Laboratory Techniques I

Oncology for Scientists I

September 8th, 2016

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Dr. Sheila Figel too!

“When we first hit the lab there are so many things to learn before we even get started that many things go unlearned” – *BiteSize Bio*

Overview

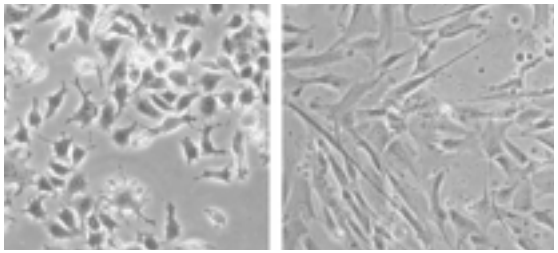
- Experimental Principles
- Molecular biology techniques, that everyone needs!
  - DNA-PCR
  - RNA-rt-PCR
  - Protein-western blotting
- Cell culture

Experimental Principles

- Controls
  - Negative
  - Positive
- Quantification
  - Qualitative vs Quantitative data
- Technical replicates vs. biological replicates

Qualitative vs. Quantitative

- Qualitative
  - “Quality”
  - A change in appearance or other characteristic has been observed
  - Descriptive
  - “Cells have become elongated and fibroblastic”



www.proteomesci.com

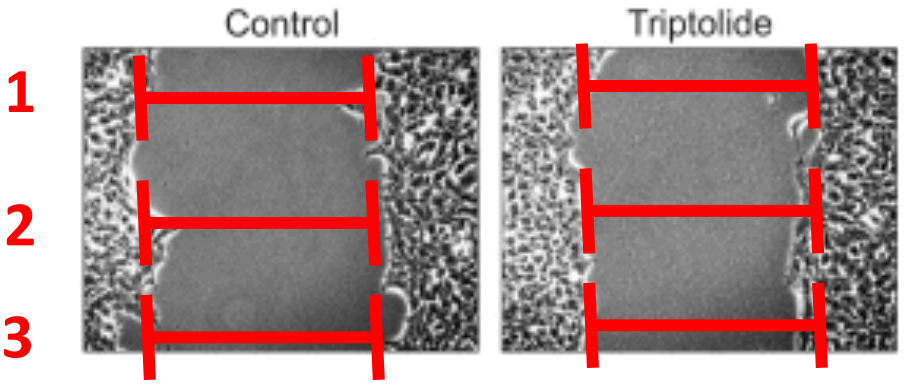
- Quantitative
  - “quantity”
  - A change in some parameter that you have measured
  - Objectively measured
  - “92% of the cells show an elongated phenotype as indicated by a length-to-width ratio of greater than 4.”

Replicates

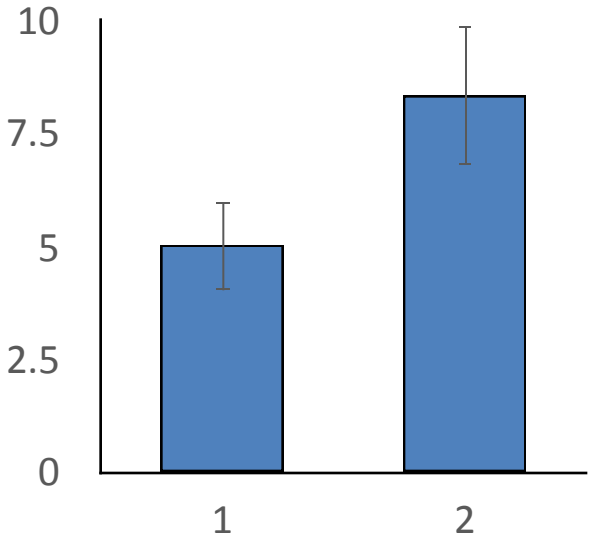
- Technical replicates
  - Within a single experiment
  - Make measurements from the same source at the same time
  - Demonstrates consistency in technique
- Biological replicates
  - Multiple repetitions of the same experiment
  - Demonstrates consistency in experimental results

Replicates

Example: wound-healing assay



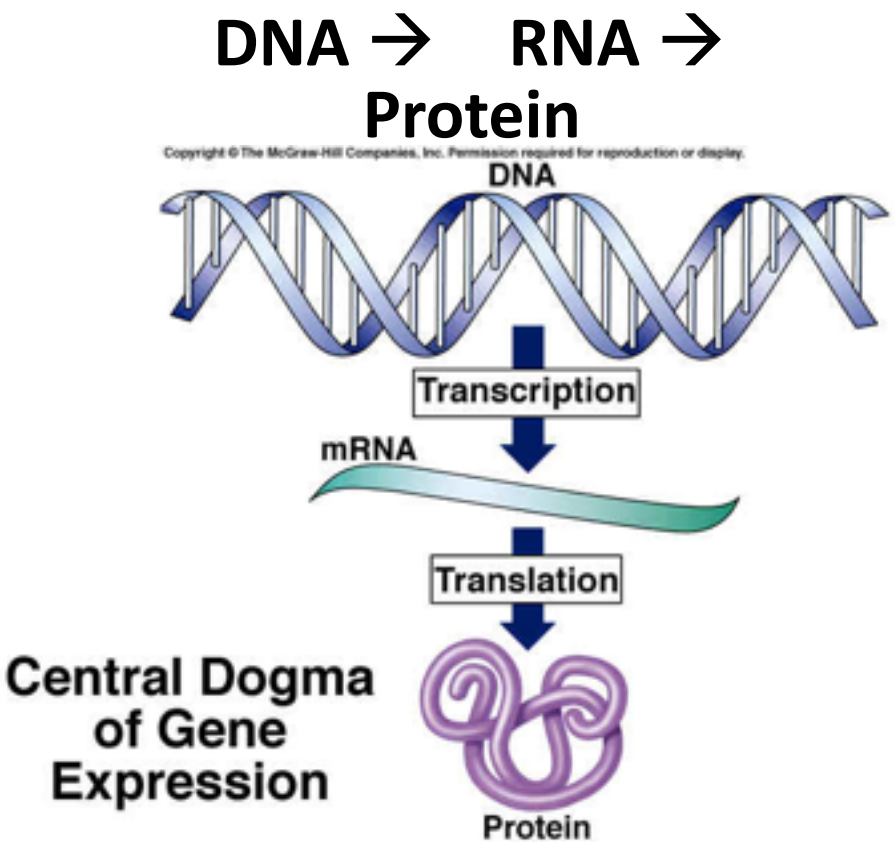
- Technical replicates
  - Three independent measurements along the length of the “wound”
- Biological replicates
  - Repeat the experiment three times



Basic molecular techniques that everyone needs!!!

