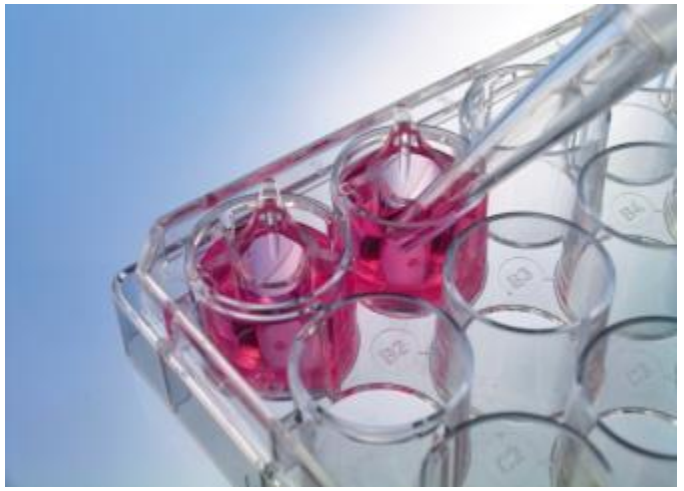
Kuliah 13

Kultur sel dan hewan coba

What is Cell Culture?

- **Cell Culture** : suatu proses kompleks dimana sel (berasal dari organ, kultur primer atau dari sel line yang didapat dengan cara pemisahan secara enzimatik, mekanik atau kimia) ditumbuhkan dalam kondisi yang terkendali.



Purpose of cell culture

- To replace the usage of animal model

  environment can be controlled , understand the exact reactions of cultured cells, tissues, or organs without hurting living beings.
- Toxicity testing
- Cancer research
- Virology
- Cell based manufacturing
- Genetic counseling
- Genetic engineering
- Gene therapy
- Drug screening

Advantages using cell culture

Kontrol Lingkungan lebih mudah dilakukan

Homogenitas Sampel

Ekonomis dalam skala dan mekanisme

Mengetahui kondisi In vivo
Dalam in vitro

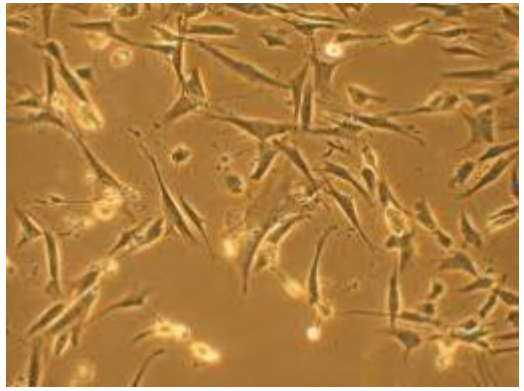
Mengetahui secara in vitro reaksi yang Terjadi sebelum melanjutkan penelitian ke in vivo
Menggurangi atau menggantikan hewan coba

Type of Cell Culture based on Morphology

Adherent Cells

Non-adherent Cells

Epithelial like-cells
flattened and polygonal in shape



Fibroblast like-cells
appears elongated and bipolar

Lymphoblast like-cells
remain in suspension with a spherical shape

Primary culture

Cells when surgically or enzymatically removed from an organism and placed in suitable culture environment will attach and grow are called as primary culture

Primary culture contains a very heterogeneous population of cells

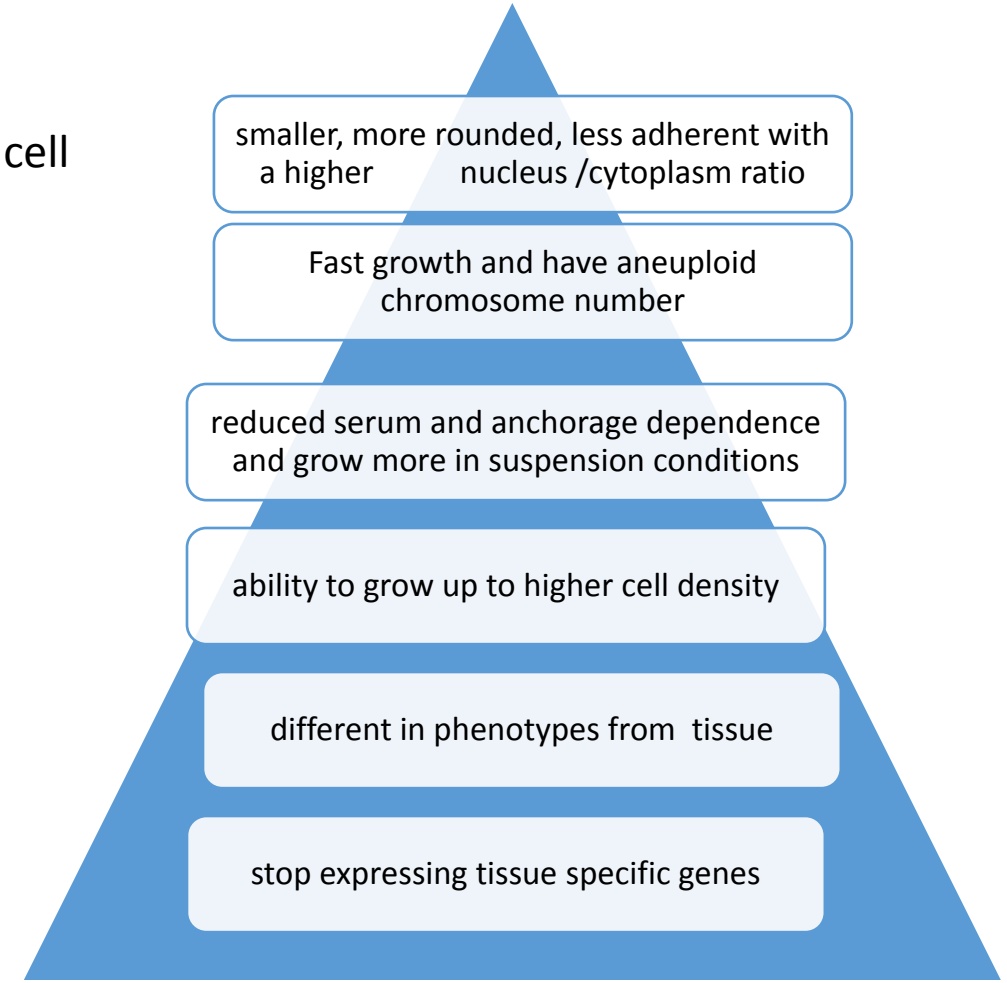
Cell lines have limited life span, they passage several times before they become senescent

Sub culturing of primary cells leads to the generation of cell lines

Continous cell lines

Cell lines which either occur spontaneously or induced virally or chemically transformed into Continuous cell lines

Characteristics of continous cell lines



Common cell lines

Human cell lines

- MCF-7 breast cancer
- HL 60 Leukemia
- HEK-293 Human embryonic kidney
- HeLa Henrietta lacks

Primate cell lines

- Vero African green monkey kidney epithelial cells
- Cos-7 African green monkey kidney cells

And others such as CHO from hamster, sf9 & sf21 from insect cells

Setting Up Lab

PASTIKAN PANEL CONTROL PRESSURE DINYALAKAN

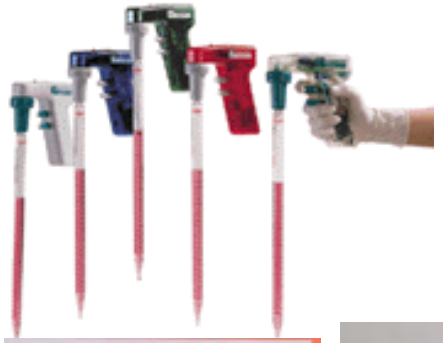
CHECK BALANCE, ROTOR, CLEAN UP

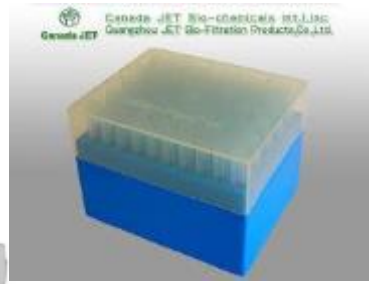
Check:
CO2 level
temperature
Water
Name our Plate

