Subculture

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This protocol describes the necessary supplies and reagents and proper sterile techniques and good practices for successful cell culture. Cell line specific tables are included. We generally work without antibiotics in our culture medium so following strict and proper sterile technique is essential.

1. Necessary Supplies and Reagents
    a. Nitrile Laboratory Gloves (Alkali Scientific # NG5010, NG5015, NG5020)
        i. Always wear gloves for every step of subculture protocol and spray and wipe hands with 70% ethanol as needed before entering the hood.
    b. 70% ethanol (Ethanol Denatured # BDH1156 diluted with distilled water)
    c. Sterile PBS (Sigma #P4417 autoclaved in distilled water)
    d. Sterile glass Pasteur pipettes (LDP #450-900 autoclaved for 30 minutes, dry cycle)
    e. Cell line specific Media (see Cell Line Table below)
    f. Cell line specific cell dissociation buffer if needed (see Cell Line Table below)
    g. Sterile culture ware (tissue culture treated flasks, dishes, plates)
2. Tissue Culture Hood Preparation
    a. Turn off the UV light (if on) and turn on the regular light, open the sash to the appropriate sash height to maintain laminar flow, and allow to run for 10-20 minutes to clean the air in the hood
        i. When placing objects in the hood, do not block the airflow in the back and leave ~6 inches clear in front of the hood
        ii. We leave the UV light on typically once a week overnight to facilitate continued decontamination. It can also be done if desired for 10-15 minutes but do not leave it on with people in the area.

b. An aspiration apparatus with a safety trap – an in-line hydrophobic filter (Whatman #6722-5001) should be inserted between the tubing connecting the in-hood vacuum outlet and the aspiration flask and replaced as needed
    i. Vacuum flask should be maintained with ~200 mL 20% bleach before use
    ii. Maximum collection volume should be no more than two-thirds full
    iii. To disinfect the liquid waste, add a sufficient amount of bleach to create a >= 10% final bleach concentration and let sit for 30 minutes before emptying into the laboratory sink.
c. Use 70% ethanol for sterilization of non-sterile equipment, reagents, and surfaces
    i. Liberally spray the work surface with 70% ethanol and wipe with a Kimwipes (preferred) or paper towels from back to front
    ii. Spray everything brought into the hood with 70% ethanol
        1. Take care if items were in the water bath to first wipe off the water completely and then spray with ethanol
    iii. Clean spills immediately with 70% ethanol to avoid cross-contamination
    iv. Never use bleach to clean the hood (corrosion)

3. Sterile Handling
    a. Always wear gloves and a lab coat.
    b. Only open sterile equipment or culture dishes inside the hood
    c. If possible always hold the lids to sterile containers in your gloved hand rather than place them on the hood surface. If placing the lid down is unavoidable, then do it face up towards the back of the hood.
    d. Once a sterile container is open or lid is exposed, never pass your hands or arms over it.
    e. Never pour from one sterile container to another; always use pipettes to transfer liquids
    f. Attach an autoclaved glass Pasteur pipette to the vacuum tubing for aspiration
        i. Ensure the tip of the Pasteur pipette never touches any surface other than the liquid it is aspirating
        ii. For extra sterility, place a clean sterile 200 uL plastic pipette tip on the end of the Pasteur pipette and change after every aspiration. Alternatively, change the Pasteur pipette after every aspiration.
    g. When opening a sterile serological pipette tip, if the tip touches any surface other than the interior of a sterile container, discard and acquire a clean pipette tip. Always open completely under the hood and discard the paper outside of the hood.
    h. Discard all tissue culture plates, flasks, and pipette tips into a biohazard waste container
    i. Cap bottles tightly before removing from the hood
    j. Spray the hood surface with 70% ethanol when finished, remove all bottles and tip boxes, and close the hood sash if no one else is using it for the day
        i. There is a typically a dedicated set of micropipettes and pipetteboy for serological pipettes that may remain in the hood.
    k. Avoid working with more than one cell line at a time to avoid cross-contamination risk.
4. Subculture for adherent cell lines

a. For typical working volumes, see the Volumes Table below.
b. NEVER let adherent cells grow to confluence. Typical subculture is done Monday, Wednesday and Friday at less than 75% confluence.
c. Pre-warm the necessary media (see Cell Line Table below) in a 37°C water bath.
d. Aspirate medium from flask and rinse monolayer with sterile PBS.
e. Add proper cell detachment buffer (see Cell Line Table below) and incubate at 37°C, checking for detachment every 5 minutes.
    i. NOTE: Some cells (e.g. MCF10A) are very strongly adherent to the culture dish and tend to form cell aggregates in suspension. To lift cells and break cell aggregates, bring trypsinized cells out of the incubator and gently tap side of culture dish until most cells detach. Using a P1000 sterile micropipette during spin down (see below) can facilitate achieving a uniform single cell suspension by pipetting up and down.
f. While cells are detaching, label new culture flasks (or dishes) with your initials, date, cell line and passage number. Increment the passage number by one.
g. To neutralize trypsin and harvest cells, add up to 10 mL warm full growth culture medium to the flask (or dish)
    i. Serum is the neutralizing component—it contains protease inhibitors.
h. Transfer the total single cell suspension to a sterile 15 mL conical tube.
i. Centrifuge at proper cell line specific time and speed (e.g. 5 minutes at 100g is typical).
j. Bring cells back inside the hood, and discard supernatant by vacuum aspirating with a sterile Pasteur pipette.
k. Break the cell pellet by gently tapping the bottom of the tube.
    i. Up and down pipetting with a P1000 is often useful here.
l. Count cells if needed (see Counting Cells protocol).
    i. This can be done after later stages too if appropriate.
m. Resuspend cell pellet in desired volume of full culture medium.
n. Add appropriate aliquots of cell suspension to new conical tube containing culture medium.
    i. To achieve a 10% seeding density, perform a 1:10 dilution of cell suspension by adding 1 mL of cell suspension to 9 mL of full culture medium (for one 10 cm culture dish or T75 flask).
o. Transfer diluted cell suspension to new culture dish or flask.
p. Put new cell culture dish back in 37°C 5% CO_2 incubator. If passage number was not recorded, do it now on the flask.
q. Discard unused cells.
r. NOTE: There is flexibility in the order of operations from steps *k*-*n*. Use judgement to determine what works for you.

