# Molecular Methods: Choosing Appropriate Tests & Test Interpretation

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## Disclosures

- None

## Learning Objectives

1. Understand the role of sensitivity/specificity in choosing the appropriate test
2. Understand variables which alter test results
3. Understand certain molecular methods used in the medial literature
4. Understand the importance of recoding data for test interpretation.

## Question 1

An infant born to a women infected with HIV has a positive Rapid HIV1 ELISA (3rd generation) at 2 months of age. What should you do first?

- A. Start HIV Antiretroviral medications as soon as possible.
- B. Repeat the Rapid HIV1 ELISA STAT.
- C. Order an HIV discriminatory test STAT.
- D. Repeat the HIV1 test test in 2 months.
- E. Recheck the specificity of the assay.

## Question 2

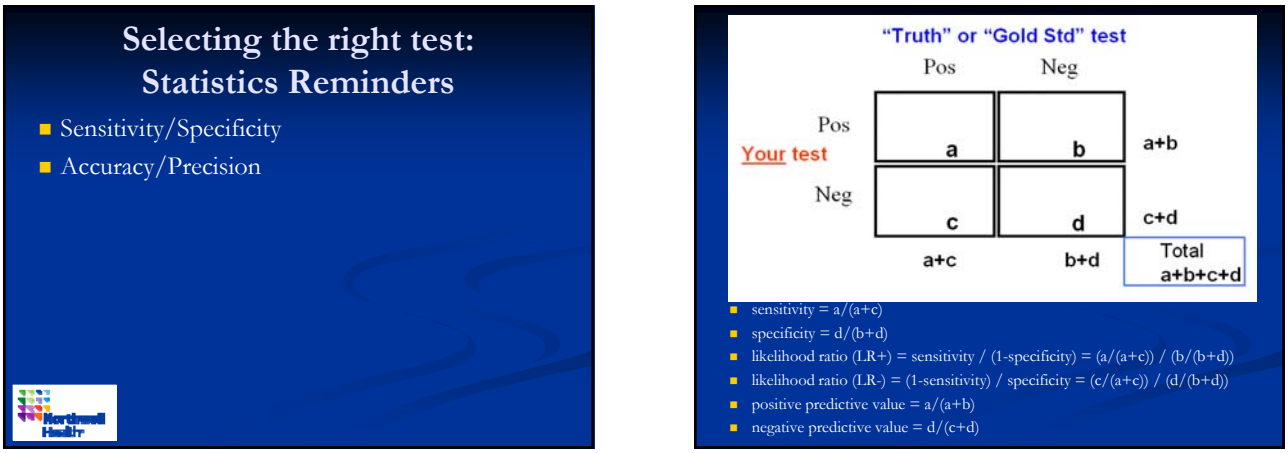
- Your research coordinator tells you that the average fasting blood glucose in your healthy general pediatric patient population (n=253) is 125 mg/dl. What should you do for patients with high glucose?
- A. Institute an evidence based medicine training session for all clinical staff to instruct them in proper diabetes screening.
- B. Look at the data to understand why the patients all have high fasting glucose.
- C. Begin treatment for DM
- D. Order oral glucose tolerance tests
- E. Order HgbA1c

## Question 3

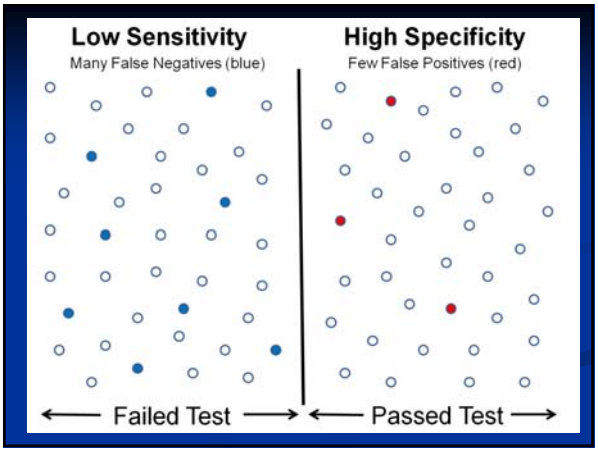
- Fifty percent of the QuantiFERON Gold Test-in-Tube (QFN) results are indeterminate. What should you do?
- A. Repeat QFN on all patients with indeterminate results
- B. Consider those patients with indeterminate results as screened for TB.
- C. Call the lab to understand how the QFN test is done.
- D. Refer all indeterminate patients to Peds ID for potential treatment.
- E. Place a PPD on all of the patients with indeterminate results.