Check Lamp, turn off after use, clean up, and cover

Setting Up Lab

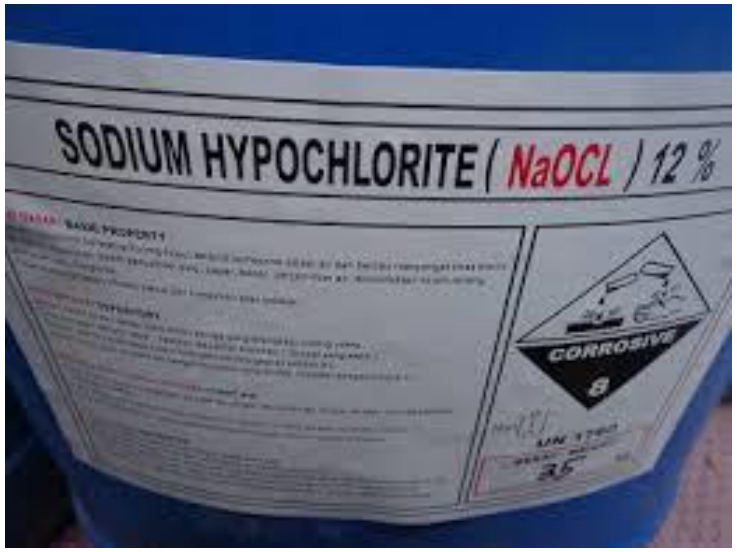
Persiapkan alat dan bahan yang akan dipakai dalam bekerja





Consumables dapat diperoleh dari lemari stok
Siapkan semuanya sebelum bekerja

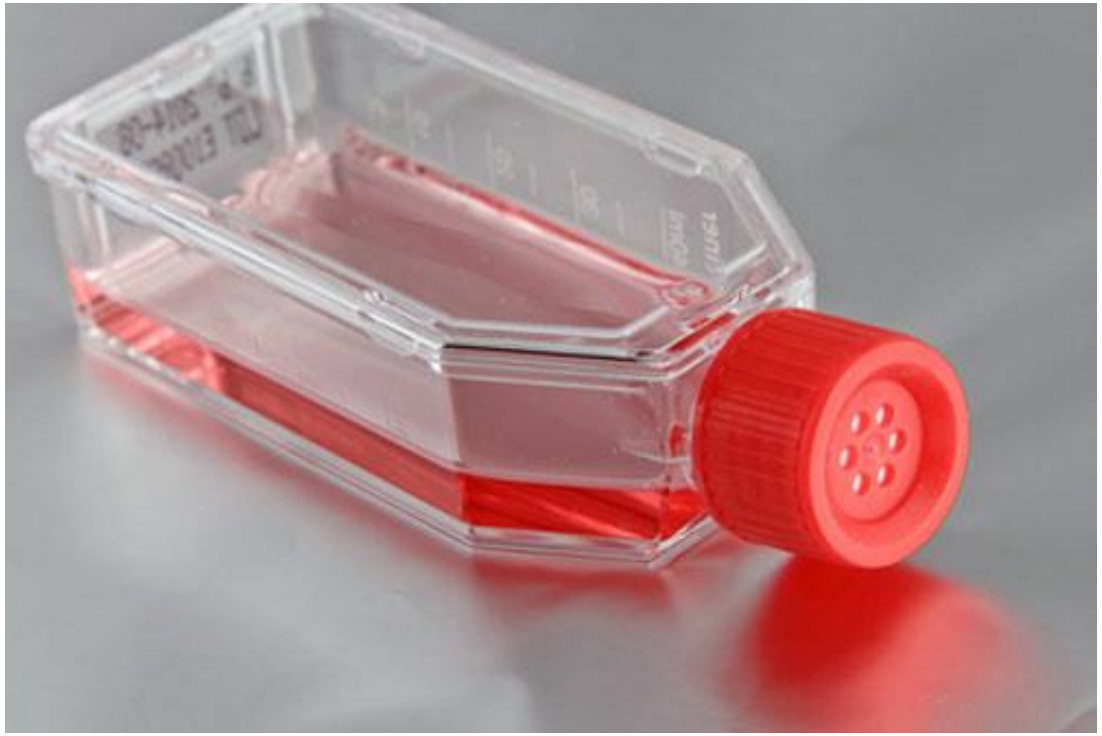

CVS Health
Alcohol
91
10 FL OZ (296 mL)


SODIUM HYPOCHLORITE (NaOCL) 12 %
CORROSIVE
8


BAYCLIN

Setting up lab

Setting up lab

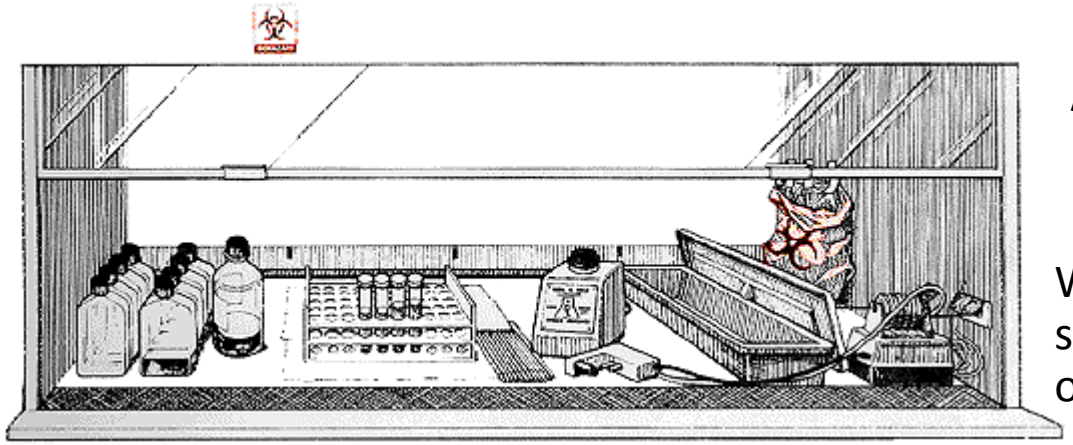


Setting up lab

Working at BSC

BERSIHKAN BENCH,
Lapisi bench dengan tisu

SIAPKAN SEMUA CONSUMABLE



ARRANGE YOUR WORK SPACE

Work either left to right or vice versa, so that all material goes to one side, once finished

Basic aseptic conditions

- Swab all bottle tops & necks with 70% ethanol
- Avoiding placing caps & pipettes down on the bench; practice holding bottle tops with the little finger (depend on personal)
- Clean up spills immediately & always leave the work place neat & tidy
- Possibly keep cultures free of antibiotics in order to be able to recognize the contamination (if necessary)
- Cell cultures which are frequently used should be subcultered & stored as duplicate strains

TEHNIK MEMELIHARA SEL

1. Persiapan media

Media atau reagen yang akan digunakan harus dalam disteril

Autoclave

Filtrasi

AIR

Air yang digunakan adalah air yang kemurniannya paling tinggi

4 cara pemurnian air : Reverse osmosis, distilasi, Deionisasi dan karbon filtrasi

IHVCB menggunakan aqua bidistilata

PBS

Buat lah stok sendiri, 5 x, 10 x

Beri label pada botol, kemudian diautoclave, gunakan strip autoclave

MEDIA

Beberapa jenis media dapat diautoclve, tapi umumnya *heat labile*

Media komersial : 1. Dalam working consentration (1X) w/o glutamin

2. Konsentrasi (10 X) without $NAHCO_3$ dan glutamin
3. Serbuk, w/o $NAHCO_3$ dan glutamin

Culture media

- Choice of media depends on the type of cell being cultured
- Commonly used Medium are RPMI,DMEM, DMEM F-12, etc.
- Media is supplemented with antibiotics ex: penicillin, streptomycin, gentamycin, fungizone.etc.
- Suplemented with $NaHCO_3$ (buffer), HEPES(osmolaritas)
- Suplemented with serum ex: FBS
- Prepared media is filtered 0,2 um and incubated at 4C

