**THAWING AND CULTIVATION OF HUMAN FIBROBLASTS**
**(Campisi Lab SOPs)**

**REAGENTS**

| Reagent | Vendor | Catalog# | Notes |
|---|---|---|---|
| Dulbecco's Modified Eagle's Medium (DMEM) (high glucose 4.5g/L, + glutamine, w/o pyruvate) + Na bicarbonate (suitable for the $CO_2$ concentration in the incubator; see preparing the medium below). | Corning Cellgro | 10-017-CV | Vendor is not critical |
| Fetal Bovine Serum (FBS) | TissueCulture Biologicals | 101 | Vendor is not critical |
| Penicillin/streptomycin (pen/strep) (10,000IU/ml 10,000ug/ml) | Corning Cellgro | 30-002-CI | Vendor is not critical |
| Trypsin/EDTA 1X | Corning Cellgro | 25-052-CI | Vendor is not critical |
| $CO_2$ and $N_2$ gases for incubators (see notes on gas composition in the incubator) | various | | Vendor is not critical |
| Edu labeling kit | Invitrogen | C10337 | |
| Senescence detection kit | Biovision | K320-250 | |
| MycoAlert mycoplasma testing kit | Lonza | LT07-701 | |

**PROCEDURES**

Preparing the medium (complete medium):

DMEM + 10% FBS + pen/strep (100 IU/ml / 100ug/ml).

DMEM contains Na bicarbonate – before purchasing, make sure the bicarbonate concentration is suitable for the $CO_2$ concentration in the incubator. $CO_2$ concentrations can be 5% or 10%; bicarbonate should be 2.2 or 3.8 g/l for 5% or 10% $CO_2$ respectively (suitable for buffering at pH 7.4). We generally use the high bicarbonate formulation and 10% $CO_2$.

Notes on gas composition in the incubator:

We generally cultivate cells in 10% $CO_2$ and 3% $O_2$ (balance is $N_2$). 5% $CO_2$ is fine; just be sure bicarbonate is appropriate for pH 7.4 buffering. Human fibroblasts will also tolerate atmospheric $O_2$ concentrations, but they fare better at 3% $O_2$.

Thawing the cells:

- Thaw cells by placing lower portion of the vial in a 37° C water bath.
- When thawed, remove cells using a wide-bore 1 ml pipet and a T75 tissue culture flask containing 15 ml prewarmed (37° C) complete medium.
- Incubate 5-6 hours at 37° C in a tissue culture incubator.
- Examine the cells under a microscope. Most should attach within 2-3 hours after thawing.
- Gently remove the medium by aspirating with a Pasteur pipet. Replace with 15 ml fresh complete medium.

Maintaining the cells:

- Senescent cells will not divide. *They are nonetheless metabolically active and medium should be replaced with fresh complete medium every 3 days or so*.
- Non-senescent cells will divide every 24-36 hours, depending on strain and passage number. *Medium should be replaced with fresh complete medium every 3 days or so*.
- In addition, non-senescent cells should be sub-cultured when 80-90% confluent.

Subculturing the cells:

- Remove the medium by aspiration.
- Wash cells with 2-3 ml pre-warmed (37° C) trypsin-EDTA solution.
- Add 2 ml trypsin-EDTA; incubate at 37° C for 2-3 minutes (cells should be rounded and detached).

- Allow cells to detach without tapping the side of the flask (this creates clumps) (check under the microscope to be sure they are)
- Add 8 ml complete medium (which will neutralize the trypsin). Keep flask upright while counting the cells.
- Remove 0.1-0.2 ml of the cell suspension and count (use hemacytometer or Coulter counter).
- Subculture by putting 2 x $10^5$ - 1 x $10^6$ cells (depends on purpose of the subculture) into a new T75 containing 15 ml warm complete medium.
- Return to incubator.

**QUALITY CONTROL**

Proliferation EdU:

Parallel cultures of senescent (and non-senescent) cells should be checked for proliferative capacity. We generally use EdU labeling (http://products.invitrogen.com/ivgn/product/C10337).

