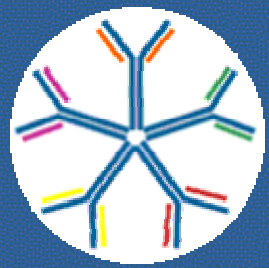
Methods for Detecting and Measuring Ag Biotech Products



AEIC

October 2004

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AEIC is pleased to provide the following slide presentation for use in educational or training applications associated with detection methods for biotech products. Due to the size of the file, this presentation is provided as a PDF, which does not allow for any changes in content. For a copy of the presentation on a CD please contact AEIC.

AEIC wishes to acknowledge the following companies and organizations for their contributions to the slides included in this presentation:

- EnviroLogix
- GeneScan
- Medallion Labs
- Monsanto
- SDI
- USFDA

Ag Biotech Crops

- **Transgenic plants have:**
  - Novel trait (e.g., herbicide resistance)
  - May express novel protein
  - Novel DNA
- **Novel DNA and protein may be found in:**
  - Plant tissues
  - Seed/Grain
  - Food ingredients and food products

Biotech Crops 2004

- **2 major traits**
  - Insect protection - *Bacillus thuringiensis* (Bt)
  - Herbicide tolerance
    - Roundup Ready (RUR)
    - Liberty Link (LL)
    - Bromoxynil tolerance (BXN)
- **4 major crops**
  - Corn - Bt, RUR, LL
  - Soy - RUR
  - Canola - RUR, LL
  - Cotton - Bt, RUR, BXN

Testing in Support of Labeling Biotech Foods

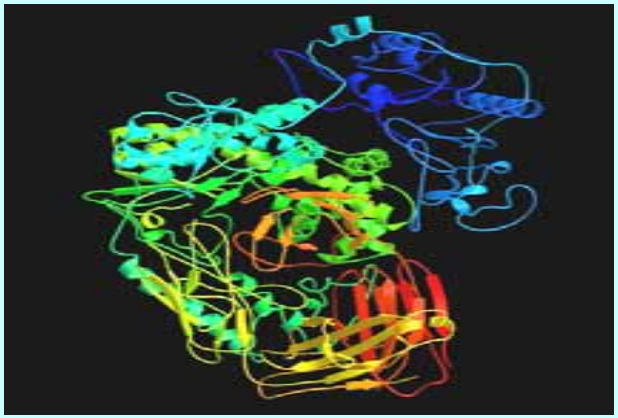
- **Consumer Choice**
- **Approved Events – Quantitative and threshold testing**
  - European Food Labeling Law
    - Labeling began April 10, 2000 and updated April 2004
    - Threshold adventitious < 0.9% - "genetically modified"
  - Japanese Food Labeling Law
    - Labeling began April, 2001
    - Threshold guidelines set at 5%
- **Unapproved Events - Detection**
  - Zero tolerance e.g. StarLink
  - European Food Labeling Law
    - Threshold adventitious < 0.5% - "genetically modified"

Determining Concentration of Biotech Ingredients in Foods

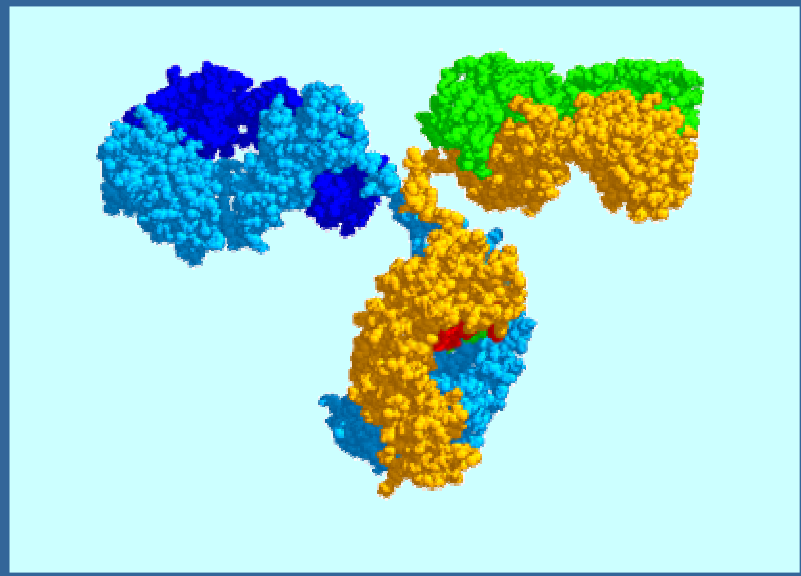
- **Results are reported in terms of % Ag Biotech**
  e.g. 1 Biotech corn kernel in 99 negative = 1%
- **Decisions are based on regulated thresholds (given in weight %)**
- **Testing is based on detection/quantitation of novel DNA or protein**
- **Ag Biotech concentrations are estimated from protein concentration**
- **DNA can be measured in relative terms, i.e. % Roundup® Ready soybeans with respect to total soybean**

Commonly Used Detection Methods

DNA-based methods
PCR

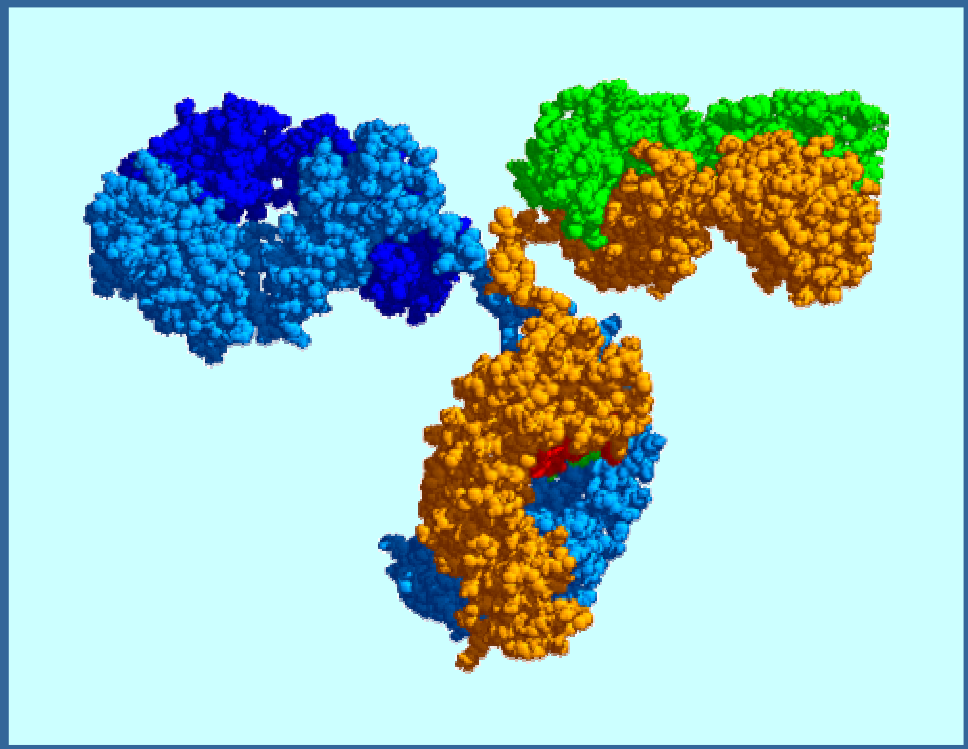


Protein-based methods
Immunoassay (ELISA)



Immunoassay

An immunoassay is an analytical method which uses antibodies as reagents to quantitate specific analytes (proteins)



Tertiary Structure of an Antibody Molecule

Immunoassays

- $6 Billion Industry Worldwide
- 2.5 Billion Tests Sold Annually
- Highly Quantitative
- Recognized by Regulatory Agencies
- Flexible Test Formats
- Diverse Markets and Applications

Principles of Immunochemistry

- **Antibodies physically bind target analytes**
- **Strength of binding determines sensitivity**
- **Specificity**
  - Broad or specific (screening or quantitative)
  - Allows detection in complex matrix
    - Minimum sample preparation
    - Field-portable tests
- **Sensitivity and specificity determined by antibody and assay conjugate pair**

Protein Detection Methods

- Capillary Electrophoresis
- Chromatography
- Mass Spectrometry
- Immunoassay

Other Immunoassay Markets

- Agricultural
- Environmental
- Veterinary
- **Food**
- Industrial
- Pharmaceutical
- Water Quality

- Microorganisms
- Allergens
- Hormones
- Toxins
- Pesticides
- Antibiotics
- Species identification
- Food adulteration
- Quality

