Animal Tissue Culture

SQG 3242

Basics of Cell Culture Media

&

its Supplements

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Introduction

- Cell Culture: "The process by which either prokaryotic or eukaryotic cells are grown under controlled conditions"
- Mainly implies to the process of culture of animal cells in vitro.
- Important tool in Cell & Molecular biology.
- Cell Based Manufacturing of various products: Vaccines, therapeutic proteins, hormones etc.
- Medium: One of the main parameters to be considered while growing cells *in vitro*
- Major factor affecting process after the Cell line.
- Various defined basal mediums have been developed in last 30 years.
- Initially Balanced salt solutions were widely used.
- Gradually replaced with various vitamins, amino acids, lipids and trace elements.
- Precise media formulations have often been derived by optimizing the concentrations of each ingredient.

Medium

- The choice of medium is empirical
- Defined Medium: Structure and concentration of every component is known
- Undefined Medium: Contain one or more unidentified components.
- Main responses: Measure the growth , cloning efficiency and expression of specific properties i.e production of desired products
- Presterilized - stable solutions may be autoclaved at 121°C for 20 min.
- Labile solutions must be filtered through 0.2 um porosity membrane filter.
- Complete Medium – contain all necessary defined constituents & supplements

Medium 199	Morgan et.al., 1950
MEM	Eagle 1959
CMRL 1066	Parker et.al., 1957
DMEM	Dulbecco 1959
Ham's F-12	Ham 1965
RPMI 1640	Moore et.al., 1967

Component	Eagle's MEM	Dulbecco's modification	Ham's F12	CMRL 1066	RPMI 1640
Amino acids					
L-alanine	-	-	8.90	25.0	-
L-arginine (free base)	-	-	-	-	200
L-arginine-HCl	126	84.0	211	70.0	-
L-asparagine	-	-	-	-	50.0
L-asparagine-H_2O	-	-15.0	-	-	-
L-aspartic acid	-	-13.3	30.0	20.0	30.0
L-cysteine (free base)	-	-	-	-	-
L-cysteine	24	48.0	-	20.0	50.0
L-cysteine. 2Na	-	-	-	-	-
L-cysteine. HCL.H_2O	-	-	35.1	260	-
L-glutamic acid	-	-14.7	75.0	20.0	66.8
L-glutamine 292	584	146	100	300	100
Glycine	-	30.0	7.50	50.0	10.0
L-histidine (free base)	-	-	-	-	15.0
L-histidine HCl.H_2O	42.0	42.0	21.0	20.0	-
L-hydroxyproline	-	-	-	10.0	20.0
L-isoleucine	52.0	105	3.94	20.0	50.0
L-leucine	52.0	105	13.1	60.0	50.0
L-lysine. HCl	73.1	146	36.5	70.0	40.0
L-methionine	15.0	30.0	4.48	15.0	15.0
L-phenylalanine	33.0	66.0	4.96	25.0	15.0
L-proline	-	-	34.5	40.0	20.0
L-serine	-	42.0	10.5	25.0	30.0
L-threonine	48.0	95.0	11.9	30.0	20.0
L-tryptophan	10.0	16.0	2.04	10.0	5.0
L-tyrosine	36.0	72.0	5.40	40.0	20.0
L-tyrosine. 2Na	-	-	-	-	-
L-valine	47.0	94.0	11.7	25.0	20.0
Vitamins					
L-ascorbic acid	-	-	-	50.0	-
Biotin	-	-	0.0073	0.010	0.200
D-Ca-pantothenate	1.00	4.00	0.480	0.010	0.250
Calciferol	-	-	-	-	-
Choline chloride	1.00	4.00	14.0	0.500	3.00
Folic acid	1.00	4.00	1.30	0.010	1.00
i-inositol	2.00	7.20	18.0	0.050	35.0
Nicotinamide	1.00	4.00	0.04	0.025	1.00
Pyridoxal. HCl	1.00	4.00	0.062	0.025	-
Riboflavin	0.10	0.40	0.038	0.010	0.20
Thiamin. HCl	1.00	4.00	0.34	0.010	1.00
Vitamin B_{12}	-	-	1.36	-	0.005
Pyridoxine HCl	-	-	0.062	0.025	1.00
Cholesterol	-	-	-	0.200	-
Para-amino benzoic acid	-	-	-	0.050	1.00
Nicotinic acid	-	-	-	-	-
Menaphthone sodium bisulphite $3H_2O$	-	-	-	-	-
Dl-Â tocopherol PO_4.2Na	-	-	-	-	-
Vitamin A acetate					
Riboflavin					
PO_4.2Na Thiamin mono PO_4,$2H_2O$					
Inorganic salts					
$CaCl_2$ (anhyd.)					
$CaCl_2.2H_2O$					
$Fe(NO_3)_3.9H_2O$					
KCl					
KH_2PO_4					
$MgCl_2.6H_2O$					
$MgSO_4.7H_2O$					
NaCl					
$NaHCO_3$					
$Na_2H_2PO_4.H_2O$					
Na_2HPO_4 (anhyd)					
$Na_2HPO_4.7H_2O$					
$CuSO_4.5H_2O$					
$FeSO_4.7H_2O$					
$ZnSO_4.7H_2O$					
$CaNO_3.4H_2O$					
Other components					
D-glucose					
D-galactose					
Lipoic acid					
Phenol red					
Sodium pyruvate					
Hypoxanthine					
Linoleic acid					

