Laboratory Techniques I
Oncology for Scientists I

September 8\textsuperscript{th}, 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”

- **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.”

www.proteomesci.com
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 → RNA → 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 hybridization (FISH)
  – Spectral karyotyping (SKY)
  – Southern blotting
• Modern techniques allow sequence analysis
  – PCR
  – Genome sequencing
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 size & charge
  – 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 $\rightarrow$ cDNA
- PCR using cDNA template

Naito et al, 2004
qPCR (Real-Time PCR)

- Quantitative 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

\[ Ct = \text{Threshold cycle} \]
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...

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
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???