge for 10 min at 200 × g at 18°C (slow acceleration and slow deceleration).

6. Remove the supernatant.

7. Break the pellet by tapping and suspend in 2 mL of medium for human PBMCs culture with Cytokines.

8. Count the cells; Mix 10μL of the cell suspension with 10μL of 0.4% trypan blue dye in a micro test tube. Gently pipet up and down ten times to mix. Pipet 10μL of the cell suspension into the either chamber on the counting slide. After loading a sample, insert a counting slide into the slide slot of the TC20 cell counter.

9. Transfer the cells to become 3×10^6 cells/well into a central well of 6-well plate.

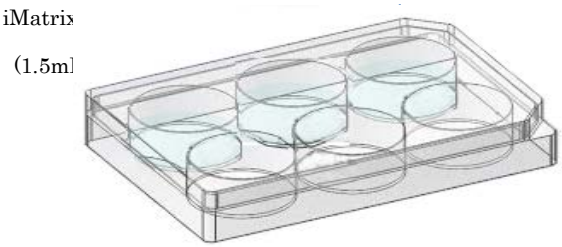
10. Culture for 7 days on 37°C/5%CO_2 incubator.

1.2.4. Generation of iPS cells

1.2.4.1. Plate coating with laminin 511 E8 (Three wells of a 6-well plate)

1. "iMatrix-511" is recombinant Laminin511-E8 Fragments. To coat a plate or dish with iMatrix-511(0.5 μg /cm^2); dilute the iMatrix-511 with sterile PBS in an appropriate size of tube. For three wells of a 6-well plate (9.6 cm^2/well), add 28.8 μL of iMatrix-511 (14.4 μg) in 4.47 mL of PBS in a 15mL size of tube (See table 1).

2. Add 1.5 mL of diluted iMatrix-511 solution to the well.



3. Incubate for 1 h at 37 °C, 3 h at room temperature, or overnight at 4 °C.

4. Add 0.75mL of StemSpan™-ACF and remove remaining fluid and medium from the coated surface.

5. Replace medium for human PBMCs culture with Cytokines and incubate at 37℃ /5%CO_2 until use it.

   * Don't allow the plate to dry.

Table.1 Amount of reagent

		12-well	6-well	60-mm	100-mm
Maker		Falcon	Falcon	IWAKI	IWAKI
Catalog Number		353043	353046	3010-060	3020-100
Area of Well or Dish	cm^2	3.8	9.6	21	55
Medium Volume	mL	0.6	1.5	3	9
Weight of iMatrix-511 Coating	μg	1.9	4.8	10.5	27.5
Volume of iMatrix-511 (0.5μg/μL)	μL	3.8	9.6	21	55

To prepare multiple wells, prepare a master mix and dispense

Coating at 0.5 μg/cm^2

1.2.4.2. Gene transfer (day 0)

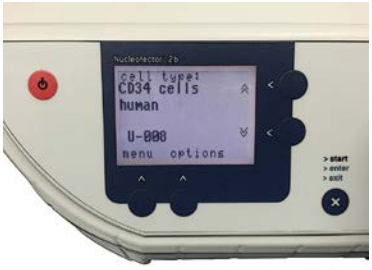
1. Collect the cultured PBMCs in a 15mL conical tube.

2. Stain the cells with trypan blue and count the number of living cells with the TC20.

3. Centrifuge for 10 min at 200 × g (slow acceleration and slow deceleration), meanwhile perform the step No.4, 5 and 6 operations

4. Add 0.8 mL of medium for human PBMCs culture with Cytokines in a 1.5 mL of micro tube.

5. Set a Nucleofector Ⅱb Device; Turn on the power of the Nucleofector Ⅱb Device and wait for the self-check to complete without abnormality. Set to program number **U-008**. And prepare cuvette and exclusive spuit beforehand.



Nucleofector Ⅱb Device **cuvette** **exclusive spuitt**

6. To prepare Gene Transfer reagents mixture, mix 81.8 μL of Nucleofectotor solution, 18.2 μL of Supplement1 solution, and 3.02 μL of plasmid solution in a 1.5mL micro tube.