And…. More importantly

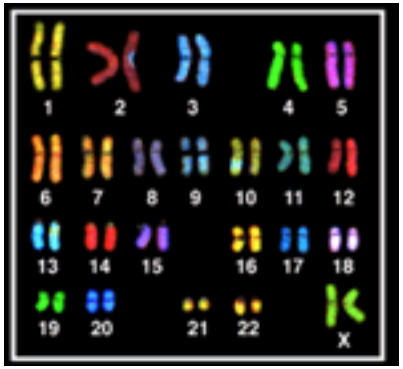
DNA, RNA and protein



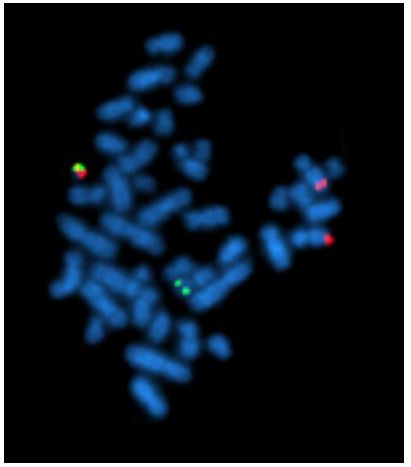
- DNA
  - Southern blot
  - PCR
- RNA
  - Northern blot
  - RT-PCR, qPCR
- Protein
  - Western blot

DNA

- Older techniques allow yes/no detection of gene copies or chromosomal rearrangements
  - Fluorescence in situ hybrization (FISH)
  - Spectral karyotyping (SKY)
  - Southern blotting
- Modern techniques allow sequence analysis
  - PCR
  - Genome sequencing



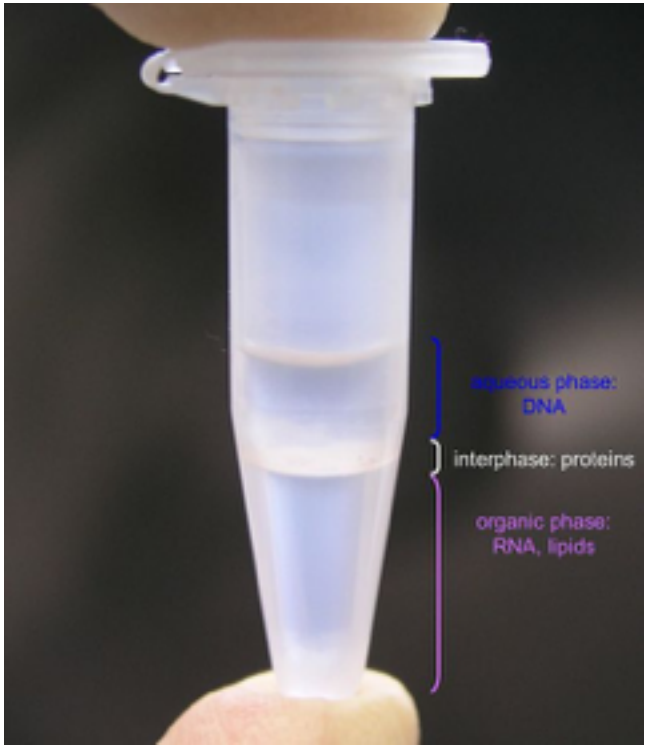
SKY



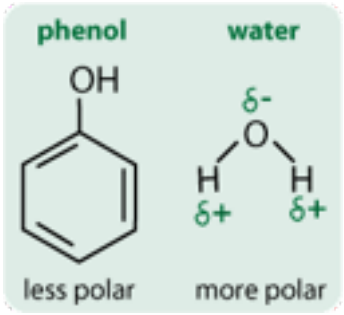
FISH

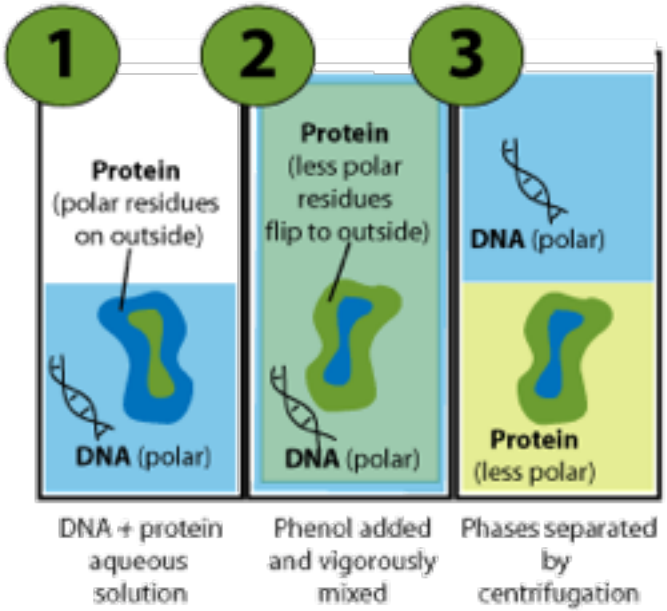
Extraction of genomic DNA

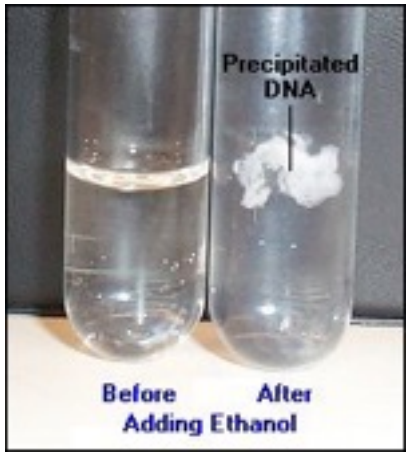
1. Cell Lysis!
   - High concentrations of chaotropic salt (HCl, guanidine thiocyanate, urea, and lithium perchlorate)
   - Detergents
   - Enzymes (Proteinase K)
2. DNA purification
   - Phenol/Chloroform extraction
     - Denatured proteins in the phenol layer
     - Ethanol precipitation of DNA in aqueous layer



How does it work!?





