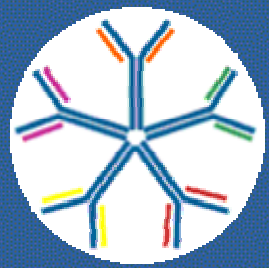
# Methods for Detecting and Measuring Ag Biotech Products



## AEIC

## October 2004

# Copyrights and Acknowledgements

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

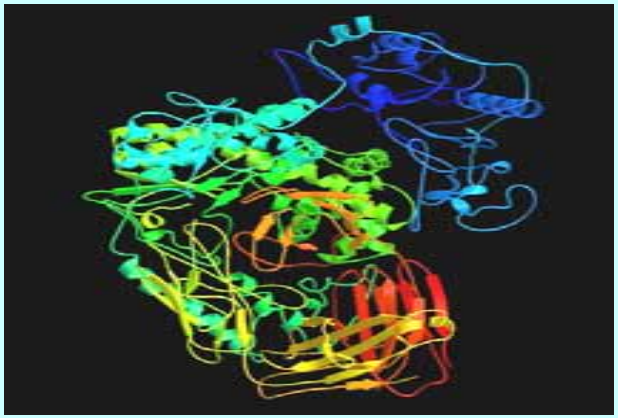
- **Consumer Choice**
- **Approved Events – Quantitative and threshold testing**
  - European Food Labeling Law
    - Labeling began April 10, 2000 and updated April 2004
    - Threshold adventitious < 0.9% - "genetically modified"
  - Japanese Food Labeling Law
    - Labeling began April, 2001
    - 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

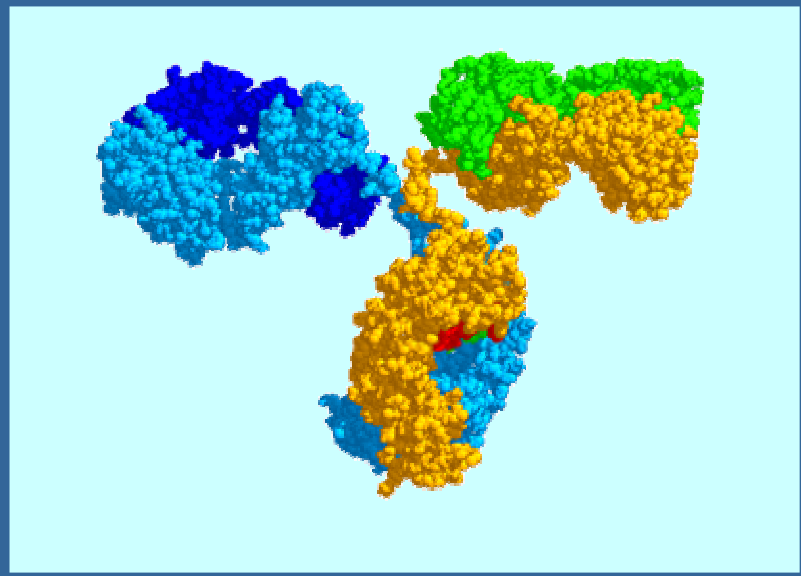
- **Results are reported in terms of % Ag Biotech**
  e.g. 1 Biotech corn kernel in 99 negative = 1%
- **Decisions are based on regulated thresholds (given in weight %)**
- **Testing is based on detection/quantitation of novel DNA or protein**
- **Ag Biotech concentrations are estimated from protein 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)**



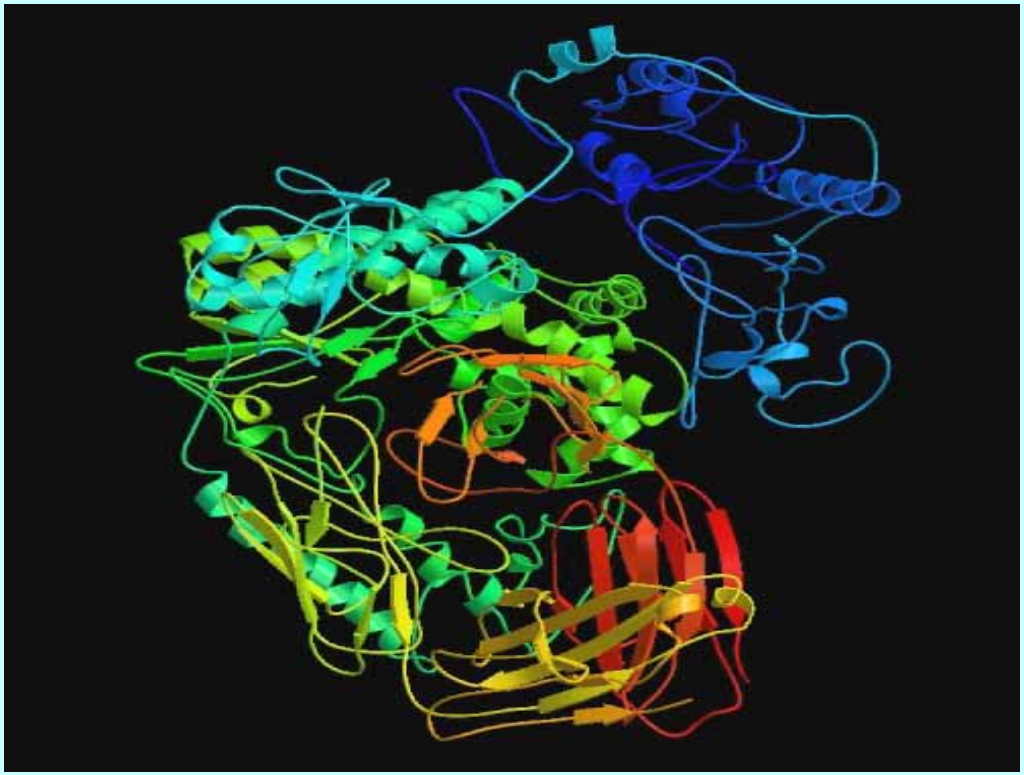
# Biotech Protein - Expression and Detection

- 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



# PCR - Polymerase Chain Reaction

Technique that allows amplification of specific DNA sequences millions of times in a few hours. DNA sequences unique to GM traits can be detected and measured.