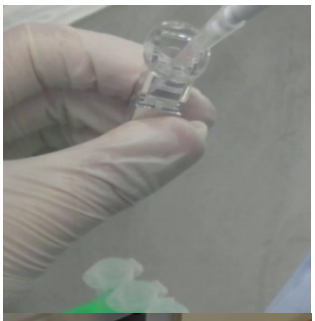
Nucleofector solution
(81.8 μ L)

Supplement1
(18.2 μ L)

plasmid DNA
(3.02 μ L)

7. Remove the supernatant as much as possible with a micropipette.

8. Suspend the cell pellets in 103 μL of gene transfer reagents mixture with micropipette, avoiding frothing.



9. Transfer the suspension to a cuvette with micropipette. Do not allow to froth

10. Place the cuvette in the Nucleofector Ⅱb Device unit and start the program.

11. Immediately collect the gene-transferred cells to the tube of step 4 with exclusive spuit and mix gently.

12. Dispense the cell suspension with necessary density (2.0×10^5 cells/well, 1×10^5 cells/well, 0.5×10^6 cells/well) into each well of the iMatrix-511-coated plates.

13. Rock the plates to achieve uniform cell density and start culture in an incubator.

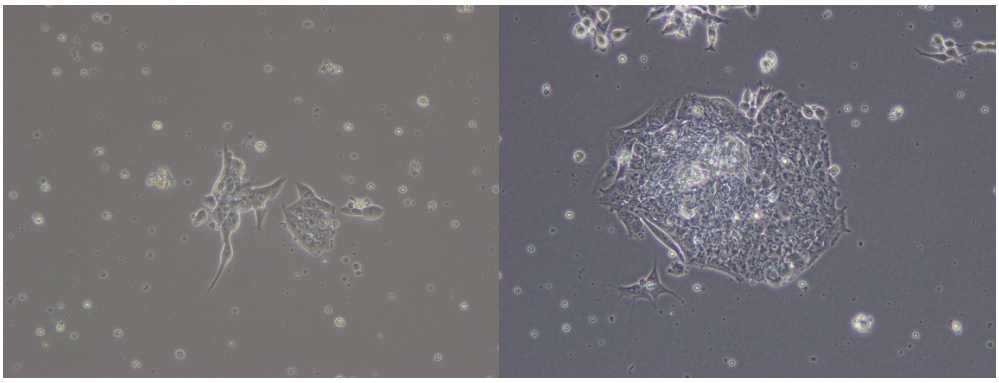
1.2.4.3. Medium addition (days 2, 4, and 6)

1. Add 1.5 mL of StemFit® AK02N medium to each well.

1.2.4.4. Medium change (day 8-)

1. Change the medium to 1.5 mL/well of fresh StemFit® AK02N medium every two days.

2. Continue medium change until the size of the iPS cell colonies exceeds 1 mm.



6 days after from introducing　　10 days after from introducing

Fig. 1 Generated iPS cell colonies from PBMC

1.2.5. olony picking

1.2.5.1. Plate coating with laminin (a well of 12-well plate)

1. Into each well of the 12-well plate to be coated, dispense 0.6 mL of PBS.

2. Add 3.8 μL/well of iMatrix-511(0.5 μg/μL) and mix well immediately (coating: 0.5 μg/ cm^2).

3. Incubate at 37°C, CO_2 5%, for at least 60 min (plates can be left overnight at 37°C).

4. Remove from the incubator after 60 min.

5. Add 0.3 mL/well of StemFit® AK02N medium and mix (this prevents drying in the next step).

6. Aspirate the medium and laminin suspension. DO NOT allow the plate to dry, or the coating will become ineffective!

7. Add 0.6 mL/well of StemFit® AK02N medium containing Y-27632 and return the plates to the incubator.

1.2.5.2. Colony picking

1. Change to fresh StemFit® AK02N medium.

2. Pick a colony from the plate under stereomicroscope using a pipette, and transfer it into 1.5mL micro tube.

3. Dissociate colonies into single cells by pipetting 10 times with a 20 μL micropipette (Adjust setting to 18 μL) in the 1.5mL micro tube.

