Level 8 Cell Culture Processing (BIO08045)
Lecture 3 – "Cell Banking & Cell Culture Media Design"

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Learning Objectives

The importance of cell banks

Cell line verification

Describe the key components in animal cell culture media

Compare and contrast serum-supplemented, serum-free media and protein free media formulations

Recommended Reading

- R.I. Freshney, *"Culture of Animal cells : a manual of basic technique"*. 8th edition, Wiley-Blackwell (2021)
- John M. Davis, *"Animal Cell Culture: Essential Methods"*, 1st Edition. Wiley, (2011)
- Michael Butler, *"Animal Cell Technology"*. 2nd edition, BIOS Scientific (2004)
- JM Davis, *"Basic Cell Culture: A Practical Approach"*. 2nd edition, Oxford University Press (2002)
- Terence Cartwright, *"Animal Cells as Bioreactors"*, Cambridge University Press, 1994

Websites/Videos

- Some useful videos of cells in culture can be viewed at:

  https://www.sartorius.com/en/products/live-cell-imaging-analysis

- American tissue culture collection : www.atcc.com
- Fundamental techniques in Cell Culture (a laboratory handbook): available from Sigma website (in pdf or hard copy)
- Cells alive website (http://www.cellsalive.com) particularly for those with little biology

Lecture Topics

- Cell Banking
- Cell Culture Media
- Defined Portion of Cell Culture Media
- Complex Portion of Cell Culture Media

Cell Line & Process Development

Early stage

- Cell line development
- Production media selection
- Clone with the highest productivity properties selection

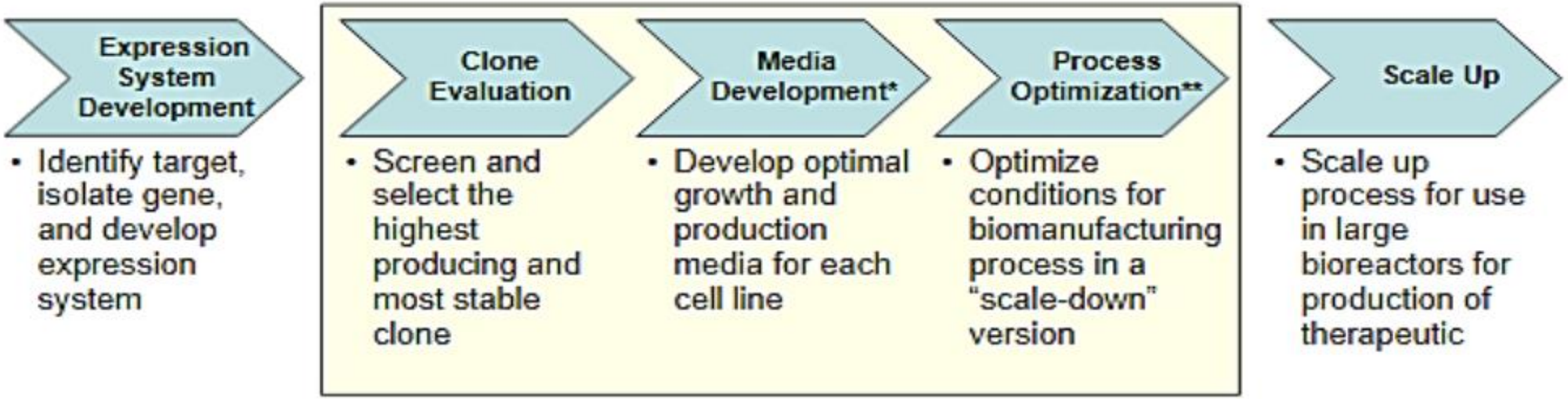
Late stage

- Feeding and suplementation strategy optimization
- Process parameters optimization
- Product titer and quality optimization

Process transfer

- Three consolidation runs in R&D before transfer
- Data alignment between R&D and Production Units

Cell Line & Process Development



- Knowing gene for the protein you want is great, but what cell line to use? What clone form that cell line is best. 100s of possibilities!
- 60 or more nutritional components in culture media, how many combinations? When to feed them? Inducers, promoters?
- What temperature? What oxygen level? CO2? pH any shifts? When to harvest?
- A strategy of multi-factorial design is the natural way to attack this type of problem, but is difficult to execute in cell culture because the parameters interact strongly-requiring a lot of experiments. This means models!

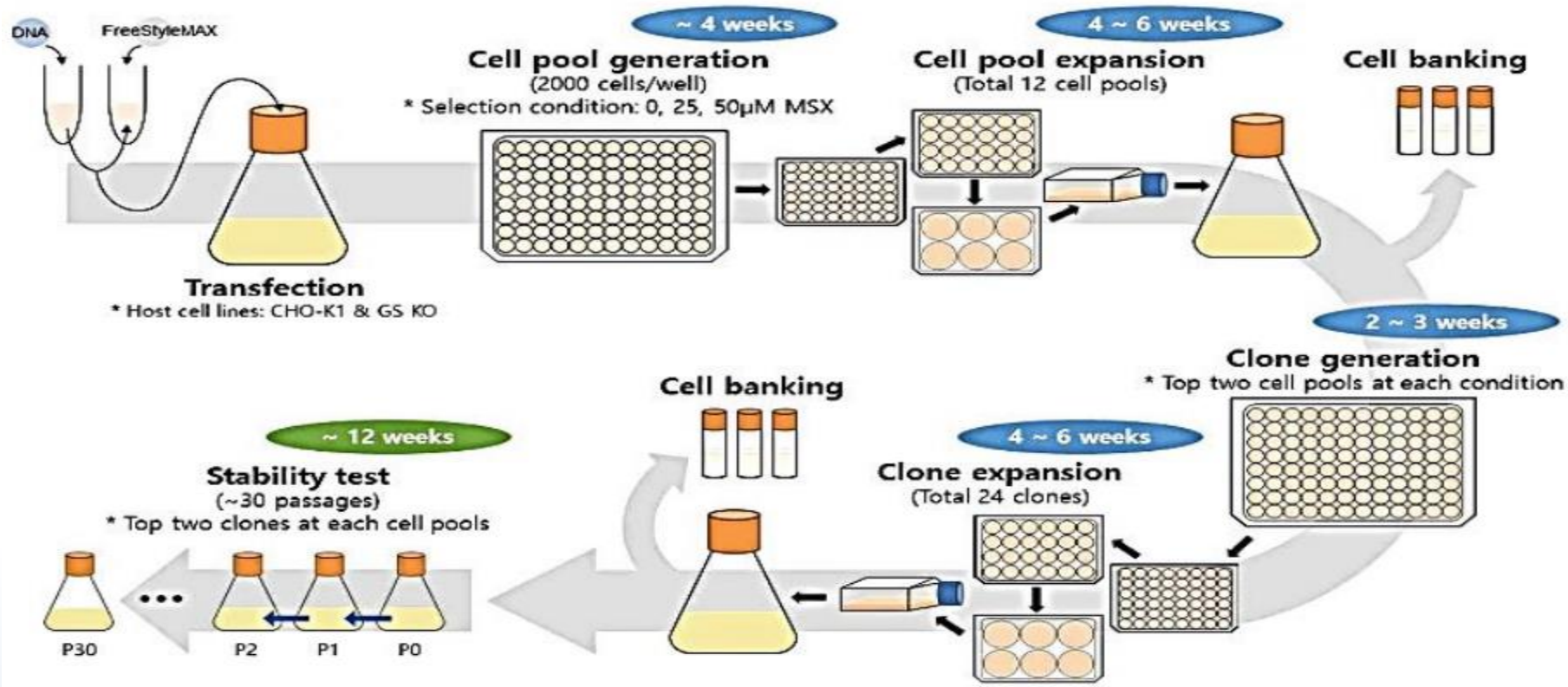
Note : ** Represent iterative processes
Source : Nature Biotechnology Vol. 22 (11) 2004

Cell Line Development to Cell Banking

From: Comprehensive characterization of glutamine synthetase-mediated selection for the establishment of recombinant CHO cells producing monoclonal antibodies

Soo Min Noh1, Seunghyeon Shin1 & Gyun Min Lee.