## Selecting the right test: Statistics Reminders

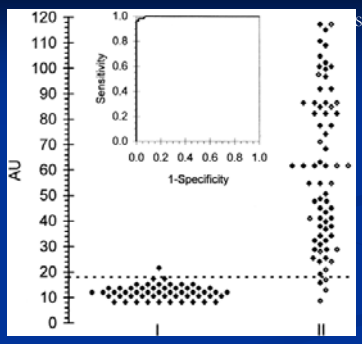
- Sensitivity/Specificity
- Accuracy/Precision




- sensitivity = a/(a+c)
- specificity = d/(b+d)
- likelihood ratio (LR+) = sensitivity / (1-specificity) = (a/(a+c)) / (b/(b+d))
- likelihood ratio (LR-) = (1-sensitivity) / specificity = (c/(a+c)) / (d/(b+d))
- positive predictive value = a/(a+b)
- negative predictive value = d/(c+d)



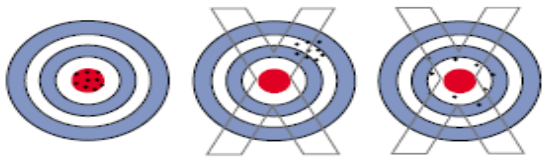

## Laboratory Testing



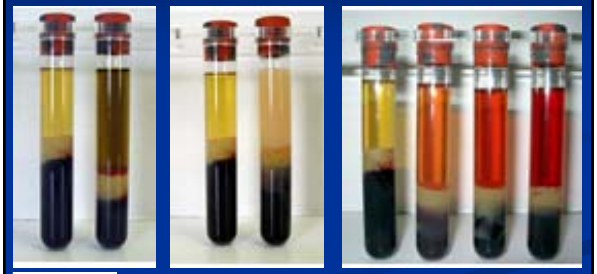

Serum concentrations of IgA antibodies against TGc in the human TGc ELISA system in controls (*I*) and in patients having CD or DH (*II*). ◦, treated CD or DH patients; ♦, controls and untreated CD or DH patients. The chosen arbitrary cutoff for positivity (*dashed line*) is drawn at 18 AU. The ROC curve for the human TGc ELISA is shown as an *inset*.

Recombinant Human Tissue Transglutaminase ELISA for the Diagnosis of Gluten-sensitive Enteropathy *Clinical Chemistry December 1999 vol. 45 no. 12 2142-2149*

## Accuracy and Precision




## Sample condition

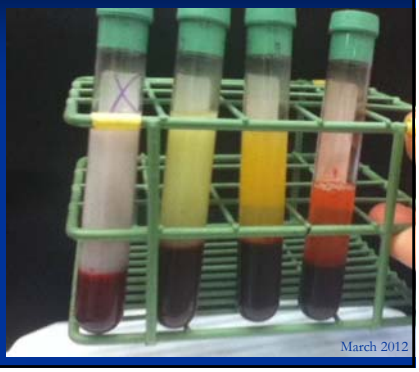


Volume of Tube
Transport temperature of Tube
Time from phlebotomy to testing

## Sample Condition

Blood from a clinical trial patient.

Obtained at the start of surgery.

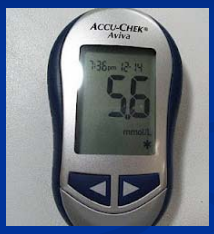


March 2012

## Selecting the right test

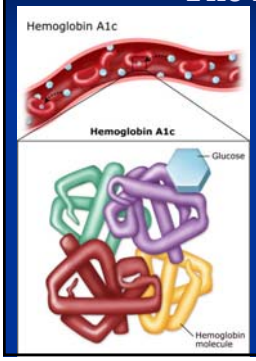
- Eg. Screening for Diabetes

## Diabetes Screening



- The right instrument

## Diabetes Screening The Right Test





## Selecting the right test

- Updated algorithms
- *In vivo* vs. *In vitro*

## Development of HIV immunoassays

| Generation | 1st | 2nd | 3rd | 4th |
|---|---|---|---|---|
| Antigen<br>Sample<br>Conjugate<br>Signal |  |  |  |  |
| ***Antigen*** | ***Lysate*** | | ***Recombinant & synthetic*** | |
| Specificity | 95–98% | >99% | >99.5% | 99.5% |
| Sensitivity | 99% | >99.5% | >99.5% | >99.8% |
| Window period | 8–10 weeks | 4–6 weeks | 2–3 weeks | 2 weeks |
| Immunoglobulin class detection | IgG | IgG | All | All |
| Approximate year of first release | 1985 | 1987 | 1991 | 1997 |
| Platforms | Plate assays<br>Particle agglutination | Plate assays<br>Automated generic platforms<br>Particle agglutination<br>Rapid assays | Plate assays<br>Dedicated instruments<br>Rapid assays | Plate assays<br>Dedicated instruments<br>Rapid assays<br>in development |

Future Microbiol. 2009;4(8):963-982.
http://www.medscape.com/viewarticle/715166_2

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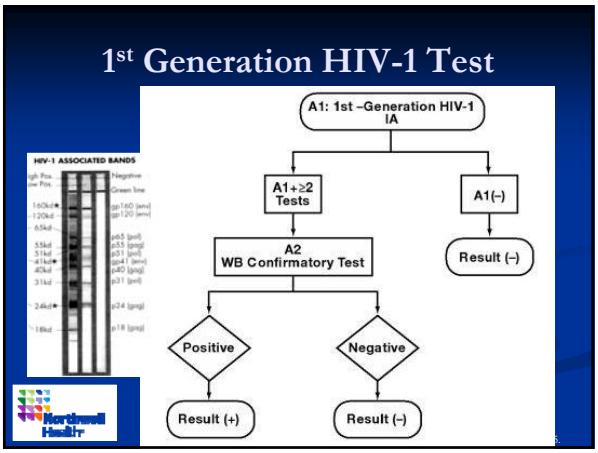
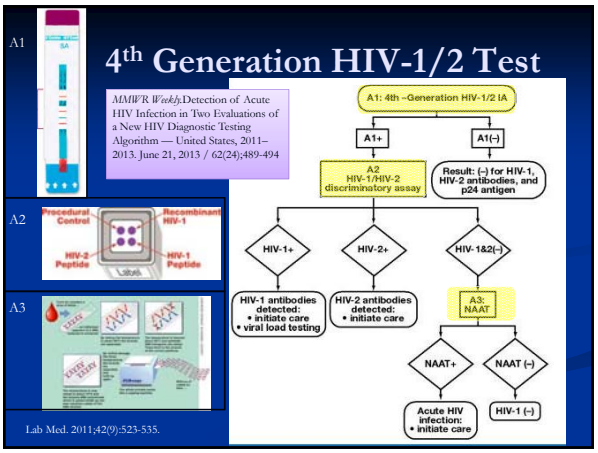
## 1st Generation HIV-1 Test