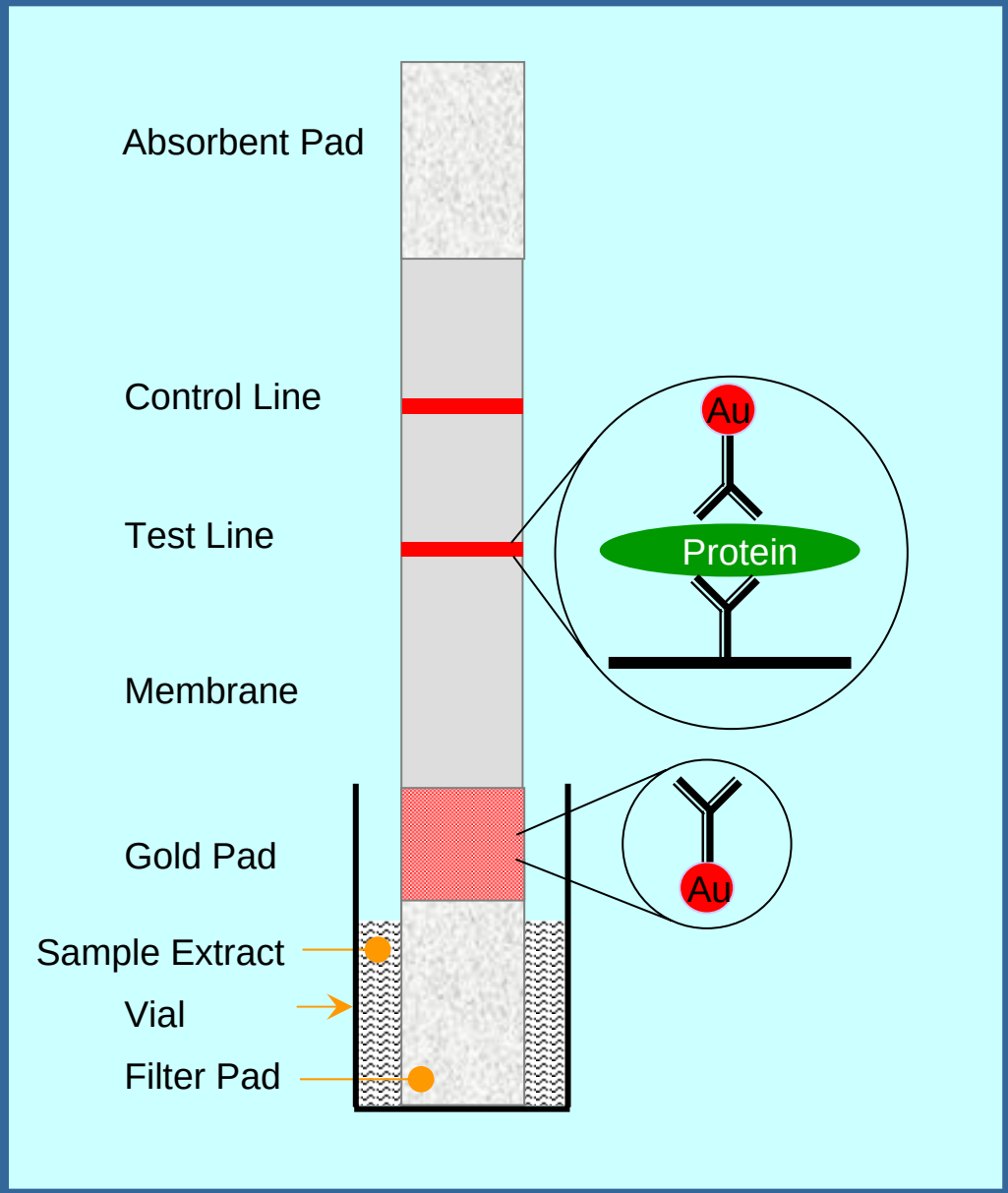
Immunoassay Formats

- **ELISA, Striptests, Western Blot**
- **Each format has advantages and disadvantages**
  - Fully automated – clinical analyzers
  - Laboratory kits - ELISA
  - Field tests – "Strip tests"
  - 'Research' methods – Western blot
- **Choice of method is determined by specific application**
  - Performance specifications
  - Ease-of-use (user training)
  - Testing location
  - Cost per test
  - Batch size, testing frequency
  - Turnaround time
  - Equipment costs