MEDIA

Tidak semua sel kultur mempunyai jenis media yang sama

Perhatikan jenis media yang akan dipakai, beri label sehingga tidak digunakan org lain

Beberapa tipe sel dapat dikembangkan di Serum Free Media, namun memerlukan
Tipe media yang lebih komplek dari media pada umumnya

MEDIA	MCD8 110	MCD8 131	MCD8 170	MCD8 202	MCD8 302	MCD8 402	MCDB
Tipe Cell	Human Lung Fibroblas	Human vaskular endotel	Mamary epitel	Chick embryo fibroblas	CHO	3T3	Keratino cytes

Serum yang digunakan adalah FBS (Fetal Bovine Serum)

Harus dilakukan inaktifasi serum terlebih dahulu :

56 C 1 jam

65 C 15 menit

Setelah di inaktivasi, serum disterilkan melalui 2x filtrasi menggunakan filter 0,45 um dan 0,2 um dan di aliquot

Serum yang akan digunakan disimpan dalam 4 C

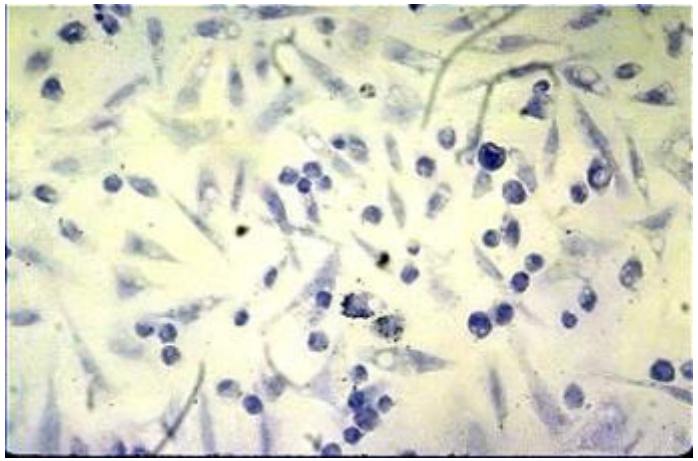
Sisa serum disimpan dalam freezer -40 C

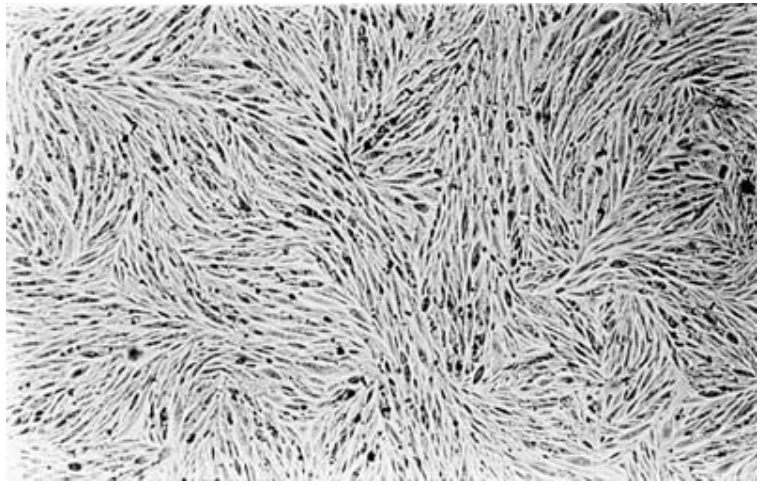
GUNAKAN SEBAIK BAIKNYA
TDK BOLEH TERKONTAMINASI !!!
TIDAK BOLEH MENGGUNAKAN FBS ORANG LAIN TANPA SEIJIN PEMILIKNYA!!!

FASE PERTUMBUHAN SEL

Confluency

- Seluruh permukaan telah ditutupi sel
- Konfluensi biasanya 70-80%
  - too low, cells will be in lag phase and won't proliferate
  - Too high and cells may undergo unfavorable changes and will be difficult to remove from plate.

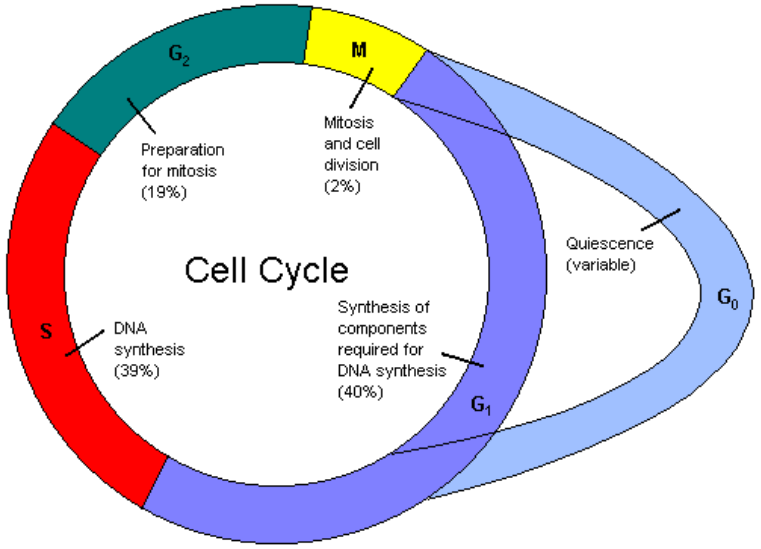




FASE PERTUMBUHAN SEL

Contact Inhibition

- Ketika sel saling bertemu, akan menurunkan pertumbuhan



Nomor Passage

- Urutan nomor atau jumlah sel yang mengalami pemindahan (split)
- Selalu tuliskan jumlah pasase yang telah dilakukan : P#

Why sub culturing.?

- Once the available substrate surface is covered by cells (a confluent culture) growth slows
- It’s the passage of cells when they reach to 80-90% confluency in flask/dishes/plates
- Enzyme such as trypsin, dipase, collagenase in combination with EDTA breaks the cellular glue that attached the cells to the surface

PROCEDUR SUB KULTUR (Adherent Cells)

Dilakukan bila konfluensi sel telah mencapai 80%

Aspirasi media sebelumnya menggunakan pipet, buang ke tempat yang sudah disediakan

Cuci media dengan PBS 1 x, kemudian bilas seluruh permukaan flask dengan PBS

Lepaskan sel dari flask dengan menggunakan tripsin 1x

Inkubasi di dalam inkubator 37C 5 menit

Bila sel sudah lepas, campurkan suspensi dengan media bebas serum sebanyak 5 ml (1:1 vol tripsin)

Sentrifuge suspensi 1000 rpm selama 10 menit

Sel kemudian dapat dihitung menggunakan hemositometer

Suspension cells

Easier to passage as no need to detach them

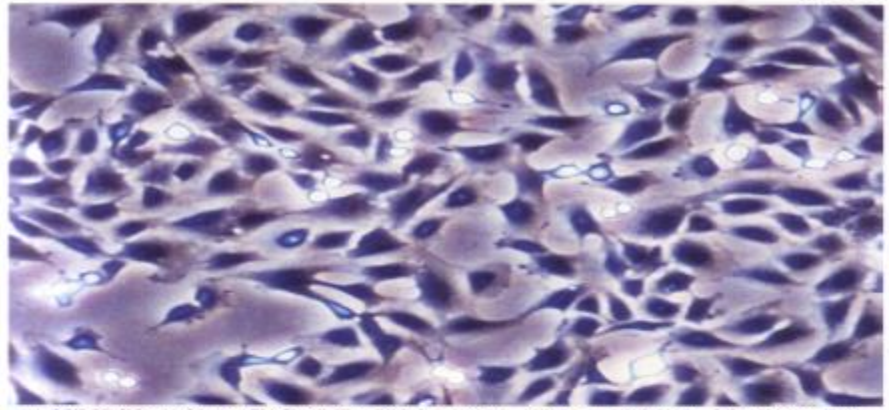
As the suspension cells reach to confluency