## 4th Generation HIV-1/2 Test



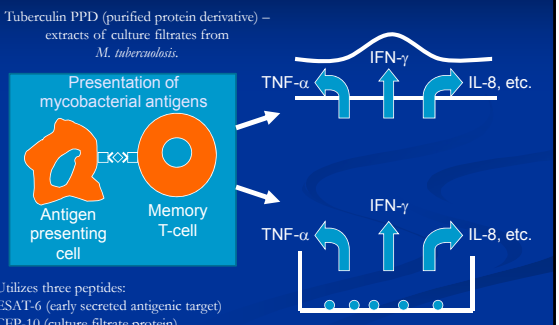

## Case: Newborn

Ex-36 wk, SGA (BW 1906 kg) born to a G6P21112 mother via C/S due to late decels

| Test | Mother | Baby |
|---|---|---|
| 1st TM HIV EIA | Neg (Oct, Nov 2013) | |
| 3rd TM HIV EIA | Neg (5/20/14) | |
| L&D HIV Rapid EIA | Pos x 2 (5/23/14) | |
| 4th Generation HIV | Neg (5/23/14) | |
| HIV Viral Load | Pos: 118,000 RNA copies | |
| HIV Multispot | Neg | |
| HIV 4th Generation EIA | | Neg |
| HIV Newborn screen | | Neg |
| HIV Newborn NYS labs | | Neg Multispot, Neg PCR |
| HIV Viral Load | Neg (5/27/14, 5/29/14) | Neg (5/26/14, 5/27/14) |

## *In Vivo* and *In Vitro* Diagnostic Tests

Tuberculin PPD (purified protein derivative) – extracts of culture filtrates from *M. tuberculosis*.




Utilizes three peptides:
ESAT-6 (early secreted antigenic target)
CFP-10 (culture filtrate protein)
TB7.7

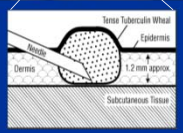
Andersen P. et al. Lancet 2000;356:1099

## Tuberculin Skin Testing

### Mantoux Method


48 to 72 hours

Interpretation depends on Patient Risk Factors




**Induration ≥5 mm**
- HIV-infected
- Recent contact with a person with TB disease
- Fibrotic changes on CXR c/w with prior TB
- H/O organ transplants
- Immunosuppressed for other reasons (e.g., taking the equivalent of >15 mg/day of prednisone for 1 month or longer, taking TNF-α antagonists)

**Induration≥10 mm**
- Recent immigrants (< 5 yrs) from high-prevalence country
- Injection drug users
- Residents and employees of high-risk congregate settings
- Mycobacteriology laboratory personnel
- Clinical conditions that place them at high risk
- Children < 4 years of age
- Infants, children, and adolescents exposed to adults in high-risk categories

**Induration≥15 mm**
- any person including persons with no known risk factors for TB.

## Whole Blood IFN-γ Assay

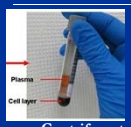
### QuantiFERON-TB Test

Stage 1 Whole Blood Culture

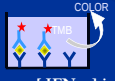
Draw blood into special tubes

Incubate → INF-γ is released from sensitized T-cells

Stage 2 IFN-gamma ELISA




Centrifuge to separate plasma from cells




Measure [ IFN-γ ] in 'Sandwich' ELISA

Computerized interpretation

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## *Quanti*FERON®-TB Gold Test
### Advantages and Disadvantages

- Advantages:
  - Only one visit required
  - Objective and reproducible; not operator-dependent
  - No cross reactivity with BCG, little cross-reactivity with non-tuberculous mycobacteria
  - Controls for low or no immune response
  - No chance of ulceration due to brisk skin test reaction
- Disadvantages:
  - Blood must be received in lab within 16 hours
  - Labor intensive for the lab
  - Not much data for some patient groups

## QuantiFERON –TB Gold In-Tube

| Grey | Red | Purple |
|---|---|---|
| NIL | TB Antigen | Mitogen |
| Neg Cntl | Test | Pos Cntl |

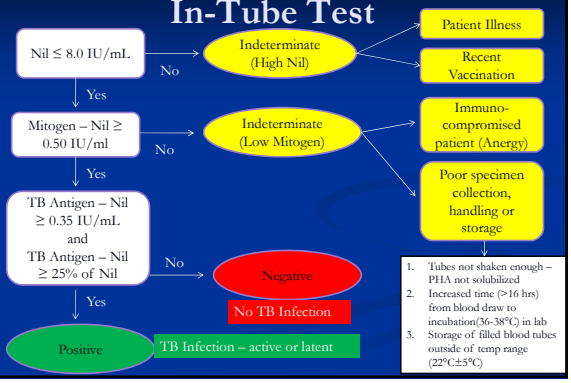
Begin → End

- All tubes have a white ring on the top of the cap.
- Draw samples in this order! "GRaPe"
- Collect 1 mL of blood directly into each of the Quantiferon-TB Gold IT blood collection tubes.
- Should always be drawn last if other blood samples are being collected.
- Blood draws slowly into tubes. Keep the tube on the needle for 2-3 seconds once the tube appears to be filled.