Culture media

- Provides all the essential nutrients
  - Amino acids, energy substrates, vitamins, minerals, salts, etc
- Maintain constant pH
  - Hepes vs CO_2/HCO_3 buffering
  - Phenol red indicator (yellow-orange-maroon) (acid>>>>>>>>>alkaline)

Basic Components of Culture Medium

Nutrient Substances	
Water	Energy Sources
Nitrogen Sources	Vitamins
Bulk Ions	Trace Elements
Lipids	Metabolites
Non Nutrient Substances	
Antibiotics	Buffers
Protective Agents	Anti Oxidants
Metabolites	High M.W Substances

Physicochemical Properties –Affecting Medium

1. **pH -** for normal cells – 7.4, for transformed cells 7.0 – 7.4
   - phenol red
2. **Buffering -**
   - Commonly used systems: CO_2 ,
   - Bicarbonate/HEPES,
3. **Oxygen - dissolved oxygen**
   - Correct O_2 tension so as to meet the requirement and avoid toxicity
   - Requirements depends upon type of culture
   - Selenium – guard against O_2 toxicity
4. **Osmolality - 280-310 mOsm/kg**
   - Measured using depression of the freezing point or elevation of the vapor pressure.
   - Helps guard against errors of weighing, dilution.
   - Addition of strong acids, bases like HEPES significantly affect.
5. **Temperature**
   - Keep at 4°C-Not use
   - Warm at 37C –before use to culture the cells

Animal Sera

- Provides various hormones/ growth factors to stimulate cell proliferation and function
- Less well defined than Serum-free culture systems
- Often difficult to culture cells without serum, but may interfere with studies of specific factors (e.g. insulin, glucose)

Serum Containing Medium

- Traditional undefined medium
- Major source of various nutrients such as growth factors, adhesion factors, minerals, lipids, trace elements etc.
- Commonly used sera: Calf, Fetal bovine, adult horse & human sera
- Horse sera: less metabolism of polyamines, more consistent batch to batch
- Human sera: only used for few cell lines, screening for HIV, hepatitis B virus.
- Promotes cell proliferation, adhesion factors, antitrypsin activity and cell attachment, source of various nutrients