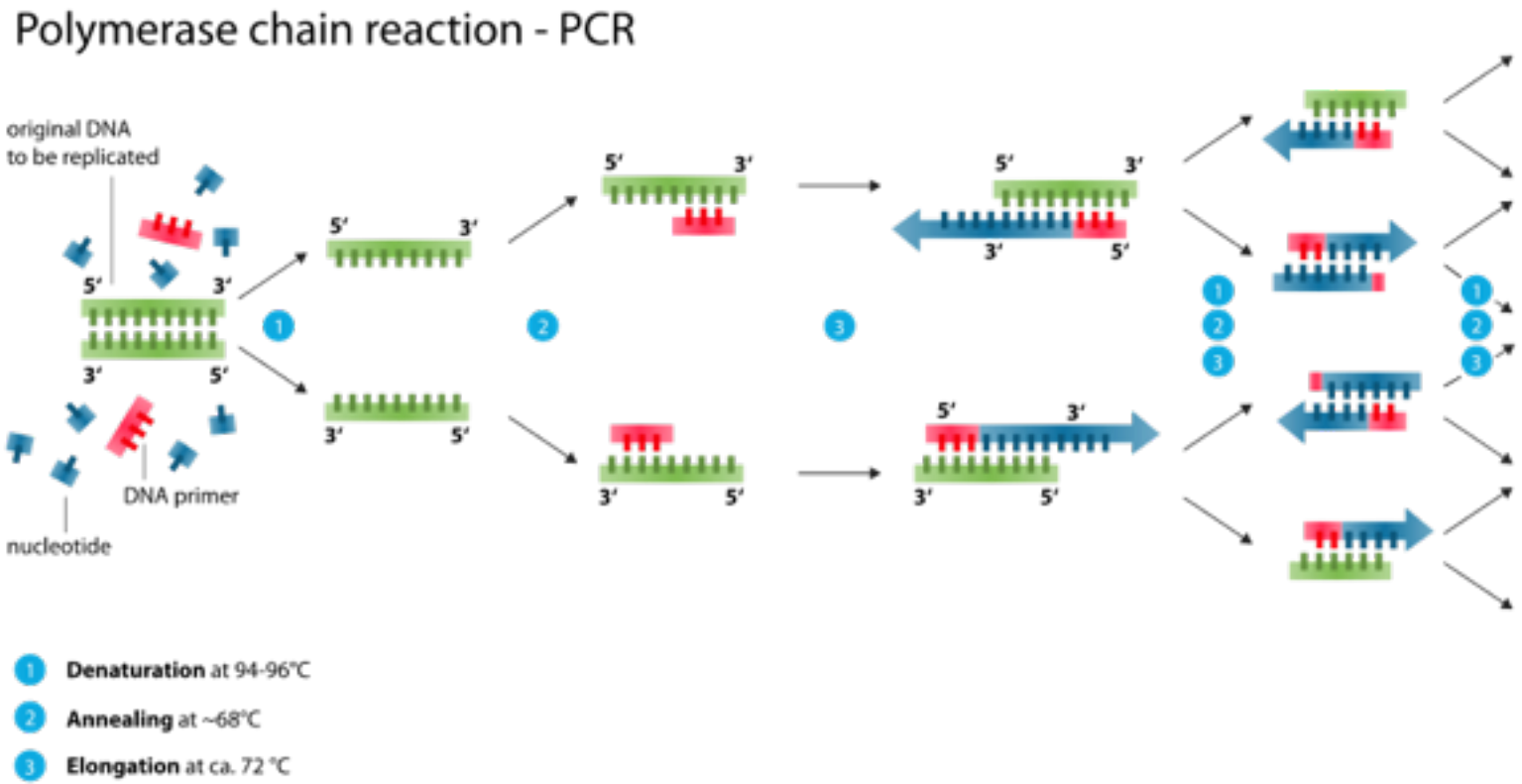


Extraction of Plasmid DNA

- Plasmids maintained in E. coli
- Alkaline lysis
  - Step 1 – resuspend bacteria
  - Step 2 – lysis/denaturation
    - NaOH/SDS – bursts cells & denatures DNA (bacterial chr. & plasmid)
  - Step 3 – precipitation of protein/bact DNA
    - Potassium acetate – plasmid DNA renatures
  - Step 4 – ethanol precipitation of plasmid DNA

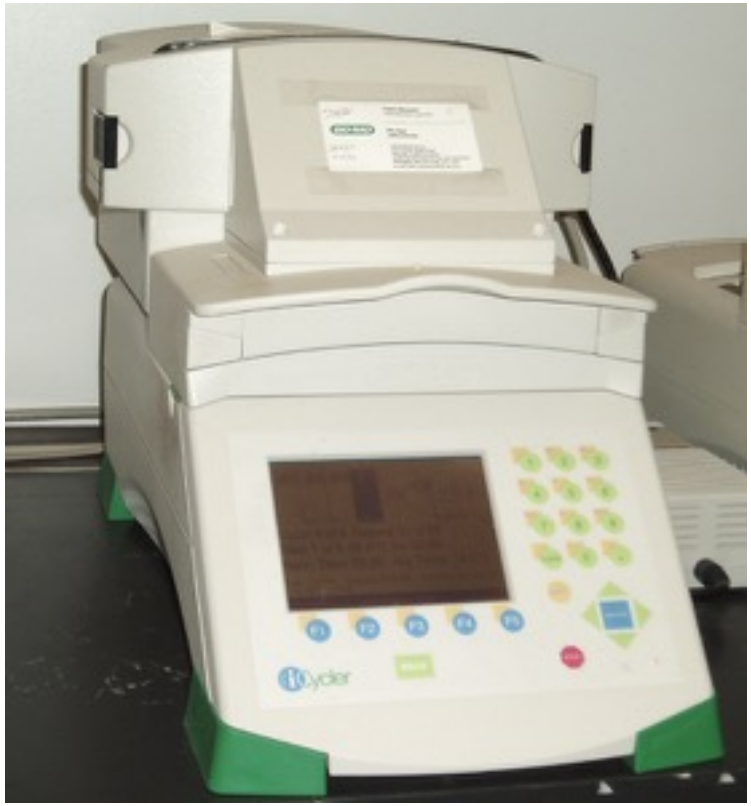
PCR

- Amplifications of small segments of DNA
- Used to identify mutations, cloning, expression (qPCR)