4. Transfer the cell suspension to one iMtarix-511-coated well of a 12-well plate.

5. Culture the plates at 37°C, 5%CO_2.

6. The next day, change to regular StemFit® AK02N medium (NOT containing Y-27632).

1.2.5.3. Medium change

1. Change the medium to 0.6 mL/well of fresh StemFit® AK02N medium every other day.

2. Continue medium change until the size of the iPS cell colonies exceeds 1 mm.

Protocols for human iPS cell Passage and Maintenance under feeder-free culture conditions

2.1. MATERIALS

2.1.1. Cells

- 80% confluent human iPS cells (6-well plate; 9.6 cm2, 100mm dish ;55cm2)(Scale down/up as required)

2.1.2. Reagents

- Phosphate buffered saline Ca,Mg free (PBS; Nacalai tesque, 14249-95)
- TrypLE Select (Gibco, 12563-011)
- 0.5 M EDTA solution (Nacalai Tesque, 689414)
- Trypanblue stain (0.4%) for use with the TC-20 automatic cell counter (BIO-RAD,1450021)
- iMatrix-511 (Laminin-511 E8) (Nippi, 385-07361)
- Culture medium (StemFit AK02N medium) (AJINOMOTO)
- 10 mM Y-27632 (Wako, 253-00511)
- STEM-CELL BANKER (NIPPON ZENYAKU KOGYO CB046)

2.1.3. Reagent setup

- **0.5X TrypLE Select Solution Preparation**

  Aliquot 250 mL of PBS (-) into the medium filtration system. Add 250 μL of 0.5 M EDTA solution. Draw the solution through the filter by vacuum (＝0.5 mM EDTA/PBS(-) solution). Aliquot 7 mL of 0.5 mM EDTA/PBS (-) into 15 mL conical tubes. To the same tubes, add 7 mL of TrypLE Select.

  *The solution can be stored at room temperature for up to 4 weeks.

- **StemFit® AK02N**

  This medium is comprised of three components, and the safekeeping temperature zone is divided into "refrigeration" and "the freezing".

  ・A solution 400mL (refrigeration, 2-8 degrees)

  ・B solution 100mL (freezing, -20 degrees or less)

  ・C solution 2mL (freezing, -20 degrees or less)

To thaw, place a solution under room temperature for several hours or under 2 to 8 °C for a night. Thaw a solution C under room temperature, add into solution B. Mix well and transfer bottle of solution A. After mix well, divide suitable tube or bottle and store -20 to -70 ° C. After preparation, use within 2weeks.

- **StemFit® AK02N medium containing 10 μ mol/L Y-27632**
  To the required amount of StemFit® AK02N medium, add 1/500 of 5mmol/L Y-27632. Use it after a preparation by the end of the day

2.1.4. Equipment

- 6, 12, 24, 96-well plates (FALCON, 353046, 353043, 353047, 351172)
- 100 mm tissue culture dish (IWAKI, 3020-100)
- 15, 50 ml conical tubes (FALCON, 352196, 352070)
- 2, 5, 10, 25 ml Plastic disposable pipette 2, 5, 10, 25 ml (FALCON, 57507, 357543, 357551, 357525)
- 1.2 ml cryotube (Nunc, 375353)
- Pipette aid (FALCON)
- Pipetman and tips (GILSON)
- 1.5 ml tube (WATSON)
- Cell scrapers (IWAKI, 9000-220)
- CO_2 incubator
- TC20™ Automated Cell Counter (BIO-RAD)
- Cell Counting Slides for TC20™(BIO-RAD, 1450015)
- Mr. Frosty (Thermo Scientific, 5000-0001)
- Water bath

2.1.5. Culture ware coating with laminin 511 E8

- **one well in a 6-well plate**

Add 1.5 mL of PBS (-) into a 15 mL conical tube and mix with 9.6 μl of iMatrix- 511 (0.5mg/mL). Add 1.5 mL of diluted iMatrix-511 into a well. Incubate at 37°C, CO_2 5%, for at least 60 min (plates can be left overnight at 37°C). Remove from the incubator after 60 min. Add 0.75 mL/well of StemFit®AK02N medium (this prevents drying in the next step). Aspirate the medium and iMatrix-511 suspension. DO NOT allow the plate to dry, or the coating will become ineffective! Add 1.5 mL/well of StemFit® AK02N medium containing 10 μM Y-27632 and return the plates to the incubator.

- **one 100 mm dish**

Add 9 mL of PBS (-) into a 15 mL conical tube and mix with 55 µl of iMatrix-511(0.5mg/mL) Add 9 mL of diluted iMatrix-511 into a dish. Incubate at 37°C, CO_2 5%, for at least 60 min (plates can be left overnight at 37°C). Remove from the incubator after 60 min. Add 4.5 mL of StemFit® AK02N medium (this prevents drying in the next step). Aspirate the medium and iMatrix-511 suspension. DO NOT allow the plate to dry, or the coating will become ineffective! Add 9 mL/well of StemFit® AK02N medium containing 10 µM Y-27632 and return the plates to the incubator.