↓

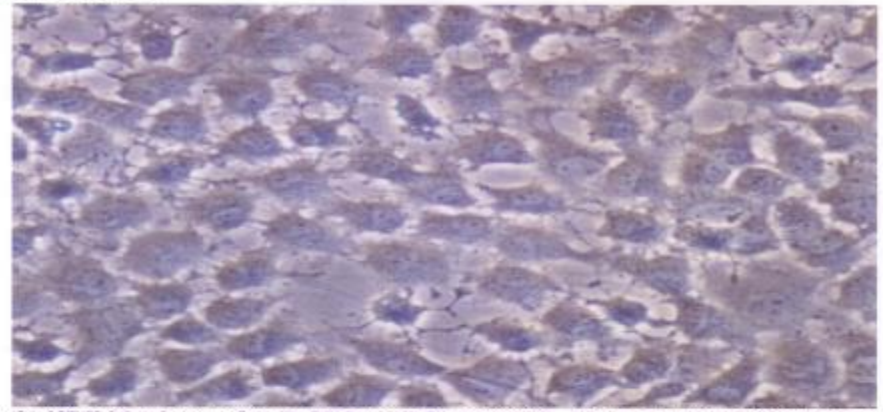
Asceptically aspirate medium, move to microcentrifuge 15 ml, sentrifuge 10 mts 1000 rpm, discard S/N

↓

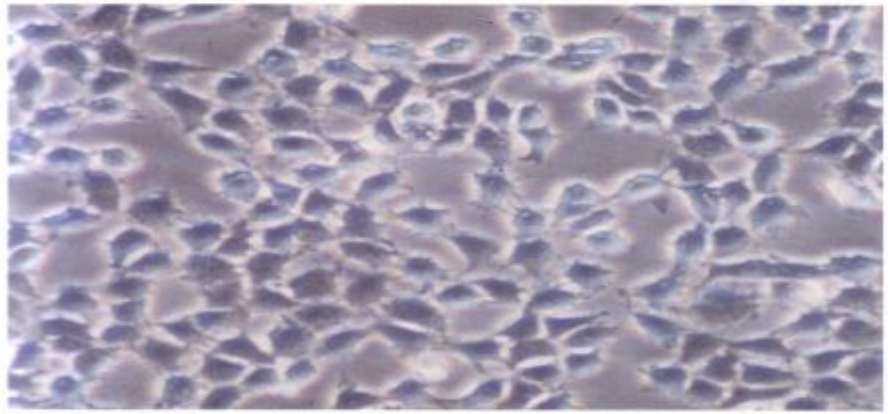
Resuspend cell pellet with the same amount of pre-warmed medium



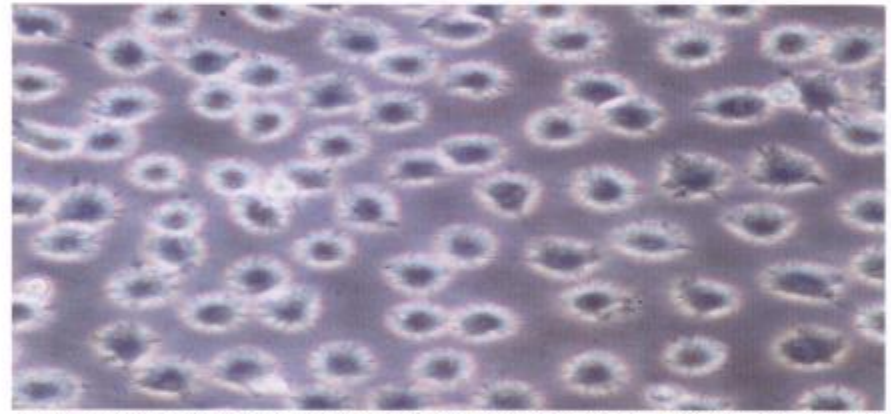
(a) ***NRK Monolayer before Trypsinization.*** *Phase contrast; 20× objective.*



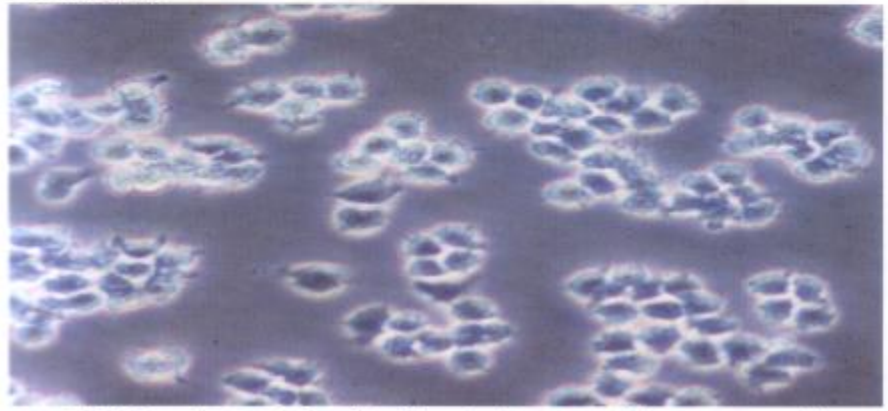
(b) NRK Monlayer after D-PBSA/EDTA Prewash. *Phase contrast; 20× objective.*



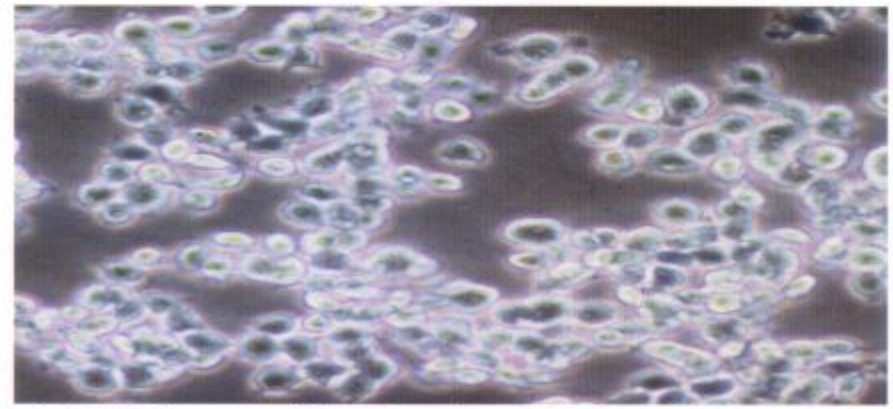
(c) ***NRK Monolayer Immediately after Trypsin Removal.*** *Phase contrast; 20× objective.*



(d) NRK Monolayer 1 min after Trypsin Removal. *Phase contrast;20× objective.*



(e) ***NRK Monolayer 5 min after Trypsin Removal.*** *Phase contrast;20× objective.*



(f) Fully Disaggregated Monolayer. *10 min after removal of trypsin and ready for dispersing and counting. Phase contrast; 20× objective.*

Plate 5. Subculture by Trypsinization.