Scientific REPOrTS | (2018) 8:5361 | DOI:10.1038/s41598-018-23720-9



A schematic diagram of the process for mAb producing clone generation and long-term culture for testing the production stability.

https://www.nature.com/articles/s41598-018-23720-9/figures/1

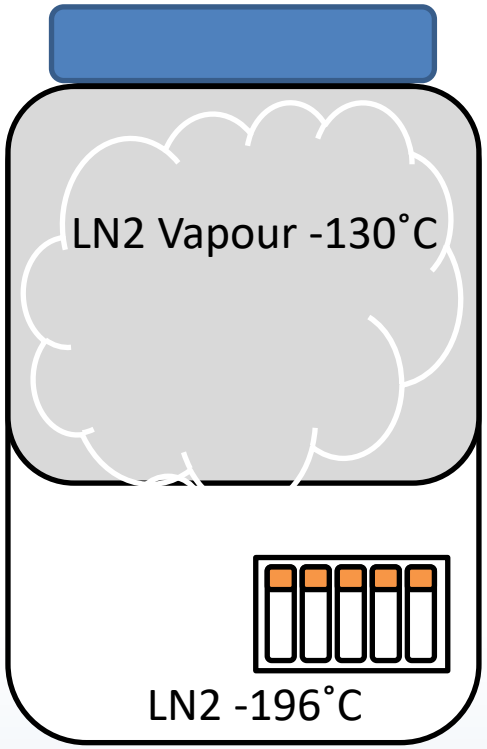
Cell Banking

- Secure, controlled long term storage of cell stocks:
  - Required to control cell lines, to have original stocks for each batch or in case of contamination.
- Required for regulation and validation of process:
  - Common starting point for each batch of product.
- Frozen stocks of cells reduces risk of contamination:
  - Stocks are identical avoiding genetic instability in cell lines.
- Vials stored in a cryofreezer or in liquid nitrogen:
  - Cells frozen in 1-2mL cryovials.
  - Sub zero temperatures are used which effectively stop any biological activity that could lead to cell death.
  - A cryoprotective agent is used to prevent damage to cells by the formation of crystals on freezing. A protective agent like DMSO (Dimethyl sulphoxide) is added to the cells before freezing.

Cell Banking

- **Cell banks are mostly stored in liquid nitrogen tanks/dewars:**
  - Cells can be stored in the liquid or in the vapour of the liquid nitrogen tank.
  - Temperature of **liquid nitrogen** -196°C.
    - ✓Constant ultra low temperature.
    - X Contamination is possible if vials are not secured.
  - Temperature of **liquid nitrogen vapour** -130° C.
    - ✓Less risk of cross contamination.
    - X Poor temperature gradient.

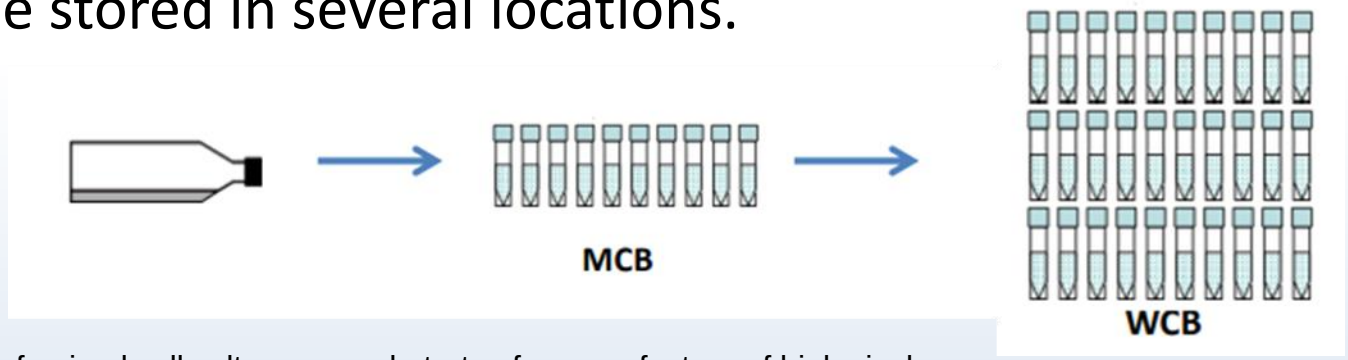
Cell Banking



- Ultra-low temperatures.
- Stops biological activity that could lead to cell death.

Cell Banking

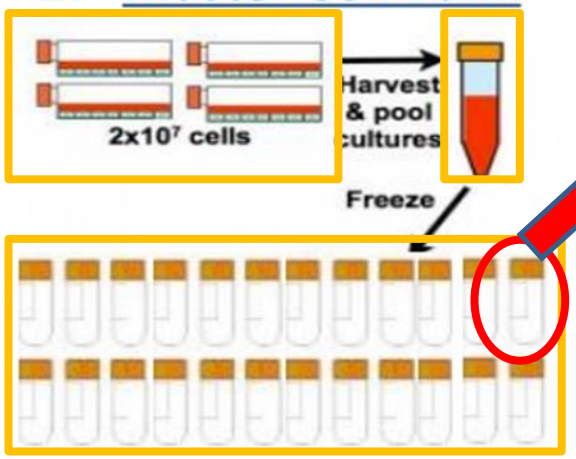
- Stocks prepared from primary culture-early passage numbers:
  - Cell bank has a 2 tier system which consists of **master cell bank (MCB)** and **working cell bank (WCB).**
  - Master stocks are prepared for the **master cell bank.**
  - From this, stocks are prepared for a **working cell bank.**
- Vials are thawed for consistency checks:
  - Cell banks can be stored in several locations.