## Interpreting the Results

- Amount of IFN-γ (IU/mL) is reported from 3 tubes:
  - Nil
  - TB Antigen
  - Mitogen
- Calculations:
  - TB Antigen-Nil
  - Mitogen-Nil
- Analysis
  - Positive
  - Negative
  - Indeterminate

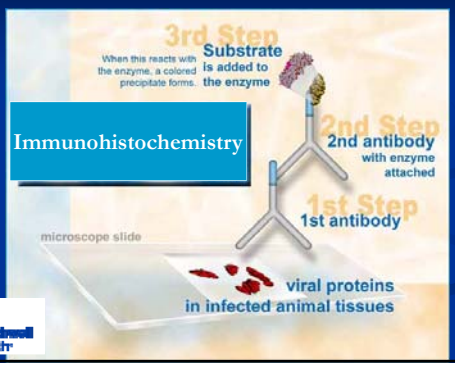
## Interpretation of QTF-TB Gold In-Tube Test



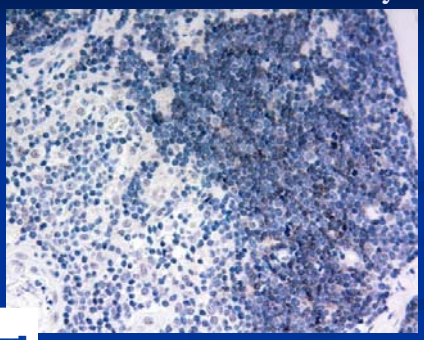

## Selecting the right Molecular Method

- Immunohistochemistry vs In situ hybridization
- PCR vs. Quantitative PCR
- Understanding ELISA results - "titers"
- Blotting – Western, Northern, Southern…

## Immunohistochemistry

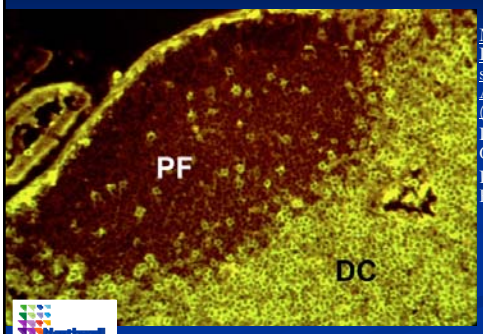


## Immunohistochemistry



CD4 staining of thymus

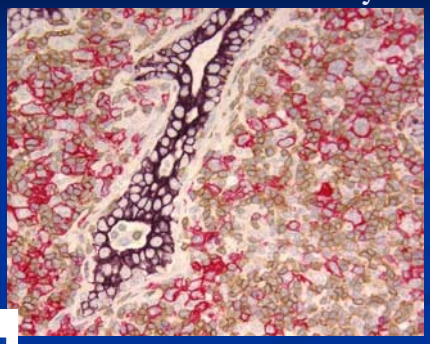
## Immunohistochemistry




Mouse Lymph Node stained with Anti-CD3 (yellow)
Diffuse Cortex (DC)
Primary Follicle (PF)

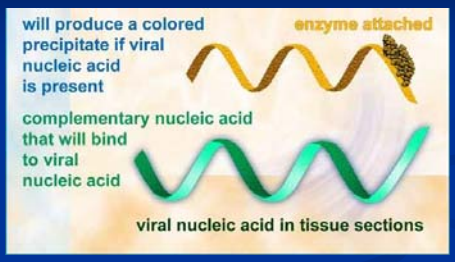
http://jeeves.mmg.uci.edu/immunology/Assays/LNanti-T.htm

## Immunohistochemistry

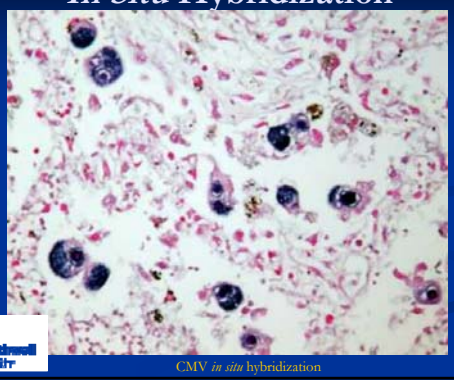


CD3=DAB (brown) T-cells ; CD20 AlkPhosRed (red) B-cells

## *In Situ* Hybridization




## *In Situ* Hybridization



CMV *in situ* hybridization

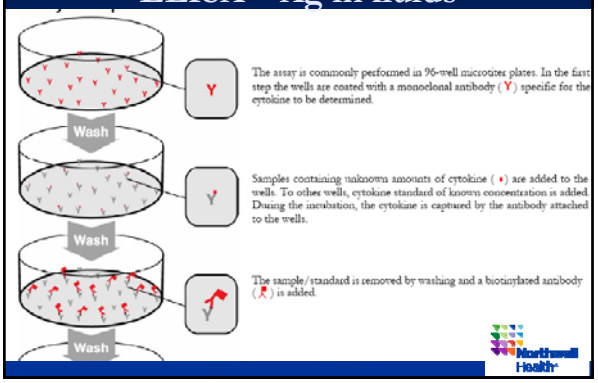
## Immunohistochemistry vs. *In Situ* Hybridization

- Both help you find which cells are making your gene/protein of interest
- Immunohistochemistry uses an antibody to find a protein in the cell
- *In Situ* Hybridization classically uses DNA or RNA (oligonucleotides) to find nucleic acids in the cell.