# PCR – Uses

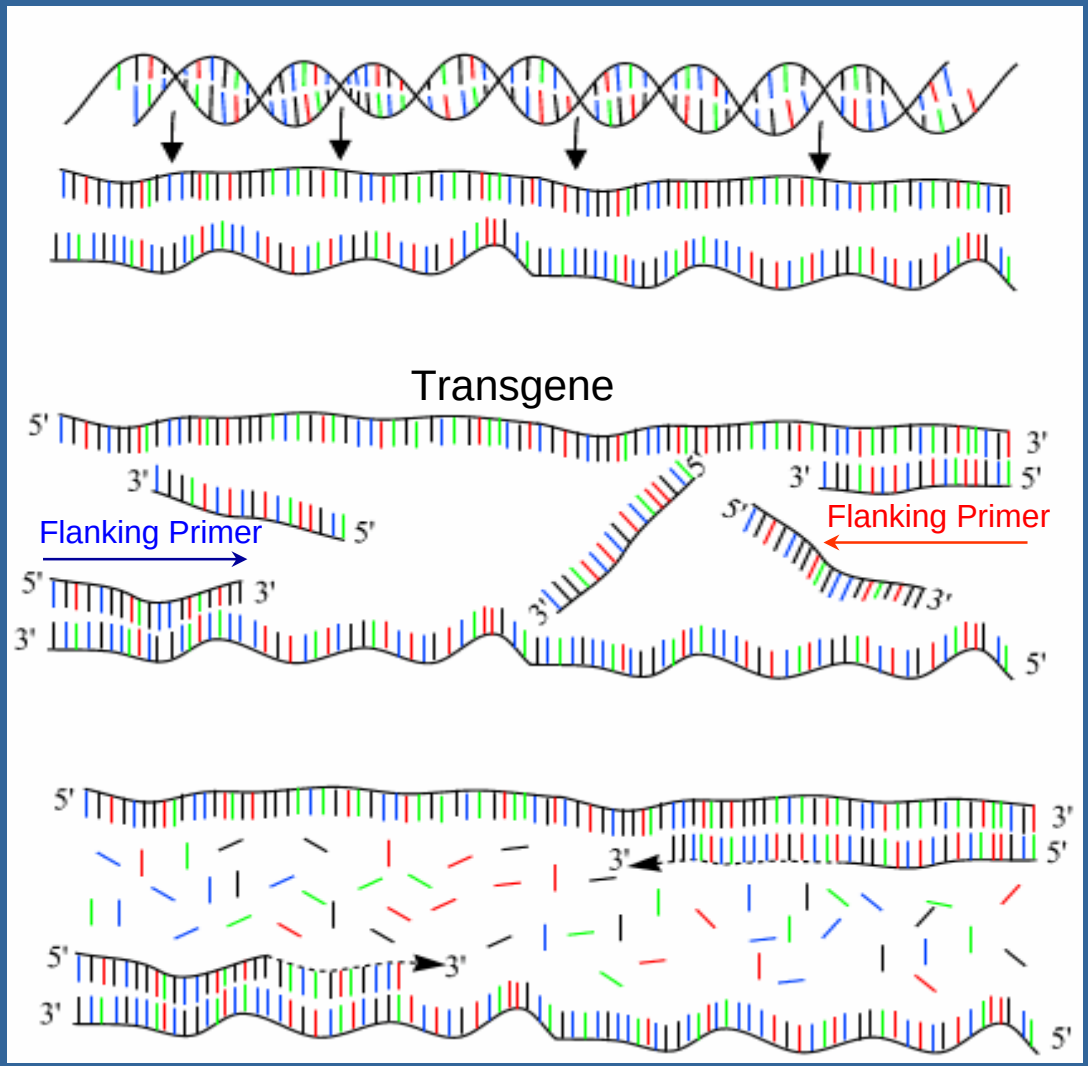
- Genetic purity testing
- Disease Diagnosis
- Forensic Medicine
- Molecular Evolution
- Gene Cloning
- DNA sequencing



# PCR – Process

- High temperature is used to separate DNA Molecules into single strands
- Synthetic sequences of single-stranded DNA's (20-30 nucleotides) serve as primers that anneal to single-stranded target DNA molecules
- Two different primers are used to bracket a target region of DNA to be amplified: one strand is complementary to one DNA strand at one end of the target; the 2nd primer is complementary to the other end of the target region
- Primers are extended by thermal stable Taq polymerase
- Target DNA sequence is amplified
- Cycle is repeated 20-40 times

# Polymerase Chain Reaction (PCR)



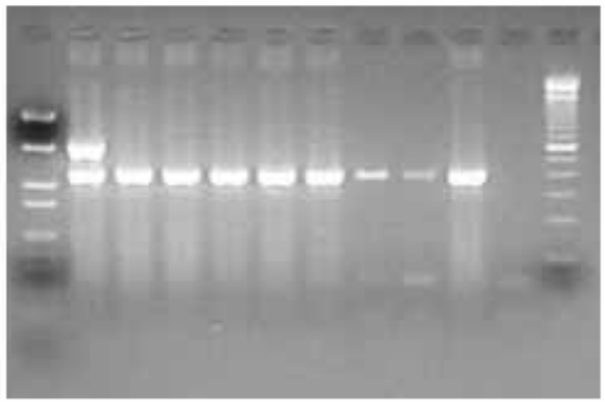

- **Denaturation Step**
  DNA denatures at high temperature (94C)
- **Anneal Step**
  Temperature lowered (50-65C)
  Primers anneal to target
- **Amplification Step**
  Temperature raised (72C)
  Taq polymerase extends annealed primers
  Target DNA is amplified

# PCR Processing Steps

DNA Extraction

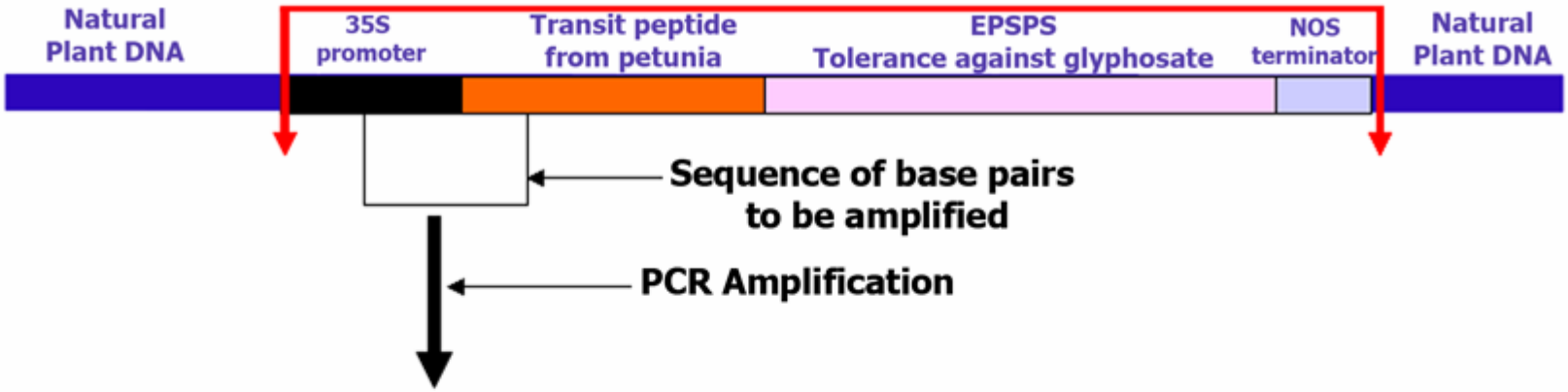
Thermal Cycle PCR

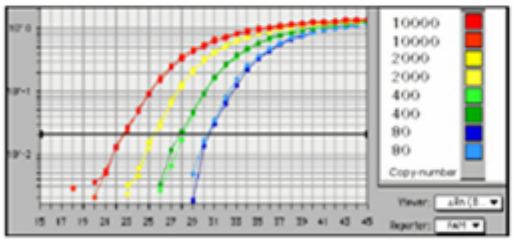
Qualitative or Quantitative Analysis
Detection e.g. by gel analysis