From: Recommendations for the evaluation of animal cell cultures as substrates for manufacture of biological medicinal products and for the characterization of cell banks. WHO 2010
http://www.who.int/biologicals/Cell_Substrates_clean_version_18_April.pdf

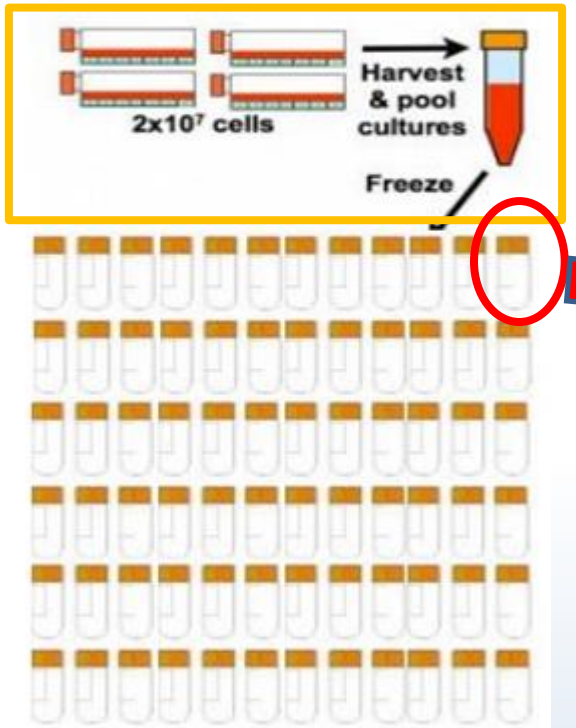
Cell Banking

1. Master Cell Bank



Grow approved modified cells and prepare vials for the Master Cell Bank

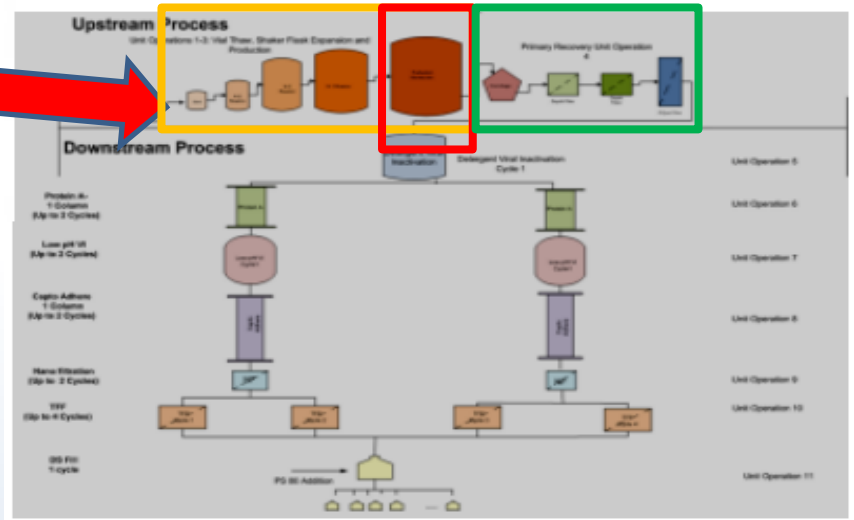
Remove Vial from the Master Cell Bank and expand culture to generate the Working Cell Bank