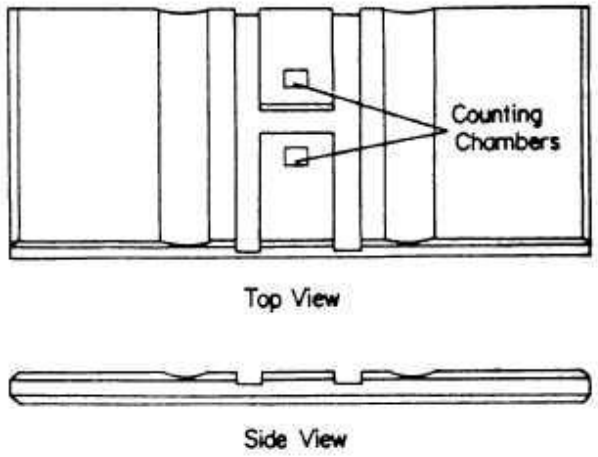
Cell viability

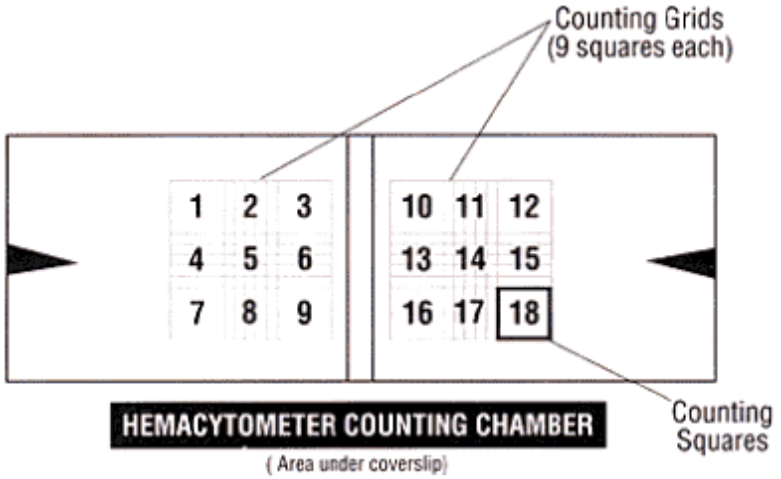
Cell viability is determined by staining the cells with trypan blue

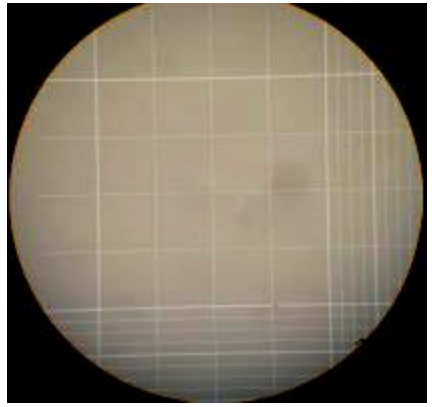
As trypan blue dye is permeable to non-viable cells or death cells whereas it is impermeable to this dye

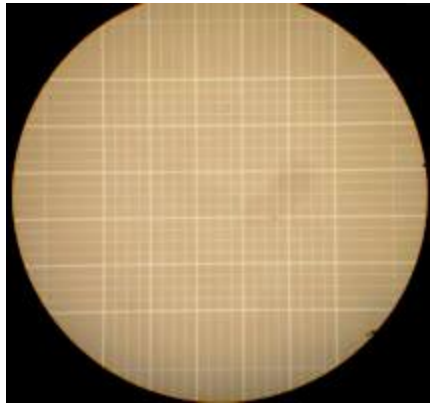
CARA MENGGUNAKAN HEMOSITOMETER

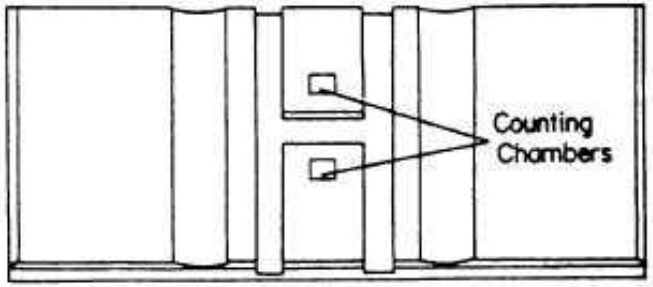
- Bersihkan cover slip & Hemocytometer dg ETOH
- Letakkan Cover slip ditengah
- Masukkan 20 ul suspensi
- Hitung sel



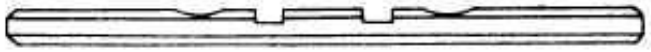




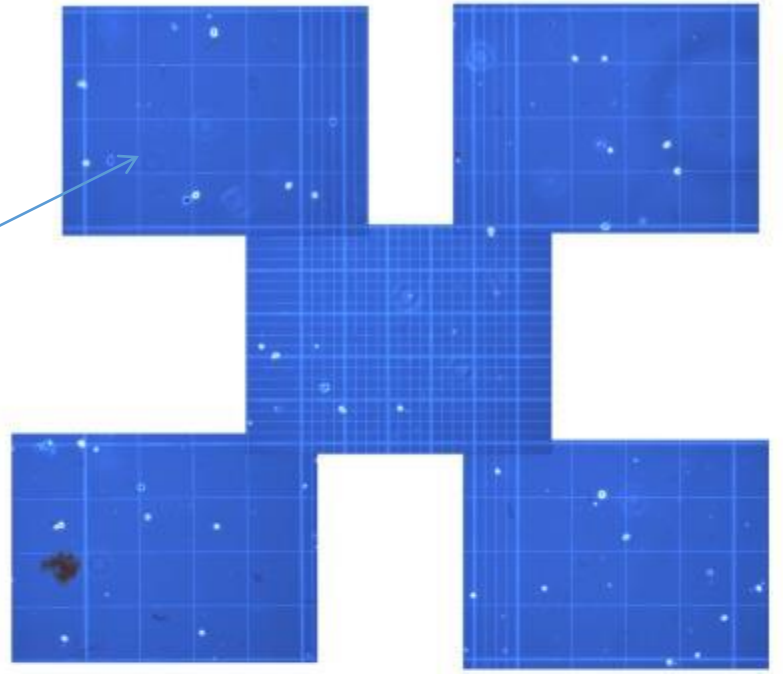


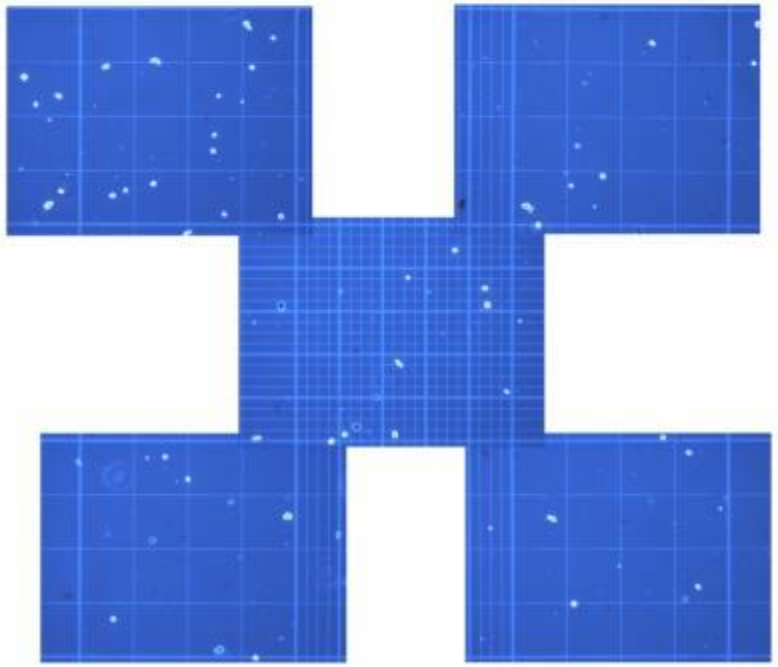


Top View



Side View





CARA MENGHITUNG SEL

$$\text{Konsentrasi sel/ ML} = \frac{N \times \text{Faktor dilusi} \times 10^4}{4}$$

N = Jumlah sel hidup yang diddapat

Faktor dilusi = faktor pengenceran suspensi sel

$10^{4=}$ Jumlah sel Didapat dari 4 kotak yang diperiksa

4 = Jumlah kotak yang dipeeriksa

PRESERVASI CELL KULTUR

Tujuan :

• Sel sangat berharga :
Menyimpan sel kultur

Kapan Menyimpan :

• 50% Konfluen

Yang Diperhatikan sebelum freezing :

1. Akuisisi : Continous cell line
   Cell transfeksi < 5 pasase
2. Standarisasi : Medium : Pilih yang optimal
   Serum : pilih batch untuk semua tahap
3. Validasi : Pencatatan : pasase, jenis sel ,jumlah keluar dan masuk