# Has The DNA Been Modified?

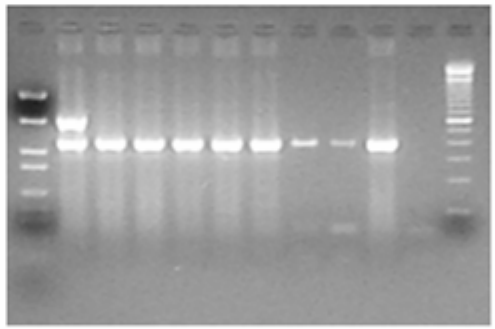
## Modified Genetic Sequence (RoundUp Ready™) Inserted Into Soy DNA



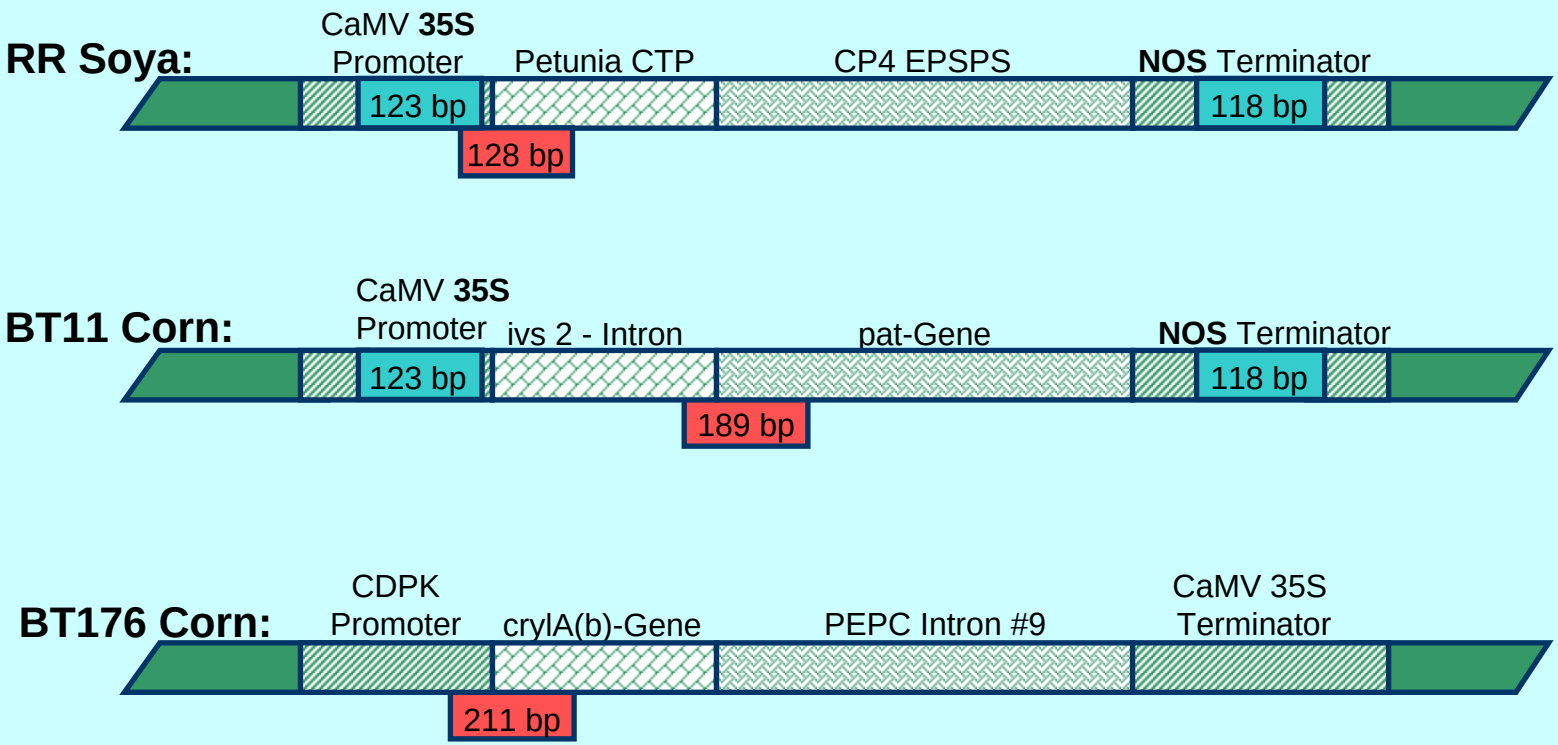

Quantification by fluorescence measurements (real-time PCR)

or



Qualitative results via gel electrophoresis

# Some Synthetic Sequences



# Applications of PCR

## Qualitative

- A "YES" or "NO" answer
- Can look for specific DNA event such as RUR, MON810, etc. or
- Can look for generic elements such as NOS, 35S
- Applicable to a zero tolerance situation