- Seed senescent and non-senescent cells at 20,000 cells per well in 4-well chamber slides.
- 24 hrs later, add EdU for 24 hrs as per the manufacturer's instructions.
- Fixed the cells and perform the assay as described by the manufacturer.
- What to expect: Around 50% of non-senescent populations should be positive (depending on cell strain and passage number), while <5% of senescent populations should be positive.

Proliferation, Clonogenic assay

- **Material:**
- -Cells: IMR90
- -6 well plate
- -Complete media: DMEM high glucose + 10% FBS
- -Crystal violet solution: 0.05% Crystal violet in 25% MeOH.
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- **Timeline:**
- -Seed 3,000 cells in 2ml of complete media in 6 well plate
- -Change media every 2-3 days
- -At day 9-12 proceed to staining (Time can be adjusted to growing speed, the goal is to have about 50 cells/colony)
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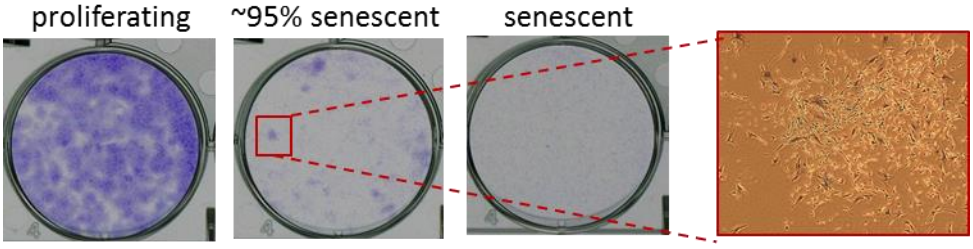
- **Staining:**
- -Wash cells twice in PBS
- -Fix cells 5 minutes with 100% MeOH.
- -Stain cells 5 minutes with Crystal violet solution
- -Wash by gently soaking the plate in a 2L beaker filled with distilled water; do a few back and forth movement. Changed water and repeat wash two more time. Remove excess of water and allow plates to dry.

- **Analysis:**
- Proliferating cells will for colonies that will be distinguishable by naked eyes. These colonies can be visualized under a microscope at 100X magnification. Plates are image under scanner.



Senescence-associated beta-galactosidase (SA-Bgal):

Senescent and non-senescent cells should be checked for SA-Bgal activity. We use the SA-Bgal kit from Biovision (http://www.biovision.com/senescence-detection-kit-2847.html).

- Seed senescent and non-senescent cells at 30,000 cells per well in 12 well plates.
- 24 hrs after seeding, performed the assay as described by the manufacturer.
- A few things to consider: 1) very confluent cells will be falsely positive; 2) the overnight incubation must be done at 37° C in air (a bacterial incubator, NOT a $CO_2$ incubator); 3) SA-Bgal is an enzyme, so cells must be fixed very lightly as recommended by the manufacturer because overfixation will destroy the enzyme and lead to false negatives.
- What to expect: Positive cells will show blue staining, mostly in the perinuclear area and other areas of the cytoplasm. Senescent populations should be 80-90% positive (intensity will vary depending on strain). Non-senescent cells should be <10% positive.

Mycoplasma testing

Cells must be free of mycoplasma and should be tested prior to use. We use the MycoAlert kit from Lonza (http://www.lonza.com/products-services/bio-research/cell-culture-products/mycoplasma-detection-and-removal/mycoalert-plus-mycoplasma-detection-kit.aspx?WT.srch=1). Collect conditioned media and test as described by the manufacturer.

**INDUCING SENESCENCE IN HUMAN FIBROBLASTS**
**(Campisi Lab SOPs)**

**REAGENTS**

| Reagent | Vendor | Catalog# | Notes |
|---|---|---|---|
| See SOP for thawing and cultivating human fibroblasts for standard tissue culture reagents | n/a | n/a | |
| X- or gamma-ray source (see Inducing senescence by ionizing radiation) | n/a | n/a | |
| Doxorubicin hydrochloride | Tocris | 2252 | |
| EdU labeling kit | Invitrogen | C10337 | |
| Senescence detection kit | Biovision | K320-250 | |

**PROCEDURES**

Inducing senescence by ionizing radiation:

*(See schematic below)*

- Plate 2.5 X $10^6$ cells in complete medium in a T175 tissue culture flask. Scale up or down depending on the number of cells needed.
- After plating, incubate at 37° C in complete medium and a 3% $O_2$-$CO_2$ incubator (see SOP for thawing and cultivating human fibroblasts for description of complete medium and choice of $CO_2$ concentration).
- 24 hours later, irradiate with 10 Gy. For X-irradiation, we use a Pantak X-ray generator (320 kV/10 mA with copper filtration) and dose rates equal to or higher than 0.75 Gy/min. If unirradiated control cells are needed, they should be mock irradiated: taken to and put inside the unpowered irradiator for the same duration as the irradiated sample.
- After irradiation (within one hour), aspirate the medium and replace with fresh prewarmed complete medium.
- 72 hours later (3 days after irradiation), aspirate the medium and replace with fresh prewarmed complete medium.
- 72 hours later (6 days after irradiation), cells can be collected and counted (see below) for analysis or treatment. *6 days post-irradiation is the minimum amount of time needed to develop a full senescent phenotype*.
- To prepare and collect conditioned medium, aspirate the medium, wash the cells twice with serum-free DMEM, add 25 ml of serum-free or 0.2% serum-containing DMEM.
- 24 hours later, collect and process conditioned medium (see below).

*Note, 24 hours after irradiation and beyond, there should be very little cell death (<10% cell loss).*


Inducing senescence using doxorubicin:

*(See schematic below)*

- Plate 2.5 X $10^6$ cells in complete medium in a T175 tissue culture flask. Scale up or down depending on the number of cells needed.
- After plating, incubate at 37° C in complete medium and a 3% $O_2$-$CO_2$ incubator (see SOP for thawing and cultivating human fibroblasts for description of complete medium and choice of $CO_2$ concentration).
- 24 hours later, add doxorubicin: 250 nM final concentration using a stock solution of 250 uM in DMSO. Note, doxorubicin should be stored at -80°C in small aliquots and thawed just before use.
- 24 hours later, remove the doxorubicin containing medium, wash with complete medium, and

replace with fresh complete medium.

- 72 hours later, (3 days after removing doxorubicin), aspirate the medium and replace with fresh prewarmed complete medium.
- 72 hours later (6 days after removing doxorubicin), cells can be collected and counted (see below) for analysis or treatment.
- To prepare and collect conditioned medium, follow protocol for inducing senescence by irradiation.

*Note, 24 hours after removing doxorubicin and beyond, there will be some cell death (<25% cell loss).*


Counting senescent cells:

*Note: Senescent cells adhere to tissue culture plastic somewhat more tenuously than non-senescent counterparts. They also are larger and therefore somewhat more fragile.*

- Remove the medium by aspiration.
- For a T175 flask, wash the cells 2-3 times with 5-6 ml pre-warmed (37° C) trypsin-EDTA solution.
- Add 5 ml trypsin-EDTA; incubate at 37° C for 3-4 minutes (cells should be partly rounded and detached).
- Allow cells to detach without tapping the side of the flask (this creates clumps) (check under the microscope to be sure they are)
- Add 10-15 ml complete medium (which will neutralize the trypsin). Keep flask upright while counting the cells.
- Remove 0.1-0.2 ml of the cell suspension and count (use hemacytometer or Coulter counter).
- Cells can be collected by gentle centrifugation (150 x g), then resuspended in complete medium to the desired concentration for replating.

Collecting and storing conditioned medium:

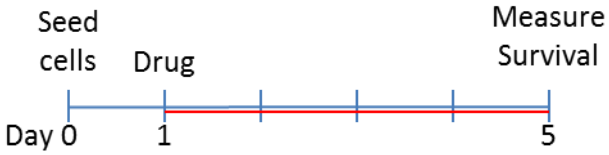
*Note: conditioned media values are normalized to cell number; thus, after removal of conditioned media, the cells should be counted or assessed by other means for cell number (remaining cells can be processed for replating or other procedures).*

- Remove the conditioned medium and transfer to a low protein binding receptacle, typically a conical plastic tube for bulk cultures. If collecting from a microtiter plate, the media can be transferred to a fresh plate. Keep on ice while counting cells.
- Centrifuge conditioned medium at 500 x g for 5-10 minute to remove cell debris. This step may not be possible for media transferred to microtiter plates.
- Transfer supernatant to a low protein binding receptacle suitable for freezing at -80° C.
- Store conditioned medium at -80° C.