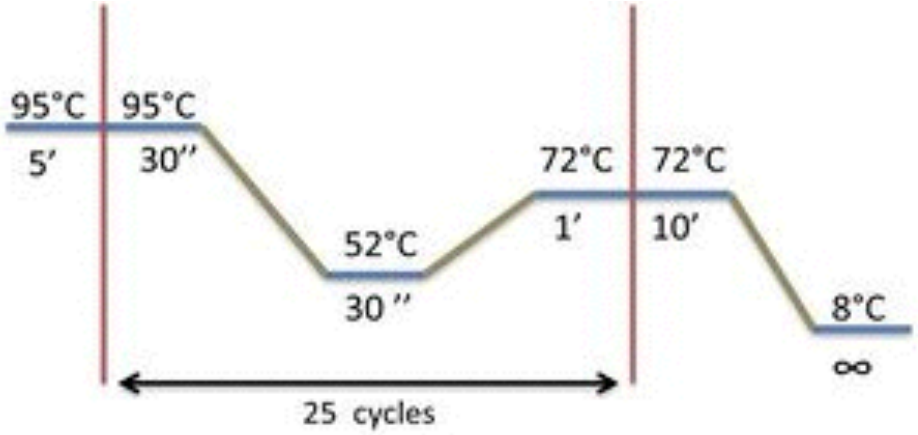
Materials for PCR

- PCR reaction mixture:
  - DNA template
    - gDNA
    - Plasmid DNA
    - cDNA (RT-PCR)
  - Primers
  - Polymerase
    - Taq
  - Buffer
  - Thermal cycler



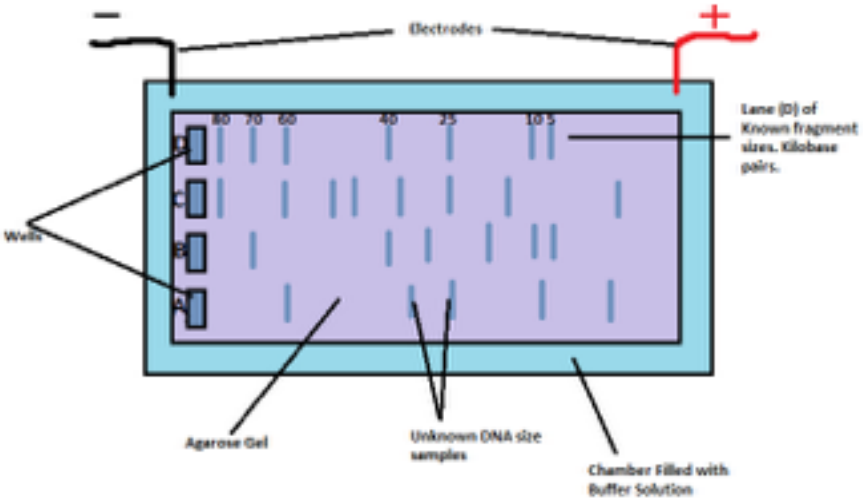
Steps for PCR

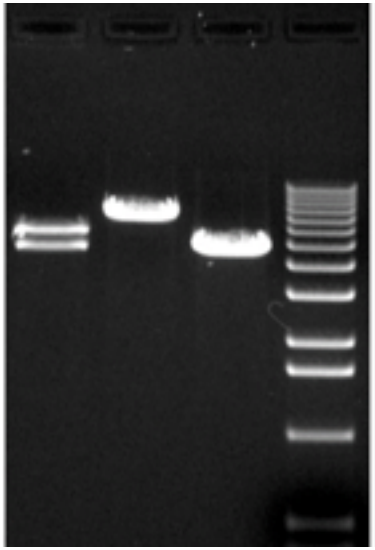
- Denaturation
  - Separate the strands of DNA
  - Heat to 95 or 98°
- Annealing
  - Primer binding to DNA
  - Temperature varies
  - Depends on the Tm of primer
  - Typically 5° less than the lowest Tm of the primer
- Elongation
  - Polymerase binds to primed DNA and adds nucleotides
- Cycles
  - Typically ~30



Agarose Gel Electrophoresis

- Electrophoresis
  - Migration of molecules through a matrix based on <u>size & charge</u>
  - Matrix is solid but porous
- Agarose
  - DNA, RNA
  - Restriction digest



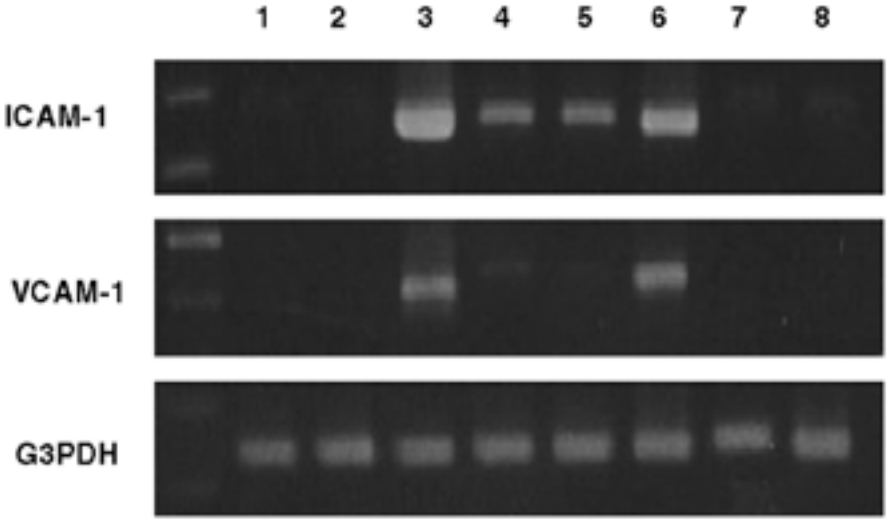


Types or PCR

1. Standard PCR
   - Amplify DNA (genomic or plasmid)
2. Reverse transcription PCR (RT-PCR)
   - Amplify **cDNA**
3. qPCR
   - *Quantitative* PCR
   - Amplify DNA or RNA