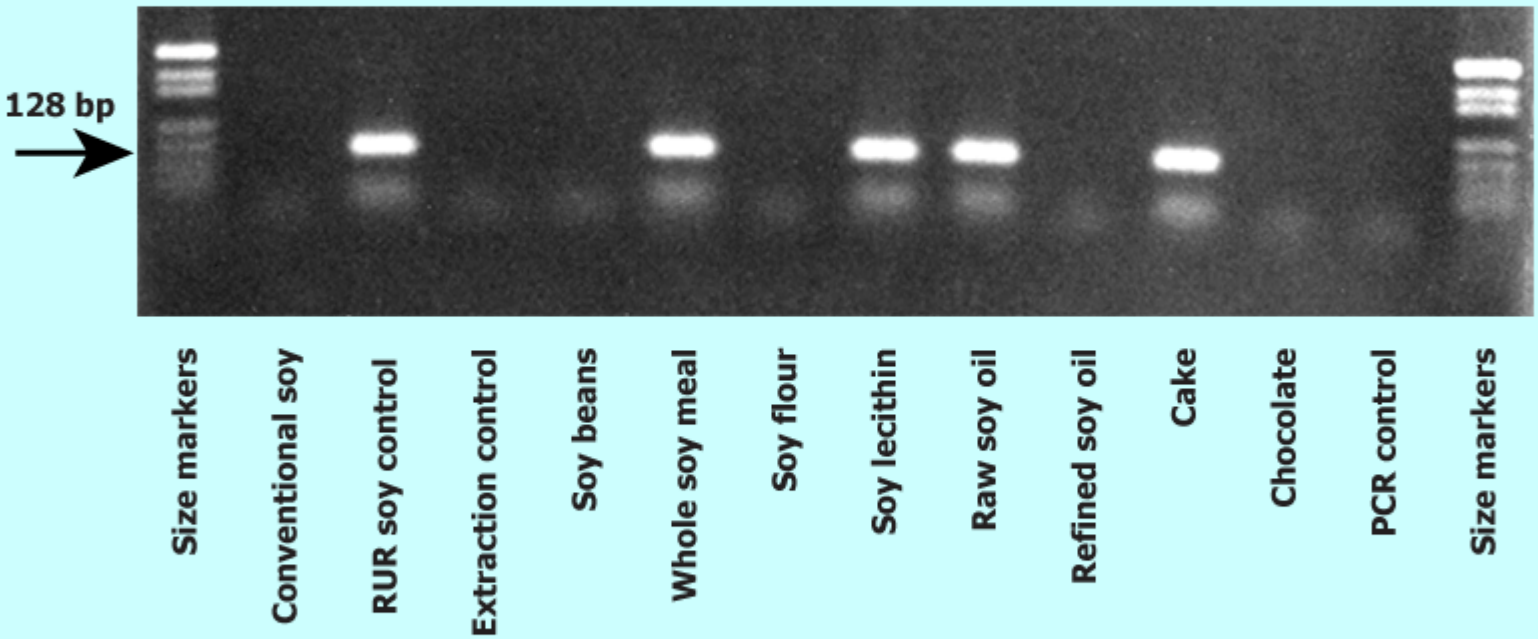
## Quantitative

- A determination of the percent of GM DNA present
- Relates amount of GM DNA to species DNA
- Real time quantitation by laser during PCR process
- Can be specific or a general screen



# Qualitative Detection

## Specific Reaction for Roundup Ready® (RUR) Soy




Detection of Roundup Ready® soy-DNA (128 bp amplicon). The samples RUR soy. whole meal, lecithin, raw oil, and cakes contain genetically modified DNA

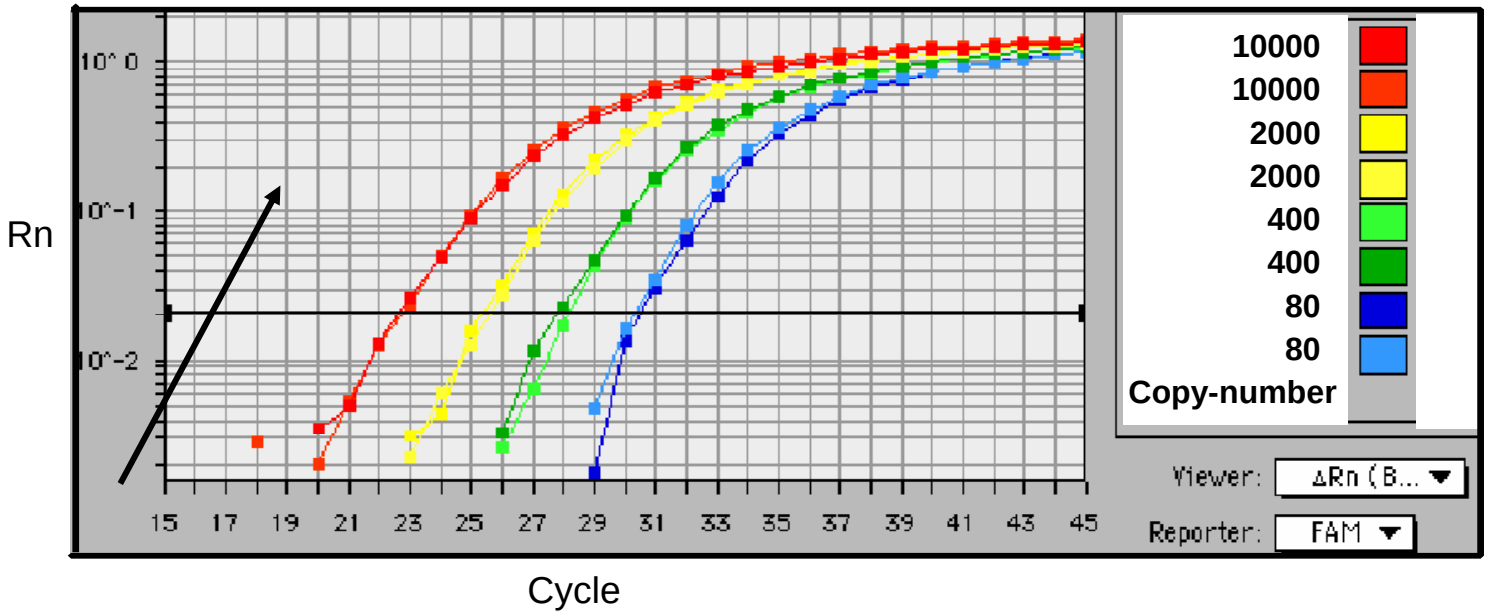
# Quantitative Detection

- Real-time PCR measures the amount of PCR product at each and every amplification cycle
- Amplification plot is a curve that represents the accumulation of product over the duration of the entire PCR reaction
- A standard curve is generated that plots the cycle threshold values against starting amounts of DNA
- Measurement of DNA is done by fluorescing DNA molecules (TaqMan™, Molecular Beacon, SYBR Green)



# Quantification By Real-time PCR

**Real-time PCR quantifies during the initial exponential phase. At higher number of cycles the fluorescence measurement reaches a plateau making a quantification impossible.**



# PCR - Advantages

- High sensitivity
- Can detect and quantify specific traits
- Capable of detecting groups of traits through the use of common genetic elements (e.g. promotor or terminator)
- Higher stability of DNA (than of proteins) permits analysis of most mixed and processed foods

# PCR - Disadvantages

- **High cost per determination**
- **Requires sophisticated equipment and procedures**
- **Requires highly skilled and well trained personnel**
- **PCR reactions can be extremely sensitive to low levels of contaminating DNA template that will result in false positive reactions.**
  - "Accidental PCR Template or amplicon carry-over"
    (e.g. Reagents, pipettes, tips, fingers)
  - 10 pg of contaminating target DNA can produce 1 μg of product (25 cycles)
- **Standardization across labs and protocols is still under discussion**
- **Labor intensive steps, needs time to complete (3 days or more)**

# False Positive Results

| Problem | Control Measures |
|---|---|
| Artifact PCR products from templates other than the target sequence in question (worst scenario: artifacts of the same size as the expected PCR product) | During validation:<br>▪ Search data base for potential unwanted (homologous) annealing sites<br>▪ Test new primer system on a wide range of DNA types that appear in food samples<br>▪ Verify the identity of the PCR product by Southern blot or restriction enzyme digestion |
| Corruption of sample, extracted sample DNA or PCR setup contains positive DNA | During routine analysis:<br>▪ Follow strict rules how to work cleanly in a molecular biology lab<br>▪ Use one way sample flow lab design to prevent contamination with positive DNA from down-stream steps (especially PCR products), strict separation of certain lab areas from each other<br>▪ Use duplicate analysis of each sample<br>▪ Use negative controls on homogenization, DNA extraction and PCR setup |