2.2. METHOD

2.2.1. Passaging procedure

2.2.1.1. 6-well plate to 6-well plate

1. Observe the cells by microscopy. If imaging is required, dead cells may be removed by replacing the medium. Culture medium is preferred over PBS as cells tend to detach with extended incubation in PBS.

2. Aspirate the medium.

3. Wash once with 1 mL of PBS and aspirate.

4. Add 300 µL/well of 0.5X TrypLE Select and spread well over the surface.

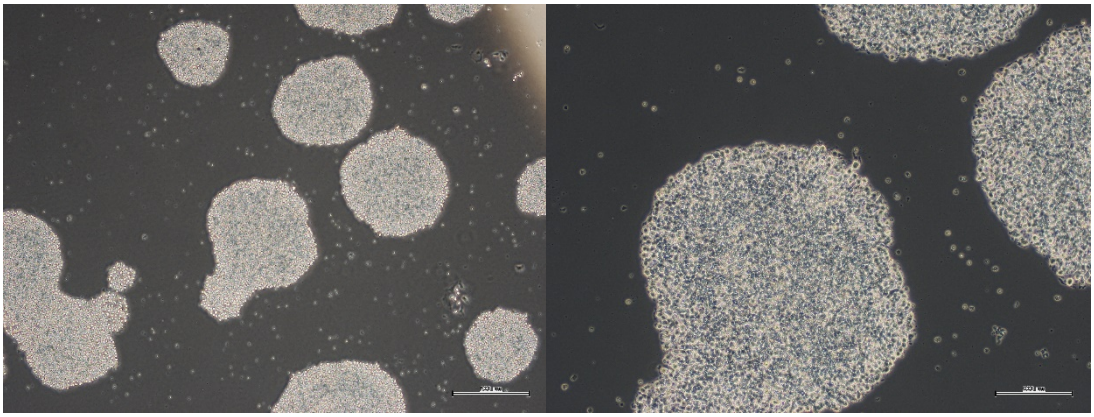
5. Incubate the plates at 37°C, CO_2 5%, for 1 min.

6. Remove the plates from the incubator after 1 min and gently re-distribute the 0.5X TrypLE Select over the surface.

7. Incubate the plates for another 3 min at 37°C, CO_2 5%, (total 4 min).

8. Remove the plates from the incubator and observe the cells by microscopy. (The cells should appear separate and rounded. During the 4min incubation, cell-cell adhesion is destroyed, but the cells remain attached to the matrix (right photo).
Caution: Longer incubation will also detach the cells.)

9. Aspirate the 0.5X TrypLE Select.

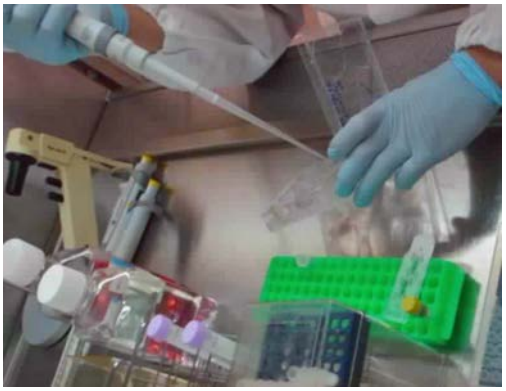


10. Wash once with 2 mL/well PBS. (Add the PBS gently as the cells tend to detach easily.)
11. Add 1 mL of StemFit®AK02N medium per well.

12. Detach the cells with a cell scraper.

13. Observe the cells under the microscope to ensure they have detached.

14. Pipette the cells 10 times to fully dissociate. Collect the cells in a 1.5 mL tube (this prevents re-attachment of the cells to the plate surface).



15. Count the cells by trypan blue exclusion using the TC20™ Automated Cell Counter. (iPSC program-Max size 30 μM, Minimum size 8 μM)

16. Plate 13,000 live cells in one well of a 6-well plate (9.6 cm^2) coated with laminin. (Immediately distribute the cells evenly over the plate surface to avoid uneven attachment.)