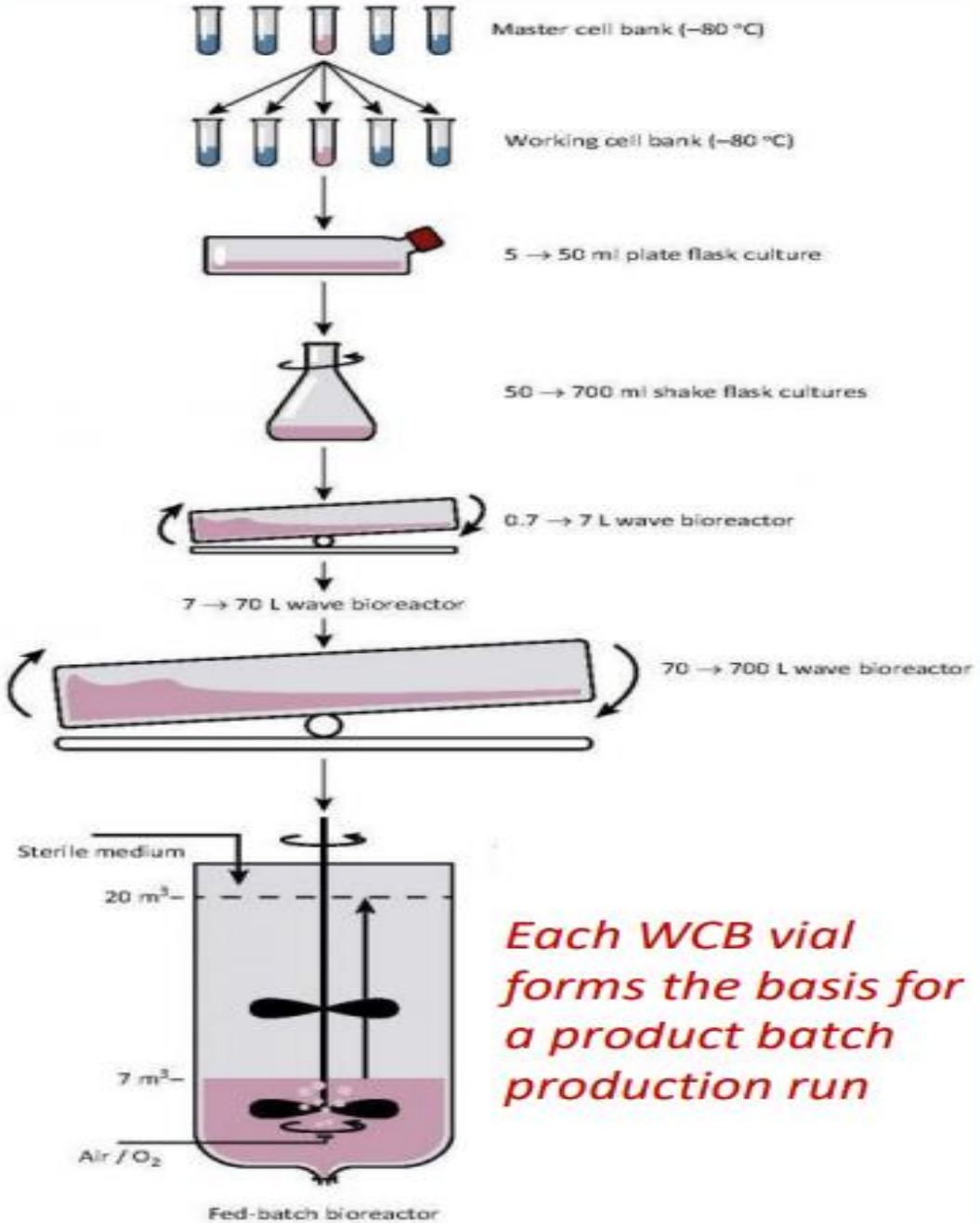


2. Working Cell Bank

3. Production Batch

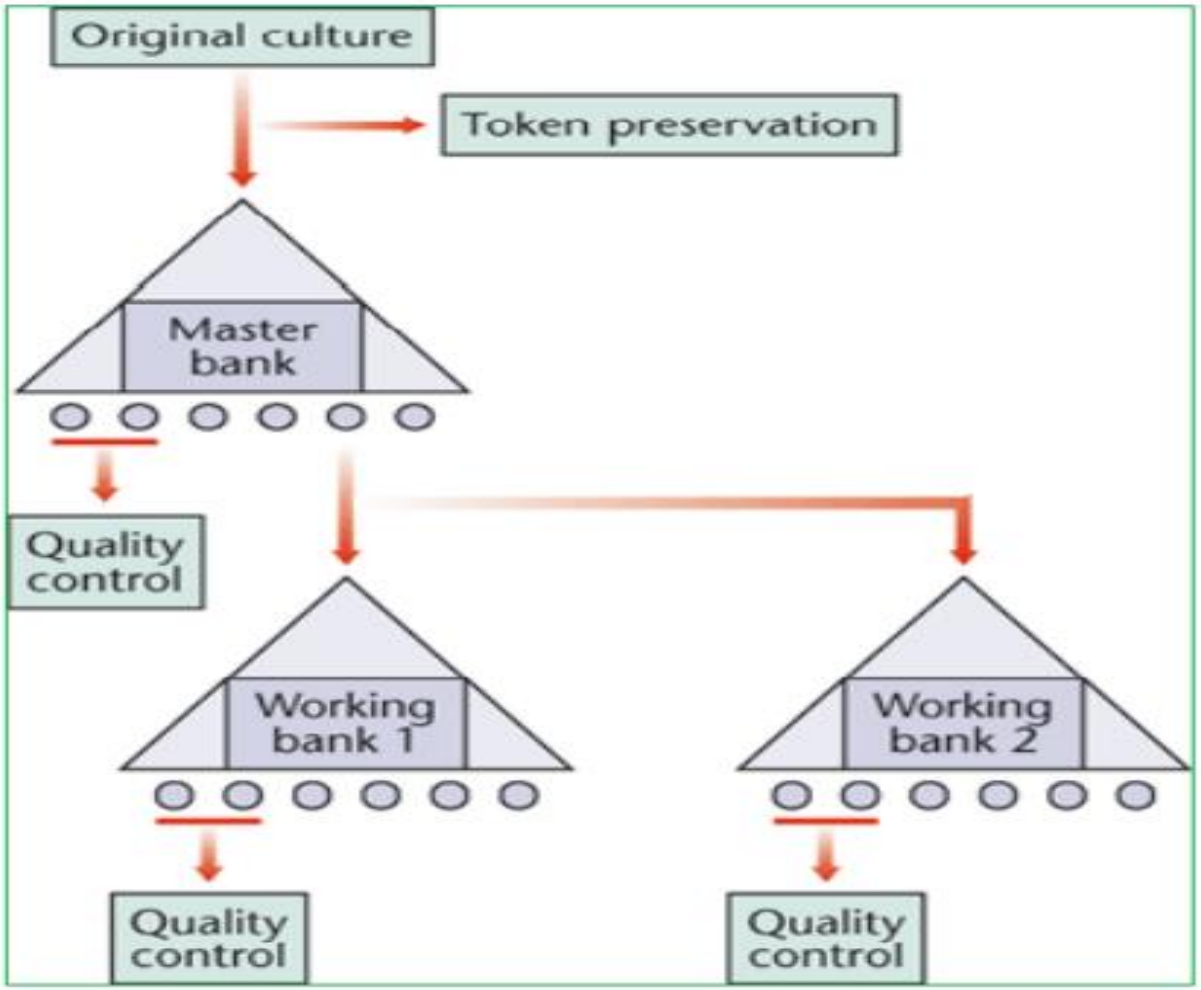
Remove Vial from the Working Cell Bank and expand culture for each production batch





Each WCB vial forms the basis for a product batch production run

Cell Banks



Principles of Good Cell Culture Practice

- In all aspects of sourcing, banking and preparing cell cultures, the principles of Good Cell Culture Practices (GCCPs) should be observed. Fundamental features to be considered in the development of cell cultures for production or testing are:

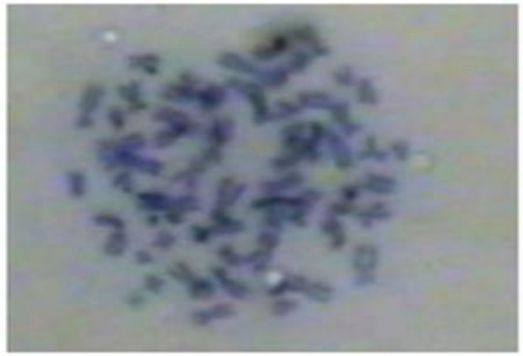
1. **Authenticity**, including identity, provenance, and genotypic/phenotypic characteristics;
2. **Absence of contamination** with another cell line;
3. **Free of microbiological contamination**, and
4. **Stability** and functional integrity on extended in vitro passage.

Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. World Health Organization 2010 http://www.who.int/biologicals/Cell_Substrates_clean_version_18_April.pdf

Cell Line Characterisation

- What is really in the vessel? MCB and WCB are among the major assets of a company.
- Need to ensure that you have good quality: check number of parameters:
  1. **Viability**: when you set up MCB must thaw vial to check viability;
  2. **Contamination**: checks for bacteria, fungi and especially for mycoplasma;
  3. **Morphology**: shape cells, attachment;
  4. **Isoenzymes**: can be used to check for species e.g. Lactate dehydrogenase;
  5. **Karyotype**: chromosomal analysis;
  6. **DNA fingerprinting**: more expensive and usually done by specialised laboratory;
  7. **Productivity/Titre**: check levels of product.

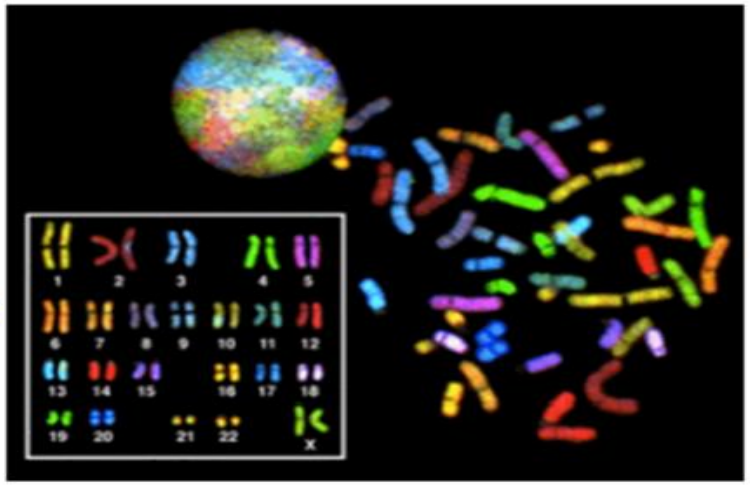
Cell Line Characterisation

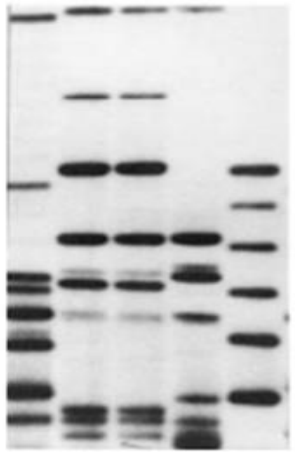


Light microscope

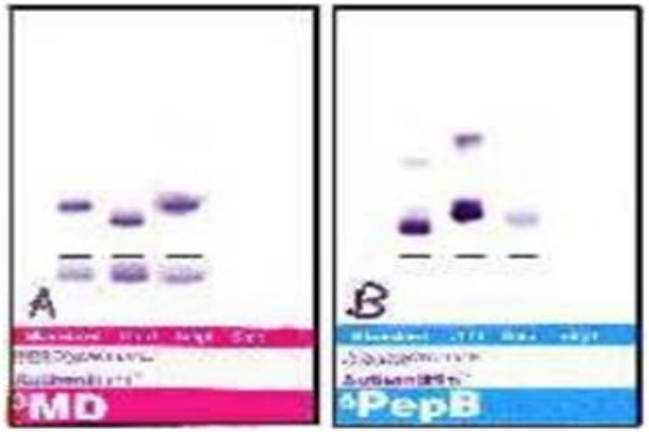
Karyotype

Spectral Ray Computer image





DNA fingerprint



Isoenzyme analysis

Cell Line Characterization Studies

	MASTER CELL BANK	MANUFACTURERS WORKING CELL BANK	END OF PRODUCTION CELLS Cells at the limit of in vitro cell age
Authenticity & Characteristics			
Identity	Yes	Optional	Yes
Growth / Morphology	Yes	Yes	Yes
Genetic Stability	Program Dependent	Program Dependent	Program Dependent
Tumorigenicity	Cell Line Dependent	No	No
Adventitious Agents			
Sterility	Yes	Yes	Yes
Mycoplasma	Yes	Yes	Yes
General Virus Screens	Yes	No	Yes
Species-Specific Virus Tests	Yes	No	No
Retrovirus Tests	Yes	No	Yes
Adventitious Agents from Raw Material	Yes	Program Dependent	Program Dependent

The table above provides an overview of the tests typically required to characterize mammalian cell lines as outlined in the worldwide regulatory guidance documents - which tests to conduct and at which stage.