# False Negative Results

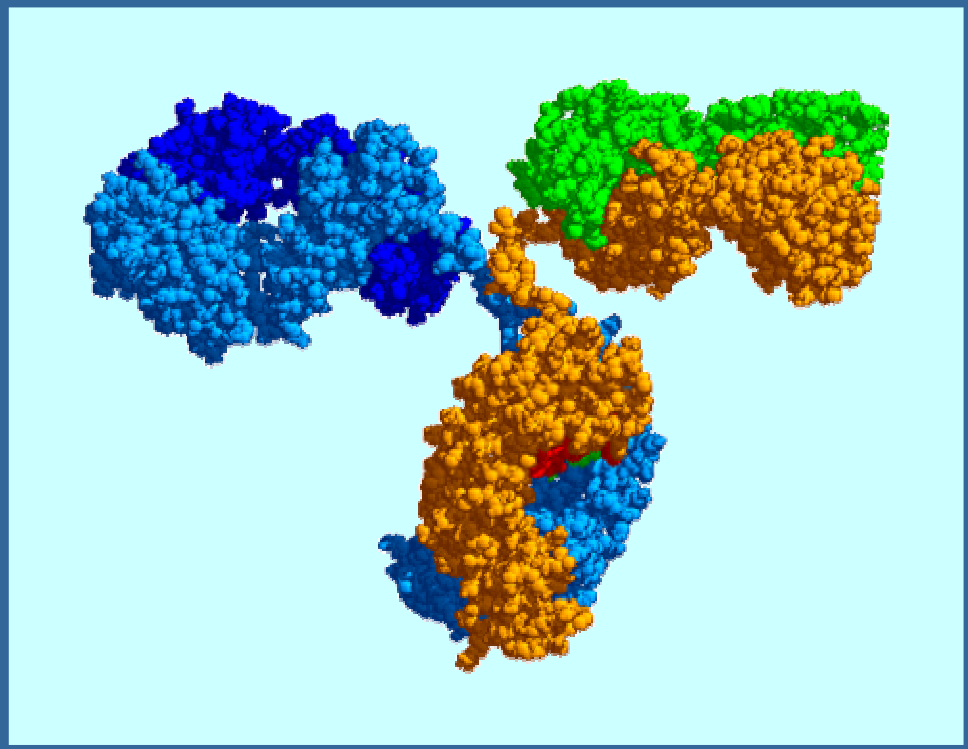
| Problem | Control Measures |
|---|---|
| Insufficient sensitivity of primer system | During design and validation:<br>▪ Use computer aided primer design<br>▪ Test the sensitivity of new primer systems for the intended concentration range |
| Inhibition of PCR reaction | During validation:<br>▪ Test primer system on sample DNA solutions known to contain inhibitory compounds<br>During routine analysis:<br>▪ Test each PCR reaction for inhibition by an individual positive control (a spiked counterpart)<br>▪ Use duplicate analysis of each sample |
| DNA extraction failed | During design and validation:<br>▪ Choose appropriate extraction protocol for sample type<br>▪ Run controls to monitor efficiency of each extraction series |

# Protein Detection Methods

- Capillary Electrophoresis
- Chromatography
- Mass Spectrometry
- Immunoassay

# Immunoassay

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



**Tertiary Structure of an Antibody Molecule**

# Principles of Immunochemistry

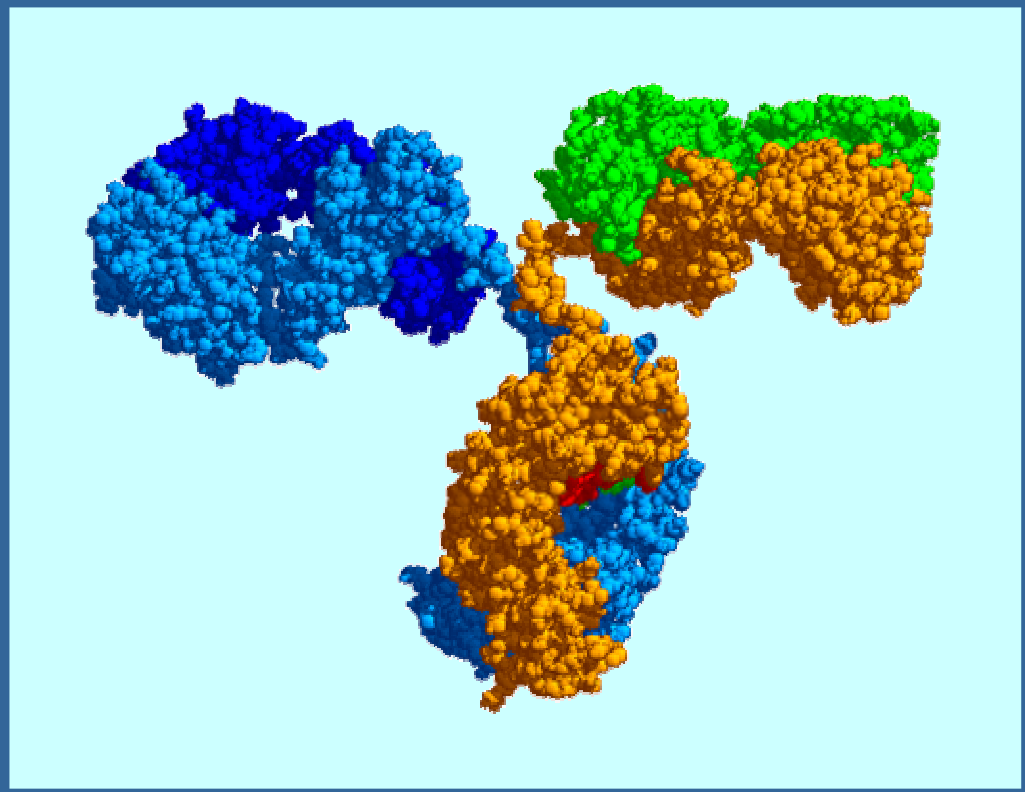
- **Antibodies physically bind target analytes**
- **Strength of binding determines sensitivity**
- **Specificity**
  - Broad or specific (screening or quantitative)
  - Allows detection in complex matrix
    - Minimum sample preparation
    - Field-portable tests
- **Sensitivity and specificity determined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