rt-PCR

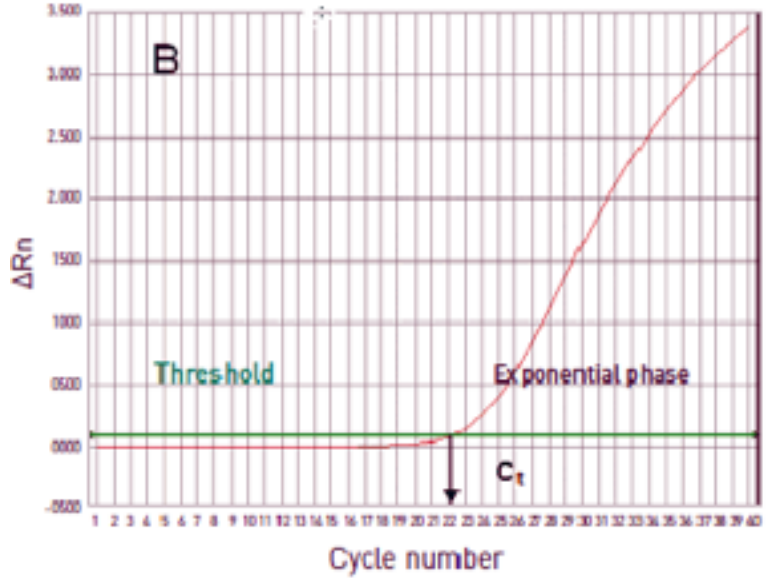
- RNA extraction (Trizol)
- Reverse transcription PCR
- Semi-quantitative – compare signals on gel
- Detect cDNAs (indicative of RNA)
- Steps:
  - Reverse transcription
    - RNA → cDNA
  - PCR using cDNA template



Naito et al, 2004

qPCR (Real-Time PCR)

- *Quanititative* PCR
- Amplify short segment representing a gene
- DNA-binding dye
  - SYBR Green
    - Excitation = 488 nm
    - Emission = 522 nm
- Fluorescence of dsDNA measured after each cycle
- Relative quantification
  - Compare amplified amt to that of housekeeping gene



Ct = Threshold cycle

Quantitative PCR

- Apply qPCR to cDNAs to quantify RNA levels
- This replaces older RNA detection techniques such as northern blotting (which is semi-quantitative)

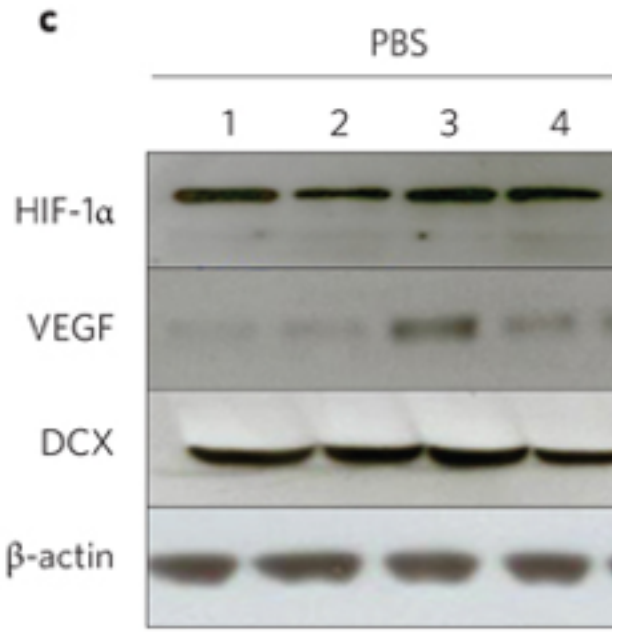
PCR experimental principles

- **Controls**
  - Amplification of housekeeping gene (RT-PCR & qPCR)
  - Positive – DNA template known to contain the correct sequence
  - Negative – Reaction mixture + water (no template)
- **Quantification**
  - Standard PCR is qualitative – presence/absence of band, sequence data
  - RT-PCR is semi-quantitative – band intensities can be compared based on equivalent control signal for each sample
  - qPCR is quantitative – Ct values can be compared

Western blot

- Allows comparison of levels of protein in samples
- *Ex: Is expression of a certain protein decreased when cells are treated with a drug?*

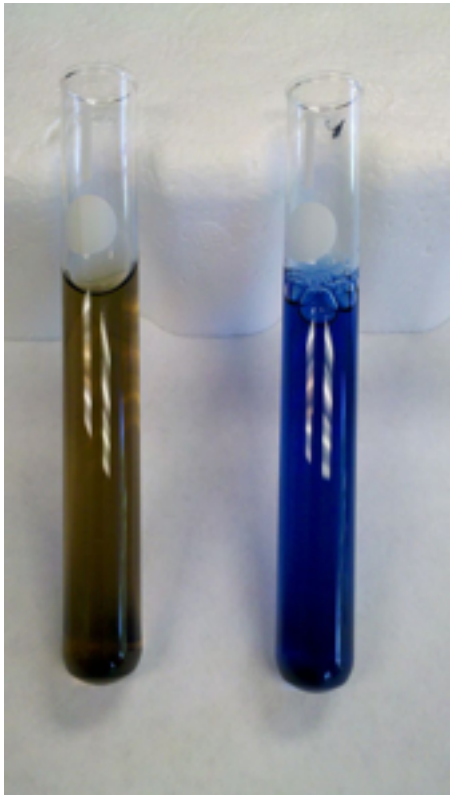
- Steps
  - Isolation of protein
  - Run lysates through SDS-PAGE gel
  - Transfer protein from gel to membrane
  - Incubate membrane with 1°/2° antibodies
  - Detect signal via film



Lee et al, 2011

WB-isolation of protein

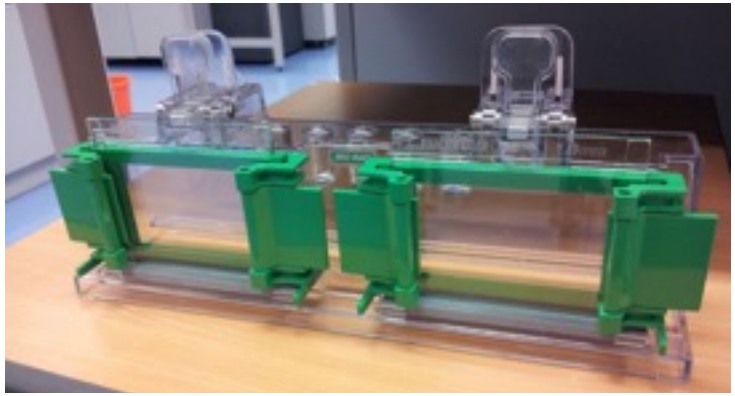
- Cells or tissue
- RIPA (radioimmunoprecipitation lysis assay buffer)
  - Contains SDS & sodium deoxycholate – ionic detergents
  - Disrupts membranes & protein-protein interactions
  - proteinase inhibitors
- Quantification of total protein in lysate
  - Bradford assay
    - Coomassie dye turns from red to blue when binding protein
    - Read A595
    - Compare to standard curve to determine protein concentration
    - ug/ul