Cell Line Characterization

- See Recommendations for the evaluation of animal cell cultures as substrates for manufacture of biological medicinal products and for the characterization of cell banks. *WHO,* 2010.

  https://www.who.int/publications/m/item/evaluation-of-cell-substrates-for-the-production-of-biologicals-revision-of-who-recommendations

Lecture Topics

- Cell Banking
- Cell Culture Media
- Defined Portion of Cell Culture Media
- Complex Portion of Cell Culture Media

Cell Culture Media

- Cell culture media must contain all the components necessary to support the growth of cells.
- Components must be present in the correct form and concentration.

Carbon/energy source + nitrogen source + other nutrients

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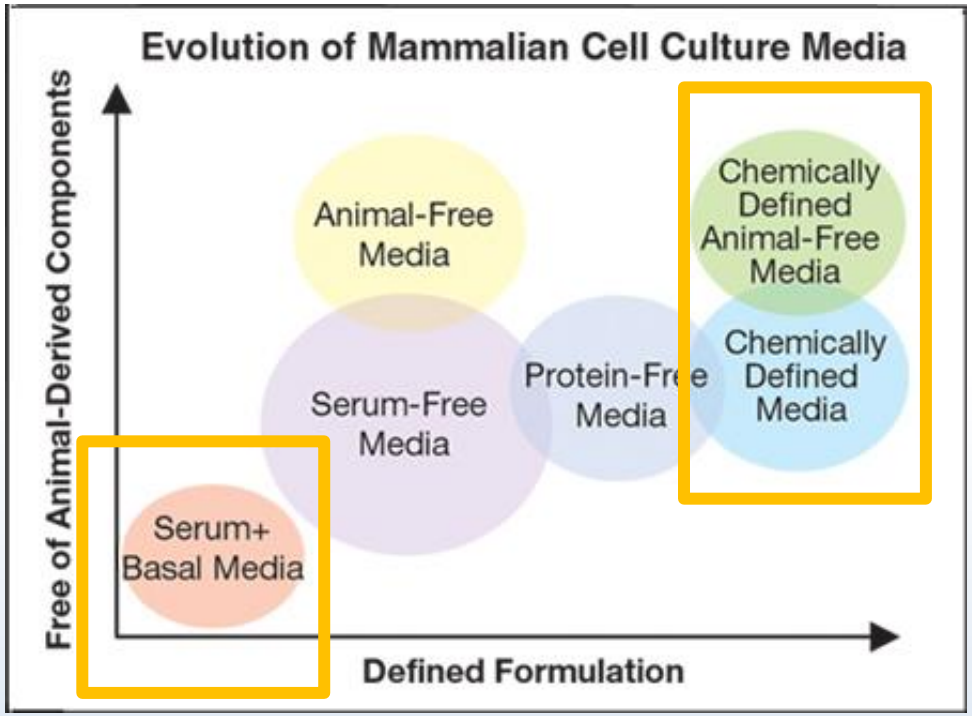
Cell biomass + product + CO_2 + H_20 + heat + metabolites



History of Cell Culture

- Pioneer scientists such as Dulbecco, Ham & Eagle determined the nutrient requirements of animal and human cells in the 1950s.
    - They did so by measuring nutrient uptake of these cell lines, and also by trial & error.
- **DMEM-F12** is one of the most popular base media, it is a 50/50 mixture of Dulbecco's Modified Eagle's Medium & Ham's Nutrient Mixture F12 (see following table for the constituents).

Cell Culture Media

- Medium optimization is a large part of the bioprocess and depends on many factors:

1. Feeding pattern-batch, fed batch, perfusion
2. Bioreactor design
3. Culture conditions-cell type, density
4. Preparation format-powder, liquid
5. Downstream purification
6. Product application
7. Business factors including cost, equipment, yield etc

Chemical environment of cells

- Growth media contains 2 parts:
  1. Complex / Non-defined portion : usually serum
  2. Defined part : glucose, amino acids, vitamins, salts etc
- First media developed by Eagle – called Eagle's minimal essential medium **(EMEM)**
- Later modified by Dulbecco – referred to as **DMEM** (Dulbecco's modification of Eagle's MEM)
- Other common media such as **HAMs F-12, M199**

Cell Culture Media Components

There are two main types of media used in cell culture processes:

Complex Media:	
Broad range of nutrients, vitamins, minerals etc.	Adventitious agents and other contaminants.
Culture of many different cell types	Batch to batch variation
High cell density and productivity	Supply may be restricted due to disease or drought
Shear protection	Difficulties in downstream processing due to large quantities of proteins etc.
Less expensive	Disliked by regulators

Defined Media:	
Defined concentration of components	Potentially lower growth
Little batch to batch variation	Cells may need to be adapted to grow without serum
Consistent quality	Optimisation of formula can be difficult
Lower risk of contamination	Higher likelihood of shear stress
Preferred by regulators	Expensive

Lecture Topics

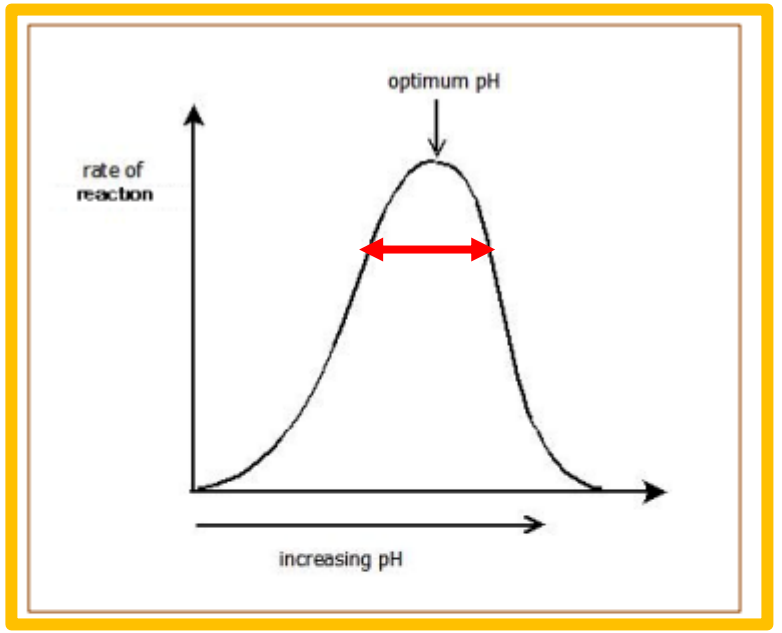
- Cell Banking
- Cell Culture Media
- Defined Portion of Cell Culture Media
- Complex Portion of Cell Culture Media