Contents of Serum

- **Protein Contents:** Albumin – carrier of lipids & minerals.
- Fetuin & Fibronectin: promote attachment
- α2 macroglobulin: inhibits trypsin
- Transferrin: makes Iron less toxic and bioavailable.
- **Growth Factors:** main role is in growth stimulation & are mitogenic E.g. PDGF, FGF, EGF, VEGF, Angiogenin etc.
- PDGF – major growth factor
- **Hormones:** Insulin: promotes uptake of glucose & amino acids, mitogenic when bound to IGF – I receptor
- IGF - 1/2: mitogenic and stimulate growth
- Hydrocortisone: promote cell attachment
- **Minerals:** Iron, Copper, Zinc, Selenium – essential trace elements required for cell growth. Selenium: important role in detoxifying free radicals by promoting glutathione synthesis.
- **Inhibitors:** TGF – β, hydrocortisone – cytostatic.
- Apart from these, Carbohydrates (1.0 2.0 mg/mL), various vitamins (10ng - 10µg/mL), amino acids are also present.

Advantages of Serum

- Provides various components
- Modulates physiological properties of medium
- Protease inhibitors
- Provides nutrients not present in basal medium
- Carrier proteins for low molecular weight substances (e.g. transferrin)
- Help in solubilization of poorly dissolved substances (e.g. apolipoprotein)
- Cell substrate attachment (fibronectin, vironectin)
- Various enzymes
- Proteins which prevent non specific adsorption (e.g. albumin)
- Neutralization of detergents
- Prevents essential nutrients e.g. fatty acids

Disadvantages of Serum in culture medium

i. Potential introduction of animal viruses

ii. Antibodies against viruses, to which host cell is exposed.

iii. Availability of high quality

iv. Undesirable contaminants

v. High running costs & capital requirements

vi. Shelf Life & Storage – always purchased in bulk

vii. Physiological variability & consistency

viii. Downstream Processing

ix. Characterization of final product laborious.

Media changes

- The purpose of media changes is to replenish nutrients and avoid the build up of potentially harmful metabolic byproducts and dead cells.
- In the case of suspension cultures, cells can be separated from the media by centrifugation and resuspended in fresh media.
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- In the case of adherent cultures, the media can be removed directly by aspiration and replaced.

- Some cells naturally live without attaching to a surface, such as cells that exist in the bloodstream.
- Others require a surface, such as most cells derived from solid tissues.
- Cells grown unattached to a surface are referred to as suspension cultures.
- Other adherent cultures cells can be grown on tissue culture plastic, which may be coated with extracellular matrix components to increase its adhesion properties and provide other signals needed for growth

Phosphate Buffered Saline - Ca^{2+} Mg^{2+} Free (PBS)

- Used to wash/remove excess serum that inhibits the function of Trypsin-EDTA.
- Must be warmed in the water bath before use so cells are not shocked by cold liquid.

Trypsin EDTA

- An enzyme used to detach the cells from a culture dish.
- Trypsin cleaves peptide bonds (LYS or ARG) in fibronectin of the extracellular matrix.
- EDTA chelates calcium ions in the media that would normally inhibit trypsin.
- Trypsin will self digest and become ineffective if left in water bath more than 20 minutes.
- Trypsinizing cells too long will reduce cell viability

Water

Quality

Types of Contaminants:

1. Inorganics: heavy metals, iron, calcium, chlorine
2. Organics: by products of plant decay, detergents
3. Bacteria: Endotoxin or pyrogens
4. Particles: Colloids or particles

Purification methods:

a. Distillation
b. Deionization
c. Reverse osmosis
d. Ultra Filtration

Energy Sources

- Most common: *glucose* Others: *Fructose, galactose, mannose, maltose* etc
- D-Glucose: main source of energy
- Metabolized by glycolysis and energy is released in form of NADH and ATP.
- Also metabolized via PPP pathway where NADPH is released.
- Stored in cells as glucose-6-phosphate/glycogen
- Cannot penetrate cell membrane, require help from transporter proteins
- Either by osmosis or by energy utilization in form of ATP.
- Mainly transferred via Glut I transporter
- 1 – 10g/L
- Higher concentration creates negative effect

High level glucose = high level of lactate = low pH = detrimental to cells = requirement of base = increase in osmolality

- Lead to glucose related oxidative and carbonyl stress.