17. Culture the plates at 37°C, CO_2 5%.

18. The next day, change to regular culture medium (NOT containing Y-27632).

*Medium change is performed every other day. If the color of the medium turns

orange or yellow, it should be changed every day.
*Should yield approximately 1 x 10^6 cells in 8 days (±1 day, 100-fold expansion).

2.2.1.2. 6-well plate to 100 mm dish

1. Same procedure as No1. to No15.

2. Plate 80,000 live cells in one well of a 100 mm dish (55 cm^2) coated with laminin.

3. (Immediately distribute the cells evenly over the plate surface to avoid uneven attachment.)

4. The next day, change to regular culture medium (NOT containing Y-27632).

2.2.2. Freezing Procedure

2.2.2.1. Make 5 cryotubes from 6-well plate

1. Observe the cells by microscopy. If imaging is required, dead cells may be removed by replacing the medium. Culture medium is preferred over PBS as cells tend to detach with extended incubation in PBS.

2. Aspirate the medium.

3. Wash once with 1mL PBS and aspirate.

4. Add 300 μL/well of 0.5X TrypLE Select and spread well over the surface.

5. Incubate the plates at 37°C, CO_2 5%, for 1 min.

6. Remove the plate from the incubator after 1 min and gently re-distribute the TrypLE over the surface.

7. Incubate the plates for another 3 min at 37°C, CO_2 5% (total 4 min).

8. Remove the plates from the incubator and observe the cells by microscopy. (The cells should appear separate and rounded. During the 4 min incubation, cell-cell adhesion is destroyed, but the cells remain attached to the matrix (right photo). Caution: Longer incubation will also detach the cells.)

9. Aspirate the 0.5X TrypLE Select.

10. Wash once with 2 mL/well of PBS. (Add the PBS gently as the cells tend to detach easily.)

11. Add 1 mL of StemFit®AK02N medium per well.

12. Detach the cells with a cell scraper.

13. Observe the cells under the microscope to ensure they have detached.

14. Pipette the cells 10 times to fully dissociate and collect them in a 15 mL tube (this prevents re-attachment of the cells to the plate surface).

15. Count the cells by trypan blue exclusion using the TC20™ Automated Cell Counter. (iPSC program-Max size 30 μM, Minimum size 8 μM)

16. Transfer the cells at least 1 x 10^6 cells to a centrifuge tube and spin at 160 X g, 22°C for 5 min.

17. Remove the supernatant and gently disrupt the pellet.

18. Resuspend the cells 1 x 10^6 cells/mL in STEM-CELL BANKER .

19. Dispense 200 μL (=2 x 10^5 cells) per cryotube.

20. Cool using a Mr. Frosty at -80°C for at least3 hrs.

21. Transfer to liquid nitrogen within a few days.

2.2.2.2. Make 30 cryotubes from 100 mm dish

1. Observe the cells by microscopy. If imaging is required, dead cells may be removed by replacing the medium. Culture medium is preferred over PBS as cells tend to detach with extended incubation in PBS.

2. Aspirate the medium.

3. Wash once with 6 mL PBS and aspirate.

4. Add 1.8 mL/well of 0.5X TrypLE Select and spread well over the surface.

5. Incubate the plates at 37°C, CO_2 5%, for 1 min.

6. Remove the plate from the incubatorafter 1 min and gently re-distribute the TrypLE over the surface.

7. Incubate the plates for another 3 min at 37°C, CO_2 5% (total 4 min).

8. Remove the plates from the incubator and observe the cells by microscopy. (The cells should appear separate and rounded. During the 4 min incubation, cell-cell adhesion is destroyed, but the cells remain attached to the matrix .
Caution: Longer incubation will also detach the cells.

9. Aspirate the 0.5X TrypLE Select.

10. Wash once with 12 mL/well of PBS. (Add the PBS gently as the cells tend to detach easily.)

11. Add 4.5 mL of StemFit®AK02N medium per dish.

12. Detach the cells with a cell scraper.

13. Observe the cells under the microscope to ensure they have detached.

14. Pipette the cells 10 times using 5000 μL Pipetman to fully dissociate and collect them in a 15 mL tube (this prevents re-attachment of the cells to the plate surface).