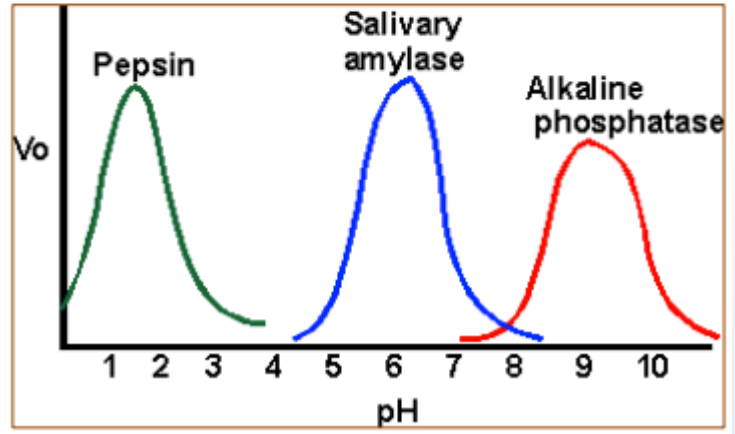
Defined Portion of Media

Carbon/ Energy source	• Carbohydrate (usually glucose)
Nitrogen source	• Ammonia and amino acids (Amino acid and protein synthesis)
Buffers	• For pH control (often bicarbonate buffer)
Vitamins	• Riboflavin, thiamine, ascorbic acid etc.
Growth Factors	• Signalling hormones (IGF-1, IL-6, G-CSF etc.)
Inorganic salts/ Trace metals	• Sodium chloride (osmolality) • Iron, zinc, magnesium, potassium, calcium etc.

Media pH

- Need to maintain a pH typically between 6.8 - 7.5 for mammalian cell cultures
  - Use phenol red as an indicator; media is initially red and changes to yellow as cells grow and metabolise
- Cytochromes – require neutral pH
  - Essential in cellular energy (ATP) production





DMEM as an example

Media formulations	DMEM	DMEM with stable glutamine
	130-091-437	130-091-438
Components	g/L	g/L
Inorganic salts		
$CaCl_2 \cdot 2H_2O$	0.264	0.264
$Fe(NO_3)_3 \cdot 9H_2O$	0.001	0.001
KCl	0.4	0.4
$MgSO_4 \cdot 7H_2O$	0.2	0.2
NaCl	6.4	6.4
$NaHCO_3$	3.7	3.7
$NaH_2PO_4 \cdot H_2O$	0.125	0.125
Other components		
Glucose	4.5	4.5
Phenol red Na	0.0159	0.0159
Sodium Pyruvate	0.11	0.11
Amino acids		
L-Arginine.HCl	0.084	0.084
L-Cystine	0.048	0.048
L-Alanyl-L-Glutamine	--	0.868
Glycine	0.03	0.03
L-Histidine HCl·H_2O	0.042	0.042
L-Isoleucine	0.105	0.105
L-Leucine	0.105	0.105
L-Lysine.HCL	0.146	0.146
L-Methionine	0.030	0.030
L-Phenylalanine	0.066	0.066
L-Serine	0.042	0.042
L-Threonine	0.095	0.095
L-Tryptophan	0.016	0.016
L-Tyrosine	0.072	0.072
L-Valine	0.094	0.094
Vitamins		
D-Ca Pantothenate	0.004	0.004
Choline Chloride	0.004	0.004
Folic acid	0.004	0.004
myo-Inositol	0.0072	0.0072
Nicotinamide	0.004	0.004
Pyridoxine.HCl	0.004	0.004
Riboflavin	0.0004	0.0004
Thiamine.HCl	0.004	0.004

Reference:
1. Dulbecco et al. (1959) Virology 8: 396-397.

DMEM-F12 Components: Inorganic Salts

Component	Amount (mg/L)
CaCl2	116.6
CuSO4	0.001
Fe(NO3)3.9H2O	0.05
FeSO4.7H2O	0.42
MgCl2	28.6
MgSO4	48.4
KCl	312
NaHCO3	**2438**
NaCl	7000
Na2HPO4	71
NaH2PO4	62.5
ZnSO4.7H2O	0.43

- **Provide metals needed for cell metabolism:**
  - Included as salts (e.g. chlorides & sulphates for improved solubility).
- **DMEM-F12 is low in iron.** Therefore, supplement with extra iron (Ferric Ammonium Sulphate or Ferrous Citrate, up to 100 mg/L).
- Sodium bicarbonate (NaHCO3) - buffering system to maintain pH. In bioreactors use CO2 sparging to complete the buffer system.
- Sodium Chloride (NaCl) conc. is often lowered to compensate for the extra supplements added to DMEM-F12 in order to maintain an osmolarity of around 320mOsm/Kg

DMEM-F12 Vitamins

- **Vitamin Amount (mg/L)**
  - Biotin 0.003
  - Ca Pantothenate 2.2
  - Choline.Cl 9
  - Cyanocobalbumin 0.68
  - Folic Acid 2.65
  - I-Inositol 12.6
  - Niacinamide 2
  - Pyridoxine.HCl 2
  - Riboflavin 0.2
  - Thiamine.HCl 2.2

- Vitamins are included because many enzymes need co-factors (such as vitamins and metal ions) to catalyze biochemical reactions.

DMEM-F12 Amino Acids

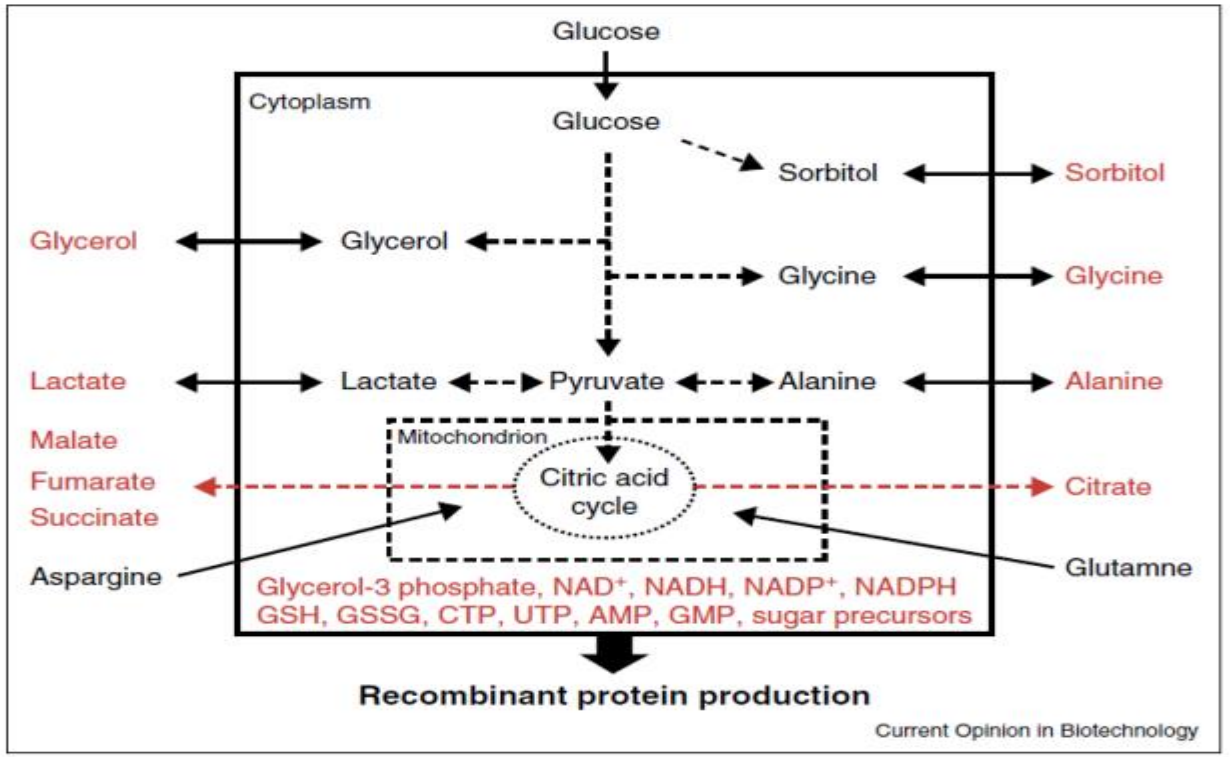
- All 20 common amino acids in L-form (mg/L):