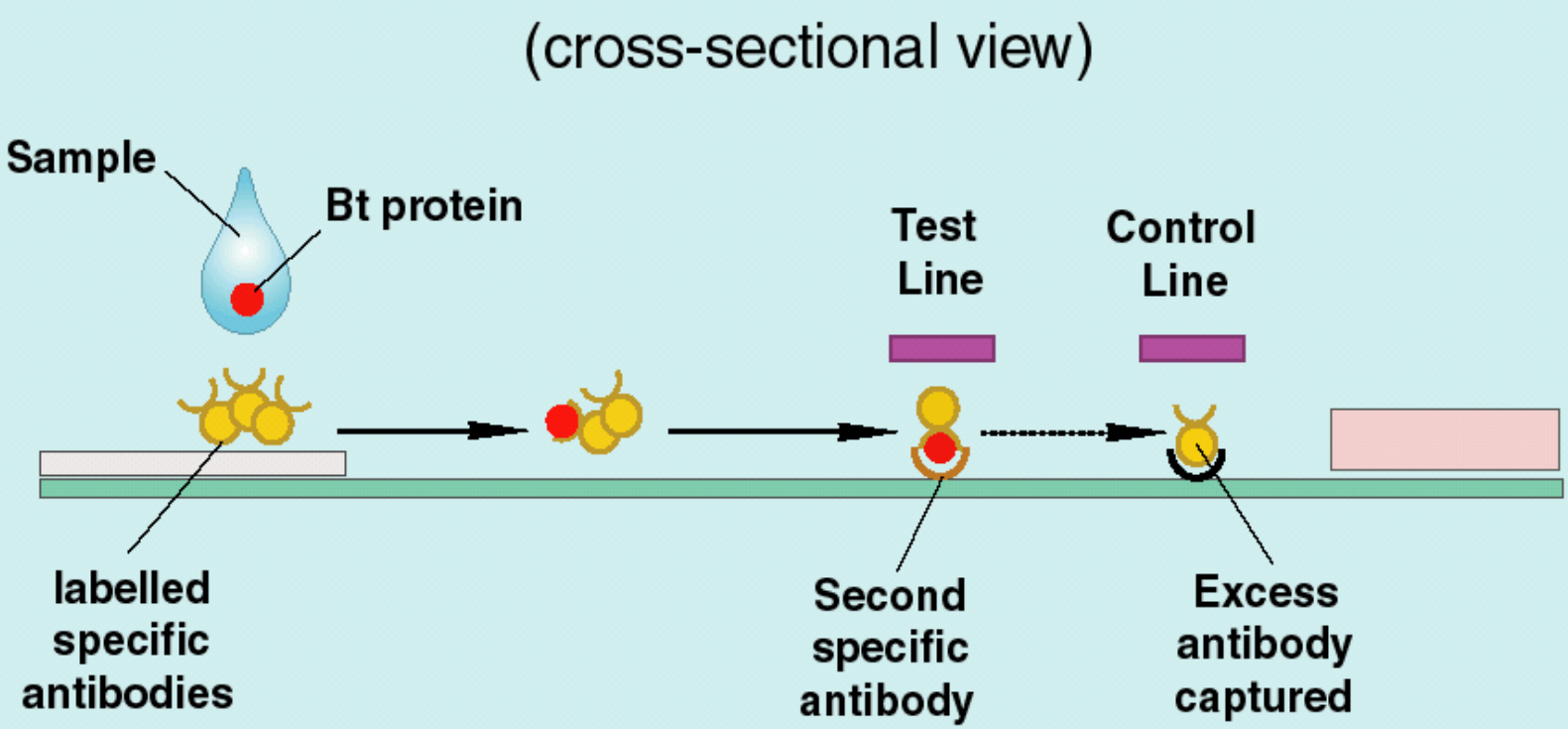
Lateral Flow Format

- 5 minute yes/no results
- Simple procedure for testing anywhere

Strip Test



Lateral Flow Format

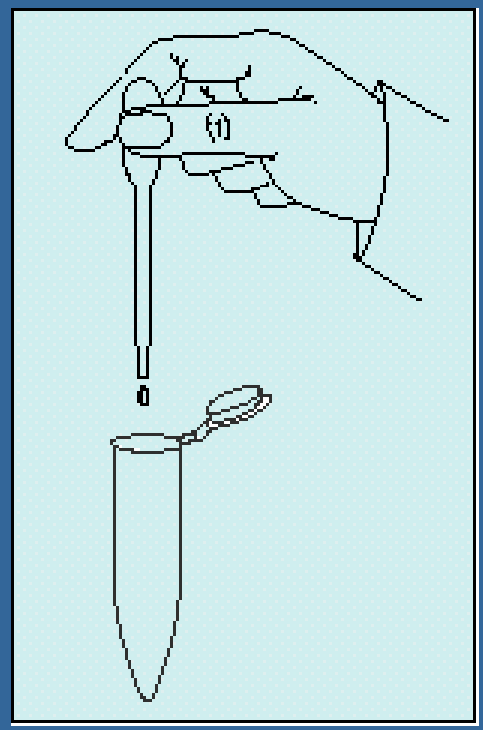


Lateral Flow Strips Testing Procedure

Extraction Step

1. Grind representative sample
   Add water (or buffer) & mix

2. Transfer extracted sample to vial



Lateral Flow Test Procedure

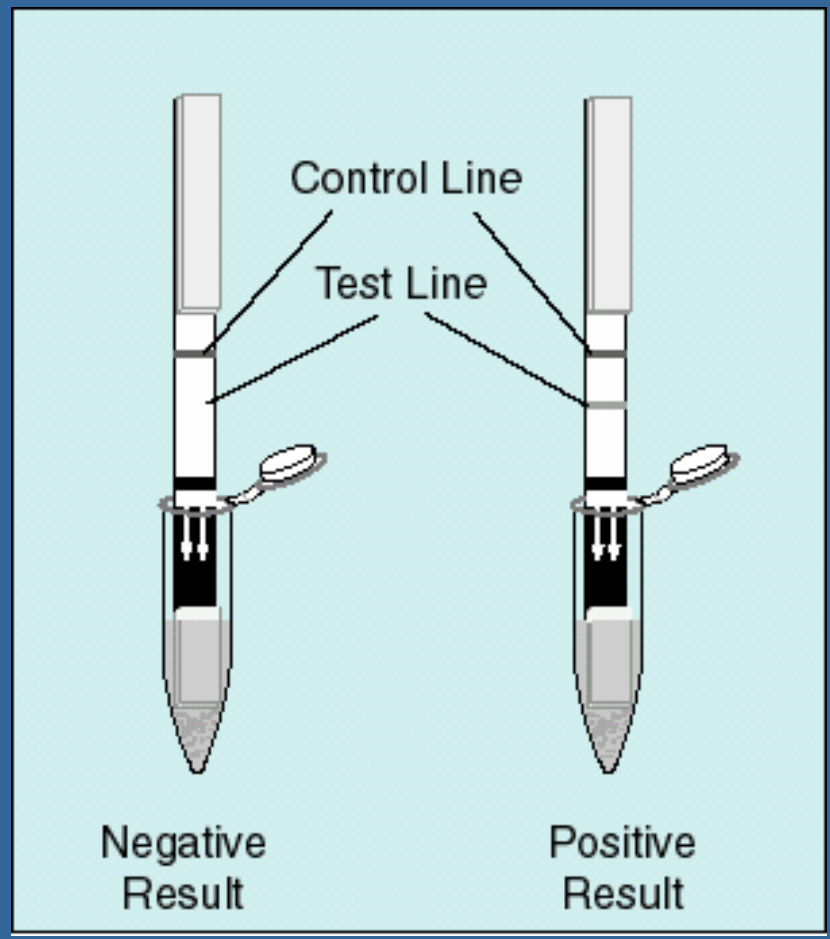
3. **Insert strip into vial**

4. **Read Results**

Negative = No test line, but control line visible

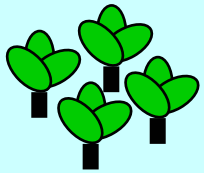
Positive = Visible test line and visible control line

Invalid test = No test line
No control line



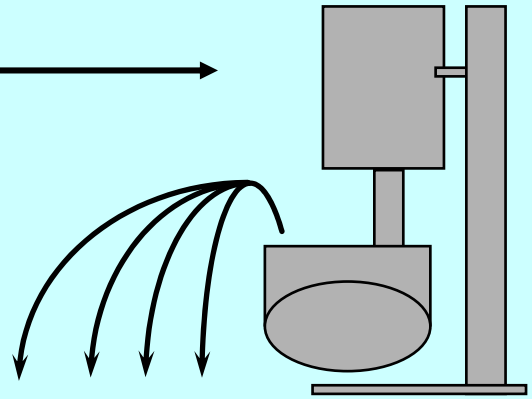
Steps in Determining Protein Expression by ELISA

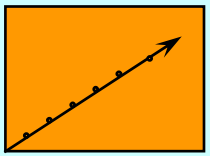
Step 1
Sample plant tissue



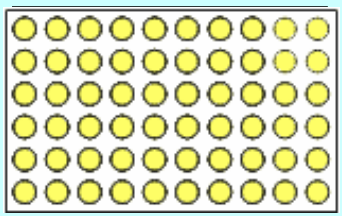
Step 2
Weigh Sample Aliquots

Step 3
Extract Samples

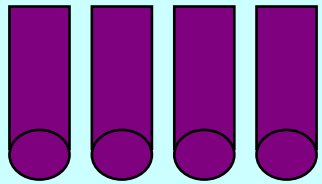




Step 6
Data analysis



Step 5
ELISA Analysis



Step 4
Aliquot extracts for analysis
and sample back-ups

Biotech Protein - Expression and Detection

- Expression of new protein ranges from ng/g to μg/g
- Cell contains many host proteins at very high concentrations
- Total protein methods not specific and not appropriate (e.g., Kjeldahl nitrogen, Near InfraRed)
- Method must be sensitive and specific

Immunoassay Development Process

- **Define Performance Characteristics**
  - Sensitivity and specificity are determined by the nature and amount of antibody and the assay conjugate
  - Format determined by application
- **Development Process**
  - Antibody and assay conjugate design and development
  - Test format and optimization
  - Validation
  - Controlled use of assay, QA/QC
- **1 to 2 Years**

Immunoassay Performance Characteristics

- **Sensitivity (LOD, LOQ) - ppb to ppt (10^{-12} M)**
  LOD - level of detection LOQ - level of quantification
- **Specificity**
  - Families of chemicals vs. single compounds
  - Commercial products
  - Metabolites, degradation products
  - Process by-products, intermediates
- **Precision - repeatability, reproducibility**
- **Accuracy - recovery and false negative/positive rates**
- **Matrix effects/interfering substances**
- **Quantitative range**
- **Stability, Reliability, Robustness**
- **Fitness for purpose**

Assay Validation/Bridging for Key Tissues

- Precision ➡ inter-plate, intra-plate, inter-assay variability
- Accuracy ➡ extraction efficiency, matrix effects, spike and recovery, robustness, ruggedness
- Sensitivity ➡ limit of detection, limit of quantitation
- Specificity ➡ matrix effects, specific for trait

Biotech Immunoassay Method Validations

- **Collaborative studies**
  - AACC
    - MON810 Cry1Ab ELISA – ground corn
    - StarLink Cry9c ELISA – corn flour and meal
  - Joint Research Centre, European Union
    - Roundup Ready® ELISA
      - IRMM ground soybean certified reference materials
      - Soy toasted meal, protein isolate, defatted flakes
  - FDA
    - StarLink Cry9c ELISA – processed food fractions
- **USDA Certification**
  - Cry9c strip tests – corn kernels
  - Cry9c ELISA - ground corn, meal, flour
  - CP4EPSPS Strip tests - ground corn, soybeans

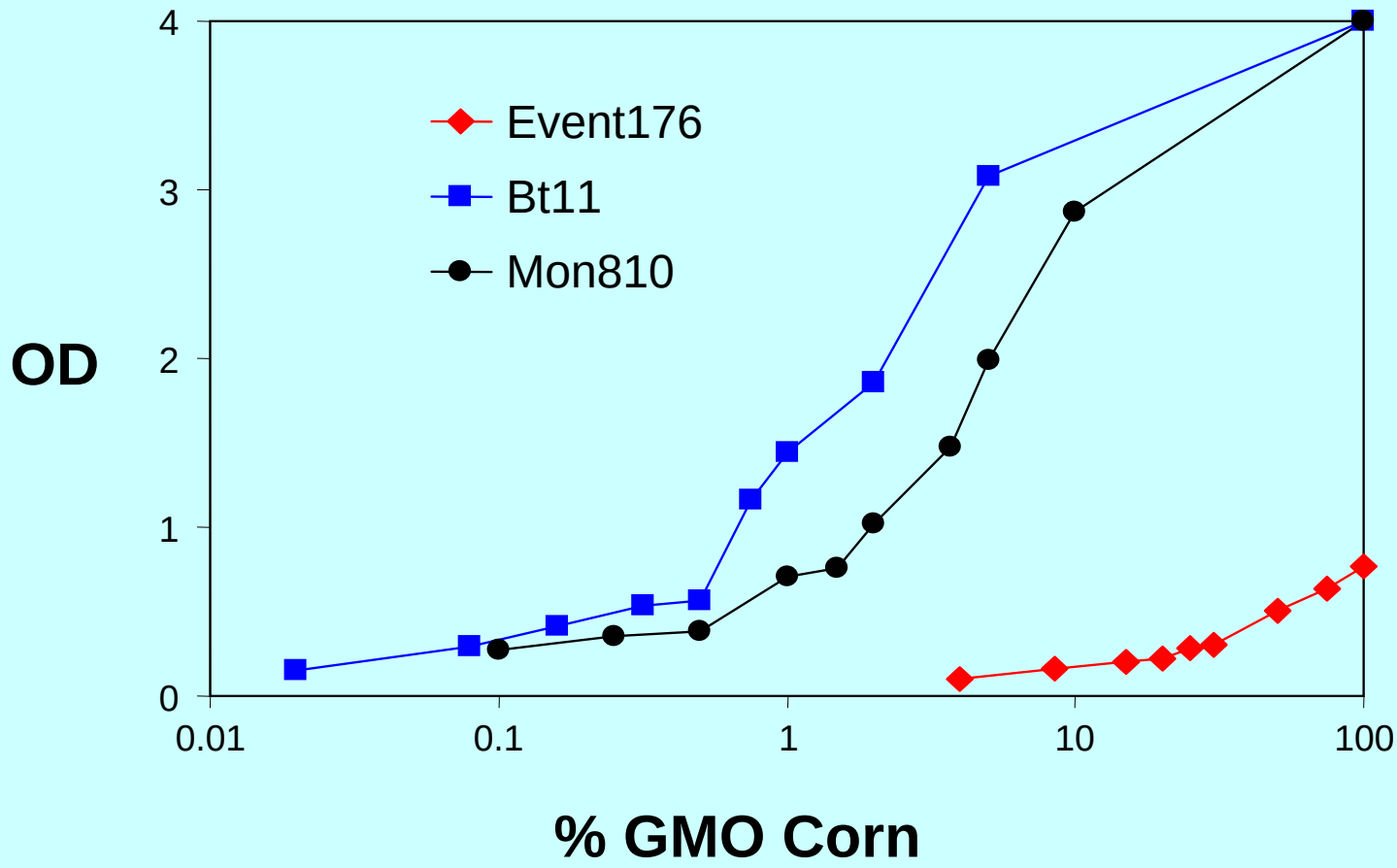
Factors Effecting Use of Protein Immunoassays