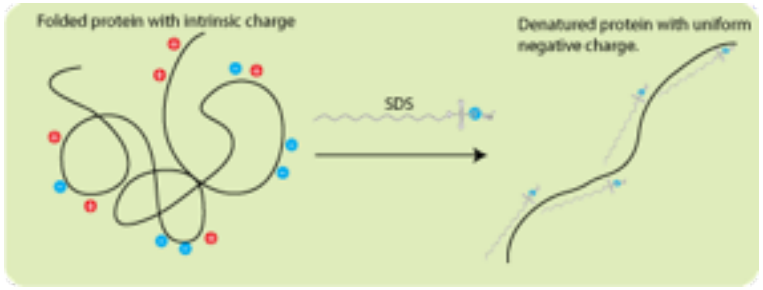
WB-SDS PAGE

- SDS = sodium dodecyl sulfate
  - Detergent
- PAGE = polyacrylamide gel electrophoresis

- **Preparation of SDS-PAGE gel**
  - Acrylamide:bisacrylamide (what polymerizes)
    - 7.5% gel, 10% gel, etc
  - SDS (denaturant)
  - Buffer (maintains pH)
  - APS/TEMED (initiates polymerization)



The elements, because this is pretty cool…



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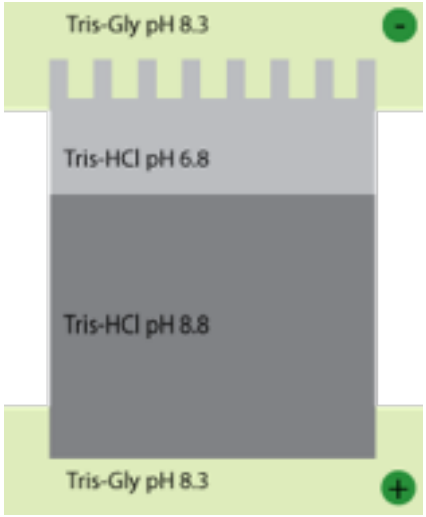
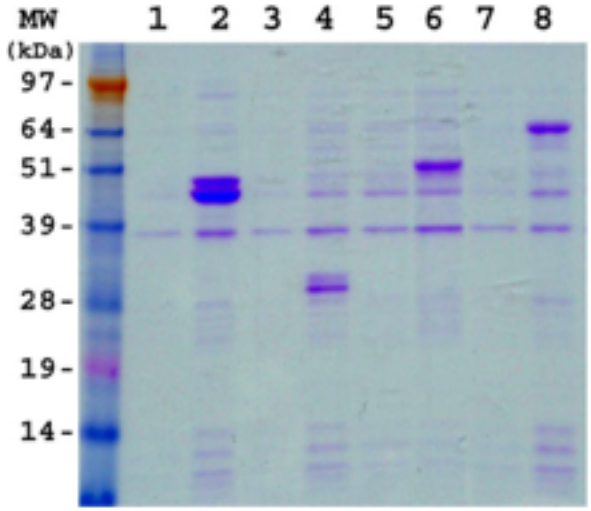


Glycine
Proteins
Cl-

↓

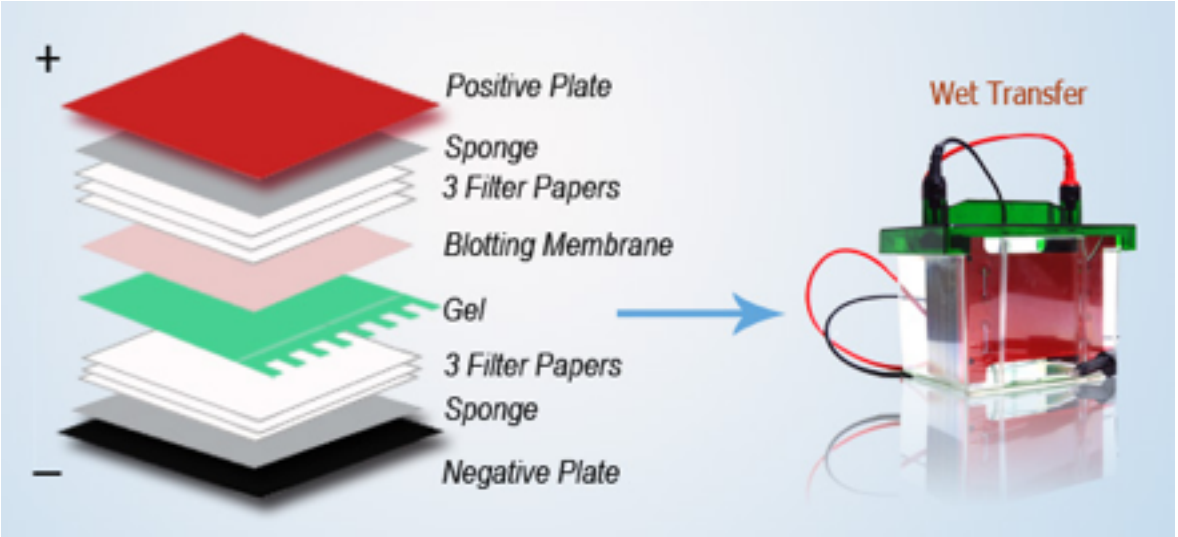
Proteins
Glycine

=



WB-transfer

- Transfer protein from gel to membrane
  - PVDF or nitrocellulose
    - Hydrophobic
  - Assemble “sandwich”
  - Electroblotting