Alanine	4.5
Arginine.HCl	147.5
Asparagine.H_2O	7.5
Aspartic Acid	6.7
Cysteine.HCl.H2O	17.6
Cystine.2HCl	81.3
Glutamic Acid	7.4
Glutamine	365
Glycine	18.8
Histidine.HCl.H_2O	31.5
Isoleucine	54.5
Leucine	59
Lysine.HCl	91.3
Methionine	17.2
Phenylalanine	35.5
Proline	17.3
Serine	26.3
Threonine	53.5
Tryptophan	9
Tyrosine.Na_2.2H_2O	55.8
Valine	52.9

DMEM-F12 Amino Acids

- **Arginine and Glutamine** are used up quickly by the cell because they feed directly into the Tri-Carboxylic Acid cycle (TCA, or Krebs cycle) and are an alternative source of energy, in addition to their structural role.
  - Many amino acids are inter-convertible through transaminase reactions.
- Glutamine is unstable in solution & breaks down to generate ammonia (an unwanted metabolite).
  - Therefore glutamine (along with glucose) is often included in bioreactor feed regimes.

Interplay of Nutrients in CHO Cell Status



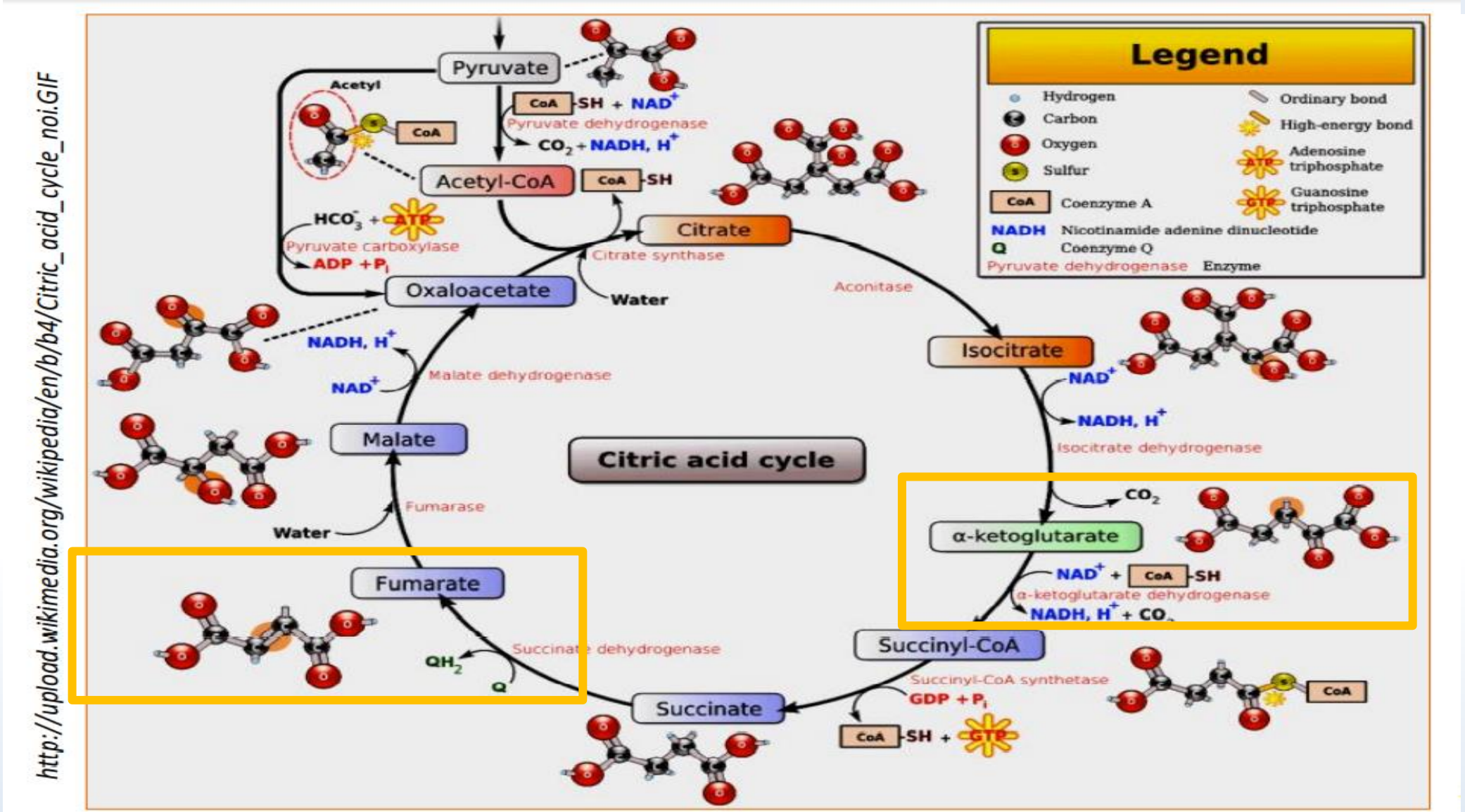
Key metabolic indicators of CHO cell function. This figure summarises the metabolites that have been defined from a variety of studies as potential indicators of CHO cell function (in terms of growth and recombinant protein production). Glucose, glutamine and asparagine are shown as the major carbon sources utilised during cell culture and other metabolites shown in black text present metabolites that accumulate during cell culture (with different timing or phases of appearance associated with the status of cell processes). Metabolites in red are produced in limited amounts at late stages (stationary and onwards) but provide markers of CHO cell status and phenotype (in both intracellular and or extracellular localisation). The intracellular metabolites shown in red present a consensus range of metabolites associated with the quality of CHO status during bioprocesses (as described within this review).