- **No protein – no Immunoassay**
- **Very low level expression (e.g. Bt 176)**
- **Crossreactivity (e.g. GA21 Roundup® Ready corn)**
  - Modified corn EPSPS – 2 amino acids of 445 different from native corn EPSPS

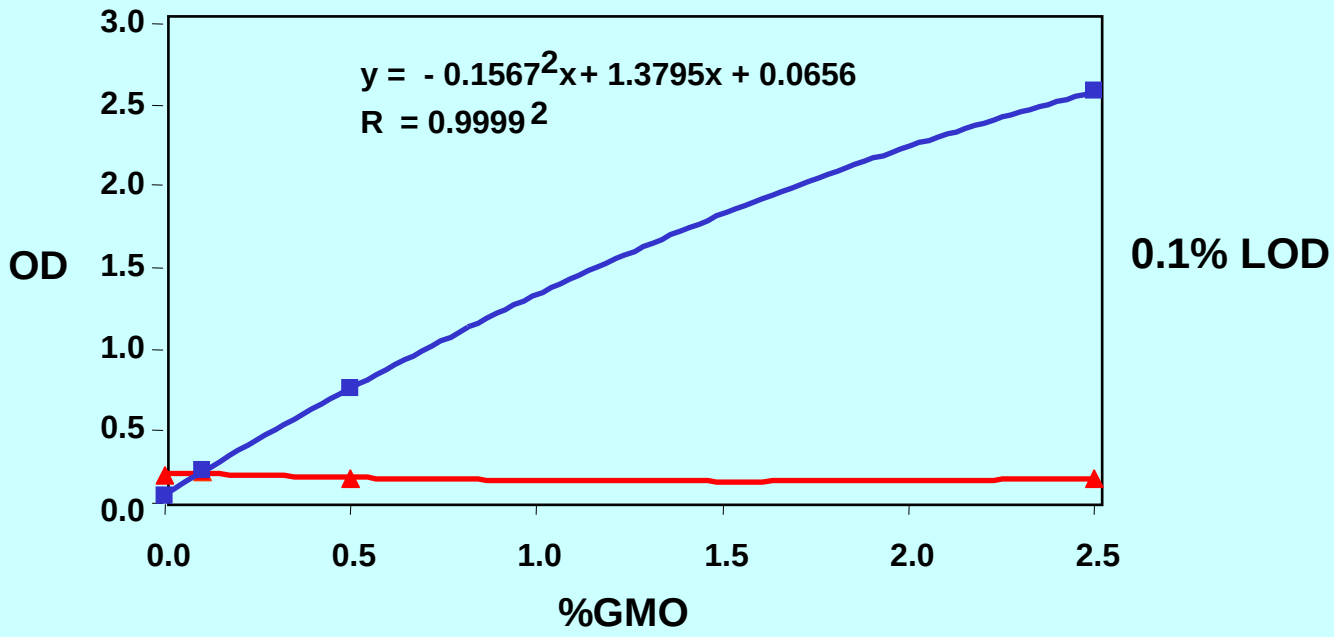
Factors Effecting Quantitation Using Immunoassays

- **Method performance characteristics (e.g. precision, accuracy)**
- **Variability of protein expression levels**
  - Within an event
  - Between events expressing same protein (e.g. Cry1Ab)
- **Varied effects of sample processing on protein conformation and antibody binding**

Reactivities of Different Varieties of Bt Corn in Cry1Ab ELISA



Reactivity of 2 Different ELISA to RUR Toasted Soy Meal



Soymeal was toasted for 60 min. at 100 ºC

Commercially Available Protein-based Tests For Commercial Ag Biotech Traits

Trait	Agdia	EnviroLogix	Neogen	SDI
CP4 EPSPS		E,S	S	E[1],S
Cry1Ab	E,S	E,S	E,S	E[2],S
Cry9C		E[3],S	E,S	E[3],S
Cry2A		E,S		
Cry3A	E			
NK603		S	S	S
	www.agdia.com	www.envirologix.com	www.neogen.com	www.sdix.com

E ELISA

S strip

[1]validated by JRC ring test for Roundup Ready® Soy bean

[2]validated by AACC ring test for YieldGard® (Mon810) Corn

[3]official method AACC international collaborative trial

Advantages of Immunoassay Methods

- Directly measure biologically active protein of interest
- Reliable
- Quantitative analysis
- Qualitative analysis
- High sample throughput
- Easy to perform and transfer to other laboratories
- Widely accepted method by regulatory agencies
- Established use in food industry
- Cost effective
- Timely Analysis

Disadvantages of Immunoassay Methods

- No single method will detect all biotechnology-derived products (DNA or protein-based)
- Methods are trait specific and must be validated for each matrix (DNA and Protein-based)
- Limited to use of protein containing processed ingredients and final food products
- Some products may not express a detectable protein in grain
- Antibodies may cross react

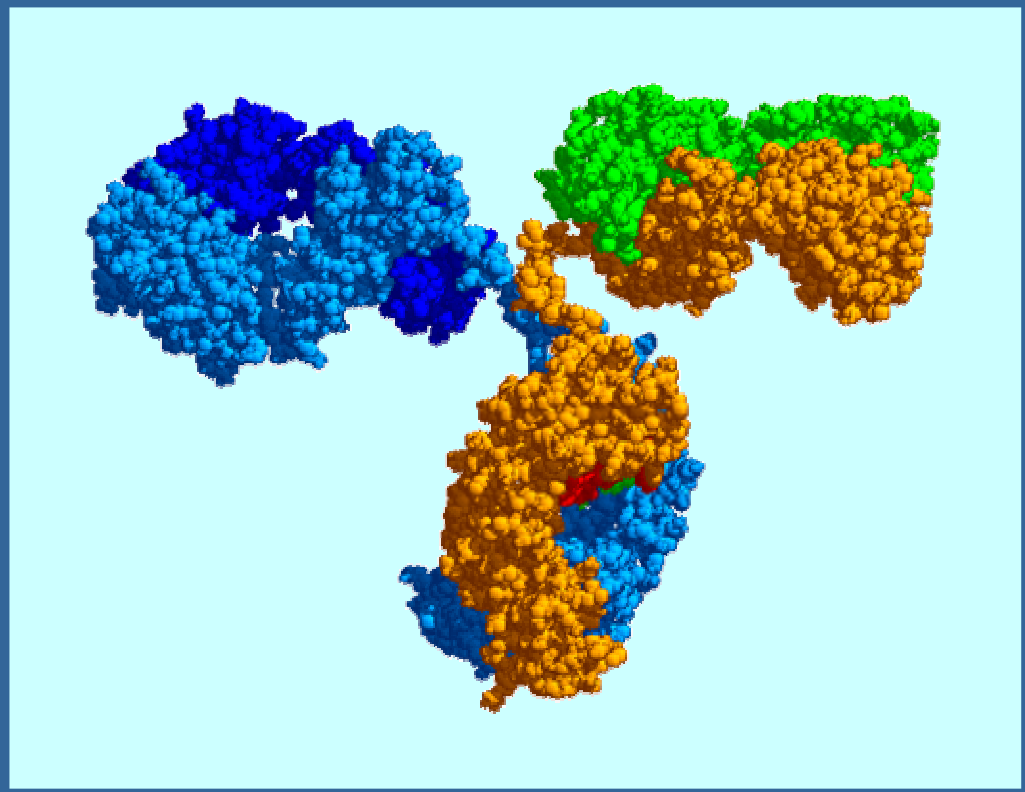
Summary of Protein Testing by Immunoassay

- **Immunoassays are quantitative analytical methods**
- **Flexible format provides for diverse applications**
- **Commercial methods are available for detection of biotech products**
  - Raw agricultural commodities
  - Processed food fractions
- **Methods have been validated internationally**
- **Accurate, reliable and timely analysis**