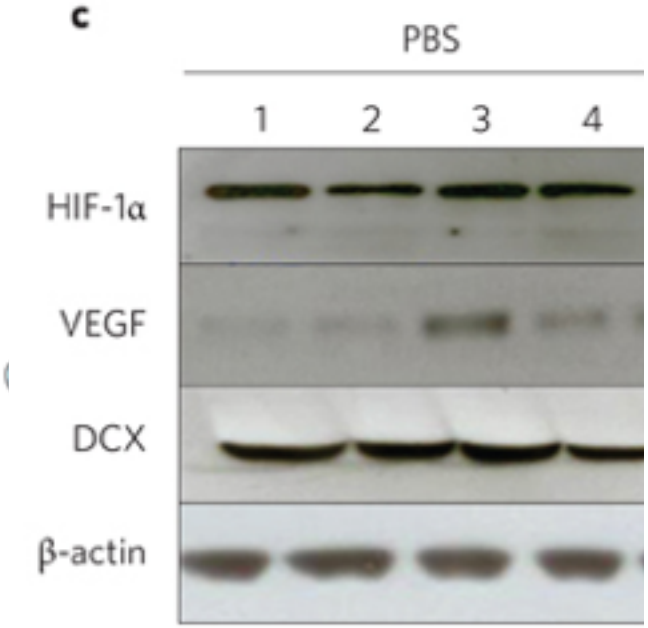


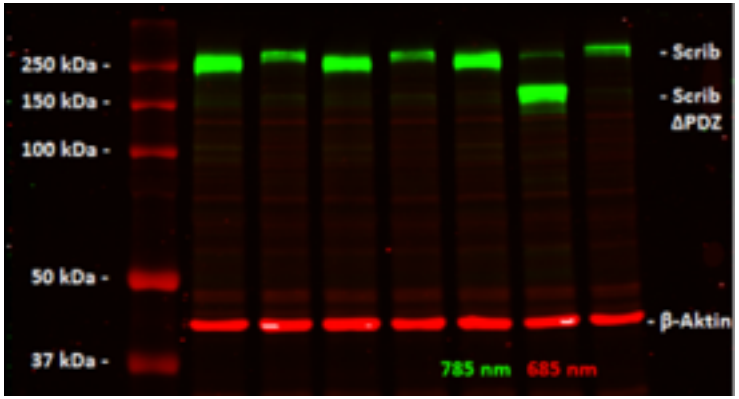
WB – detection via 1°/2° antibodies

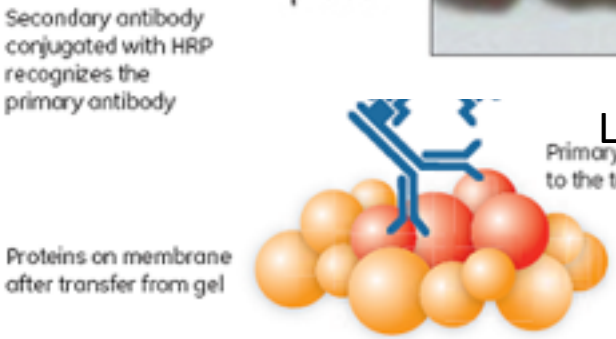
- Block membrane
  - Prevent non-specific binding of antibodies
  - BSA, non-fat milk
- 1° antibody
  - Specific for protein of interest
- 2° antibody
  - Specific for the species of the 1° Ab
  - Conjugated to a fluor or to HRP (horseradish peroxidase)

WB – detection via 1°/2° antibodies

- Fluor-conjugated 2° Ab
  - Scan membrane & detect fluorescence
- HRP-conjugated 2° Ab
  - Incubate membrane with ECL reagent
  - Expose membrane to X-ray film







Lee et al, 2011

WB experimental principles

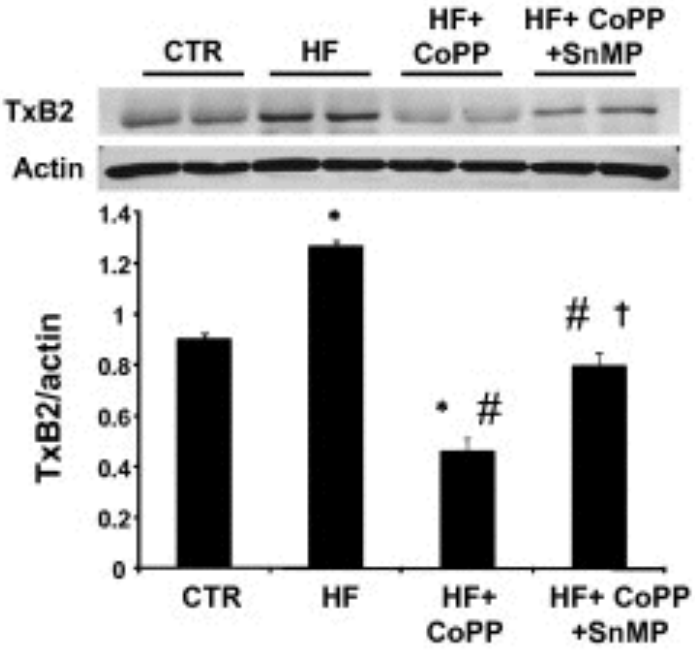
- **Controls**
  - Immunoblotting (same membrane) for housekeeping gene (ex: GAPDH)
    - Loading control
  - Positive – Protein lysate from known positive sample
  - Negative – Dependent on experiment
    - Ex: Treatment of control cells with PBS instead of drug
    - Ex: Treated cells at “0 h” (before drug can have an effect)
- **Quantification**
  - WB is semi-quantitative – band intensities can be compared based on equivalent control signal for each sample
  - In order to quantify, apply densitometry analysis

Densitometry

- Allows quantification of images based on pixel intensity
- A way to quantify rt-PCR and WB

Using Image J:

- Measure pixel intensity of all bands
- Normalize the value of each band to the value of its associated actin band
- Compare normalized values



Cao et al, 2011

Cell Culture

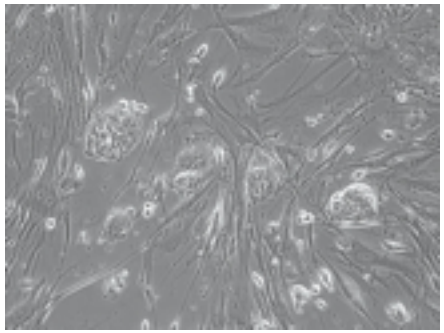
- 1900s – tissue culture
  - Harrison & Carrel
  - “a method for studying the behavior of animal cells free of systemic variations that might arise in the animal both during normal homeostasis and under the stress of an experiment”
- 1952 – development of continuous human tumor cell line
  - HeLa
- Uses of cultured cells:
  - Development of antiviral vaccines
  - Production of monoclonal antibodies
  - Production of cell products
    - Insulin, HGH, interferon
  - Understanding of neoplasia