Current Opinion in Biotechnology 2014, **30**:73–79 www.sciencedirect.com

34

From: Dickson , A.J. Enhancement of production of protein biopharmaceuticals by mammalian cell cultures: the metabolomics perspective. Current Opinion in Biotechnology 2014, 30:73–79 http://dx.doi.org/10.1016/j.copbio.2014.06.004

Cell Metabolism



DMEM-F12 Other Components

- **Other Components (mg/L)**

Glucose	2151	Energy
Na.Pyruvate	55	
Na.Hypoxanthine	2.4	Precursors for DNA synthesis
Thymidine	0.37	
Linoleic Acid	0.04	Essential fatty acids
Lipoic Acid	0.1	
Putrescine.2HCl	0.08	
(Phenol Red)	(8.6) – used in laboratory media as a pH indicator	

DMEM-F12 Other Components

- **Glucose is the main energy source for the cell.**
  - However, CHO cells are not good at completing glucose metabolism to generate the 38 ATP molecules of full oxidative metabolism.
  - They often stop at glycolysis, yielding lactate.
  - Hence amino acids are supplemented to provide other energy sources.
- Sodium pyruvate readily enters the TCA cycle.
- Arginine and Glutamine feed directly into the Tri-Carboxylic Acid cycle (TCA, or Krebs cycle) and are an alternative source of energy

Other Supplements & Notes (1)

- Base medium is often supplemented with **insulin (10mg/L)** to increase glucose uptake and promote cell growth (putrescine does this also).
- Plant or yeast hydrolysates (extracts that are digested by proteases or acid hydrolysis) are a good source of small peptides, which are often taken up by cells more easily than free amino acids. These are added at 5-10 g/L.
- **Trace elements** such as vanadium, cobalt & iodide are often supplemented in very small amounts (<0.0001 mg/L) to DMEM-F12 as they are co-factors to some enzymes. However, they are toxic to cells at higher doses

Other Supplements & Notes (2)

- **Today, most media used in manufacturing do not contain animal-derived components such as serum, albumin or transferrin, for safety reasons.**
  - Chemically defined and protein free media – more later!
  - In the lab, media often come as sterile liquids
- At manufacturing scale, media is shipped as a powder or granular (AGT) format, which is re-solubilised on site with sterile, pure water.
- At manufacturing scale no antibiotics (such as penicillin or streptomycin) are used in the medium.
  - For cost and allergy reasons, but does put the onus on all operators to ensure really good sterile techniques for cell culture.

Lecture Topics

- Cell Banking
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- Defined Portion of Cell Culture Media
- Complex Portion of Cell Culture Media

Undefined Portion of Media

- Serum regarded as the black box of tissue culture media!
  - Usually makes up 5-20% of media – depending on cell line
- Serum contains many things including the following:
  1. **Hormones** – e.g. Growth hormone which increases growth, Insulin which promotes uptake of glucose
  2. **Growth factors** – e.g. Epidermal growth factor (EGF), nerve growth factor (NGF)
  3. **Proteins** can act as carriers for lipids and hormones e.g. α2 macroglobulin inhibits trypsin
  4. **Lipids** – e.g. oleic acid, stearic and linoleic acid
  5. **Attachment factors** e.g. collagen, fibronectin and laminin
  6. **Trace elements** e.g. like Cu, Zn may act as enzyme co-factors and Se can inactivate free radicals

Advantages and Disadvantages of Serum

Advantages	Disadvantages
Allows the culture of many different cell types in non-defined media **(Some cells will only grow in media with serum)**	Quality of serum differs from batch to batch **(Majority is foetal calf serum, also calf & horse serum)**
Cells generally grow well and get high production	Supply may be restricted due to disease or drought
Serum offers protection against shear in bioreactor	Serum may act as a toxin to cells due to presence of viruses and mycoplasma
	Changing batches requires extensive testing
	Presents difficulties in downstream processing

Different Types of Media Available

1. **Serum-free Media (SFM):**
   - These media do not require supplementation with serum but may contain discrete proteins or bulk protein fractions. In most cases the protein has been kept to a minimum and the media has been optimised for a specific cell type.
2. **Protein-Free Media (PFM):**
   - These are a step closer to a chemically-defined formulation as compared to serum-free. However, they may still contain undefined components of animal or plant origin such as various hydrolysates that contribute low molecular weight peptides.
3. **Chemically-Defined (CD) Media:**

Contains no proteins, hydrolysates or components of unknown composition. All components have a known and well-defined chemical structure. This is the 'gold standard' of media available.

Different Types of Media Available

- **Generally cells can be adapted to grow in serum-free media**
  - Gradually over period of time, reduce amount of serum: can take several weeks or months
  - Can be more drastic and eliminate serum from media and select cells that grow in serum-free
- **Due to lack of serum require Pluronic®F68**
  - Pluronic is a non-ionic surfactant that is used to protect animal cells from damage caused by shear and the effects of sparging
  - Pluronic acts by creating an artificial layer around cells or is absorbed into membrane thus strengthening it
- Cells growing in serum-free media may not reach same cell densities as in serum containing media.

Advantages and Disadvantages of Serum-Free

Advantages	Disadvantages
Easier downstream processing : don't have to worry about getting rid of proteins in serum (e.g. albumins)	More expensive! : even though serum is costly, individual components of serum-free also costly
	Some cells more sensitive to shear in serum-free (use Pluronic F68)
Less problems with FDA as no risk of animal-derived products (viruses, prions)	Serum may act as a toxin to cells due to presence of viruses and mycoplasma
	Cells may not reach as high density as with serum
	Cells may be harder to freeze down

Protein-free Media

- **An increasing emphasis from regulatory authorities for the removal of animal-derived raw materials from antibody producing processes.**
- Number of groups have developed protein-free media:
  - Most of these are formulated for specific cell type : different for each cell type
- A number of successful formulations:
  - Protein-free medium for NS0 (hybridoma cell line) contains zinc instead of insulin, amino acids, ethanolamine, tropolone, nucleosides, glutamine
  - Protein-free medium for BHK : e.g. SMIF6
  - Many companies will use one media for growth of cells and then switch to another media for production

Protein-free Media

- Serum is a complex source of proteins, albumins, lipids, vitamins, growth factors, low molecular weight components, mineral ions etc.
- Serum proteins protect cells from shear, from protease attack & also facilitates transfer of vitamins through cells:
  - This reduces the risk of introducing adventitious agents and other contaminants. The main concerns relate to BSE (bovine spongiform encephalopathy) and CJD (Creutzfeldt -Jakob disease).
  - Makes process optimisation easier if poorly-defined additives such as serum and hydrolysates are avoided.
  - Improves process consistency
  - It reduces the protein load for downstream purification

Summary

- Cell culture media must contain all elements necessary to allow growth of cells including energy source, macro and micro nutrients
- Growth media usually contain 2 parts: defined part and non-defined portion:
  - Significant move towards fully defined media
- Defined part – key components:
  - Energy – glucose, pyruvate, Glu and Arg
  - Buffers – $NaHCO_3$, CO_2
  - Amino acids – for building proteins, -source
  - Vitamins & essential fats
  - Inorganic ions – metals like Ca, Mg, Fe, Zn etc.
- Undefined portion - serum

Sample Questions

- Explain the structure of a cell bank system and the relationship between master and working banks.
- What steps are taken in the characterisation of cells for a cell bank?
- Discuss the following points: Characterisation tests include:
  - Viability (>90% viable) / Contamination free of microbes and a pure culture / Morphology / Isoenzyme profiles / Karyotype / DNA fingerprinting / Productivity - Titre
  - Master bank - failsafe - only access as a last resort. All cells stored derived from a single cell clone.
  - Use 1 master cell bank vial to expand and create multiple copies to act as working bank.

Sample Questions

- What are the major energy sources for mammalian cell contained in culture media? What concentration is each normally provided at? What impact would addition of additional sugar have on the medium – explain why?
- Discuss the following points:
  - Carbon sources: Glucose / Na-Pyruvate / Glutamine
    - Levels: Glucose – 4.5 to 6.0 g/L ; Na-Pyruvate –
    - 0.11g/L; L-Gln – 0.584g/L; L-Arg – 0.084g/L
  - Excess glucose – upsets the osmolality of the medium
    - Low Glucose media = 324-333 mOsm
    - High Glucose media = 400 mOsm
  - If hypertonic, then cells risk dehydration and cell death.

Questions?