Biomanufacturing Training Program

CELL CULTURING AND PREPARATION IN BIOMANUFACTURING

Designed By:

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OBJECTIVES AND USAGE - PREFACE

Biomanufacturing industries are one of the steadily growing industries all over the world and predicted to be growing steadily and consistently over next decades. The needs of healthcare and its associated products and services are expected to rise proportionately with the growth of global human population. Biomanufacturing-based products are among the most highly cost-, labor-, skill- and knowledge- in healthcare products. This requires extensive training for the personnel to gain sufficient competency to operate.

This handbook marks the upstream part of Biomanufacturing, covering the initiation of making cell culture stock and increase it up to the production levels, until the cell banking stage. The model cell culture described in this handbook is Chinese Hamster Ovary (CHO) cells that are commonly used as mammalian cell-culture based system and production.

To enhance the proficiency of the personnel in cell culturing. Practise and guidelines in laboratory setting is also provided in this handbook. Basic aseptic techniques should be properly mastered before as one of the prerequisites to effectively acquire the learning outcome delivered in this handbook.

This handbook is designed for Community Service program served to establish, improve and enhance biomanufacturing process with our partner company, PT Kalbio Global Medika. Implementation and development of methods described in this handbook is possible in many other related cell-culture based application in biomanufacturing settings. The proficiency level required to fully understand this book is intended for user(s) who is/are familiar with basic biological and chemical science and technology.

Chapter 1: CELL CULTURING INTRODUCTION

1.1 INTRODUCTION

- Cell culture is the process by which prokaryotic, eukaryotic or plant cells are manipulated under controlled condition.
- It refers to the culturing of cells derived from animal cells (some are plant cells: protoplasts) (Figure 1).
- Cell culture was first successfully undertaken by Ross Harrison in 1907.
- Usually carried out in controlled environment like sterile tissue culture cabinets. (Figure 2)

Figure 1 Animal-based adherent cell culture (left). Plant-based dish grown cell culture (right).

Figure 2 Conducting cell-culture based work in biosafety cabinet

1.2 CELL CULTURE AND APPLICATION

- Model systems
  Studying molecular cellular biology, interactions between infectious diseases and cells, effects of drugs on cells, process and triggering of various cellular effects.

- Toxicity testing
  New drug study, drug-drug interaction, short/ long side effects of drugs

- Virology
  Cultivation of virus for models, protein recombinant production, vaccine production.

- Cancer research
  Studying changes in oncogenes/ tumor suppressor genes in cancer cells.

- Genetic Engineering
  Production of commercial proteins: insulin, interferons, etc.

- Gene therapy
  Alteration of genes using plasmids (or other carriers) cultivated in cells before transfecting/ transforming into the new cells.

- Tissue/ Organoid cell culture
  Cultivation and production of various model tissues, forming organoids.

EXAMPLES TYPES OF CELLS FOR MAMMALIAN CELL CULTURING

1. Lymphoblast like- cells do not attach remain in suspension with a spherical shape,
2. Epithelial like- attached to a substrate that appears flattened and polygonal in shape,
3. Fibroblast like- cells attached to a substrate that appears elongated and bipolar in shape.
4. Neuronal like- attached to surface and appeared to be dendritic in shape.

The image under microscope of each mammalian cell lines can be seen at Figure 3.

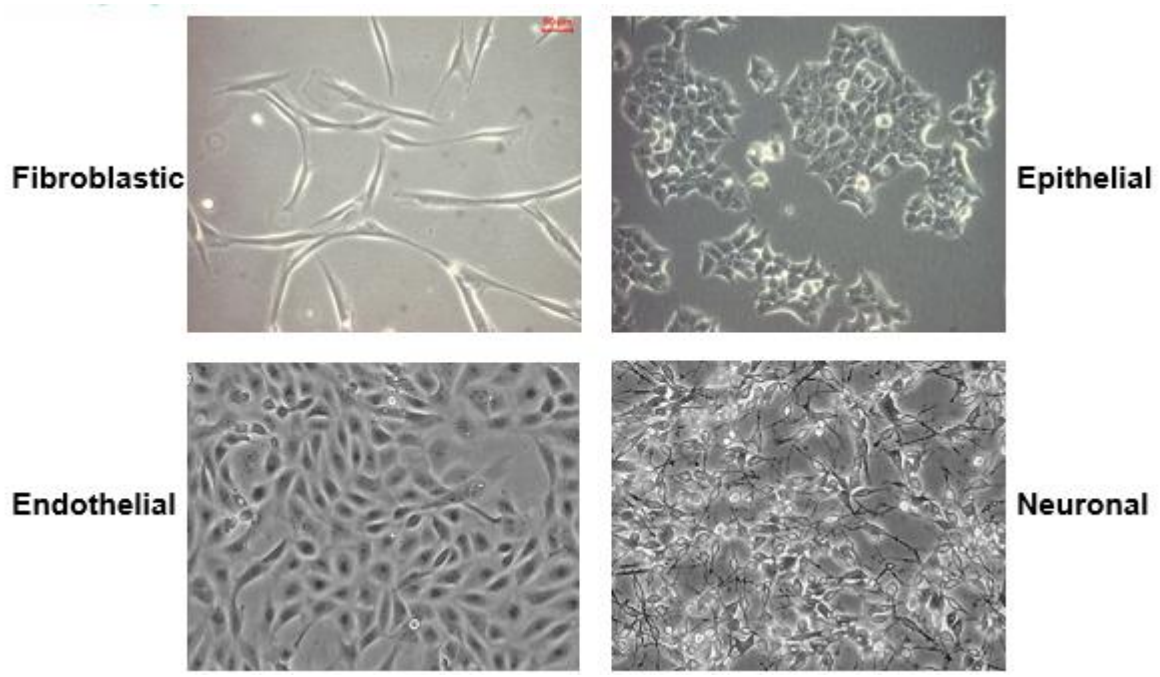


Figure 3 Visualization of various animal-based cell culture under Phase-contrast Microscope (Magnification 100x)

1.3 PROCESSING IN TISSUE CULTURE CULTIVATION AND UPSCALING

- The creation and initiation of tissue culture as biomanufacturing vessel candidate from resected tissue are commonly performed.
- Generally requires series of subculturing (Figure 4 in blue box) for proper adaptation to become in-vitro cell line.
- The end product of tissue culture can be either transformed cell line, immortal cell line, clonal cell line or can be carried over via successive sub culturing.