TSRI – Cenexys HTS Protocol

- Human primary fibroblasts (IMR90, ATCC #CCL-186)
  - Doubling time: +/- 30-40hrs (at 3% $O_2$)
  - Medium: DMEM, high glucose, + glutamine, w/o pyruvate, Pen/Strep, 10% FBS (change media every three days).
  - All primary human fibroblasts lose telomeric DNA with every doubling, and so will eventually senesce. IMR90 can proliferate up to +/- 60 divisions. They should be used at as early passage as possible, i.e. below population doubling (PD) level 45. Cells should be counted every passage to determine the PD level.

- Screen 96 wells plate - To be miniaturized for 1536 wells
  - Number of cells/well: 10,000
  - End point: Survival as determined by CellTiterGlo Promega




  - Note: During the counterscreen, quiescent cells will be put in 0.2% FBS – they should last 4-5 days (for human cells). 4 days of exposure is a good compromise for now, but could be adjusted if needed.

Concentration response curve example as for secondary screen

- Plate: 96 Well Flat Clear Bottom White Polystyrene TC-Treated Microplates, Corning (3610)
- Cells: 1M Frozen Sen(IR) IMR90
- Complete Media: DMEM + 10%FBS + Pen/Strep
- Drug: Doxorubicin hydrochloride Tocris bioscience (2252)
- Assay: CellTiter-Glo® Luminescent Cell Viability Assay, Promega (G7571)
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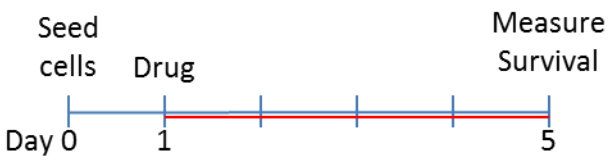
- **Procedure**:
- Senescent and Non-senescent cells are tested in parallel in two FBS concentrations; 10% and 0.2%. 0.2% FBS allows to study the effect of the drug while cells are Quiescent.
  - Cell seeding: Thaw a vial of Frozen cell in a water bath @ 37°C and dilute it in 10ml of serum free media and centrifuge 5min @150Xg. Aspirate the media and resuspend in 10ml of complete Media (10% or 0.2% FBS) to obtain 100,000 cell/ ml (split cells and or adjust volume on the need). For the drug titration, 10,000 cells / well will be plated. Prepare enough for 9 drug concentrations including in triplicate control). Save three wells with media without cells (marked ''-'' in the plate design bellow) for CTG blank.
  - Drug addition: 24 hrs following cell plating, 100 µL of 2X concentrated drug diluted in complete media is added to each well. Dilute doxorubicin stock solution in DMSO in order to make a stock solution of roughly 1000X. Prepare serial dilution of Doxo in complete media (10% or 0.2% FBS) starting at 2 µM (2X). Add to wells. See plate design bellow.
  - CTG: Proceed as describe by manufacturer.
- Timeline:




- Plate design:

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PBS | PBS | PBS | PBS | PBS | PBS | PBS | PBS | PBS | PBS | PBS | PBS |
| PBS | 2 | 1 | 0.5 | 0.25 | 0.125 | 0.0625 | 0.03125 | 0.015625 | 0 | - | PBS |
| PBS | | | | | | | | | | | PBS |
| PBS | | | | | | | | | | | PBS |
| PBS | 2 | 1 | 0.5 | 0.25 | 0.125 | 0.0625 | 0.03125 | 0.015625 | 0 | - | PBS |
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| PBS | PBS | PBS | PBS | PBS | PBS | PBS | PBS | PBS | PBS | PBS | PBS |

Drug (Doxorubicin) titration starting at 2 µM. Titration 1/2. ‘’-‘’ No cells no drug

Proliferating cells (Non-Senescent cells in complete media with 10% FBS)

Quiescent cells (Non-Senescent cells in complete media with 0.2% FBS)

*Repeat the same plating for senescent cells with both 10% and 0.2% FBS