NOV 23, 2023

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Protocol Citation: Allan JW Lui 2023. Standard Cell Culture Practices. **protocols.io** https://protocols.io/view/standard-cell-culture-practices-byinpude

Protocol status: Working
We use this protocol and it's working

Created: Sep 25, 2021

Last Modified: Nov 23, 2023

PROTOCOL integer ID: 53550

Standard Cell Culture Practices

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ABSTRACT

Basic cell culture techniques

PROTOCOL MATERIALS

Trypsin-EDTA (0.25%), phenol red **Thermofisher Catalog #25200-056** In 2 steps

DMSO **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D8418** Step 16

Trypsin-EDTA (0.5%) no phenol red **Thermo Fisher Scientific Catalog #15400054**

In 2 steps

BEFORE START INSTRUCTIONS

Cell culture numbers:

	Flasks/plates	Surface area	Media volume (ml)	Wash volume (ml)	Trypsin volume (ml)
	6-well	9.6	2 - 3	2	1
	12-well	3.5	1 - 2	1	0.5
	24-well	1.9	0.5 - 1.0	0.5	0.25
	48-well	1.1	0.2 - 0.4	0.2	0.125
	96-well	0.32	0.1 - 0.2	0.1	0.05
	T-25	25	5	5	1
	T-75	75	10 - 15	10	1-2
	T-175	175	25 - 35	20	5

Cell culture prep

15m

1 Pre-warm media (and reagents to make complete media) +/- trypsin in 37 °C water bath

Note

Use weights to ensure media bottles do not tip over and for the mouth of the bottle to contact water!

2 While reagents are warming up:

- Wipe down microscope table using paper towels and 70% ethanol
- Wipe down microscope stage using Kimwipes and 70% ethanol
- View cells under microscope
- Switch on biosafety cabinet, wipe down using paper towels and 1% Chemgene, then with 70% ethanol
- Fill a 1L plastic beaker with ~100-200ml 2% Virkon
- Add a tablet of Virkon into a aspirator collection bottle and fill with 250-500ml tap water, then fit into vacuum aspirator and turn on system

Note

When viewing cells under microscope, record confluence and presence of floating cells, dying cells & debris

2.1 **Any time** before placing into the hood:

- Pipettes: Wipe down using paper towels and 70% ethanol
- All racks and containers (of liquids, pipette tips, etc but not cells): Spray down with 70% ethanol
- All items in water bath: Wipe down using paper towels and 70% ethanol, then spray down with 70% ethanol

Spray hands with 70% ethanol frequently

3 Wipe down flasks containing cells with 70% ethanol before handling, at least once per week

Thaw cells

15m

4 Request cells from RICS on or before 9am on the day of planned thaw

5 Confirm items are in the hood pre-thaw:

- **Warm** media
- 15ml centrifuge tube
- 1ml pipette & tips
- Waste bin
- Aspiration pipettes
- Motorised pipette controller

6 Prepare covered bucket of dry ice
Retrieve vials of frozen cells from -80C freezer and place into dry ice bucket and cover

7 Thaw vial in 37 °C water bath , observing closely until only a small crystal is left, then transfer to hood

8 Using 1ml pipette, transfer contents of vial into a 15ml centrifuge tube 5m

Add 9 mL media **dropwise**
Gently mix by inversion

Pelleting:
Centrifuge at 200 rcf, Room temperature, 00:05:00
Aspirate supernatant using aspiration pipette connected to vacuum aspirator

8.1 During centrifugation, transfer cell culture flasks to be used to the hood

> **Note**
>
> If doubling time or recommended seeding density of cells unknown, and cells <= 3x10E6: thaw into 1x T25; if >= 3x10E6 cells thaw into 1x T75
>
> Seeding densities vary based on cell size and doubling time, seeding densities seen on cell culture reference charts often may not work for slowly dividing or small cells

8.2 Using alcohol-resistant markers, label with cell line name, passage number, date & initials

> **Note**
>
> Passage numbers: if thawed from a vial frozen by someone else: can continue numbering or append numbers (i.e. pXX+1)
>
> Each transfer to a new cell culture surface constitutes a passage, including after a thaw. Freezing into a vial does not count as a passage

9 Flick pellet to loosen

9.1 (Optional) Resuspend cells in 5 mL media
Count cells and assess viability.

Note

If using Vi-cell for counting, dilute 260 µL cell suspension in 260 µL media first to reduce number of cells taken for counting.

9.2 Alternatively, resuspend cells in final volume required for culture vessel(s)
Transfer into culture vessel

9.3 Check cells under microscope, observing for dead cells or large clumps
Transfer to 37 °C incubator (5% CO2, humidified)

10 1-2 days later, Check on cells to assess recovery/attachment and change media

Subculturing cells

30m

11 Inspect cells under the microscope .
Cells are best trypsinised while in the exponential phase of growth, generally between 40-90% confluence.
Usually, cells are trypsinised near the end of the exponential growth phase, at 70-90% confluence.
Cells may also be subcultured to remove debris not detached during media changes.