Mr Frosty

Freezing cells for storage

Remove the growth medium, wash the cells by PBS and remove the PBS by aspiration

Dislodge the cells by trypsin-EDTA. Dilute the cells with growth medium

Transfer the cell suspension to a 15 ml conical tube, centrifuge at 200g for 5 mts at RT and remove the growth medium by aspiration

Resuspend the cells in 1-2ml of freezing medium

Transfer the cells to cryovials, incubate the cryovials at -80 C overnight. Next day transfer the cryovials to Liquid nitrogen

Working with cryopreserved cells

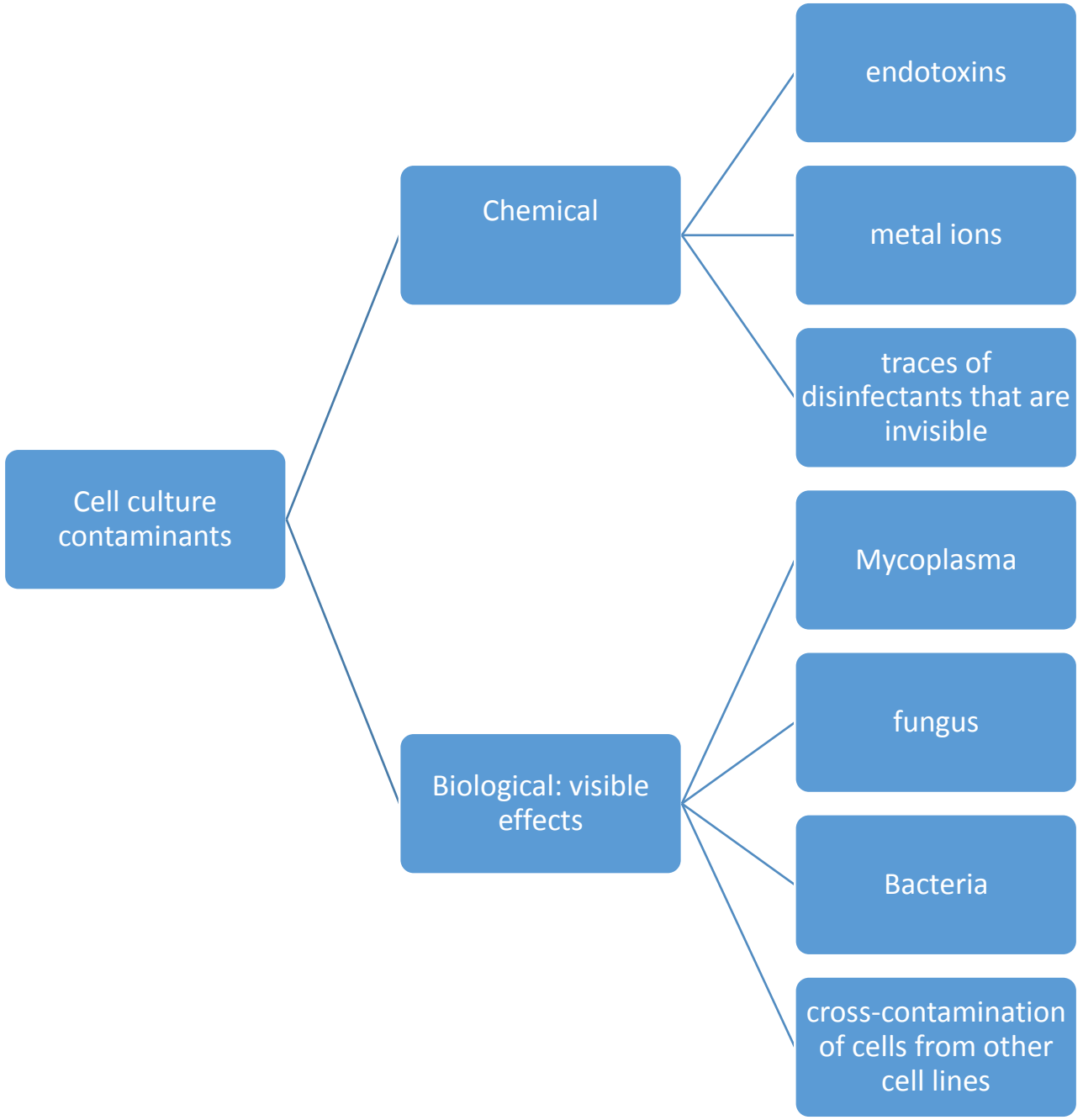
Vial from liquid nitrogen is placed into 37 C water bath, agitate vial continuously until medium is thawed

wipe top of vial with 70% ethanol. Aspirate thawed suspension cell to centrifuge tube 15 ml, add complete medium and sentrifuge 1000 rpm, 10 mts, discard S/N

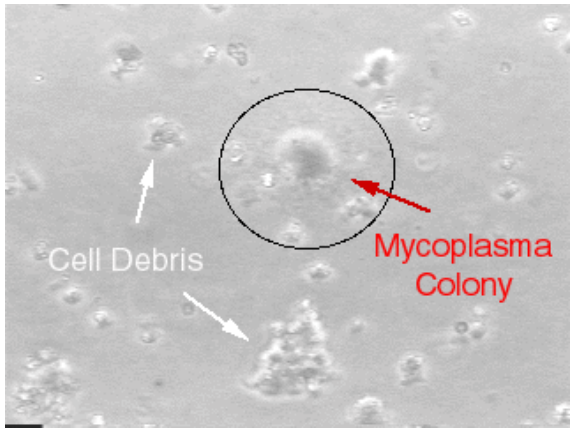
Resuspend the cell pellet in 1 ml of complete medium with 20% FBS and transfer to properly labeled culture plate containing the appropriate amount of medium

Check the cultures after 24 hrs to ensure that they are attached to the plate

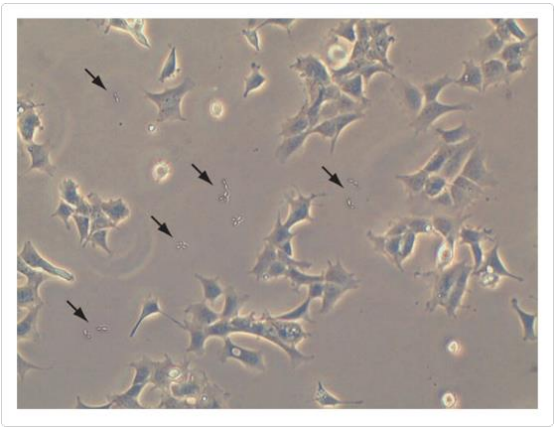
Change medium as the colour changes, use 20% FBS until the cells are established


Cell culture contaminants
Chemical
endotoxins
metal ions
traces of disinfectants that are invisible
Biological: visible effects
Mycoplasma
fungus
Bacteria
cross-contamination of cells from other cell lines

