BNRF GENERAL CELL CULTURE PROTOCOL

Location: WVU – Engineering Sciences Building – Room: G75E

WVU – Chemistry Research Laboratory building – Room: 381

Primary Staff Contact: Dr. Huiyuan Li (304) 293-0747

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1) ASEPTIC TECHNIQUE

Good cell culture and aseptic practice will prevent contamination from bacterial, fungi, mycoplasma and cross-contamination with other cell lines.

Sterilizing the hood

- Make sure the sash is fully closed and turn on the UV light (circled in red, Figure 1) in the hood. Let the hood sterilize for 20-30 minutes. While the hood is sterilizing, warm your cell culture solutions in the 37°C water bath.

Figure 1. Biosafety cabinet.

- After 20-30 minutes, turn off the UV light and turn on the regular light (circled in yellow, Figure 1). And open the front window to marked position.
- Wearing gloves, spray the inside of the hood with 70% ethanol and wipe with a paper towel. Discard the paper towel in regular trash can.
- Personal attire should be worn at all time, especially **powder free gloves**.
- Sanitize your gloves by spraying them with 70% ethanol[1] and allowing to air dry for 30 seconds before commencing work in the cabinet.
- Sanitize the cabinet using 70% ethanol before commencing work.
- Equipment that will be taken into the cabinet during the cell culture (media bottles, pipette tip boxes) should be wiped with 70% ethanol soaked tissue prior to use.
- While working, do not contaminate your gloves by touching anything outside the cabinet (especially face and hair). If gloves become contaminated, re-sanitize them with 70% ethanol before proceeding.
- Discard gloves after handling contaminated cultures and at the end of all cell culture procedures.
- After completing work, spray and wipe the work area in the cabinet with 70% ethanol.
- All waste (pipette tips and culture dishes) should be discarded in biohazard trash can, and then our staff will autoclave and discard it as biological waste with proper label.

Cleaning Up after experiment done:

- Put media, trypsin, PBS, etc back in fridge.
- Throw away all cell plates, used pipette tips, media containers, etc. in biohazard trash.
- Spray hood with 70% ethanol and wipe down.
- Spray vacuum pipette with 70% ethanol (inside and out). Wipe off outside of pipette.
- Turn off vacuum and close hood.

Turn off the light in the hood and turn UV on. The UV light will be off automatically after 1 hour.

[1] 70% ethanol solution should be directly prepared from 200 proof (absolute) Ethanol (CAS 64-17-5)

2) CELL CULTURE MEDIA

Check the ATCC website for your specific cell line culturing condition. List the base medium and additives.

There are two common mediums, Dulbecco's Modified Eagle's Medium (**DMEM**) and Roswell Park Memorial Institute medium (**RPMI-1640**) in our lab. Depends on the type of mammalian cell lines that you are working with, different concentration of Fetal Bovine Serum (FBS), L-glutamine and Penicillin-Streptomycin (P/S) will be used as their desired culture media. To prevent excess freezing-thawing steps and contamination, the aliquot of each supply (**see below**) will be prepared by BNRF staff for your convenience.

- FBS: **25mL/tube**
- L-glutamine: **5mL/tube**
- Penicillin-Streptomycin: **5mL/tube**

To prepare **DMEM with 10%FBS, 1% L-glutamine, and 1% P/S**:

1. Grab two tubes of FBS, one tube of each L-glutamine and P/S from –20ºC freezer and grab one bottle of DMEM from the 4ºC refrigerator, and thaw them in 37ºC water bath (Figure 2).

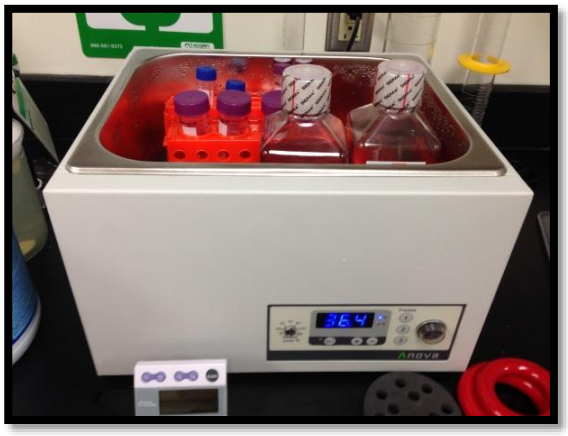


Figure 2. Warm the media and other supplements in 37 water bath.