15. Add 5.5 mL of StemFit®AK02N medium per dish and rince a surface one time.

16. After rince, collect them in a 15 mL tube. (total 10 mL)

17. Count the cells by trypan blue exclusion using the TC20™ Automated Cell Counter. (iPSC program-Max size 30 μM, Minimum size 8 μM)

18. Transfer the cells at least 8 x 10^6 cells to a centrifuge tube and spin at 160 X g, 22°C for 5 min.

19. Remove the supernatant and gently disrupt the pellet.

20. Resuspend the cells 1 x 10^6 cells/mL in STEM-CELL BANKER .

21. Dispense 200 μL (=2 x 10^5 cells) per cryotube.

22. Cool using a Mr. Frosty at -80°C for at least3 hrs.

23. Transfer to liquid nitrogen within a few days.

2.2.3. Thawing procedure

1. Warm the water bath to 37°C and the medium to room temperature.

2. Add 5mL of StemFit®AK02N medium to a 15 mL conical tube.

3. Remove the cells from the liquid nitrogen and thaw immediately in the water bath (~1 min, until only small ice particles remain).

4. Transfer the cell suspension to the conical tube prepared in Step 2 with gentle pipetting (1-2 times).

5. Spin the cells at 160 x g , 22°C, for 5 min to pellet.

6. Aspirate the supernatant.

7. Tap the pellet gently to break, then resuspend in 0.5 mL of StemFit®AK02N medium.

8. Count the cells by trypan blue exclusion using the TC20™ Automated Cell Counter.

(iPS program-Max size 30 μM, Minimum size 8 μM)

9. Plate 65,000 live cells to one well of a laminin-coated 6-well plate.

10. Immediately distribute the cells evenly over the plate surface to avoid uneven attachment.

11. Culture the plates at 37°C, CO_2 5%.

12. The next day, change to StemFit®AK02N medium (NOT containing Y-27632).

* Medium change is performed day after thawing, then every other day until day 6 or 7 afterthawing. When the cells become more confluent, feed them every day.
* If the color of the medium turnsorange or yellow, it should be changed every day.
* Should yield approximately 8 days.

Protocols for evaluation ofhuman iPS cell

3.1. Alkaline phosphatase staining for human iPSCs

3.1.1. Preparation of reagents

- Paraformaldehyde solution; 4% paraformaldehyde solution (Wako, Cat. No. 163-20145)
- ALAKLINE PHOSPHATASE(AP), LEUKOCYTE Kit (SIGMA, Cat.No. 86-R)

3.1.2. Fixation and Staining of human iPSCs

1. Add 2-5 ml of paraformaldehyde solution to a culture dish and incubate cells for 10 minutes.

2. Wash cells with distilled water.

3. Mix 1 ml of Sodium Nitrite Solution and 1 ml of FRV-Alkaline Solution in ALAKLINE PHOSPHATASE(AP), LEUKOCYTE kit by inverting tubes, and incubate for 2 minutes. Add 45 ml of distilled water and 1 ml of Naphthol AS-BI Alkaline Solution as a substrate solution.

4. After wash, add the solution prepared in Step 3 and incubate at room temperature for 15 minutes.
5. Aspirate the substrate solution and wash cells with distilled water. After aspirating water, add fresh distilled water and incubate 2 minutes. After shaking gently, dry up the cells (Figure 2).