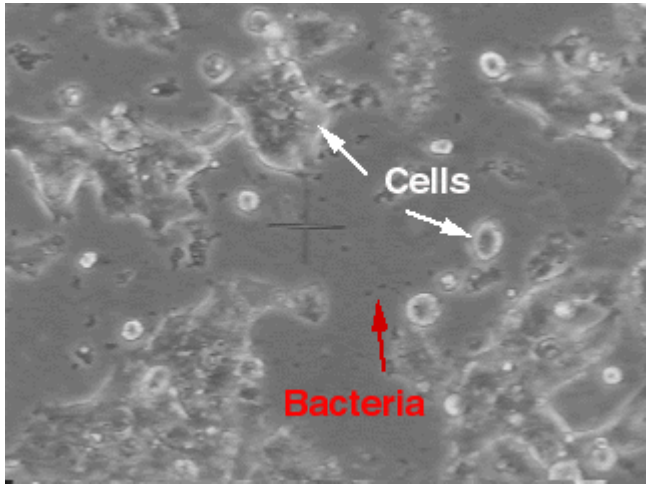
Mycoplasma



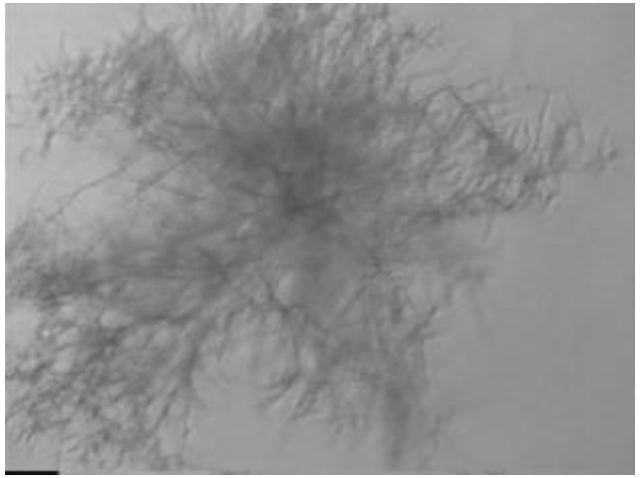
yeast



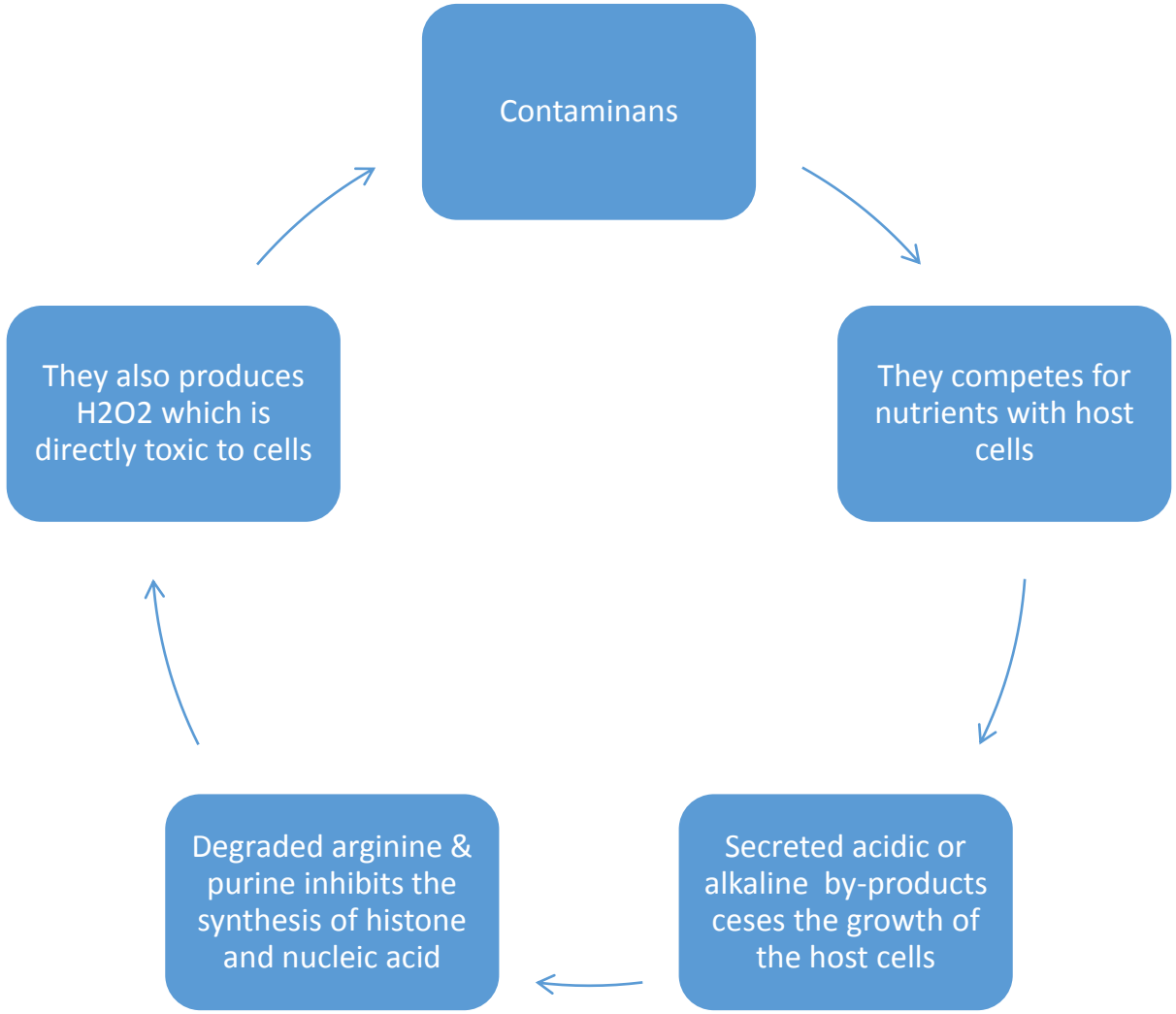
Bakteri



Jamur



Effects of Biological Contamination



Detection of contaminants

In general indicators of contamination are turbid culture media, change in growth rates, abnormally high pH, poor attachment, multi-nucleated cells, graining cellular appearance, vacuolization, inclusion bodies and cell lysis

Yeast, bacteria & fungi usually shows visible effect on the culture (changes in medium turbidity or pH)

Mycoplasma detected by direct DNA staining with intercalating fluorescent substances e.g. Hoechst 33258

Mycoplasma also detected by enzyme immunoassay by specific antisera or monoclonal abs or by PCR amplification of mycoplasmal RNA

The best and the oldest way to eliminate contamination is to discard the infected cell lines directly

Antibiotics in Mammalian Cell Culture

Electrophoresis

	Anti-microbial Spectrum[1]	Mode of action[1]	Solubility[2,3,4]	Conc. (µg/ml)[5,6,7]		Stability 37 °C (days)[6,8]
				Recom.	Toxic	
Inhibitors of Cell Wall Synthesis:						
a) β-Lactams						
Ampicillin Trihydrate	G+, G-	Inhibit the last step in cell wall synthesis, the crosslinking of different peptidoglycan strands	1p. in 150 p. H_2O; soluble in diluted acids and bases; insoluble in alcohols	100 U		3
Carbenicillin, Na_2-Salt	G+, G-		1p. in 1,2 p. H_2O; 1P- in 25 p. 96 % ETOH	100 U		3
Penicillin G, K-Salt	G+		> 20 mg/ml H_2O; > 20 mg/ml MEOH; 10 mg/ml ETOH	100 U	10,000	3
Ampicillin Sodium	G+, G-		1p in 2 p H_2O	100 U		3
Inhibitors of Cell Membrane Function:						
a) Polyene Macrolides						
Amphotericin B	F, Y, M	Form complex with cholesterol. Cause leakage of glucose. Only act on membranes containing cholesterol, hence having no effect on bacteria	2-4 mg/ml DMF; 30-40 mg/ml DMSO; soluble in H_2O with Na-deoxycholate	2,5	30	3
Nystatin	F, Y		freely soluble in DMF; slightly soluble in H_2O and MEOH	50	600	3
b) Peptides						
Polymyxin-B Sulfate	G-	Causes changes in membrane structure resulting in leakage of small molecules	> 20 mg/ml H_2O; 0,3 mg/ml MEOH;0,1 mg/ml ETOH	50	3000	5
Inhibitors of Protein Synthesis						
a) Aminoglycosides						
Gentamycin Sulfate	G+, G-, My	Bind to the 30S subunit of the bacterial 70S ribosome and block the initiation complex	Freely soluble in H_2O; insoluble in alcohol and acetone	50	3000	5
Kanamycin Sulfate	G+, G-, My		1p. in 1p. H_2O; insoluble in alcohol and acetone	100	10,000	5
Neomycin Sulfate	G+, G-		1p. in 3 p. H_2O; insoluble in acetone	50	3000	5
Streptomycin Sulfate	G+, G-		> 20 mg/ml H_2O; 0,85 mg/ml MEOH; 0,3 mg/ml ETOH	100	20,000	3
b) Tetracyclines						
7-Chlortetracycline-HCL	G+, G-	Inhibit bacterial protein synthesis by preventing aminoacyl-RNA binding to the A-side of the 30S ribosomal subunit	8,6 mg/ml H_2O; 17,4 mg/ml MEOH; soluble in sol. of alkali hydroxide and carbonate; insoluble in acetone	10	80	1
Oxytetracycline-HCL (7-Hydroxy-Tetracycline)	G+, G-		6,9 mg/ml H_2O; 16,3 mg/ml MEOH; 11,9 mg/ml ETOH	5	25	3
Tetracycline-HCL	G+, G-, My		10,9 mg/ml H_2O; > 20 mg/ml MEOH; 7,9 mg/ml ETOH	10	35	4
c) Macrolides						
Erythromycin Base	G+, My	Binds to the ribosomal 50S subunit and interferes with the peptidyl transferase activity	2,1 mg/ml H_2O; > 20 mg/ml MEOH; > 20 mg/ml ETOH	100	300	3
Tylosin Tartrate	G+, My		1p. in 10 p. H_2O; soluble in $CHCl_3$; slightly soluble in alcohol	10	300	3
d) Others						
Chloramphenicol	G+, G-	Inhibits prokaryote but not eukaryote protein synthesis by preventing the peptidyl transferase reaction	4.4 mg/ml; H_2O; > 20 mg/ml in MeOH	5	30	5