2. Make sure you have strictly followed the antiseptic procedure (**see Part I**).
3. Transfer 440 mL DMEM, 50 mL FBS, 5 mL L-glu, and 5 mL Pen-strep to 500 mL filter bottle. (Make sure you vertex/mix the L-glutamine and P/S before transferring to DMEM, since they tend to precipitate over time).
4. Filter the solution through the filter bottle membrane under vacuum pressure.
5. Label the media bottle with **content of supply**, **date**, and **your initial**.

To prepare **RPMI-1640 with 10%FBS, 1% L-glutamine, and 1% P/S**:

Follow the steps described above using **RPMI-1640 medium.**

Table 1: Cell line and growth media in BNRF.

Cell Lines and Growth Solution					
Cell Type	**Cell Name**	**Media**	**FBS**	**Trypsin**	**Note***
Breast	MCF-7	DMEM	10%	0.25%	
	MDA-MB-231	DMEM	10%	0.25%	
	MDA-435	DMEM	5%	0.25%	w/o L-Glu
Lung	NCI-H460	RPMI	10%	0.25%	
	A549	DMEM	10%	0.25%	
Cervical	HeLa	DMEM	10%	0.25%	
Colon	HT-29	DMEM	10%	0.25%	
Ovarian	OVCAR-3	RPMI	20%	0.25%	w/o L-Glu

3) RESUSCITATION OF FROZEN CELL LINES

To maintain the viability of cell culture, it is essential that cells are thawed quickly and diluted in culture medium to minimize the toxic effects from cryoprotectant, such as DMSO.

1. Prepare the complete growth media as described in Part 2.
2. Put 10 mL – 12 mL complete media into a 25 cm^2 flask, and place it into incubator. And balance the condition for 10-15 min.
3. Remove a cryogenic vial from Liq. N_2 cryogenic freezer.
4. Constantly swirling the vial in a 37°C water bath for 1 to 2 minutes until a small amount of ice remains in the vial. (Thawing should not go over 2 minutes).
5. Wipe the outside of the vial with a tissue moistened with 70% ethanol before it is transfer into cabinet.
6. Transfer cells into a 15ml centrifuge tube, and slowly dilute the cells with either PBS or pre-warmed growth medium dropwise to avoid osmotic shock.[2]
7. Centrifuge the tube at 1,000 rpm for 5 minutes.
8. Gently aspirate and discard the supernatant. Be careful not to disturb the cell pellet.
9. Pipette 1 mL of serum-containing medium to the side of tube and resuspend the cell pellet. Transfer the cell suspension to the pre-incubated 25 cm^2 flask. Gently move the dish in this pattern (↔, ↑↓) to evenly spread out the cells.
10. Incubate cells overnight at 37°C and 5% CO_2 humidified incubator.
11. Examine cells confluence and morphology by inverted microscopy after 24 hours and sub-culture the cell if necessary.

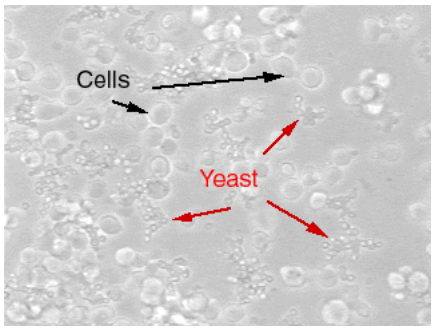
Tips: Thaw fast, avoid shocking and be patient on your cells.

[2] Alternative procedure could skip centrifugation and directly culture the cell after thawing. But need to change to new complete growth media within 24 hours.

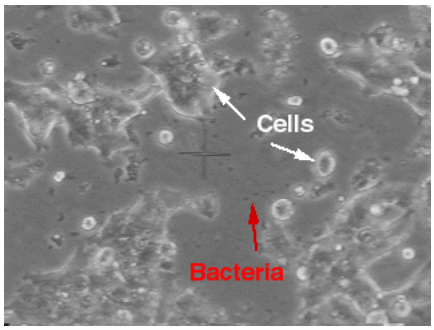
4) SUBCULTURE OF CELL LINES

Adherent cell lines will grow *in vitro* until they reach 100% confluency or the medium is depleted of nutrients. Prior to this point, the cell lines should be sub-cultured in order to prevent the culture from dying by cell-cell contact inhibition. Unless otherwise notices for specific cell lines, trypsin proteases are generally used to detach the adherent cell and prepare cell suspension for subsequent cell passaging. Notices that prolong exposure of trypsin will reduce cell viability.