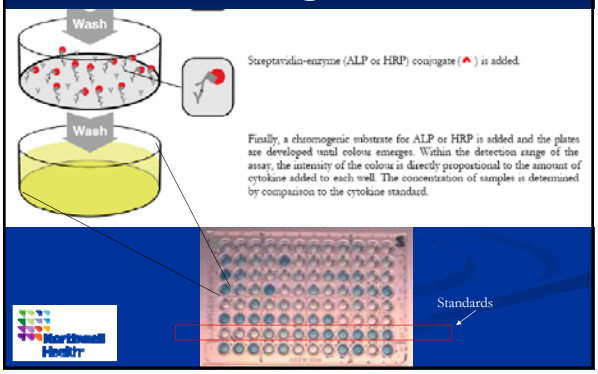
## ELISA

ELISA measures the amount of antigen in liquid phase
ELISPOT measures the number of cells with antigen on them

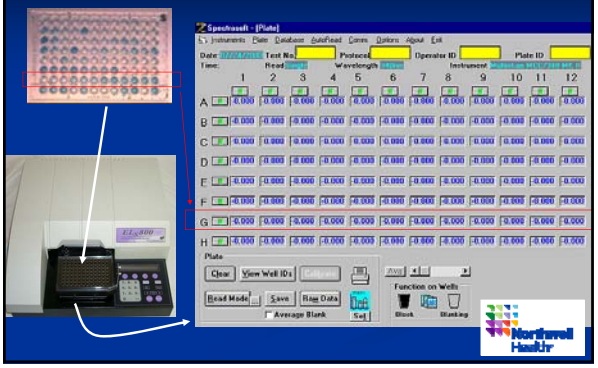
## ELISA – Ag in fluids



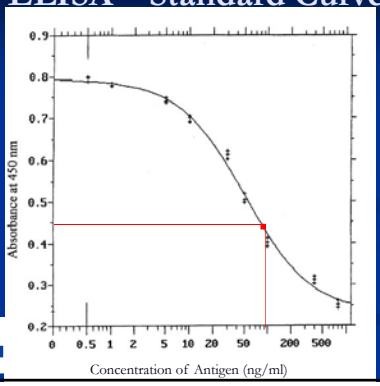

## ELISA - Ag in fluids




## ELISA



## ELISA – Standard Curve

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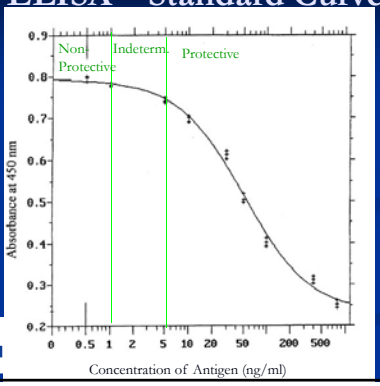


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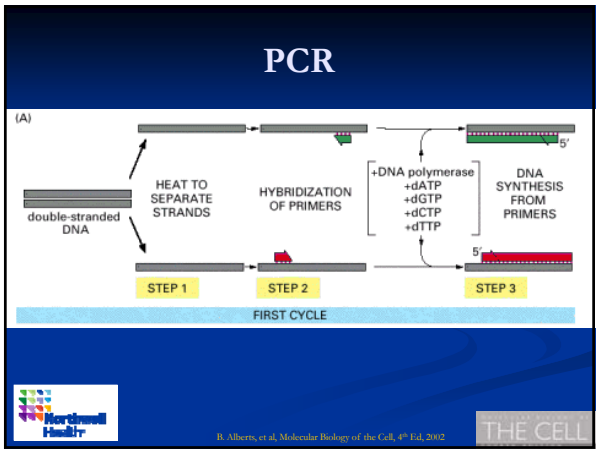