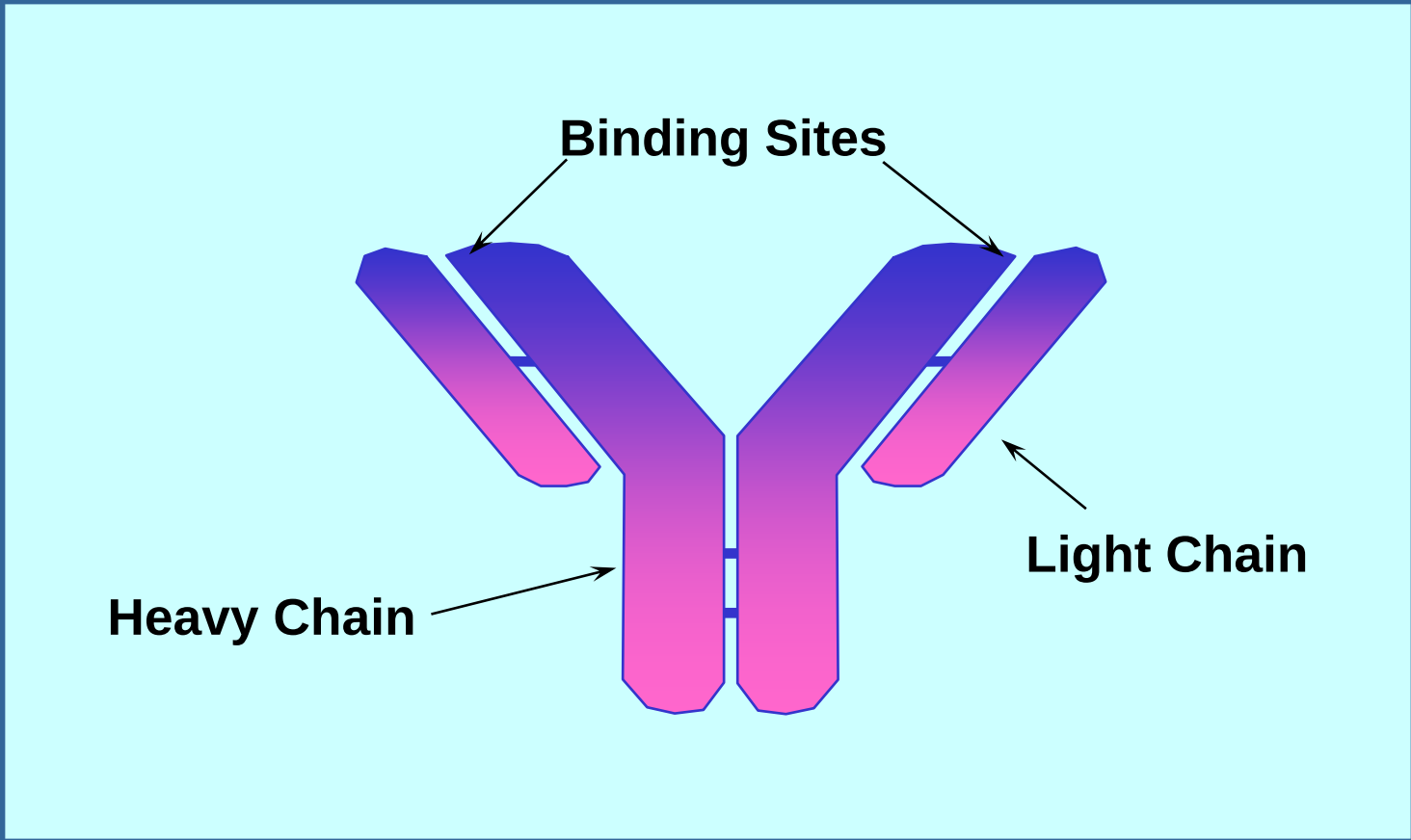
Antibodies

- **Key reagents in all immunoassays**
- **Proteins produced by immune system of higher animals**
  - Produced by specific white blood cells
  - In response to recognition of "foreign" substances
  - Examples:
    - Vaccinations
    - Response to natural infections (mumps, chicken pox)
- **Chemically bind to "Antigens"**
- **Tightly bind only to specific structure in substance which elicited production (Specificity)**
- **Strength of binding (Affinity) determines sensitivity of method**
- **Specificity allows detection in complex matrix - minimum sample preparation**

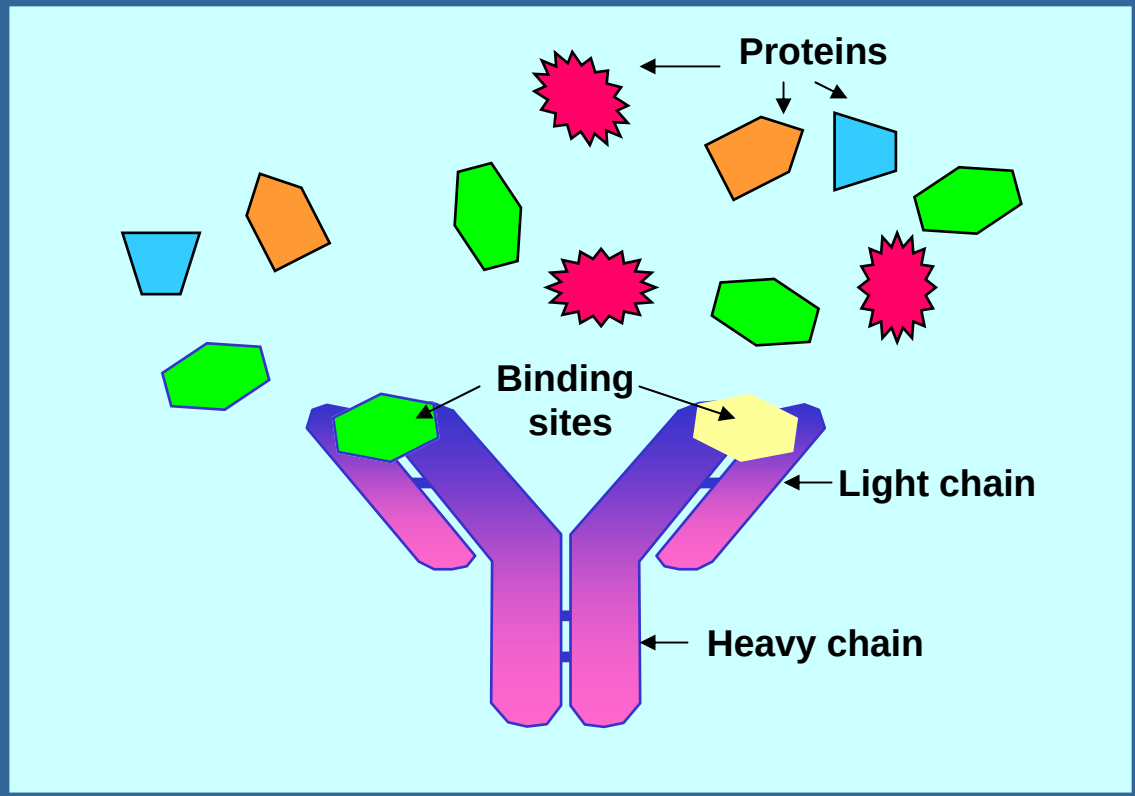
Antibody Structure



Antibody Structure



Antibody-Antigen Specificity



Antibodies only react with the epitope that elicited their production. Antigens must "fit" into the binding site.

Polyclonal Antibodies

- Polyclonal = "many cells"
- Animals (e.g. rabbits) are injected with target protein
- Many different antibody-producing cells make many different antibodies with many specificities
- Each antibody producing cell makes only one antibody with a single specificity
- Polyclonal antibodies purified directly from blood

Monoclonal Antibodies

- Monoclonal = "one cell"
- Mice are injected with the analytical target
- Antibody producing cells are taken from the animals
- Antibody-producing cells are fused with cells that grow continuously in culture to form "Hybridomas"
- A single hybridoma produces only one antibody
- A single hybridoma divides to produce a large population of 'clones' all making the same "Monoclonal" antibody
- Living hybridomas are frozen indefinitely in liquid nitrogen
- Indefinite supply of uniform consistency reagent

Monoclonal vs. Polyclonal Antibodies

Monoclonal	Polyclonal
• Lot-to-lot consistency	• Lot-to-lot variability
• Indefinite supply	• More broadly reactive
• Highly specific	• Often more sensitive
• Longer lead time	• Shorter lead times
• Higher initial costs	• Lower initial costs

Selection is based on application, time and money

Immunoassay Reagents and Test Components

- **Antibodies are purified and attached to a 'solid phase' to provide a means for separating the target protein from the sample**
  - Plastic wells, tubes, capillaries
  - Membranes
  - Magnetic particles

- **Antibodies are 'labeled' with detectable substances to provide a means for detecting and quantifying the presence of the target protein e.g.**
  - Colored particles (e.g., colloidal gold, latex)
  - Enzymes
  - Fluorescent molecules
  - Chemiluminescent molecules