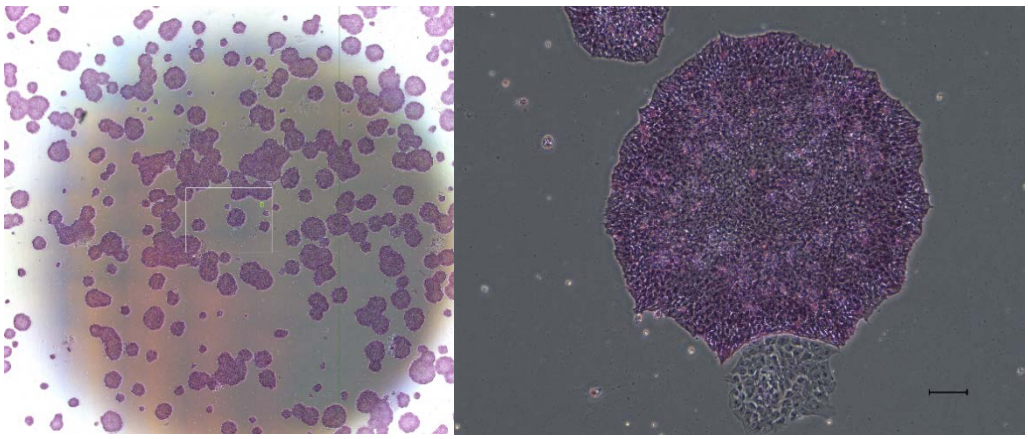


Figure 2. Human iPSCs with alkaline phosphatase staining

3.2. SSEA-4 staining for human iPSCs

3.2.1. Preparation of reagents

- Paraformaldehyde solution; 4% paraformaldehyde solution (Wako, Japan, Cat. No. 163-20145)
- Goat serum (Funakoshi, Japan, Cat. No. S-1000)
- Mouse-anti human SSEA-4 antibody (Santa Cruz Biotechnology, Inc, Cat. No. sc-21704)
- Alexa Fluor® 488 Goat Anti-mouse IgG, 2 mg/ml (Invitrogen, Cat. No. A11029)
- DAPI (Invitrogen, Cat. No. 03571)

3.2.2. Fixation and immune-taining of human iPSCs

1. Wash cells with PBS.

2. Fix the cells with 4% paraformaldehyde solution for 10 minutes.

3. Remove the 4% paraformaldehyde solution.

4. Wash the cells with PBS at 3 times.

5. Block the cells with 10% goat serum solution for 30 minutes. Meanwhile, dilute mouse-anti human SSEA-4 antibody 1:50 in 1.5% goat serum solution.

6. Remove the 1.5% goat serum solution.

7. Add the diluted mouse-anti human SSEA-4 antibody solution and incubate for 1

hour at room temperature. Meanwhile, dilute Alexa Fluor® 488 Goat Anti-mouse IgG, 2 mg/ml 1:50 in 1.5% goat serum solution.

8. Remove the diluted mouse-anti human SSEA-4 antibody solution.

9. Wash the cells with PBS at 3 times for 5 minutes.

10. Add the diluted Alexa Fluor® 488 Goat Anti-mouse IgG solution and incubate for 45 minutes at room temperature.

11. Remove the diluted Alexa Fluor® 488 Goat Anti-mouse IgG solution.

12. Wash the cells with PBS at 3 times for 5 minutes.

13. Add 1μg/ml DAPI solution and incubate for 5 minutes at room temperature.

14. Remove the DAPI solution.

15. Wash the cells with PBS at 3 times.

16. Observe the cells under immunofluorescence microscopy.

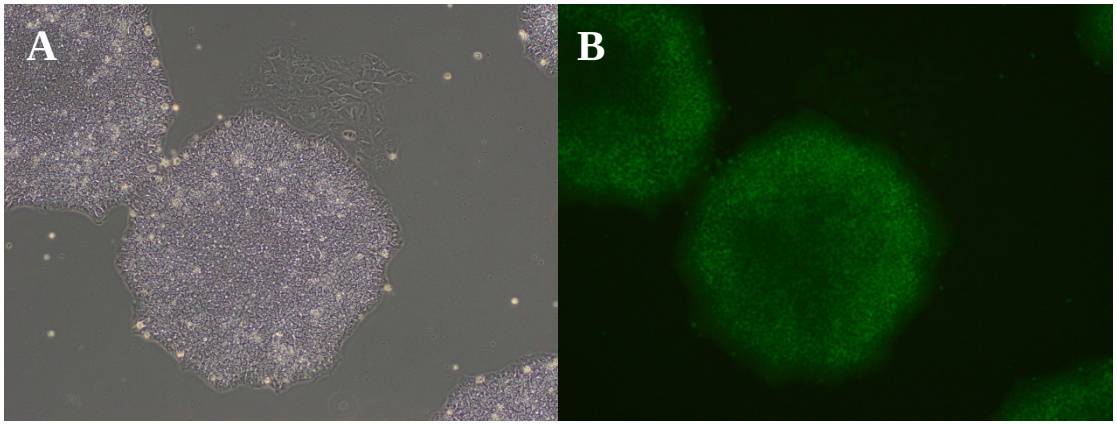


Figure 3. Immunofluorescence staining with SSEA-4
A: Phase-contrast image, B: SSEA-4 staining

3.3. Mycoplasma detection

3.3.1. Preparation of reagents

- MycoAlert® Mycoplasma Detection Kit (Lonza, Cat. No. LT07-118; 10 tests)