5. Subculture for suspension cell lines
    a. For typical working volumes, see the Volumes Table below.
    b. Suspension cells can be maintained by the addition of fresh medium every 2 to 3 days (depending on cell density). Alternatively, cultures can be maintained by centrifugation with subsequent resuspension at 1×10^5 viable cells/mL.
        i. Do not allow cell density to exceed 3×10^6 cells/mL. Maintain cultures at a cell concentration between 1×10^5 and 1×10^6 viable cells/mL.
    c. Pre-warm the necessary media (see Cell Line Table below) in a 37°C water bath.

d. Place total volume of suspension cells in full growth medium in a 15 mL conical tube.
e. Centrifuge at proper cell line specific time and speed
f. Bring cells back inside the hood and discard supernatant by vacuum aspirating with a sterile Pasteur pipette.
g. Count cells.
h. Gently resuspend cells in appropriate volume for your desired flask size and cell density.
i. Label the flask with your initials, cell line, date, and passage number.
j. Put new cell culture vessel back in the 37°C, 5% CO_2 humidified incubator.

6. Cleaning the hood
    a. Follow good sterile technique (as described above) to clean the hood. Discard all remaining items as appropriate, remove all remaining items from the hood, clean as when starting with 70% ethanol, and close the sash if you are the last one for the day.
    b. If appropriate, restock serological pipettes as needed. I will find you and make you feel shameful if you are a repeat offender.

Cell Line Table

Cell Line	Media	Additives	Cell Detachment	Centrifuge
MCF10A	DMEM/F12 (Gibco#11330032)	5% Horse Serum(Gibco#16050122), EGF(20ng/ml)(Pepro Tech#AF100-15), Hydrocortisone (0.5 µg/ml) (Sigma#H0888), Cholera Toxin(100ng/ml)(Sigma#C8052), Insulin (10 µg/ml)(Sigma#I1882)	0.25% Trypsin, 2.21 mM EDTA (25-053-CI)	5 minutes, 100g
U87	DMEM (Gibco#10313021)	10% FBS (Gibco#10082139), L-Glutamine (2mM) (Corning #25-005-CI)	0.05% Trypsin, 0.53 mM EDTA (25-052-Cl)	5 minutes, 100g
HEK293-T	DMEM (Gibco#10313021)	10% FBS (Gibco#10082139), L-Glutamine (2mM) (Corning #25-005-CI)	0.05% Trypsin, 0.53 mM EDTA (25-052-Cl)	5 minutes, 100g
MCF-7	DMEM (Gibco#10313021)	10% FBS (Gibco#10082139), L-Glutamine (2mM) (Corning #25-005-CI)	0.05% Trypsin, 0.53 mM EDTA (25-052-Cl)	5 minutes, 100g

BT-474	DMEM (Gibco#10313021)	10% FBS (Gibco#10082139), L-Glutamine (2mM) (Corning #25-005-CI), Insulin (10 µg/ml) (Sigma#I1882), 2 mM HEPES (Sigma#H4034)	0.25% Trypsin, 2.21 mM EDTA (25-053-CI)	5 minutes, 100g
MDA-MB-231	DMEM (Gibco#10313021)	10% FBS (Gibco#10082139), L-Glutamine (2mM) (Corning #25-005-CI)	0.05% Trypsin, 0.53 mM EDTA (25-052-CI)	5 minutes, 100g
Jurkat	RPMI (Gibco#11875093)	10% FBS (Gibco#10082139), L-Glutamine (2mM) (corning #25-005-CI), Sodium pyruvate (1 mM) (Gibco#11360070)	N/A	5 minutes, 100g
Jurkat (MSKCC Ming Li Lab alternative recipe)	RPMI (Gibco#11875093)	10% FBS (Gibco#10082139), L-Glutamine (2mM) (Corning #25-005-CI), 50µM β-mercaptoethanol (Sigma#M3148), NEAA (#11140050), 1 mM Sodium Pyruvate (Gibco#11360070), 2 mM HEPES (Sigma#H4034)	N/A	5 minutes, 100g

Volumes Table

Plastic	**Media Volume**	**Trypsin Volume**
T25 Flask (Falcon#353108)	3 mL	0.5 mL
T75 Flask (Falcon#353110)	10 mL	1 mL
T175 Flask (Falcon#353112)	15 mL	1-3 mL
100 mm dish (Falcon#353003)	10 mL	1 mL
60 mm dish (Falcon#353002)	5 mL	0.5 mL
35 mm dish (Falcon#353001)	2 mL	200 µL
6 well plate (Falcon#353046)	2 mL	200 µL
12 well plate (Falcon#353043)	1 mL	100 µL
24 well plate (Falcon#353226)	0.5 mL	100 µL
96 well plate (Falcon#353072)	100 µL	10 µL