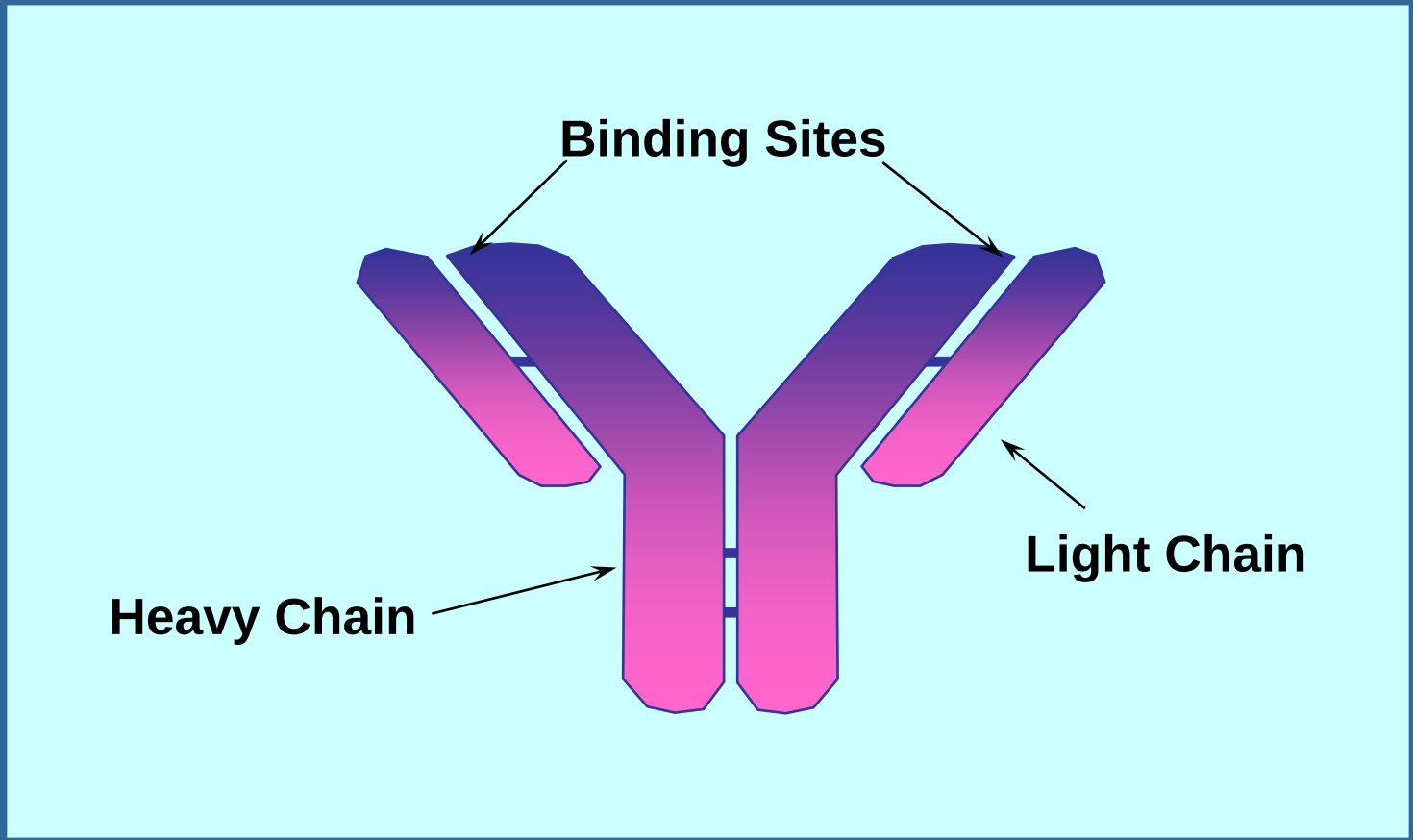
# Immunoassays

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

# Antibody Structure



# Antibody Structure



# Immunoassay Formats

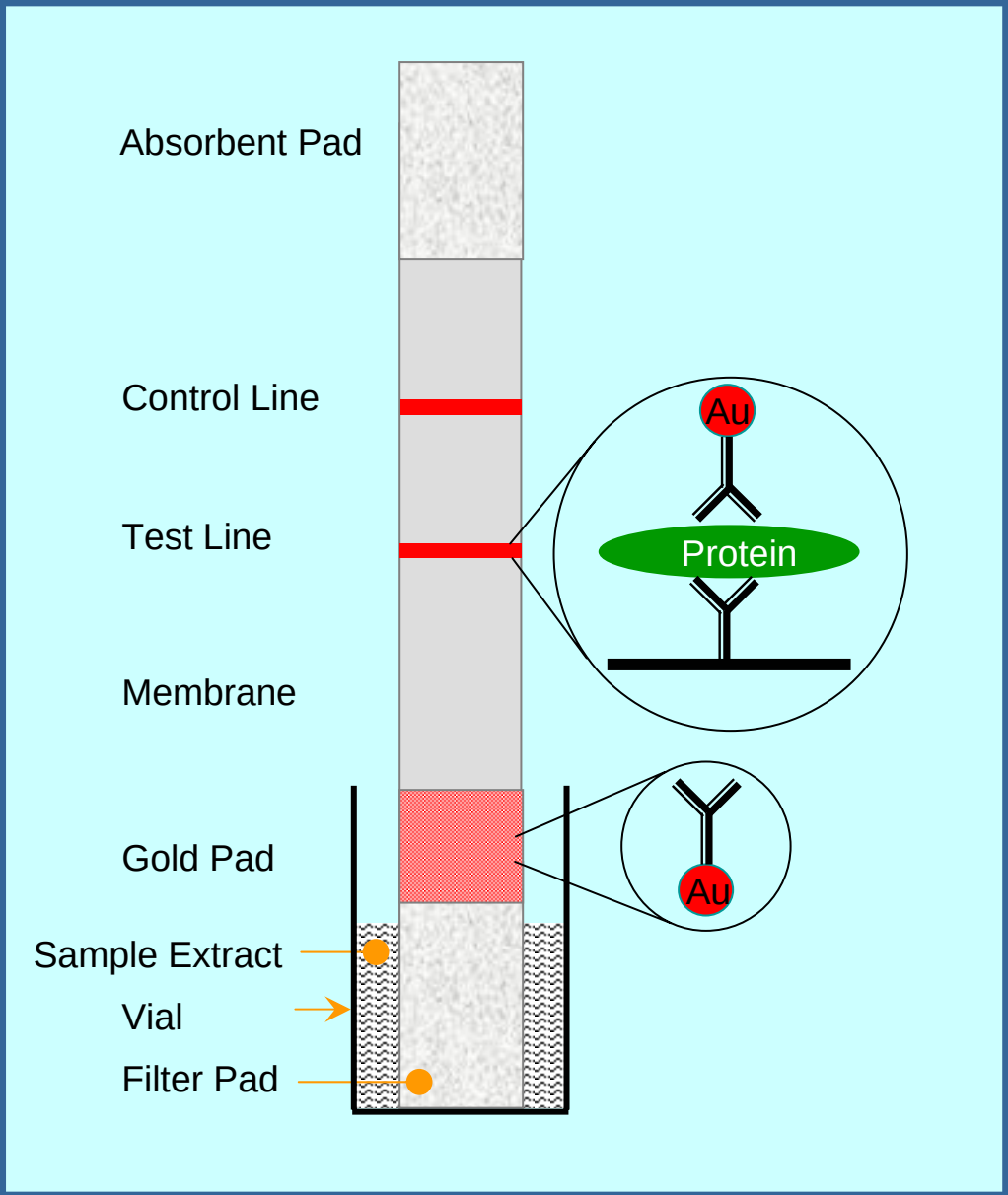
- **ELISA, Striptests, Western Blot**
- **Each format has advantages and disadvantages**
  - Fully automated – clinical analyzers
  - Laboratory kits - ELISA
  - Field tests – "Strip tests"
  - 'Research' methods – Western blot
- **Choice of method is determined by specific application**
  - Performance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