- **Choice of format determined by application**

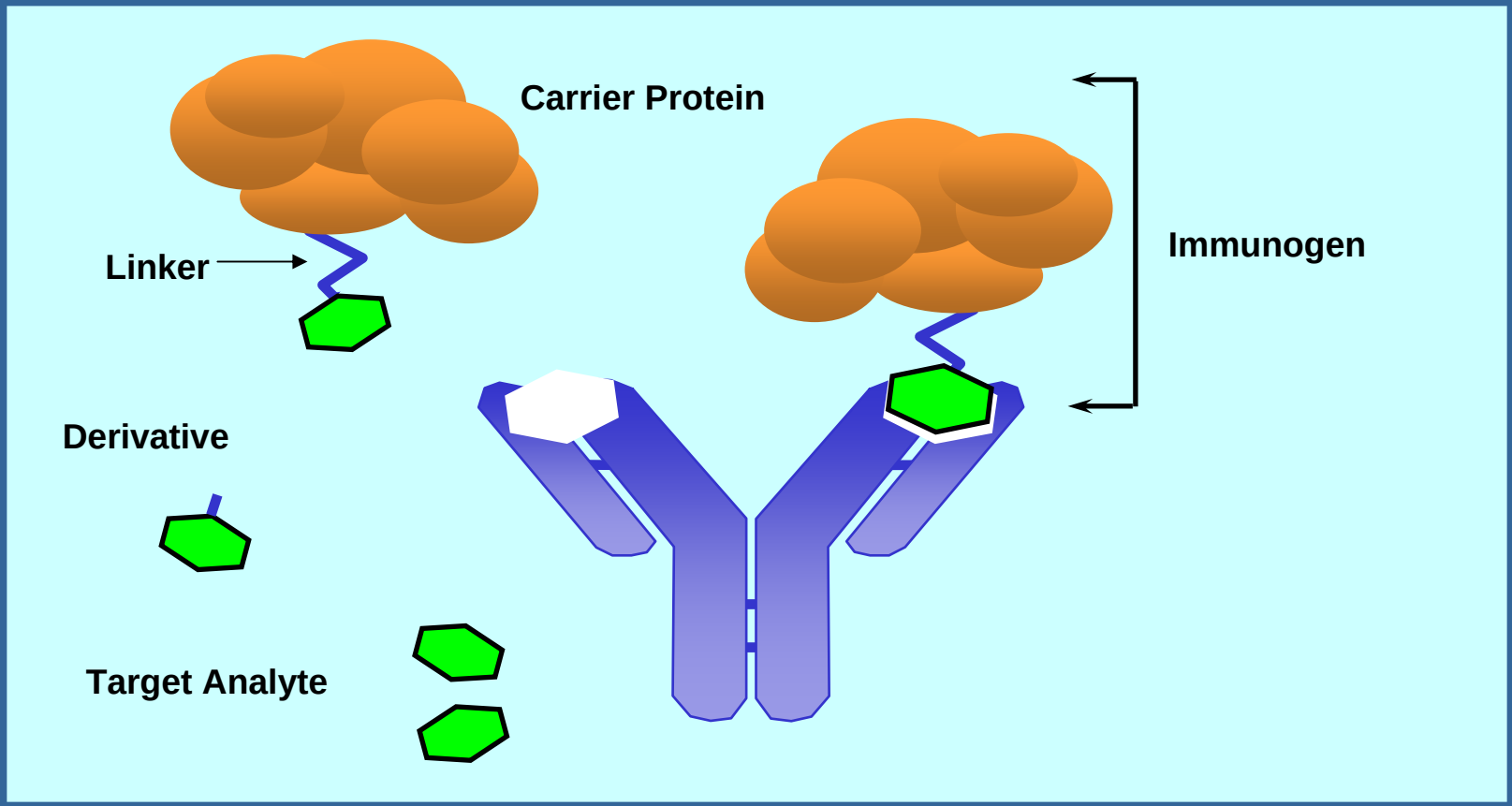
Types of Immunoassays

- Competitive
- Double Antibody Sandwich

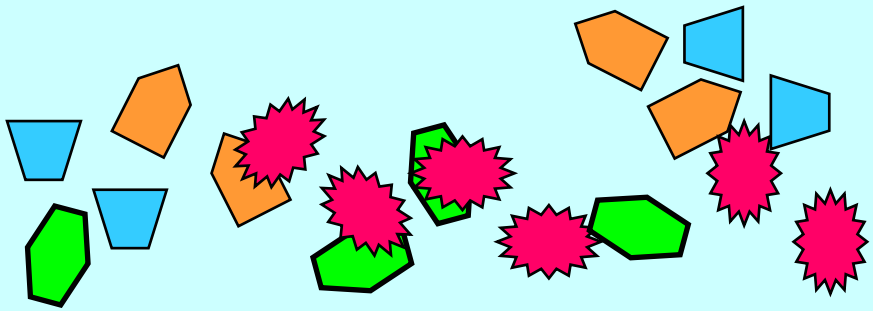
Antibody Development

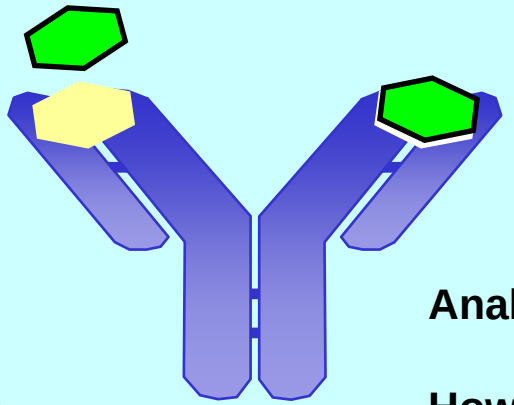
- **Immune system responds best to high molecular weight "Immunogens"**
  M.W. Typically > 10,000
- **Agrochemicals and environmental pollutants mostly small molecules**
  M.W. Typically < 1,000
- **Agrochemicals require preparation of suitable immunogen**
  Couple chemical to carrier protein

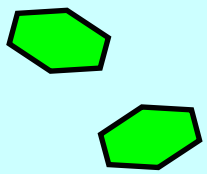
Immunogens



Immunoassay Visualization







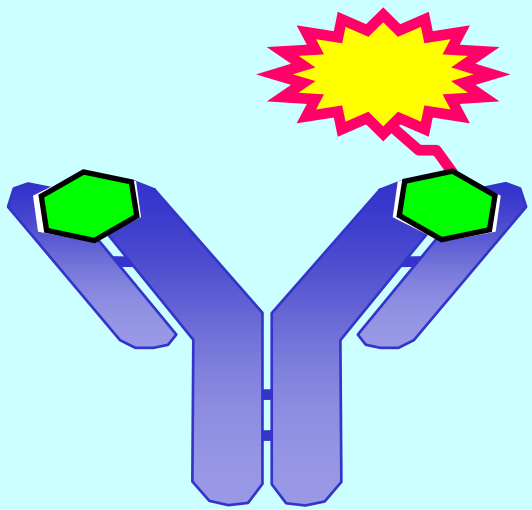
Target Analyte

Analyte bound by antibody

How to detect binding?