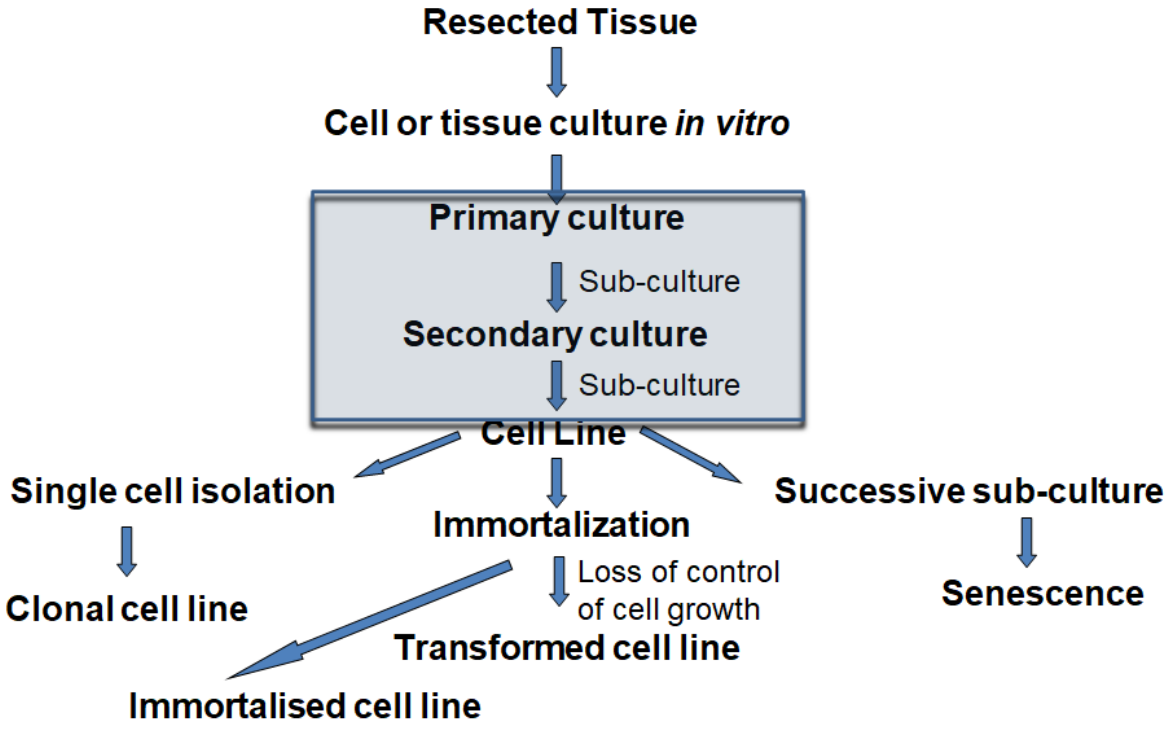


Figure 4 The flowchart of processing tissues from animal into established cell line

1.4 TYPES OF CELL CULTURES IN-VITRO

Primary Cell Culture

- Derived directly from animal tissue.
- Cultured either as tissue explants or single cells.
- Initially heterogeneous – become overpopulated with fibroblasts.
- Finite life span in vitro.
- Retain differentiated phenotype.
- Mainly anchorage dependant.
- Exhibit contact inhibition.

Secondary Cell Cuture

- Derived from a primary cell culture.
- Isolated by selection or cloning.
- Becoming a more homogeneous cell population.
- Finite life span in vitro.
- Retain differentiated phenotype.
- Mainly anchorage dependant.
- Exhibit contact inhibition.

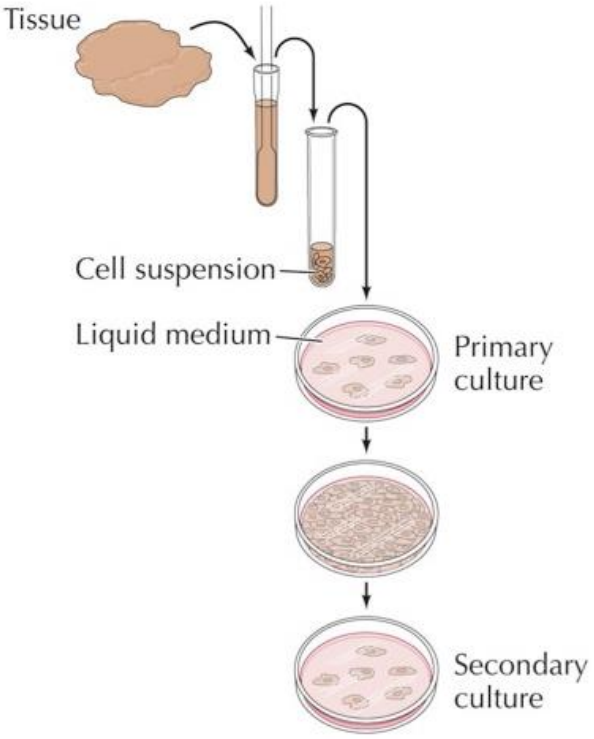


Figure 5 Primary Culture and Secondary Culture in Tissue Culturing

1.5 CELL CULTURE PLATFORM

The choice of platforms are important to properly grow and cultivate the tissue culturing and provide good model for downstream application.

Different platform used is shown in Figure 6.

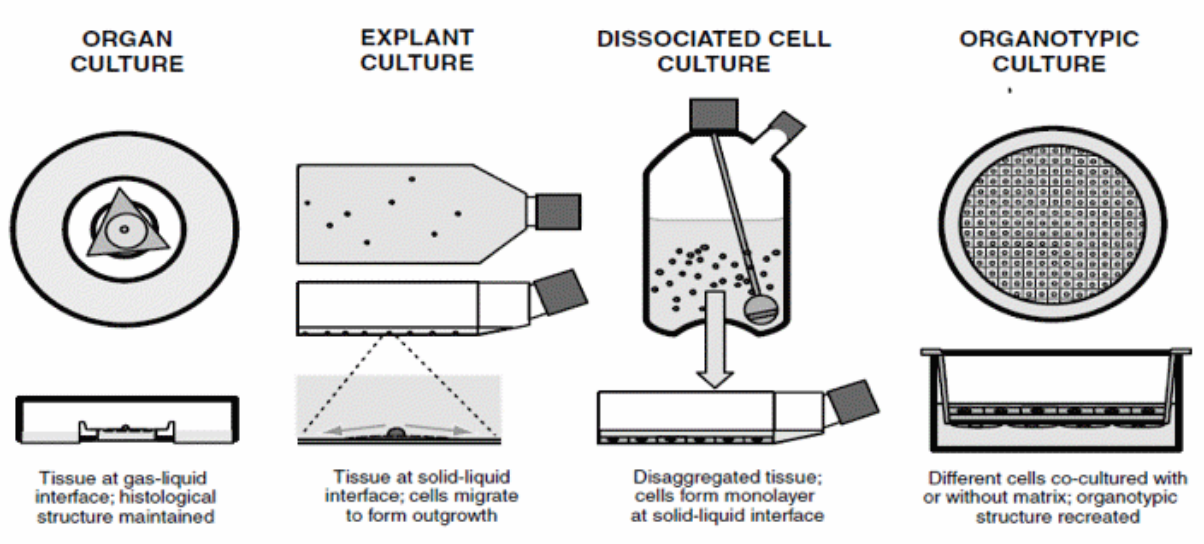


Figure 6 Tissue Culture Platforms and Their Uses

TYPES OF PRIMARY AND SECONDARY ANIMAL CELL CULTURES

- **Adherent cells:**

require attachment for growth. Usually derived from tissues of organs, such as kidney where they are immobile and embedded in connective tissue

- **Suspension cells:**

do not require attachment for growth. Derived from liquid-based cellular system such as blood.