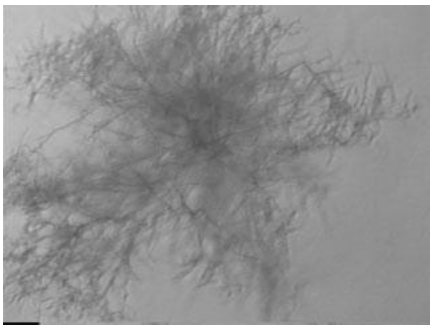
1. Examine the cells under the inverted microscope for the following assessments:
   - **Cell attachment**
     - Good: Cells are well attached and evenly spread out. However, it is normal to see a small amount of cancer cells floating, since cancer cells are known to metastasize.
     - Bad: Floating cells indicate over confluency or dead cells. If the majority cells are floating, cells are severely traumatized and no longer healthy for cell culture.
   - **Percent confluency** (% of area coverage of the dish by the cells)
     - Good: Always keep cells at or below 80% confluency for both sub-culture and cell seeding when performing an experiment.
     - Bad: Over confluency will inhibit cell growth via contact inhibition and may lead to genetic drift of cancer cells, which will drastically change its behavior and affect your result on cytotoxicity assays.
   - **Cell morphology**
     - Good: Unless cells undergo division, cancer cells are usually appeared in a doublet cluster
     - Bad: Swollen or not growing cell (dying) are usually appeared in round shape and in a not crowded environment
   - **Bacterial, Fungal and Mycoplasma contaminant (Figure 3)**



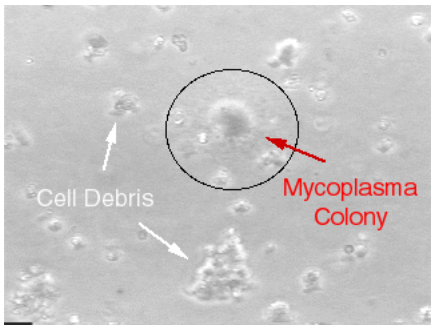
Yeast (multi-branched particles)



Bacteria (small black dot)



Fungal (fuzzy clumps of spores)



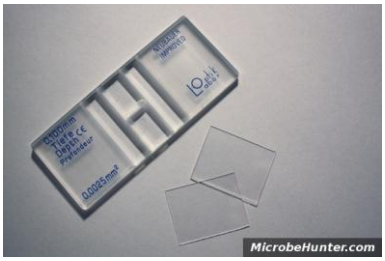
Mycoplasma (egg-like colony)

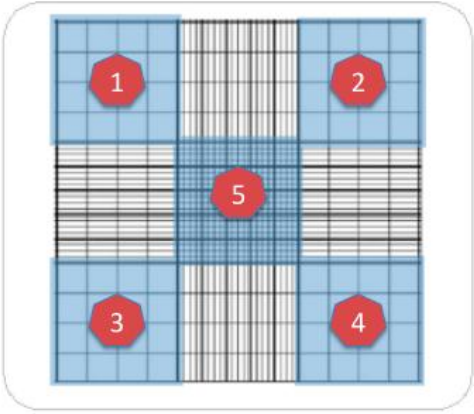
Figure 3. Common contaminations in cell culture

2. Aspirate spent medium.
3. Wash the cell with ~10ml of PBS[3] and aspirate the spent PBS (Avoid touching the pipette to the plate to prevent cross-contamination).
4. Trypsinize the cell with 3 ml of trypsin[4] and incubate for 2 minutes at 37°C and 5% CO_2 incubator. (Notice that incubation time is subject to change based on the cell line. Prolong trypsinization will lead to cell death, and shortage of trypsinization will lead to cell insufficiency).
5. Before adding serum to quench and stop the trypsinization, gently tap the culture dish to help detaching the remaining cells. (You should also check the cells under inverted microscopy to ensure 90% cell detachment).
6. Pipette 7 mL of serum-containing medium to inactivate the trypsin, and resuspend the cells by carefully pipetting up and down the cell suspension to break and disaggregate any cell clumps. (Be careful not to aspirate your suspension into the pipet aid. If this happens, the pipet aid must be cleaned with 70% ethanol, and gently tap the pipet aid against a tissue paper to soak out all the media that are trapped inside the pipet aid)
7. Transfer cells solution into 15 mL centrifuge tube.
8. Pipette 50 µl of suspension into a 0.5ml Eppendorf tube that contain 50 µl of trypan blue stain. Resuspend the mixture (~100µl) and pipette 15µl of stained suspension into each side of the cell counter plate (hemocytometer) (Figure 4, left). (Be gentle when pipetting the cells into cell counter plate. If not, the cells will not be evenly distributed in the plate, which will lead to an inaccurate result of cell counting)

[3] Phosphate Buffered Saline (PBS) without Ca or Mg

[4] 0.25% Trypsin with EDTA•4Na or other concentration of trypsin depends on the cell lines





Cell counts = Total cell counts in 5 area/ 5

Cells density = cell counts × dilution rate × 10^4 cells/ mL

Figure 4. Hemocytometer, counting area, and calculation.