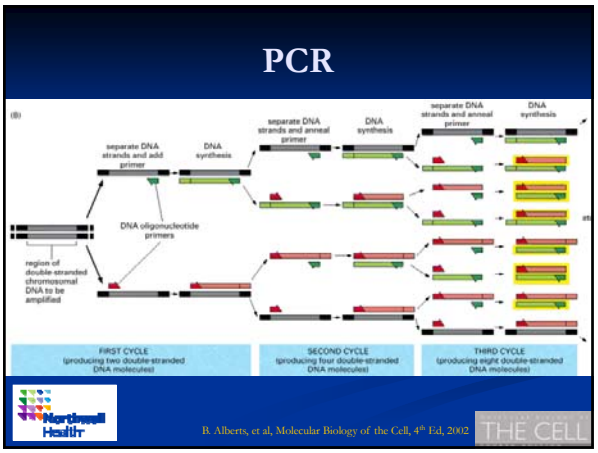


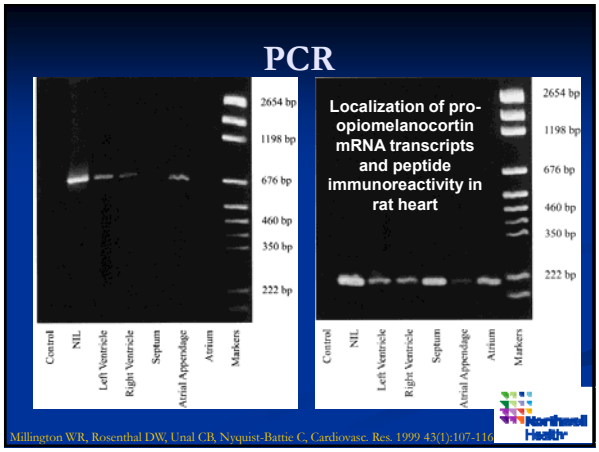


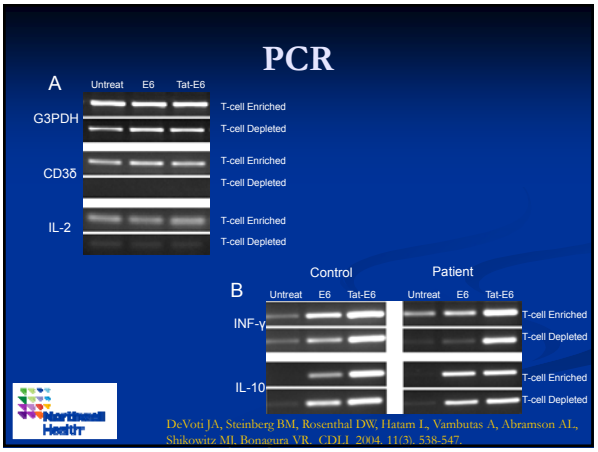


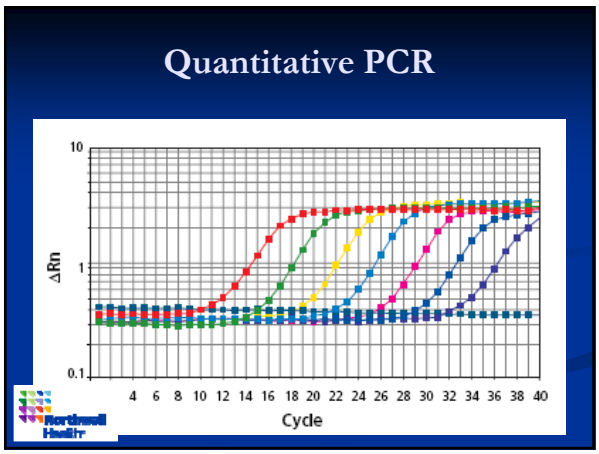


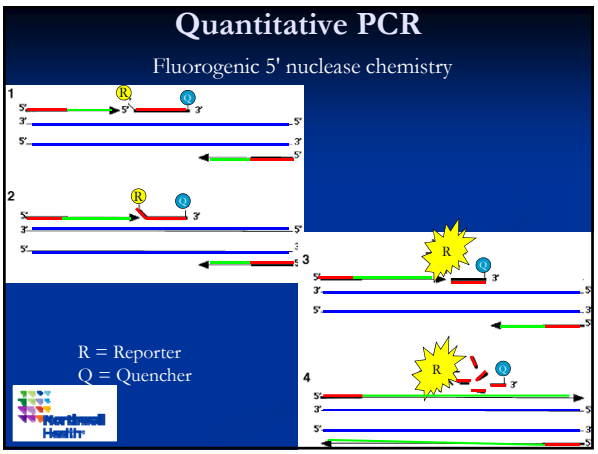


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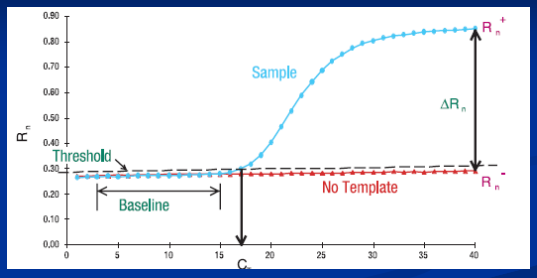
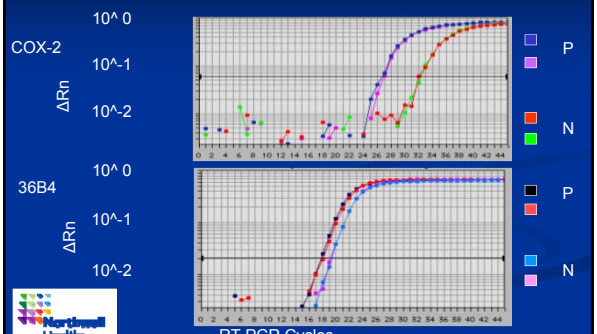
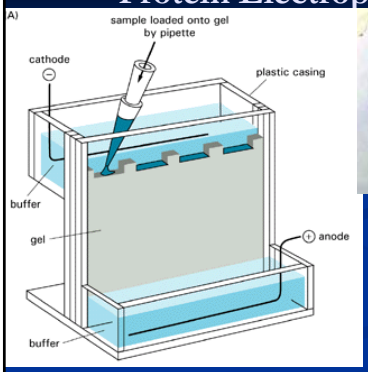
## Quantitative PCR