Cell Culture

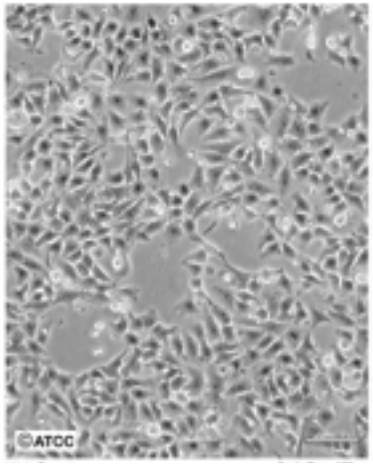
- Benefits
  - Can carefully control environment
  - Preservation
  - Avoid using animals
  - Rapid, relatively cheap
- Disadvantages/limitations
  - Expertise
  - Identification of cell type
  - Genetic & phenotypic instability
  - Quantity

Cell Culture-Types of Cells

- Primary culture
  - Cells isolated from tumors or organs
  - Non-immortalized
  - Replication limit → senescence
- Immortalized cell lines
  - Have evaded senescence
  - Sources
    - Cancer – ex, HeLa, A549
    - Stable expression of a gene which de-regulates cell cycle
      - Adenovirus E1 in HEK293
      - Telomerase



Primary cells – mouse embryonic fibroblasts



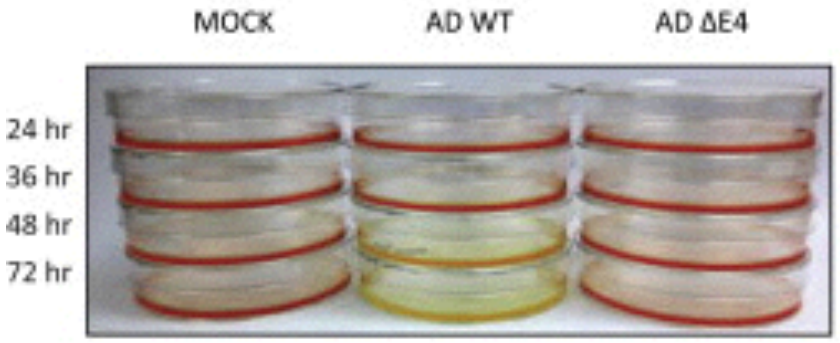
Immortalized cells – HeLa

Cell Culture-Types of cells

- Adherent
  - Epithelial & fibroblast
- Non-adherent
  - Hematopoietic cells
  - Jurkat cells – human T lymphocytes

Cell Culture Basics

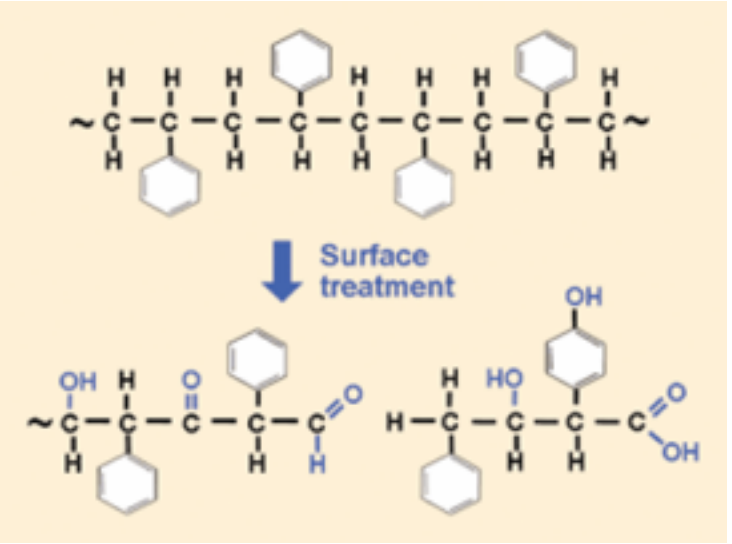
- Growth media
  - DMEM, MEM, RPMI 1640
    - Amino acids, vitamins, glucose
  - Phenol red – pH indicator
  - Serum – FBS, FCS, BS
    - Proteins & polypeptides, growth factors, amino acids, lipids, carbohydrates, polyamines, urea, inorganics, hormones, vitamins
    - Contains antitrypsin activity



Thai et al, 2014

Cell Culture Basics – adherent cells

- Plates/flasks
  - "Tissue culture treated"
    - Plates are made of polystyrene which is hydrophobic
    - TC treatment (many types) → make surface hydrophilic/negatively charged
    - Cell adhesion proteins (ex, vitronectin & fibronectin) can coat plate



Cell Culture basics – adherent cells

- Subculturing
  - Trypsin/EDTA
  - Trypsin
    - Serine protease
    - Cleaves adhesion proteins (integrins)
    - Optimal activity at 37°
  - EDTA
    - Chelating agent – Calcium & Magnesium
  - Neutralize with complete (serum-containing) medium

Aseptic Technique

- Cell culture must be kept sterile
- Free of microorganisms
  - Bacteria
  - Fungi
  - Viruses
- **Aseptic technique** – designed to create a barrier between the sterile cell culture & microorganisms in the environment
  - Sterile work area
  - Good personal hygiene
  - Sterile reagents & media
  - Sterile handling

Summary I

- Cell & molecular techniques allow scientists to examine subcellular components
- Each experiment should include positive & negative controls
- Measurements can be qualitative or quantitative
- Technical replicates ensure consistency within an experiment; biological replicates provide confidence in experimental results
- DNA may be extracted using phenol/chloroform or (for plasmids) alkaline lysis

Summary II

- PCR allows us to amplify pieces of DNA or cDNA
- Western blotting allows detection of proteins within cells or tissue
- Densitometry enables quantification of otherwise semi-quantitative data through analysis of image pixel intensity
- Cell culture is a way to study cells in the laboratory
- Aseptic technique is critical in cell culture

Thank you!
Questions???