Figure 7.

Figure 7 Different Animal Cell Culture Platform. Adherent Cell Platform (Left) and Suspension Cell Platform (Right)

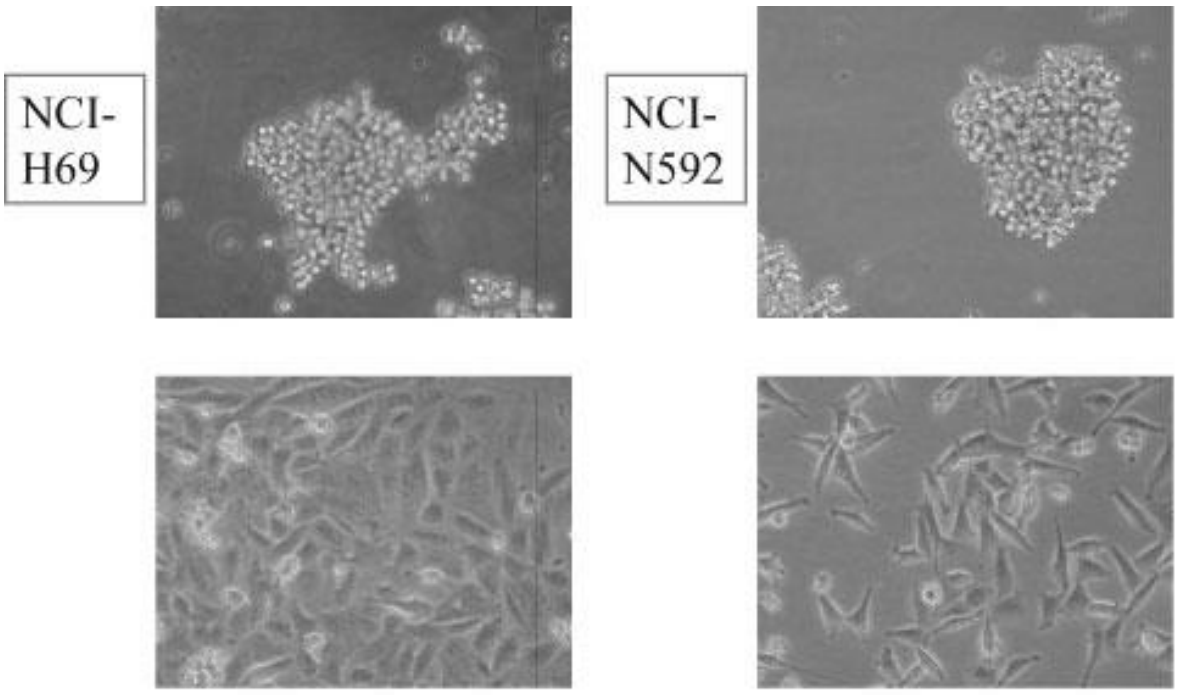


Figure 8 Different forms of cell culture.. Suspension from of NCI-H69 (top left) and NCI-N592 (top right) in comparison with adherent form of NCI-H69 (bottom left) and NCI-N592 (bottom right)

1.6 BASIC EQUIPMENTS USED IN CELL CULTURE

Biosafety Cabinet

- Aseptic condition and protection for both user and samples,
- HEPA filters and controlled air flow at the front, back and above.

Incubator:

- Usage parameter varies,
- For mammalian cells, 37 ± 2 ºC with ~5% CO2 is commonly used,
- Relative humidity is also maintained.

Cold Storage:

- Liquid media kept at 4ºC (refrigerating temperature): enzymes (e.g. trypsin), medium both liquid and powder form.
- Media supplements (e.g. glutamine & serum) at -20 ºC, can be both standing freezer or chest freezer
- Storage for cell culture stocks, to be stored in -80oC freezer for short term < 6 months and Liquid N_2 in long term.

Microscope

- An inverted microscope with 10x to 100x magnification with pedestal to put cell culture platforms: disk, flasks, plates, bottles.

Figure 9 Instrumentation in Cell Culture. Biosafety Cabinet (Left) and Cell Culture Incubator, usually connected to gas tank. (Right)

Figure 10 Cold Storage Units used in Cell Culturing: Refrigerator (Top Left). Stand Freezer (Top Right), Chest Freezer (Bottom Left) and Liquid Nitrogen Tank (Bottom Right).

Figure 11 Inverted Microscope with maximum of 100x Magnification

1.7 CELL CULTURE REQUIREMENT FOR PROLIFERATION

1. Substrate or liquid inside vessels (flasks, dishes) that are tissue-culture treated Sterile, pyrogen-free and enable cell-adherence.

2. Environment (CO2, temperature 37oC, humidity)

3. Sterility (aseptic technique, antibiotics and antimycotics)

4. Basal Medium, which contains:
   - Inorganic Salts
     - Maintain osmolarity
     - Regulate membrane potential (Na^+, K^+, Ca^{2+})
     - Ions for cell attachment and enzyme cofactors
   - Keto acids (oxalacetate and pyruvate)
     - Intermediate in Glycolysis/Krebs cycle
     - Keto acids added to the media as additional energy source
     - Maintain maximum cell metabolism
   - Carbohydrates
     - Energy source
     - Glucose and galactose
     - Low (1 g/L) and high (4.5 g/L) concentrations of sugars in basal media
   - Vitamins
     - Precursors for numerous co-factors
     - B group vitamins necessary for cell growth and proliferation
     - Common vitamins found in basal media is riboflavin, thiamine and biotin
   - Trace Elements
     - Zinc, copper, selenium and tricarboxylic acid intermediates

5. Supplements
   - L-glutamine
     - Consists of Essential amino acid (not synthesised by the cell)
     - Acts as energy source (citric acid cycle), used in protein synthesis
     - Unstable in liquid media - added as a supplement

6. Non-essential amino acids (NEAA)
   - Supplemental to basic media compositions
   - Energy source for protein synthesis and translation
   - Primarily used to reduce metabolic burden on cells

7. Growth Factors and Hormones (e.g.: insulin)
   - Stimulate glucose transport and utilisation
   - Uptake of amino acids
   - Maintenance of differentiation

8. Antibiotics and Antimycotics
    - Penicillin, streptomycin, gentamicin, amphotericin B
    - Reduce the risk of bacterial and fungal contamination
    - Cells can become antibiotic resistant – changing phenotype
    - Preferably avoided in long term culture

9. Foetal Calf/Bovine Serum (FCS & FBS)
   Consists of:
    - Micronutrients, Growth factors and hormones, with functions:
    - Aids cell attachment
    - Binds and neutralise toxins
    - Encourage cell growth and maintenance
    - Heat Inactivation (56oC for 30 mins) of Foetal Calf/ Bovine Serum:
        a. Destruction of complement and immunoglobulins
        b. Destruction of some viruses (also gamma irradiated serum).