## Quantitative PCR



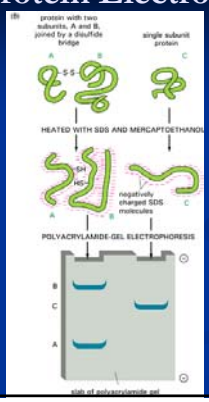

## Protein Electrophoresis

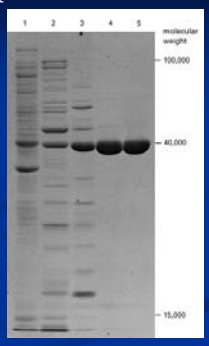



Commassie Blue stain (Steven Burg, Winona State Univ.)

Alberts *et al.* Molecular Cell Biology , figures 4-42 and 4-43

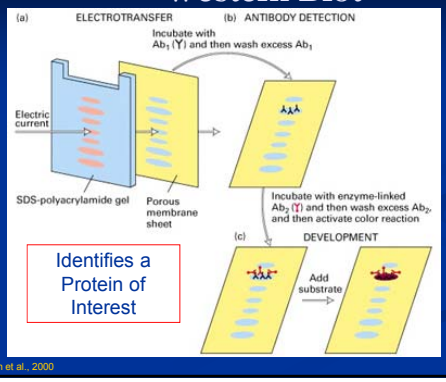
## Protein Electrophoresis




Alberts *et al.* Molecular Cell Biology , figures 4-42 and 4-43

## Western Blot



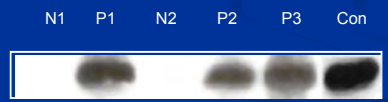

Identifies a Protein of Interest

Western Blots
1. Electrophoresis
2. Transfer
3. Block
4. Primary Ab
5. Secondary Ab
6. Substrate
7. Develop

Lodish et al., 2000

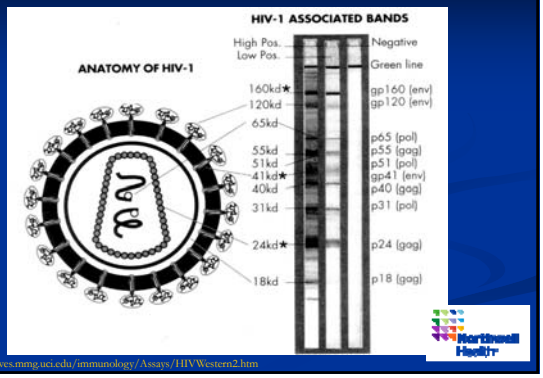
## Western Blot

Serial dilutions of HSP 70 (initially diluted 1/5) were electrophoresed on a 4% to 20% SDS Page gel. The gel proteins were transferred to a nitrocellulose membrane, blocked, and reacted with mouse anti-HSP 70 (1:50,000), followed by a 1:50,000 dilution of HRP labeled goat anti-mouse IgG. The membrane was washed and incubated in BioFX chemiluminescent HRP substrate for 5 minutes prior to film exposure.




Rosenthal DW, Devoti JA, Wu R, Steinberg BM, Bonagura VR, HPV-6 Up-Regulates Cyclooxygenase-2 in Recurrent Respiratory Papillomatosis (RRP) AAAAI 2004 Poster Presentation