9. Calculate cell density as the formula shown in Figure 4. Multiply the cell density by the volume of cell culture media, you will get the total cells number in your test tube.
10. Calculate and pipette the required amount of cells and serum-containing medium to a new centrifuge tube, and resuspend the cell before seeding on the plate. Incubate the cells at 37°C and 5% CO_2 humidified incubator. (**See the table below for seeding density**)

	6 well plate	12 well plate	24 well plate	48 well plate	96 well plate
Volume (ml/well)	3	2	1	0.4	0.1
Concentration (cell/well)	500,000	250,000	100,000	50,000	10,000

(Note: Seeding density may change to different cell lines. Check the literature or manufacture's guide for cell density for your specific experiment.)

5) FREEZING CELL

Maintaining a stock of cell lines, especially working with new cell line, is an important policy to follow in order to store a good portion of cells for future use. Ideally, each cell lines should be stocked up to 6 vials minimum, in which vials should be better prepared from different culture dish in case some cells are not as healthy as other batch. Generally, you will want to freeze around $1x10^6$ cell per each 1 mL cryogenic vial. Rule of thumb is that cells must be healthy and should not be over 80% or less than 50% confluency prior to the freezing.

1. Thaw 10% FBS growth media or Recovery™ Cell Culture Freezing Medium, PBS and Trypsin[5] in 37ºC water bath.
2. Aspirate the spent medium.
3. Wash the cell with 10 mL PBS and aspirate the spent PBS.
4. Trypsinize the cells if necessary.
5. If trypsin was used, inactivate the trypsin after detachment of cells with 10% FBS growth media. Centrifuge at 1000 rpm for 5 minutes and aspirate the supernatant. (Don't let the pellet get dry)
6. Resuspend the cell pellet with freezing media[6] (1 mL freezing media per cryogenic vial)
7. Place the cryogenic vial in the rack (Figure 5). Pipette 1 mL of cell suspension to a well-labeled pre-chilled cryogenic vial. (Cryogenic vial should be clearly labeled with the **cell name**, **freezing date**, **passage number**, **cell density** and **person's initial**)

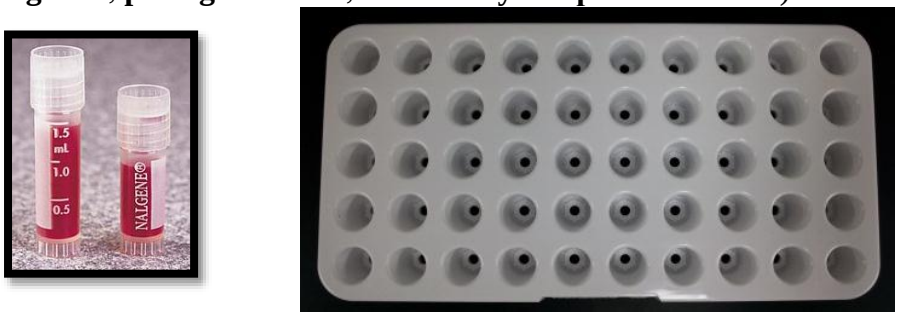


Figure 5. Cryogenic vial and vial rack.

8. Place the cryogenic vials into freezing container (filled with isopropanol) (Figure 6) and then store in –80ºC freezer overnight.[7]

[5] Different concentration of Trypsin should be used based on the cell lines.

[6] Freezing media should be prepared with fresh FBS with 10% sterilized filtered DMSO. Add 2.5ml DMSO into 25ml FBS to make a 10% DMSO/FBS freezing solution.

[7] Alternate approach is using Mr. Frosty directly at –80ºC freezer overnight.

Figure 6. Cell freezing container

9. Transfer the vials to the liquid nitrogen dewar, where it will be stored until further use.
10. To verify the cell viability of the frozen cell, thaw one of the vials after storage for a week in liq. N_2 dewar by following the thawing procedure (**see Part III**).

Tips: Freeze slow, and freeze cells at a higher density (i.e., At least one confluent dish per vial)