10. pH Indicator – Phenol Red
    - Optimum cell growth approx. pH 7.4
    - Useful to detect growth abnormalities and contamination.

11. Buffers (Bicarbonate and HEPES)
    - Maintains buffer for optimal condition for cell growth
    - Bicarbonate buffered media requires CO2 atmosphere
    - HEPES buffered media does not require CO2 atmosphere

1.8 TYPICAL LABORATORY SETTING FOR TISSUE CULTURING

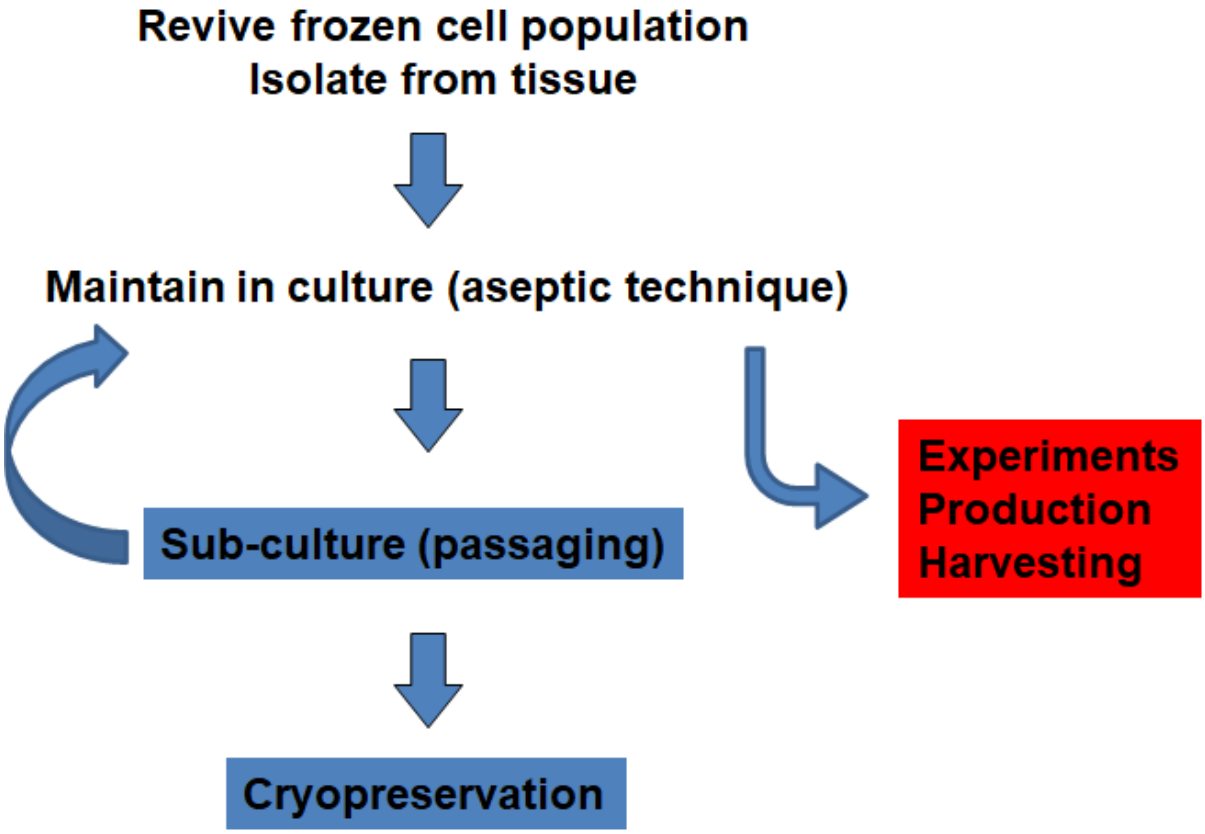


Figure 12 Typical of cell culturing routines in Biomanufacturing

PASSAGING (SUB-CULTURING) CELL CULTURE

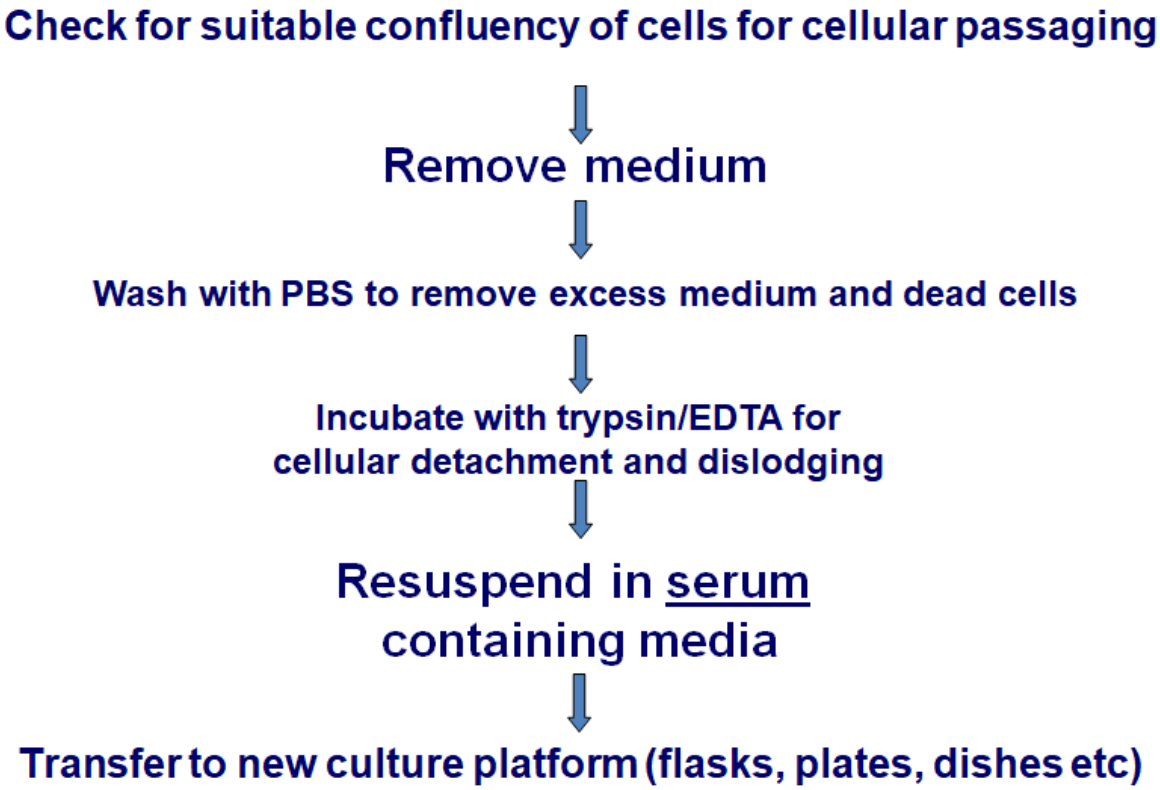


Figure 13 Workflow of passaging and maintaining cells in Biomanufacturing setting