L – Glutamine （1-20mM）

- Major Carbon Source in case of low glucose levels & Nitrogen source
- Support cell growth and amino acid uptake
- Metabolic hydrolysis to glutamic acid
- Spontaneously decomposes, overproduction of ammonia
- In vivo ammonia produced is converted to urea
- Invitro ammonia produced due to glutamine degradation accumulates
- At physiological pH, inorganic ammonia converted to organic nitrogen either in the form of amine of glutamate or amide of glutamine which act as primary reservoir for nitrogen.
- Acts as precursor of glutamate – role in transamination
- Most commercial medias free from glutamine – included either in basal formulation or in liquid formulation at time of use.

Exercise

- At what temperature serum and tyrpsin can be stored ?
- Why?

Bulk Ions

- Basal Salt Mixtures mainly containing$CaCl_2$,*$MgCl_2$, KCl_2, Potassium Phosphate Monobasic (Anhydrous),$NaCl_2$, Sodium Phosphate (Dibasic)*
- Provide bulk ions for cell metabolism e.g Na^+, K^+
- Osmotic balance maintenance & provide buffering system
- Cofactors in various enzymatic reactions
- Cell adhesion (Ca^{2+}, Mg^{2+})
- Binding of Iron to transferrin (HCO_3^-)
- Contribute to Osmolality of medium (280-310 mOsm/kg)

Trace Elements

Iron, Manganese, Zinc, Molybdenum, Selenium, Vanadium, Copper

Lipids & Phospholipids Precursors

- Cholesterol, Fatty acids
- Phospholipids – phosphotidyl choline, phosphotidyl ethnaolamine, sphingomyelin
- Choline, Inositol, Ethanolamine.

Non Nutrient Substances

Antibiotics: more inhibitory to bacteria then to cell culture cells

- Commonly used antibiotics: Gentamycin ,Fungizone Penicillin streptomycin
- Cautious selection

Buffers: Sodium Bicarbonate 44mM in DMEM, 12mM in F12, 26mM in circulatory blood.

- 5-10% CO_2 required as media containing bicarbonate becomes alkaline very rapidly due to loss of CO_2
- Low pKa
- Alternate buffers: Sodium beta glycerophosphate, HEPES – osmolality
- Phenol Red: pH indicator, might be estrogenic

Protective Agents: Pluronic Surfactants – Polyoxyethylene group

- Toxicity effect.
- 0.01 – 0.1% working concentration.
- Commonly used pluronics: F68, F88, F77

Antioxidants

- Superoxide radicals & Hydrogen Peroxide – harmful
- Generated during normal respiratory metabolism by xanthine oxidase/photo oxidation of riboflavin,tryptophan.
- Commonly used Vitamin E, Taurine, Bilirubin, transferrin, various amino acids, selenium, catalase, glutathione.

Metabolites & Conditioning Factors

- Produced by cells usually of 2 types:
  - (i) Growth Inhibitory: Lactate, ammonia, CO2, TGF beta etc
  - (ii) Growth Stimulatory: TGF alpha, IGF – 1, IL – 2, PDGF

2. Insulin Like Growth Factor – one order less as compared to insulin

- Can replace insulin, bind to IGF-IR
- IGF-1 affects protein & carbohydrate metabolism
- Also stimulates glycolysis and protein synthesis
- Increase DNA synthesis, affect cell proliferation & differentiation

3. Transferrin – 1-30µg/ml

- 80KDa witn N/C terminal iron binding domains
- Binds with high dissociation constant
- Can be replaced with hemoglobin, ferrous sulfate etc