Principles (quote a MycoAlert® Mycoplasma Detection Kit manual)
The MycoAlert Assay is a selective biochemical test that exploits the activity of certain mycoplasmal enzymes. The presence of these enzymes provides a rapid screening procedure, allowing sensitive detection of contaminating mycoplasma in a test sample. The viable mycoplasma are lysed and the enzymes react with the MycoAlert Substrate catalyzing the conversion of ADP to ATP.
By measuring the level of ATP in a sample both before and after the addition of the

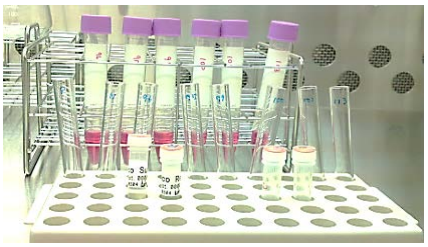
MycoAlert® Substrate, a ratio can be obtained which is indicative of the presence or absence of mycoplasma. If these enzymes are not present, the second reading shows no increase over the first, while reaction of mycoplasmal enzymes with their specific substrates in the MycoAlert Substrate, leads to elevated ATP levels. This increase in ATP can be detected using the following bioluminescent reaction.

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[\text{Mg 2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{LIGHT}$$

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer. The assay is conducted at room temperature (18°C-22°C), the optimal temperature for luciferase activity.

3.3.2. Procedure (quote a MycoAlert® Mycoplasma Detection Kit manual)

1. Bring all reagents up to room temperature before use.



2. Reconstitute the MycoAlert Reagent and MycoAlert Substrate in MycoAlert Assay Buffer. Leave for 15 minutes at room temperature to ensure complete rehydration.

3. Transfer 2 ml of cell culture or cell culture supernatant into a centrifuge tube and pellet any cells at 1,500 rpm (200×*g*) for 5 minutes.

4. Transfer 100 µl of cleared supernatant into a luminescence compatible plate. White walled plates provide best sensitivity with the MycoAlert Assay.

5. Program the luminometer to take a 1 second integrated reading.

6. Add 100 µl of MycoAlert Reagent to each sample and wait 5 minutes.

7. Place plate in luminometer and initiate the program (Reading A).

8. Add 100 µl of MycoAlert Substrate to each sample and wait 10 minutes.

9. Place plate in luminometer and initiate the program (Reading B).

10. Calculate ratio = Reading B/Reading A.

3.3.3. Interpretation of results (quote a MycoAlert® Mycoplasma Detection Kit manual)

The ratio of Reading B to Reading A is used to determine whether a cell culture is contaminated by mycoplasma.
The speed and convenience offered by the MycoAlert Kit means that it provides a unique method for screening cultures for the presence of mycoplasma. As such, it is ideally suited to routine testing of cells in culture. Frequent use of the MycoAlert Assay will indicate when a cell line becomes infected allowing prompt remedial action to be taken. The MycoAlert Assay can also be extended to incoming cell lines and the commonly used constituents of complete media.
The interpretation of the different ratios obtained, within each experimental situation, may vary according to the cell types and conditions used. However, the test has been designed to give ratios of less than 1 with uninfected cultures. Cells which are infected with mycoplasma will routinely produce ratios greater than 1.

Table 1. Interpretation of MycoAlert Assay results illustrating examples of healthy and infected cell lines.

Cell Line	MycoAlert Assay ratio	Conclusions
Infected cells		
K562	123.26	Positive
A549	4.10	Positive
U937	8.26	Positive
HepG2	1.27*	Borderline, quarantine and retest in 24 hours
Healthy cells		
HL60	0.72	Negative
COS-7	0.46	Negative

References

1. M. Nakagawa et al., A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. Scientific Reports, 4:3594 (2014) DOI: 10.1038/srep03594
2. Okita, K ., et al. An Efficient Non-viral Method to Generate Integration-Free Human iPS Cells from Cord Blood and Peripheral Blood Cells. Stem Cells. 2012 Nov 29
3. Okita, K ., et al. A more efficient method to generate integration-free human iPS cells. Nat Methods. 8, 409- 12 (2011) .
4. Mack, AA., et al. Generation of Induced Pluripotent Stem Cells from CD34+ Cells across Blood Drawn from Multiple Donors with Non-Integrating Episomal Vectors. PLoS One. 6, e27956 (2011).
5. Takahashi, K, et al. Induction of pluripotent stem cells from adeult human fibroblasts by defined factors. Cell 131, 861-872 (2007)