G+ active against Gram-positive Bacteria; **G-** active against Gram-negative Bacteria;
F active against Fungi; **My** active against Mycoplasma; **Y** active against Yeast

KONTAMINASI

SUMBER SUMBER KONTAMINASI :

1. Faktor Operator : Manipulasi, Pipeting, Mencuci, Permukaan yang tidak steril karena terkena tumpahan media
2. Permukaan bench : Debu dan tumpahan
3. Rambut, Tangan, pakaian
4. Reagen
5. Inefektive sterilisasi
6. Inkubator yang tidak dijaga sterilisasinya

Monitoring Kontaminasi

1. Check kultur yang dipelihara secara reguler
2. Bila masih suspect, bersihkan inkubator and bench, kultur diobservasi bila positif, bersihkan kembali inkubator, dll, gunakan peralatan keesokan harinya
3. Catat semua kontaminasi yang terjadi
4. Untuk kontaminasi tidak menyebar maupun sudah terjadi, buang : kultur, media, tripsin (dekontaminasi)
5. Test sampel semua media, serum sebelum bekerja
6. Bila kontaminasi sangat menyebar : dekontaminasi ruangan

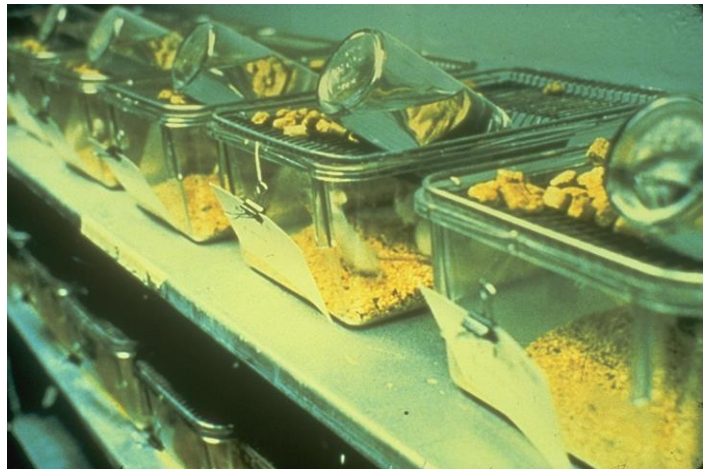
Memilih Hewan Coba

- Relevan → Sesuai dengan kondisi asli
- Reliabilitas → Memberikan data yang konsisten
- Simpel → tidak memerlukan variable kontrol yang tidak perlu
- Akses Mudah didapat dan tidak mahal

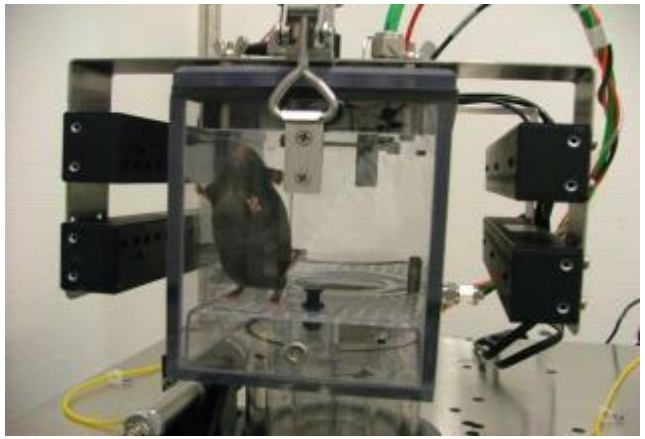
5’s Freedom of Animal

- Freedom from thirst, hunger and malnutrition
- Freedom from discomfort due to environment
- Freedom from pain, injury and disease
- Freedom from fear and distress
- Freedom to express its normal behaviour

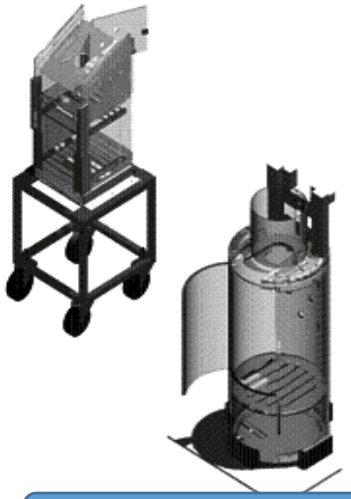
KANDANG HEWAN COBA



Peralatan dan Kandang Khusus



Metabolic cage

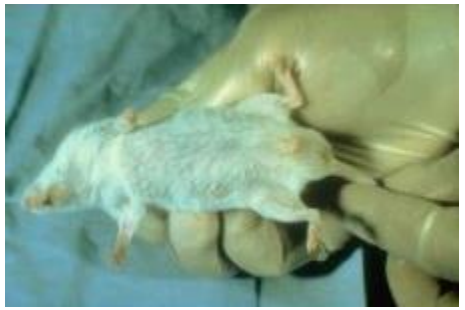
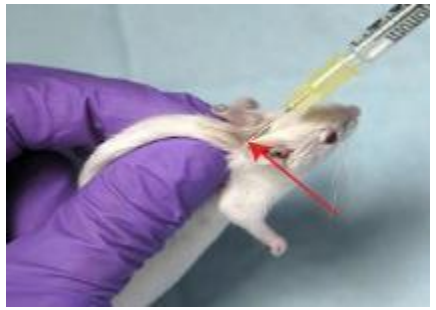


Kursi primata

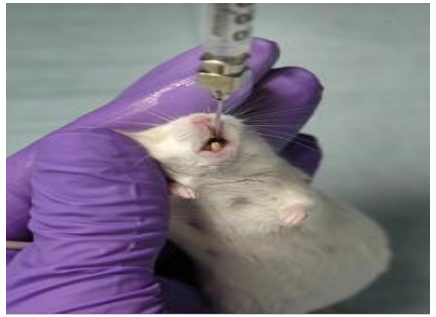
Gnotobiotic animal cage

Pintu Untuk Hewan Besar

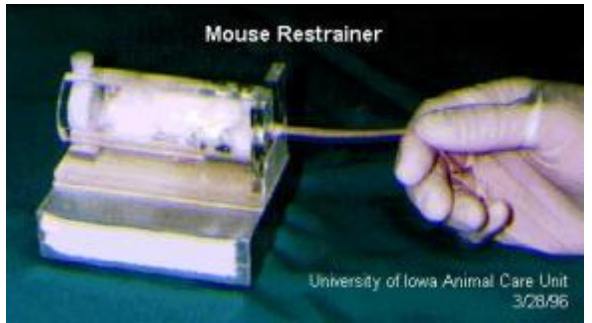
INOKULASI





Restrain

TERMINASI LIMBAH LAB HEWAN COBA

Soil Animal Bedding

Collected twice a week
Autoclaved before put
at garbage reservoir

Animal Carcasses

Sharp Waste