Assays for gene expression and protein production

Module 3, Lecture 5

20.109 Spring 2011
Topics for Lecture 5

- Measuring protein levels
- Measuring transcript levels
- Imaging assays
Module overview: 2nd half

1. Enzymatic digestions
   Test for collagen proteins (by ELISA) and for proteoglycans (with dye)

2. EDTA-citrate dissolution

Purify mRNA from cells → Prepare complete cDNAs → Run qPCR to measure CN II, CN I, and 18S RNA.
Antibodies are specific and diverse

- **Specificity**
  - variable region binding, $K_D \sim \text{nM}$
  - linear or conformational antigens

- **Diversity**
  - gene recombination

- **Production**
  - inject animal with antigen, collect blood
  - hybridomas (B cell + immortal cell)
Day 5-7: protein analysis by ELISA

- ELISA: enzyme-linked immunosorbent assay
  - specific
  - sensitive
  - multiple kinds

“blocking” step also needed

△ = protein of interest

**Indirect ELISA**
- Bind antigen
- Add 1° antibody
- Wash
- Add 2° antibody, wash

**Sandwich ELISA**
- Bind capture antibody
- Add antigen
- Wash
- Add detection antibody, wash
Protein gels: SDS-PAGE

- Polyacrylamide gel electrophoresis
  - separates proteins
  - by size, shape, charge
- Sample preparation
  - SDS to coat with negative charge
  - β-Me to break disulfide bonds
  - boiling to further denature
- Visualization: Coomassie stain
  - binds certain AA

protein ladder, bio-rad.com
Common protein-level assays

• PAGE
  – simple and low cost
  – Coomassie detection limit ~ 0.3-1 ug/band
    (2-5 ng/band for silver staining)
  – cannot distinguish two proteins of same MW

• Western blot
  – identifies specific protein
  – detection limit ~1 pg (chemiluminescent)
  – only simple for denatured proteins

• ELISA
  – detects native state proteins
  – quantitative
  – high throughput

Current Protocols in Cell Biology, Molecular Biology
Common transcript-level assays

• RT-PCR (end-point)
  – simple, low cost
  – can be semi-quantitative

• Microarrays (end-point)
  – high cost, need specialty equipment
  – complicated and fraught analysis
  – high throughput

• q-PCR (real-time)
  – some special equipment, medium cost
  – highly quantitative
  – multiplexing potential
  – requires optimization (primers)
End-point RT-PCR

• Co-amplification in one tube
  – Collagen + GAPDH

• Optimize primers
  – no cross-hybridization
  – similar signals (vary [primer])
  – similar efficiency

• Reliability issues
  – must be in exponential phase
  – sensitive to change in [RNA]

• Visualize on a gel
  – measure band intensity/area
  – low dynamic range

Which sample is from chondrocytes, and which from stem cells?
Introduction to qPCR

• Real-time tracking of [DNA]
• Uses probes that fluoresce
  – when bind to any DNA
  – when bind to specific DNA (FRET)
• How and why does [DNA] change during PCR?
  – first plateau
  – exponential phase
  – second plateau
  – detection limit
  – competition, reagent limits, inhibition
• Starting point for analysis: threshold cycle $C_T$

Current Protocols in Cell Biology, Molecular Biology
Interlude: intersection of science and commerce

Patenting genes

“Judge invalidates human gene patent”
*NY Times* March 2010

“Metastasizing patent claims on BRCA1”
*Genomics* May 2010
Day 5-6: image analysis

• Imaging data is often high throughput
  – 4D: time, x-y-z
  – requires computation, and
  – human design/interpretation

• Many available analysis packages
  – some ~ $20-30K
  – NIH ImageJ = free

• Your analyses
  – automated cell counts
  – optional: explore other features

Fluorescence microscopy

- **Light source**
  - Epifluorescence: lamp (Hg, Xe)
  - Confocal: laser (Ar, HeNe)
  - 2-photon: pulsed laser

- **Filter cube**
  - Excitation
  - Dichroic mirror
  - Emission
  - Band-pass vs. long-pass

- **Detection**
  - CCD camera

Specifications for Day 3 imaging

- Live/Dead Dyes
  - Green 490 ex, 520 em
  - Red 490 ex, 620 em
- Excitation 450-490 nm
- Dichroic 500 nm
- Emission 515\(^+\) nm

Images from: Nikon microscopy website: www.microscopyu.com
Types of microscopy

- Epifluorescence: noisy due to out-of-plane light
- Confocal: pinhole rids out-of-plane light
- 2-photon: femtoliter volume excited; good depth (IR)

Epifluorescence

Confocal
Confocal uscopy permits 3D reconstruction
Lecture 5: conclusions

- Antibodies to diverse targets (e.g., proteins) can be made and used for detection/measurement.
- Trade-offs exist (e.g., between simplicity and accuracy) for different transcript-level assays.
- Fluorescence imaging is a powerful tool for studying cells and materials.

Next time: cartilage TE, from *in vitro* and *in vivo* models to the clinic; qPCR analysis.