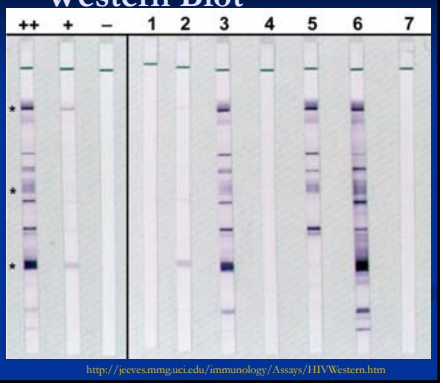
## Western Blot




http://jeeves.mmg.uci.edu/immunology/Assays/HIVWestern2.htm

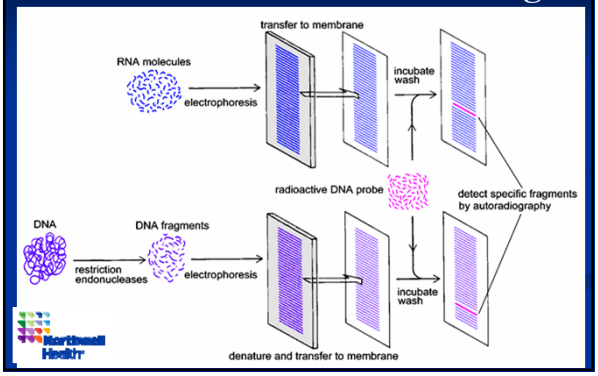
## Western Blot

- HIV
  3 bands

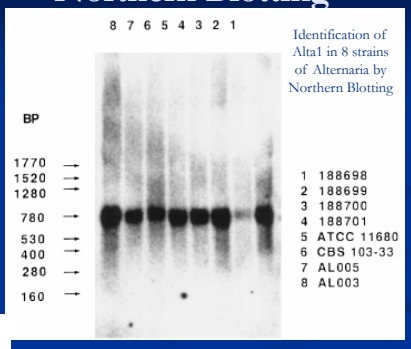



http://jeeves.mmg.uci.edu/immunology/Assays/HIVWestern.htm

## Southern and Northern Blotting




## Northern Blotting




Identification of Alta1 in 8 strains of Alternaria by Northern Blotting

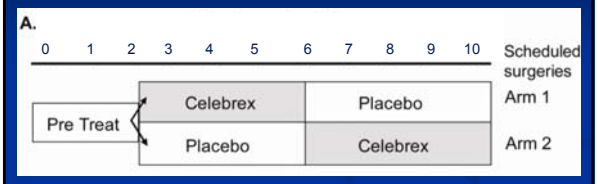
Rosenthal et al, Ann All Asthma 1998, 80: 471-5

## Blotting… how to avoid confusion

- Dr. EM **Southern** first described "Detection of specific sequences among DNA fragments separated by gel electrophoresis." (J Mol Biol. 1975)
- Thus it was Blotting is described as:
  - Southern=DNA
  - Northern=RNA
  - Western=Protein

## Study Design and Interpreting the Data

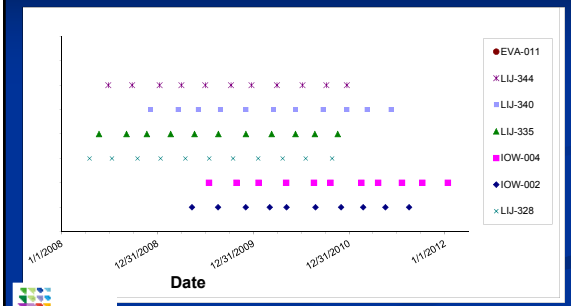
## Clinical Trial Example



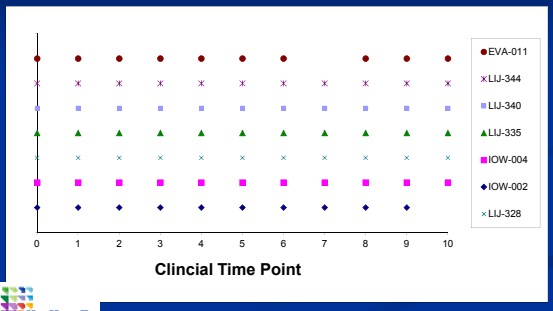

- Each Study visit is 3 months apart from the previous one

Adapted from Lucs A et al, Molecular Medicine, 2012

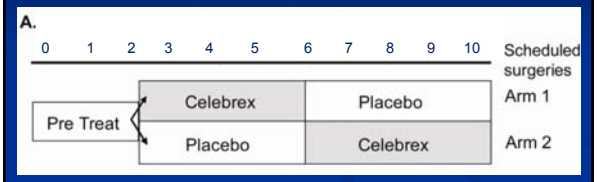
## Data Transformations




## Data Transformations




## Clinical Trial Example

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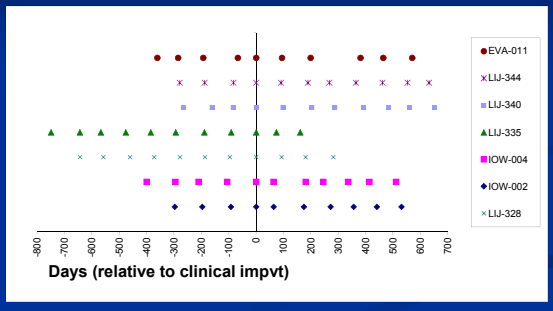


## Question 1

An infant born to a women infected with HIV has a positive Rapid HIV1 ELISA (3rd generation) at 2 months of age. What should you do first?

- A. Start HIV Antiretroviral medications as soon as possible.
- B. Repeat the Rapid HIV1 ELISA STAT.
- C. Order an HIV discriminatory test STAT.
- D. Repeat the HIV1 test test in 2 months.
- E. Recheck the specificity of the assay.

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## Question 2

Your research coordinator tells you that the average fasting blood glucose in your healthy general pediatric patient population (n=253) is 125 mg/dl. What should you do for patients with high glucose?

- A. Institute an evidence based medicine training session for all clinical staff to instruct them in proper diabetes screening.
- B. Look at the data to understand why the patients all have high fasting glucose.
- C. Begin treatment for DM
- D. Order oral glucose tolerance tests
- E. Order HgbA1c

## Question 3

Fifty percent of the Quantiferon Gold Test-in-Tube (QFN) results are indeterminate. What should you do?

- A. Repeat QFN on all patients with indeterminate results
- B. Consider those patients with indeterminate results as screened for TB.
- C. Call the lab to understand how the QFN test is done.
- D. Refer all indeterminate patients to Peds ID for potential treatment.
- E. Place a PPD on all of the patients with indeterminate results.

Northwell Health Division of Allergy/Immunology

Adult, Adolescent and Pediatric Allergy, Asthma, and Clinical Immunology

- HIV/AIDS (Age ≤ 30 years old)
- Primary Immune Deficiency
- Allergic Rhinitis
- Urticaria / Angioedema
- Food / Medication Allergy
- Eczema/Atopic Dermatitis
- Asthma

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