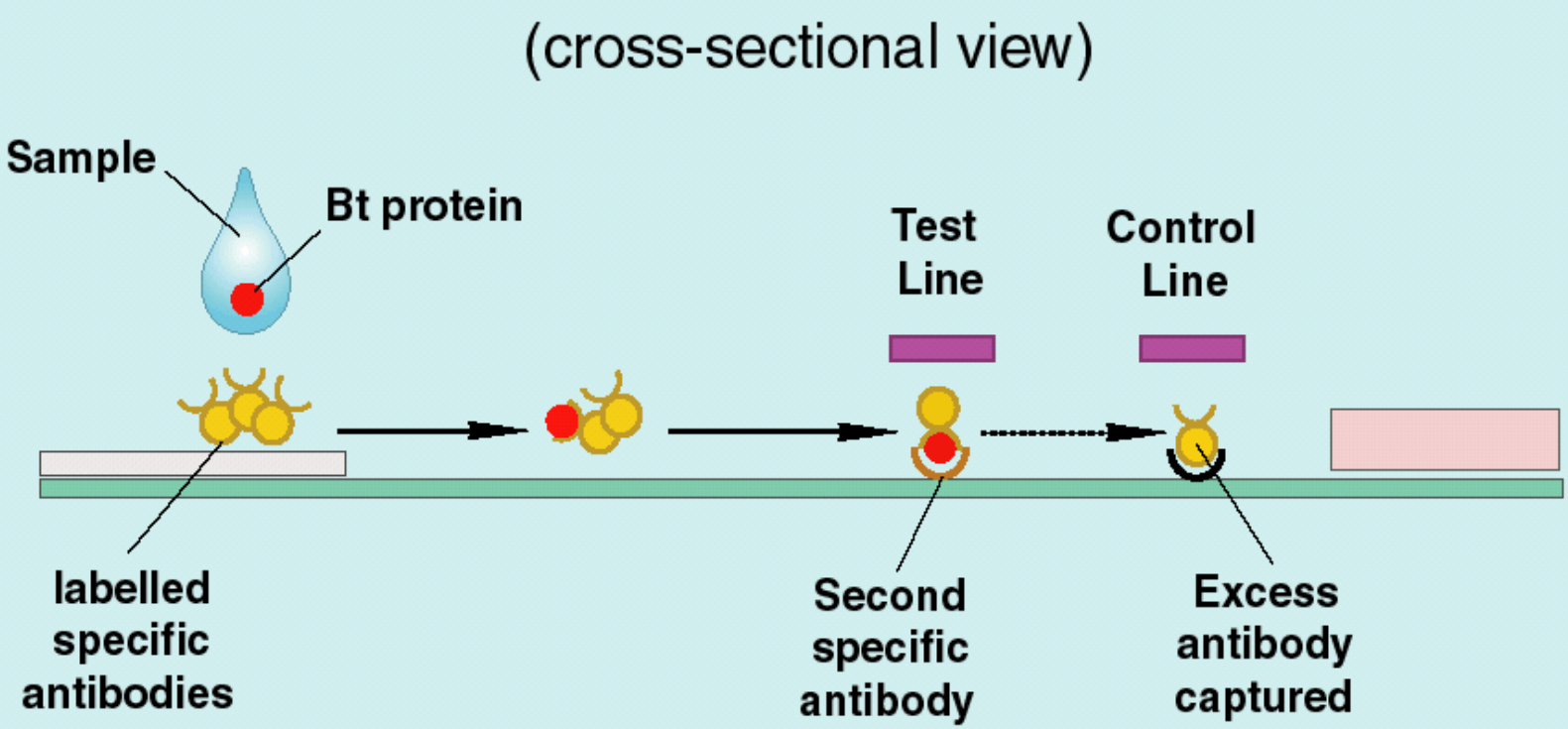
# Lateral Flow Format

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

# Strip Test



# Lateral Flow Format

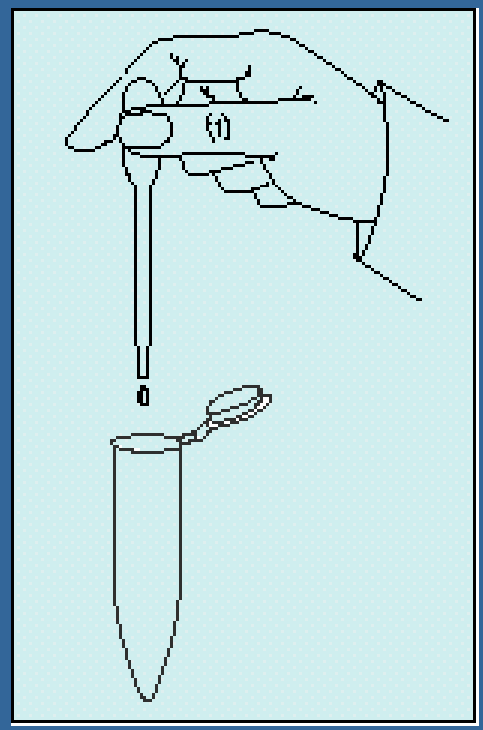


# Lateral Flow Strips Testing Procedure

## Extraction Step

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

2. Transfer extracted sample to vial



# Lateral Flow Test Procedure

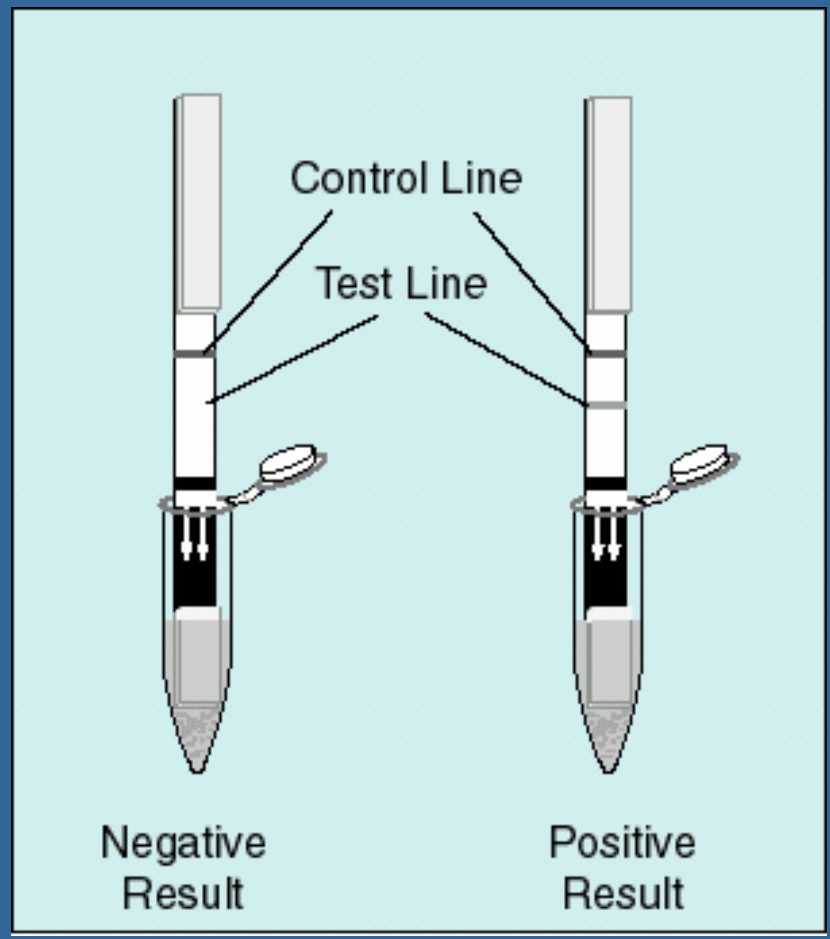
3. **Insert strip into vial**

4. **Read Results**

Negative = No test line, but control line visible

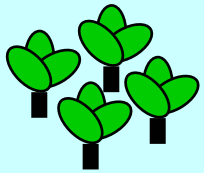
Positive = Visible test line and visible control line