Immunoassay Conjugates Detecting Binding

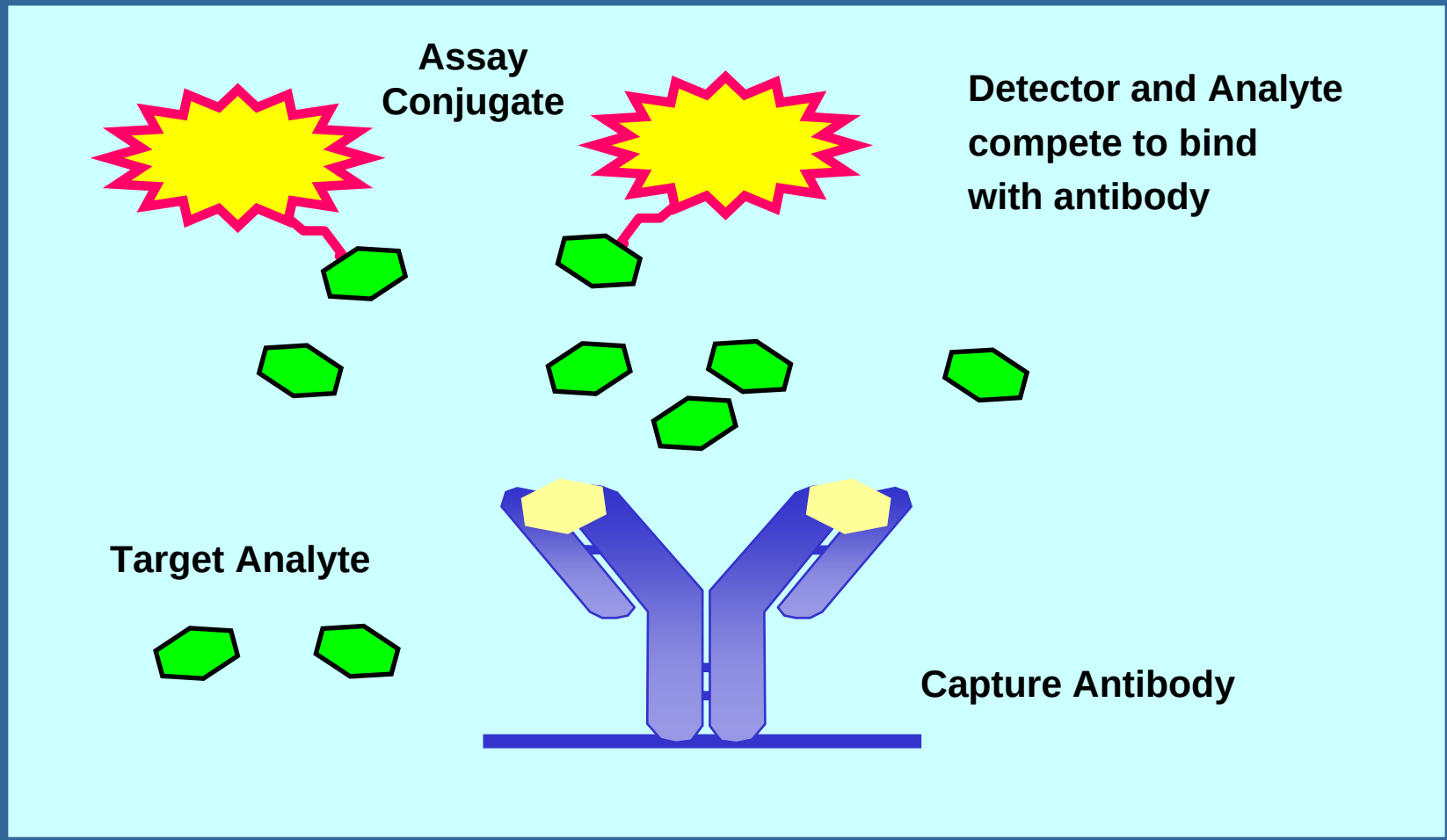
Immunoassay Conjugate



Detectable Label

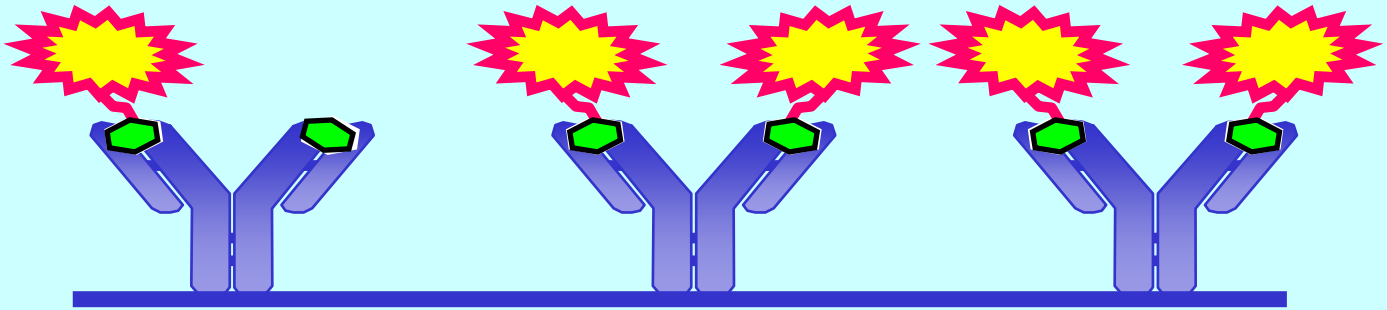
- Radiolabel (RIA)
- Enzyme (EIA)
- Fluorescence (FIA)
- Luminescence
- Electrochemical
- Visual
  - Colloidal gold
  - Colored latex

Competitive Immunoassay

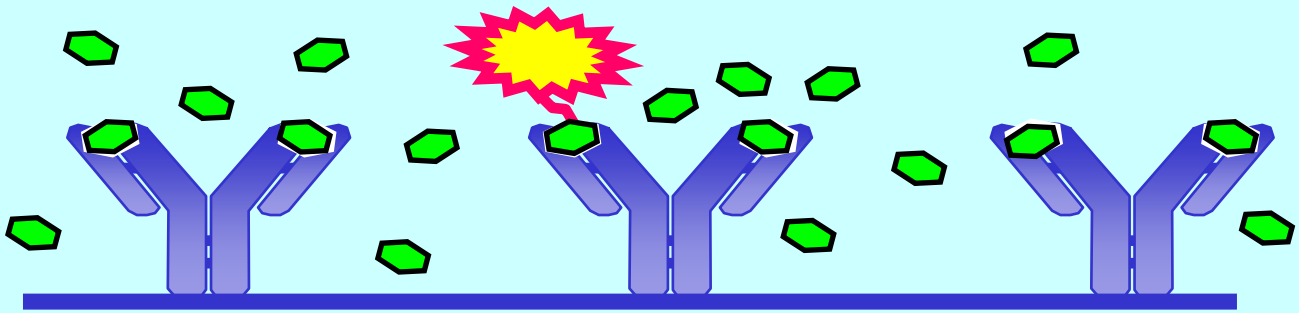


Competitive Immunoassay

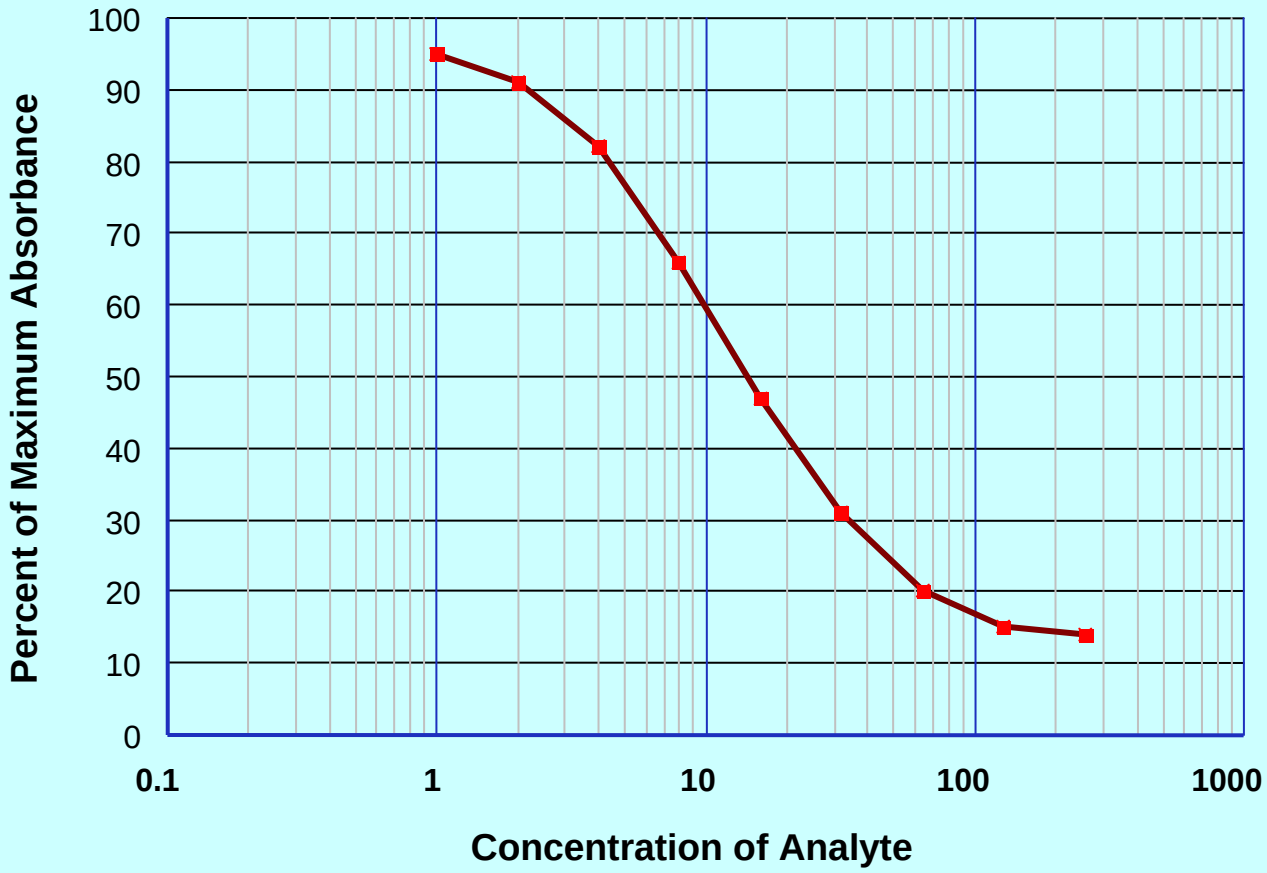
I. No Analyte - high detection signal



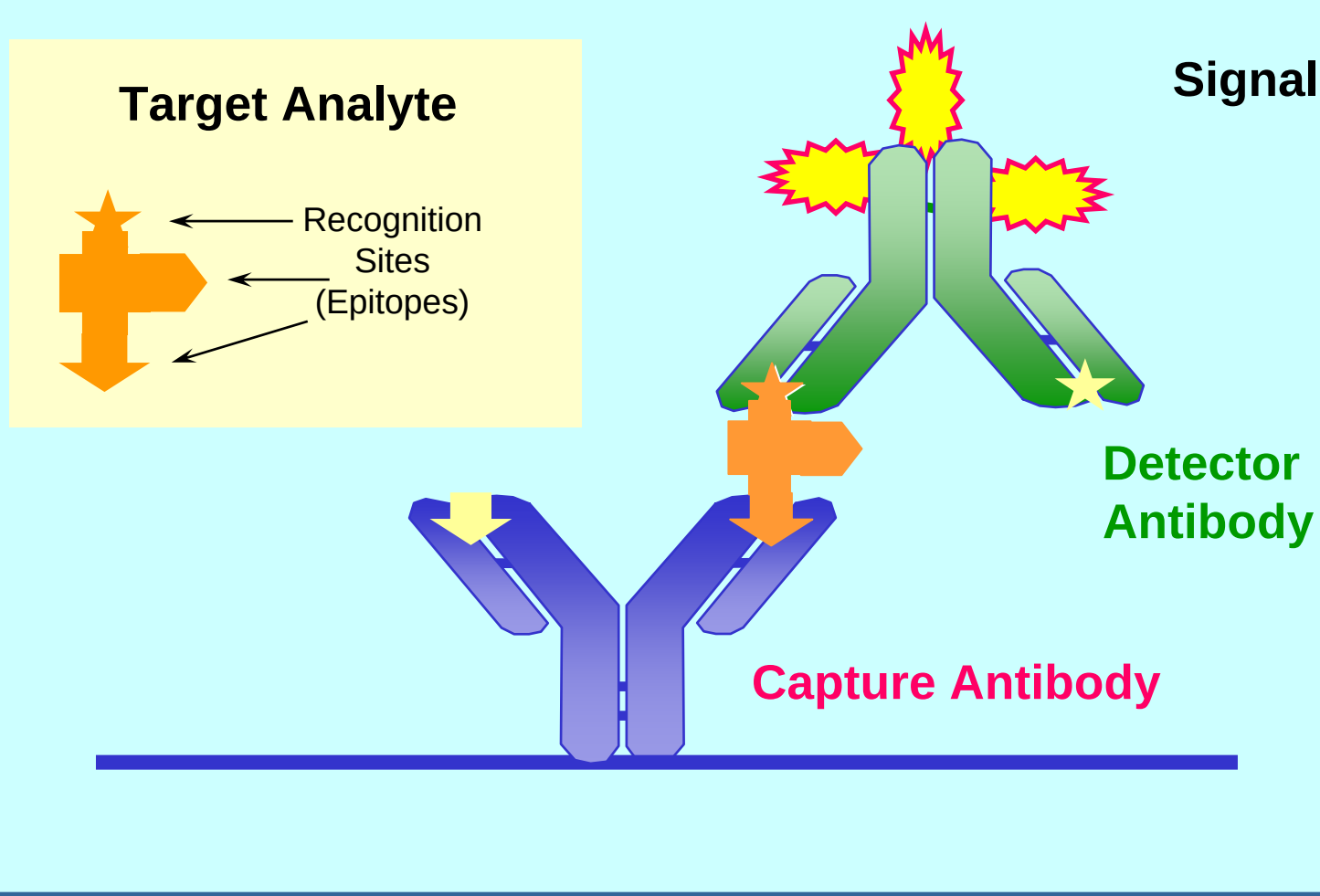
II. Analyte present - detection signal reduced