Note

Some cell lines grow in multilayered clusters (i.e. HCC1500) and never reach high confluence

12 Prepare items:

- **Warm** media
- **Warm** trypsin Trypsin-EDTA (0.25%), phenol red **Sigma Aldrich Catalog #25200-056** or Trypsin-EDTA (0.5%) no phenol red **Sigma Aldrich Catalog #15400054** (for firmly adherent cells)
- 15ml / 50ml centrifuge tube
- 1ml pipette & tips
- Waste bin

- Aspiration pipettes
- Motorised pipette controller

13 Wash cells wiith PBS 1-2 times 10m
Add the appropriate volume of trypsin for the flasks used.
Tilt plates to ensure trypsin evenly coats all cells
Incubate in 37 °C incubator (5% CO2, humidified) for up to 00:10:00

> Note
>
> **Work with up to 4 flasks at a time**

13.1 Check under microscope whether cells have detached 3, 5, 8 & 10 minutes after placing into incubator, particularly the edges.
Tapping the sides of the flask may help to detach cells but also cause clumping.

14 Once the vast majority of cells have detached, inactivate trypsin w/ media, using at least double the volume of trypsin. 5m
Pellet cells: 200 rcf, Room temperature, 00:05:00

14.1 During centrifugation, transfer cell culture flasks to be used to the hood & label

15 Flick pellet to loosen
Resuspend cells in media

15.1 (Optional) Count cells on Vi-cell

> Note
>
> For slow growing cells, it is advisable to correlate cell count with confluence to provide an indication of the number of cells to plate to achieve 20-40% confluence

15.2 Dilute cells as necessary to achieve the desired subcultivation ratio and volume, and transfer to

culture vessels
For culture plates: shake back and forth, then side to side to distribute cells evenly across wells. Do not swirl.

Transfer to 37 °C incubator (5% CO2, humidified)

> **Note**
>
> Refer to supplier's (e.g. ATCC) guidelines for subcultivation ratios if doubling time of cell lines have not been determined.
>
> In general:
> Doubing time <2 days: 1:4 - 1:10 subcultivation ratio (aim for 10-15% confluence)
> Doubing time 2-4 days: 1:2 - 1:6 subcultivation ratio (aim for 15-20% confluence)
> Doubing time >4 days: 1:2 - 1:3 subcultivation ratio (aim for 20-30% confluence)

Freezing cells

5m

16 Prepare freezing media: [M] 10 % (v/v) DMSO in media On ice

DMSO **Sigma Aldrich Catalog #D8418**

> **Note**
>
> Freezing media can be aliquoted and stored at -20C long term (refer to expiry date of FBS)
> At 4C, freezing media can be stored for 1 month

17 Inspect cells under the microscope . 5m
Cells should be frozen while in the exponential phase of growth, generally between 40-90% confluence.
Trypsinise and pellet cells (steps 11-13) 200 rcf, Room temperature, 00:05:00

18 Prepare items:

- **Warm** media
- **Warm** trypsin Trypsin-EDTA (0.25%), phenol red **Sigma Aldrich Catalog #25200-056** or Trypsin-EDTA (0.5%) no phenol red **Sigma Aldrich Catalog #15400054** (for firmly adherent cells)
- 15ml / 50ml centrifuge tubes
- 1.5ml centrifuge tubes

- Cryovials
- 1ml pipette & tips
- Waste bin
- Aspiration pipettes
- Motorised pipette controller
- Freezing containers (cooling rate of <= 1 C/min) at Room temperature

19 Flick pellet to loosen
Resuspend cells in media
Transfer 550 µL cell suspension to a 1.5ml centrifuge tube
Count cells on Vi-cell

20 Calculate the total number of cells remaining after count.
Determine volume of 5*10^5 cells for Mycoplasma testing , or 1*10^6 cells for STR + Mycoplasma testing
Determine volume of cells to subculture, if any.
Determine volume of cell suspension and freezing media to use

Note

Aim for 1-4 x 10E6 cells/ml of freezing media

Ideally: aim to freeze down enough cells to thaw into at least a T25 flask.
(For slow growing cells, aim to freeze enough cells per vial to yield ~20-30% confluence in a T25)

General guideline:
Fast growing cells: 1 x 10E6 cells for 1x T25, 2 x 10E6 for 1x T75
Slow growing cells: 2-3 x 10E6 cells for 1x T25

If establishing cell stock:
1. Freeze >=15 vials of cells, replate enough cells at the usual subcultivation ratio to replace the number of flasks used during this freeze.
2. Keep cells in culture until good recovery and absence of bacterial, fungal or mycoplasma contamination are confirmed (see step 24)

20.1 Create labels for vials
- cell line name,
- current passage,
- cell number/vessel to thaw cells into
- date,
- initials/name

21 **Mix cell suspension well**

21.1 Transfer cells needed for mycoplasma +/- STR testing into a 1.5ml centrifuge tube
Label tubes
Place On ice

21.2 Replate cells as needed

21.3 Pellet cells to be frozen 200 rcf, Room temperature, 00:05:00 5m
Label cryovials during centrifugation
Resuspend cells in freezing media
Transfer cells in freezing media to cryovials; screw cap tightly

> **Note**
>
> Use 1ml pipette if freezing only a few vials
> Use 5ml serological pipette with reverse pipetting technique if freezing >=5 vials

22 Transfer cryovials into freezing container and freeze at -80C

23 Pellet cells 200 rcf, Room temperature, 00:05:00 and submit for mycoplasma +/- STR testing, kee 5m
extracted DNA if possible

24 If establishing cell stock, thaw a vial of cells (steps 4-10) after >1 day of freezing to confirm good recovery and absence of bacterial and fungal contamination.

If recovery is poor or contamination+, discard frozen vials and freeze new batch of cells.

25 Transfer vials to liquid nitrogen storage at least 2 hours after freezing and when mycoplasma-negative.

Keep vials on dry ice during transfer.