Nitrogen Sources （Amino Acids）

- Mainly divided as essential & nonessential
- Wide range of optimal concentration and play important role in primary protein structure
- Semi log plot of growth response vs. nutrient scale
- Initially only essential amino acids but now both are supplied.
- Amino acid hydrolysates an important substitute for serum, can be autoclaved but are undefined. E.g bactopeptone, tryptose etc.
- Some important amino acids are:
- Proline: required by mutant CHO cells
- Serine: required for high clonal densities
- Aspargine: required mainly by tumor producing cells
- L-cysteine: active role in protein synthesis; important in glutathione synthesis thereby prevent cells from oxidative stress
- Glycine: mainly used with methotrexate and aminopterin which leads to folic acid deficiency.

Vitamins

- Eagle's MEM contains only water soluble vitamins, rest derived from serum.
- Affect cell survival & growth rates.
- Usually added empirically till the actual effect determined

Ascorbic acid	Synthesis of collagen
Vitamin A	Growth & differentiation of some cells
Vitamin K	Many Vitamin K dependent proteins
Vitamin E	Antioxidant
Vitamin D	Regulation of Ca^{2+}, important to maintain at appropriate levels.
Thiamine pyrophosphate	Catalyst in the transfer of carboxyl group, transaldolases, transketolases
Pyridoxal phosphate	Catalyses transamination
Biotin	Carrier of CO_2, required for functioning of pyruvate dehydrogenase, pyruvate carboxyase & fatty acid synthesis
Vitamin B12	Important for methylation, degradation of amino acids & fattyacids

Summary

- One of the major factors considered in cell culture is the choice of Medium
- Type of Medium & its constituents affect culturing & passage of cells.
- Till date, most of the selection is done Empirically.
- Depending upon the previous data obtained & medium used for different cell lines.
- Selection of energy & nitrogen source plays a critical role along with its concentration.
- Adaptation of a cell line from serum containing to serum free medium is carried out using serial subcultures by gradually reducing the serum concentration.
- Concentration & effect of the amino acids used should be priorly optimized.

Conclusion:

Although relative simplicity of retaining serum in medium is lucrative, requirements of governing bodies & various disadvantages of serum have lead to the development of serum free compositions. The main intention being to simplify the process of cell culturing, protein purification and to eliminate all potential sources of infection from the medium.

Phenol red indicator

- Alkaline?
  - Colour and pH
- Acidic?
  - Colour and pH

Contamination

- Normally:
  - Bacteria
  - Yeast
  - Virus
  - Pleuropneumonia group
  - Protozoa *

Detection

- Inverted microscopic
  - Bacteria
    - Can be detected immediately
    - Takes about 24-48 hours
    - Easily found using the microscope/ naked eyes??
  - Yeast
    - Slow growing
    - Takes few days to be detected using the microscope
    - Worst- naked eyes
  - Virus
    - Difficult to traces using the microscope. Size smaller than bacteria
    - Can takes ages to be detected.
    - How we know there are virus in the cell media?

- Cells stressed
- Media colour change quickly-cells still not confluent
- Debris occurs

– Pleuropneumonia group
  - Known as PPLo/mycoplasma
  - Serious problem of chronic contamination
  - Present in many animal tissue

– Protozoa
  - Contaminating the fresh isolated tissues
  - Easily recognised on microscopic examination

Sterility Test

- To find out which chemicals being infected
  - Supplemented media –incubator
  - Supplemented media+trypsin-incubator
  - Supplemented media +PBS-incubator
    - Check everyday for a week
- Or
  - Spread about 1ml of supplemented media/tyrpin/PBS on agar plate
  - Leave in incubator –check for microorganism growth

Elimination of contamination

- Do not rescue the contaminated cell lines
- Discard any contaminated cells (flasks, plates)-autoclave ASAP
- Check the sterility of the chemicals
- Incubator cleaning, biohazard/laminar flow cleaning, change/wash lab coat, change water bath water /+ waterbath treatment water

Video

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