Chapter 2: LABORATORY WORK IN CELL CULTURE

2.1 CRYOPRESERVATION OF CELL CULTURES FOR STORAGE

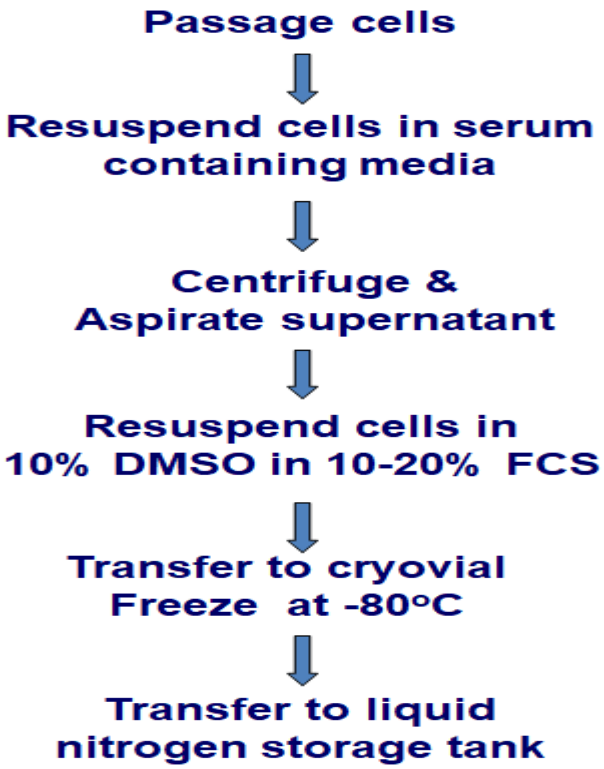


Figure 14 Workflow of passaging and maintaining cells in Biomanufacturing setting

2.2 CELL CONDITION AND CONFLUENCY

- Confluency: Percentage of cell occupation in designated platform.
- Most cells should be passaged when they reach approximately 80-90% confluency, unless recommended otherwise.
- ATTENTION: Different confluency of cells may have different cellular signalling, genotypes and phenotypes.

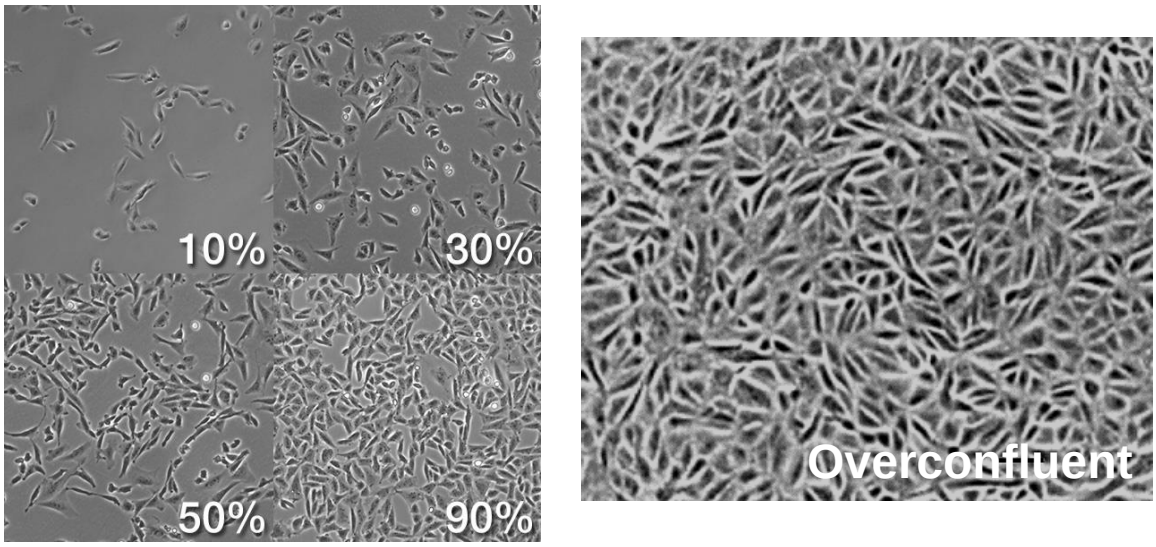


Figure 15 State of confluency in Fibroblast type cells, which percentage of confluency denoted on bottom right of each inzet.

2.3 CELL SUB-CULTURING, WASHING AND TRYPSYNIZING

- Washing: Rinsing and removing excessive cell debris and medium for better trypsinization process.
  - Using either tissue culture grade PBS, Hank's Balanced Salt Solution (HBSS) or Trypsin-EDTA in PBS.
- Trypsinizing: Detaching and dislodging cells from container.
  - Using 0.05% - 0.25% Trypsin-EDTA in PBS
  - For more delicate cells, the utilization as low as 0.01% can be done.
  - For trypsin sensitive cells, cell scrapping and/or tapping to dislodge cells can be considered.