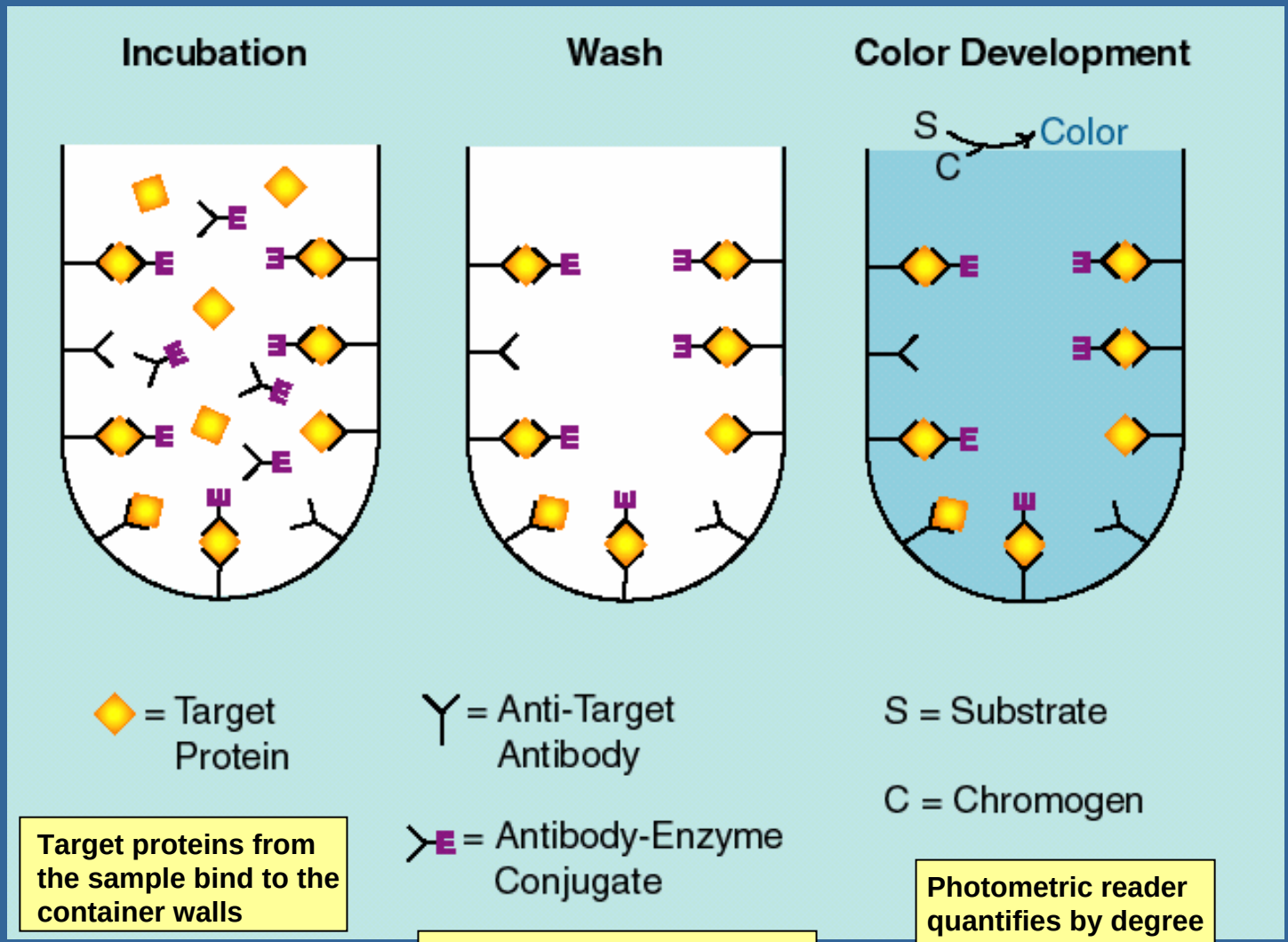
Competitive Immunoassay Data Format



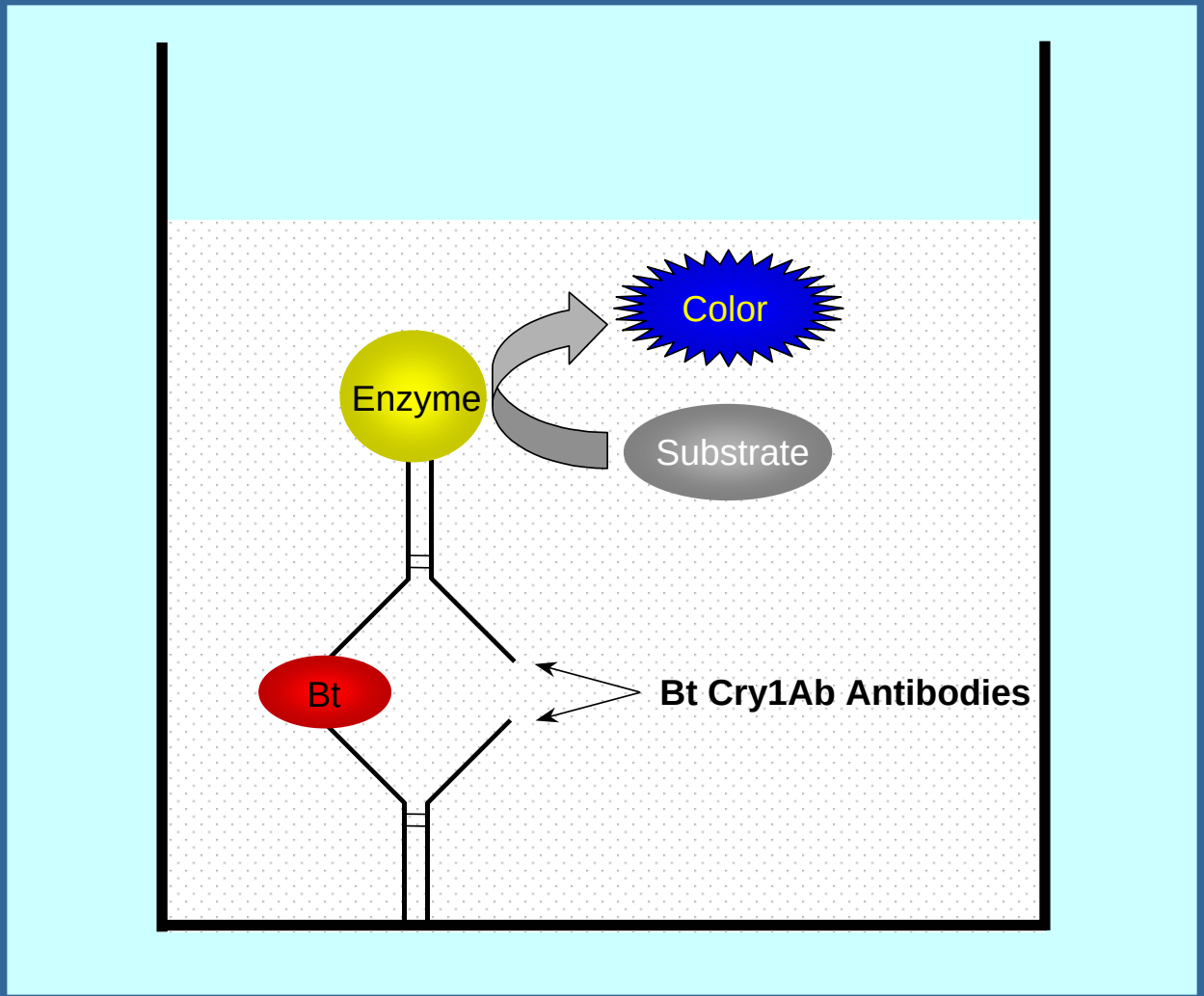
Double Antibody Sandwich Immunoassay



Sandwich Immunoassay Principle



Bt Cry1Ab Sandwich ELISA



Microwell Plate Format

- Quantitative
- 1-2 hours
- Plate reader required

Coated Tube Format

- Qualititative or semi-quantitative
- Visually read color reactions (no equipment required)