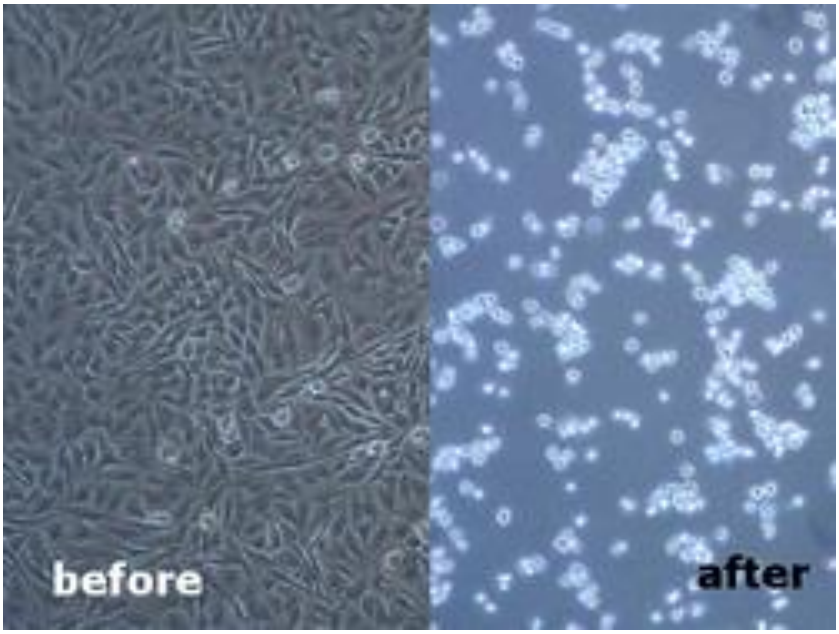


Figure 16 Trypsination process of MDCK cells, before (left) and after 2 mins of trypsinization (right)

2.4 CELL SPLITTING TO NEW PLATFORM

- After trypsinization, trypsin-EDTA should be inactivated by adding approximately 5-10x the amount of 10% FBS supplemented media.
  - For example: in 1 ml of trypsin-EDTA solution, we should add 9 ml more [medium+10% FBS]
- In passaging, we use split-ratio to show the proportion of the cells transferred into new flasks. The denomination is 1 : X, means that we will transfer : (total medium divided by X)
  - Split ratio **1 : 5 (1 in 5)** means that out of **10 ml** medium in old flask, we will transfer (10 ml : 5 = **2 ml)** to the new flask (same size!!).
  - IF different flask container is used, adjust the number accordingly.
  - Example: If we transfer 5 ml of cells in 10 ml medium in 25 cm^2 flask into 75 cm^2 flask, it is **NOT 1 : 2**, but 1 : [2 x (75/25)] = **1 : 6 split ratio**.

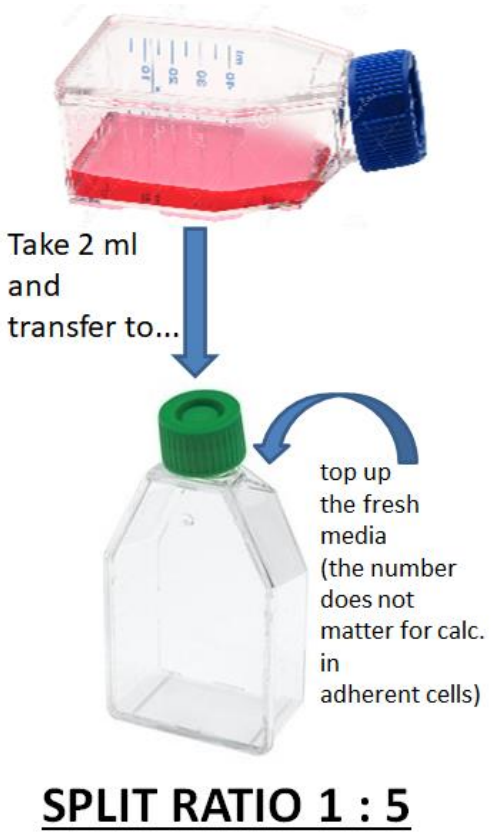


Figure 17 Cell splitting and calculation for transferring cells into the new media

2.5 PASSAGING DIFFERENT CELL LINES

- Each cell line has different optimum characteristics for maintaining and passaging such as:
    - Optimal Growth Condition
    - Suitable platforms for growth
    - Different supplements and nutrients
    - Resistances to trypsin
    - Passage-life per isolate
- It is important to test and determine all of above-mentioned condition for maximum performance of cell line.
- The information can usually be retrieved from the sample (In Figure 18, ATCC)

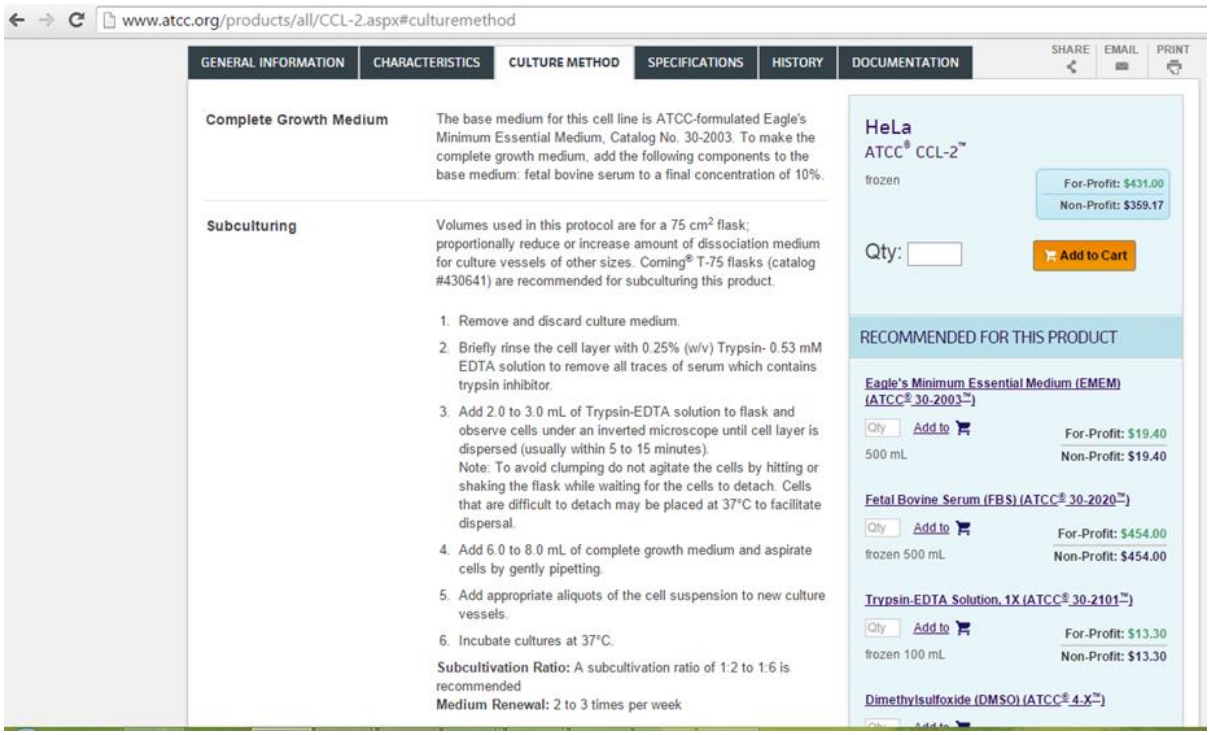


Figure 18 The direction of subculturing as shown in ATCC website

2.6 CELL SEEDING AND COUNTING

- Cell seeding: Transferring cells into new containers with precise amount number of cells.
- Denoted with $x.x \times 10^{x}$ cells per volume.
- Cell numbers are calculated using hemacytometer (Figure 19) with grids (Figure 20)
- Many application have different recommended cell seeding density. Consult with the publications, finding and optimization.

Figure 19 Hemacytometer

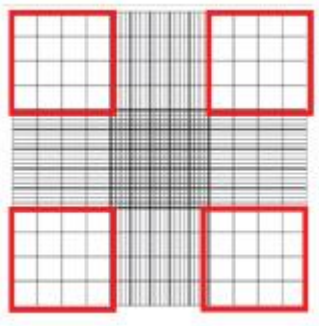


Figure 20 Hemacytomere grid

2.7 CELL COUNTING PROTOCOL USING HEMACYTOMETER

- Counting ideally is conducted on all 4 squares illustrated in Figure 21.
- In each box, we can use either top left method (include cells that overlap in top or left line) or bottom right method (include cells that overlap in bottom or right line) or others two overlapping lines (top right or bottom left). Illustration of left bottom method can be seen in Figure 22.

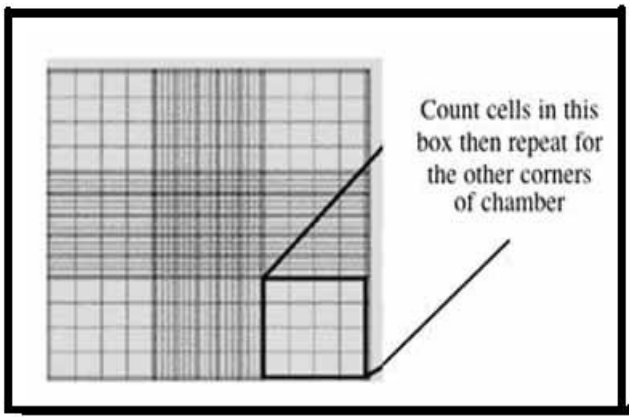


Figure 21 Diagram represent cell count using hemocytometer

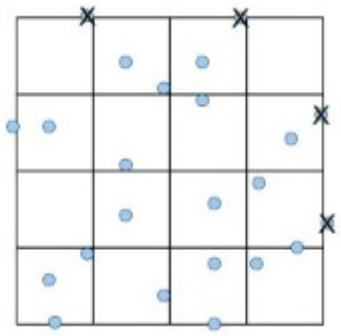


Figure 22 Cell counting inclusion method of bottom left methods

$$cell\ count\ = \frac{cells\ counted\ in\ 4\ areas}{average\ viewing\ areas\ (4)} dilution\ factor\ \times\ 10^4\ [\frac{cells}{ml}]$$

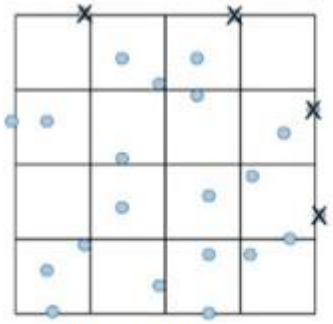
PS:

If trypan blue is mixed with cells with 1 in 1 ratio, the dilution factor will be 2.

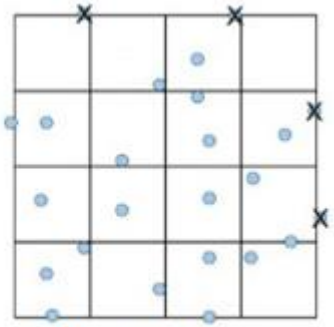
Use 20 µl per side when loading hemacytometer.

ILLUSTRATION OF CELL COUNTING:

We split our cells in a flask and trypsinize them using 0.1% Trypsin-EDTA, upon dislodging, cells are resuspended into medium and put into hemacytometer with mixture of 1:1 Trypan Blue. The result can be seen as follows:



Area 1



Area 2

Since above counted areas, therefore we average the cells in both areas:

$$cell\ count\ = \frac{cells\ counted\ in\ 2\ areas}{average\ viewing\ areas\ (2)} \times dilution\ factor\ \times\ 10^4\ [\frac{cells}{ml}]$$

$$cell\ count\ = \frac{38}{2} \times 2\ \times\ 10^4\ \left[\frac{cells}{ml}\right] = 1.9 \times 10^5\ \left[\frac{cells}{ml}\right]$$

ILLUSTRATION OF CELL SEEDING:

We have :

1.9×10^5 cells/ml

We need to seed:

4 T-25 flasks
with 5 ml medium each
containing
3×10^5 cells/flask

If we need to seed 3 x 10^5 cells in 4 T-25 flasks (each flask contains 5 ml of medium) with the cell number as above. We will proceed with protocol and formula as follows:

Use dilution formula V1 . M1 = V2 . M2 formula (Adjustment of unit is necessary and must pay a lot of attention, as a rule of thumb, ALWAYS change all units to become **ml and cells/ml**).

M1 = 1.9×10^5 cells/ml

V2 = 4 flasks × 5 ml per flask = 20 ml (to compensate for pipetting error, we should increase by appr. 10-20%, in this case, we will increase by 10%, therefore, V2 will be 22 ml)

M2 = 3×10^5 cells/flask = 3×10^5 cells/ 5 ml = 6×10^4 cells/ml

V1 = ???

V1 . M1 = V2 . M2

V1 . 1.9 x 10^5 cells/ml = 22 ml . 6 x 10^4 cells/ml

V1 = [22 ml . 6 x 10^4 cells/ml] / 1.9 x 10^5 cells/ml = 6.94 ml

It means that we need **6.94 ml** of cells inoculum to be mixed with [22 – 6.94 = **15.06 ml**] of additional fresh medium and dispersing 5 ml into each flask.

1.9×10^5 cells/ml

We take out 6.94 ml of cell stock into

We take out 15.06 ml fresh medium into

Container

Container containing
6.94 ml cell + 15.06 ml medium

Container

Seed 5 ml from the container into:

X 4 flasks

T-25 flask

2.8 Cell Upscaling

- Cell upscaling: Increasing cell stock with proper planning organization to working volume.
- Make sure that the upscaling is not too sparse or too dense for optimal growth.
- Usually it is included in the ATCC guideline (see Figure 23).
- For example: if the recommended is 1:2 to 1:6 ratio, then it is recommended to increase the stock 6 times at one round at maximum.

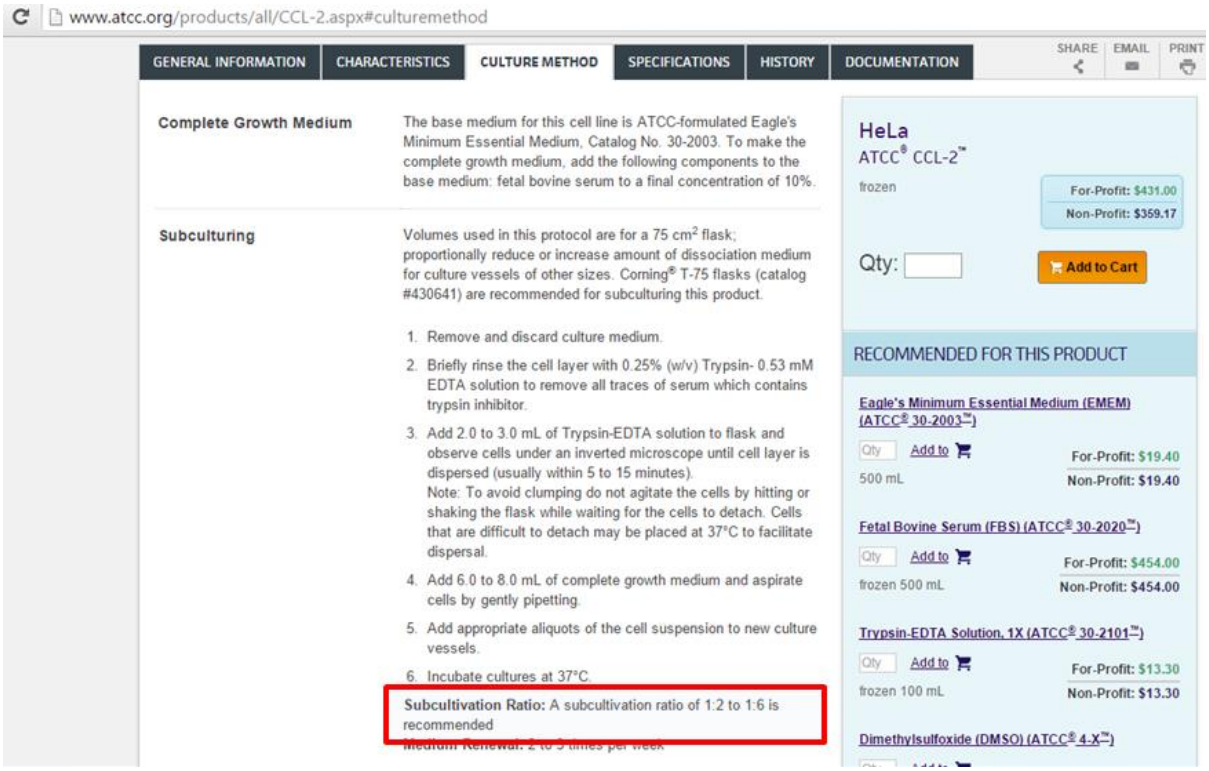


Figure 23 Recommended subcultivation ratio as recommended by ATCC

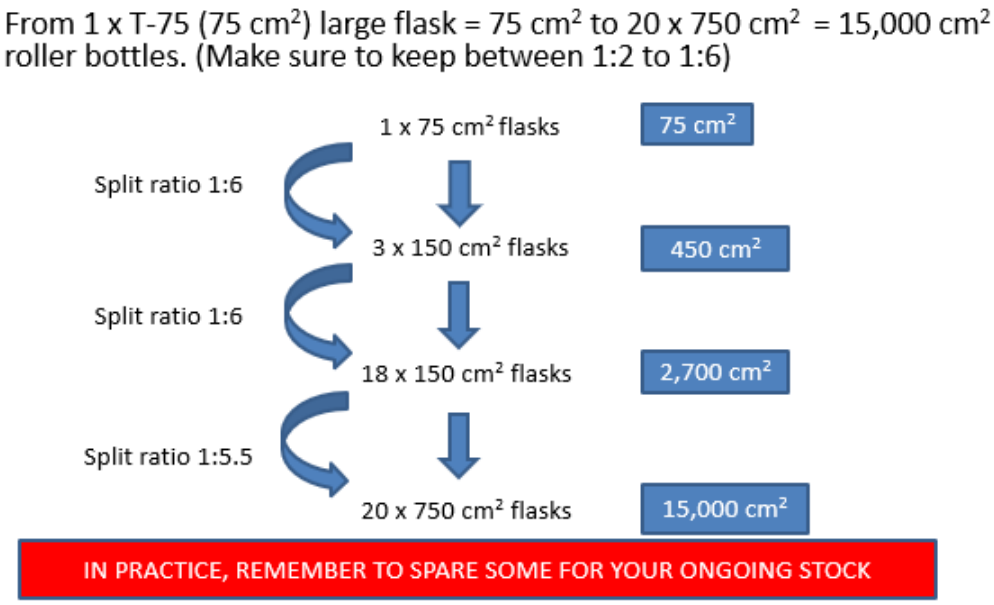
ILLUSTRATION OF UPSCALING

If currently I have one confluent T-75 flasks of HeLa and I wish to upscale my product into 20 roller bottles (equivalent of 750 cm^2) per bottle. What are the steps?

Answer:

1. Check the recommended sub-cultivation ratio by the ATCC (IT is written 1:2 to 1:6 split ratio, which means I can use within this range).
2. It can be done **multiple ways**, but try to stick to the "least" recommended amount to save time and possibly amount of medium spent, unless instructed otherwise according to the needs.
3. All must be done **simultaneously and synchronously**, DO NOT upscale only some part. It will cause uneven distribution of the cells.

The step can be done as follows:



2.9 QUALITY CONTROL OF CELL LINES MAINTENANCE IN BIOMANUFACTURING

Quality Control in Cell Culture: To make sure cultured-cells still possess desirable properties and qualities.

In Biomanufacturing, usually consists of 3 elements of QC:

- **Morphology**

  Compared between initial stock morphology with current stock

  If significant changes or abnormalities are found in morphology, discard and change into new stocks

- **Cell doubling time**

  Cell doubling time is the amount of time required for cells to double

  Cell doubling time :

  $$The\ amount\ of\ cells\ after\ 24\ hours\ = The\ seeded\ amount\ \times\ 2^{\boldsymbol{time}}$$

  We need to find out the **time** required. It will go down as we continuously passage the cells.

- **Biological products**

  The measurement of the biological products

  Measured for its metabolites, antibodies, proteins, etc.

Currently I have 1 x 150 cm^2 flask of CHO-K1 (ATCC® CCL-61™) (cell number of confluency = 2 x 10^7 cells/flask), 1 flask = 25 ml medium, and I wish to expand and seed it into:

1. 10 x 750 cm^2 roller bottles for production.
2. 4 wells in 24-well plate with (1 x 10^5 cells per well) (1 well = 3 ml medium) for QC-doubling-time test.
3. 40 wells in 96-well plate with (1x 10^4 cells per well) (1 well = 100 µl medium) for QC-ELISA test.

All of the procedures above must be done in **same day and same set of experiment**, write a complete SOP from splitting, seeding, and upscaling.

References

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