Invalid test = No test line
No control line



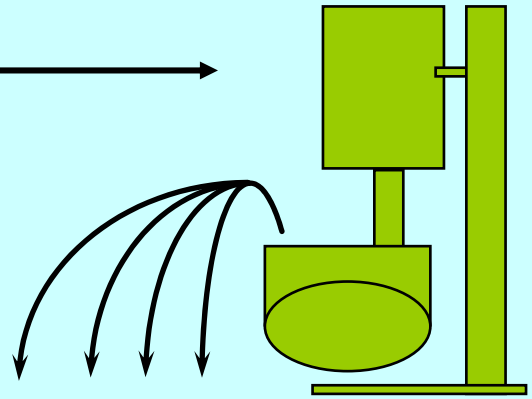
# Steps in Determining Protein Expression by ELISA

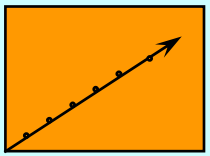
**Step 1**
Sample plant tissue



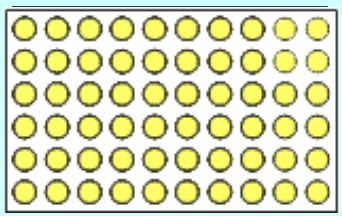
**Step 2**
Weigh Sample Aliquots

**Step 3**
Extract Samples

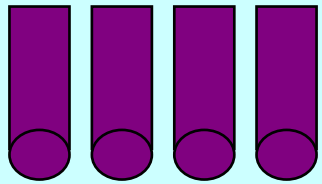




**Step 6**
Data analysis



**Step 5**
ELISA Analysis



**Step 4**
Aliquot extracts for analysis
and sample back-ups

# 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**



# 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

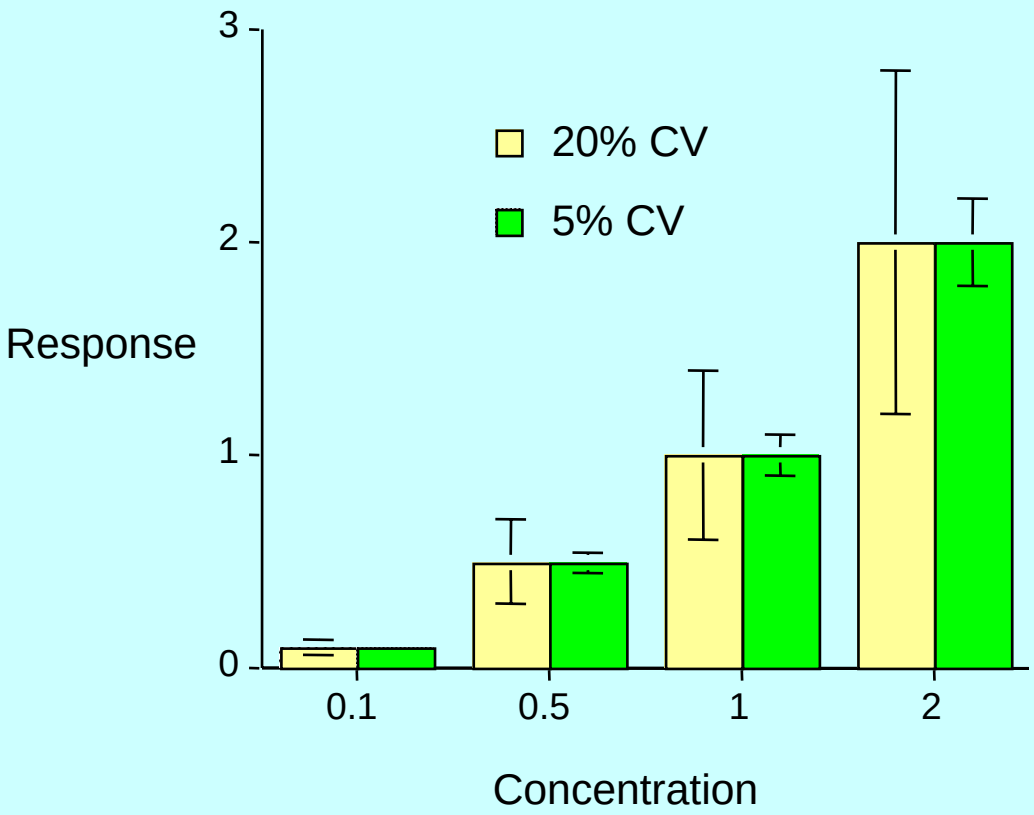
- Precision ➡ inter-plate, intra-plate, inter-assay variability
- Accuracy ➡ extraction efficiency, matrix effects, spike and recovery, robustness, ruggedness
- Sensitivity ➡ limit of detection, limit of quantitation
- Specificity ➡ matrix effects, specific for trait

# Precision

- The closeness of agreement between independent test results obtained under stipulated conditions
- The amount of variability due to random error
- Expressed as the standard deviation (sd) or coefficient of variation (cv)
- Determined by testing replicates of samples
- The precision of the final concentration must include variability of sample preparation
- Good precision provides ability to distinguish closely related concentrations
- Repeatability (reproducibility) is precision under repeatability (reproducibility) conditions
  - Repeatability conditions, test results obtained in the same lab, same method, same conditions, same operator, within short period of time
  - Reproducibility conditions, test results with the same method in different labs, different operators and different equipment



# Effect of Precision on Ability to Discriminate Between Concentrations




**Error bars for concentrations determined by the method having a 20% CV overlap.**
**Both methods give the same concentration but it is not possible to say that adjacent concentrations differ from each other in the assay with the 20% CV within the given confidence interval.**

# Accuracy

- **The closeness of agreement between the (or a) reported result and the accepted reference value**
- **Quantitative methods**
  Expressed as '% Recovery' of 'true' value
- **Qualitative methods**
  Expressed as rate of false positive and negative results
- **Influenced by**
  Precision
  Bias
  Matrix effects



# Sample Extraction

- **Matrix effects**
  - Sample matrix - Extraction of the protein also extracts other substances from the sample
  - Interfering substances - Substances affecting assay performance
- **Extraction efficiency**
  - The % of target protein extracted by the method protocol
  - Does not need to be 100% - must be consistent
  - Balance
    - Sensitivity
    - Cost
    - Time
    - Ease-of-use



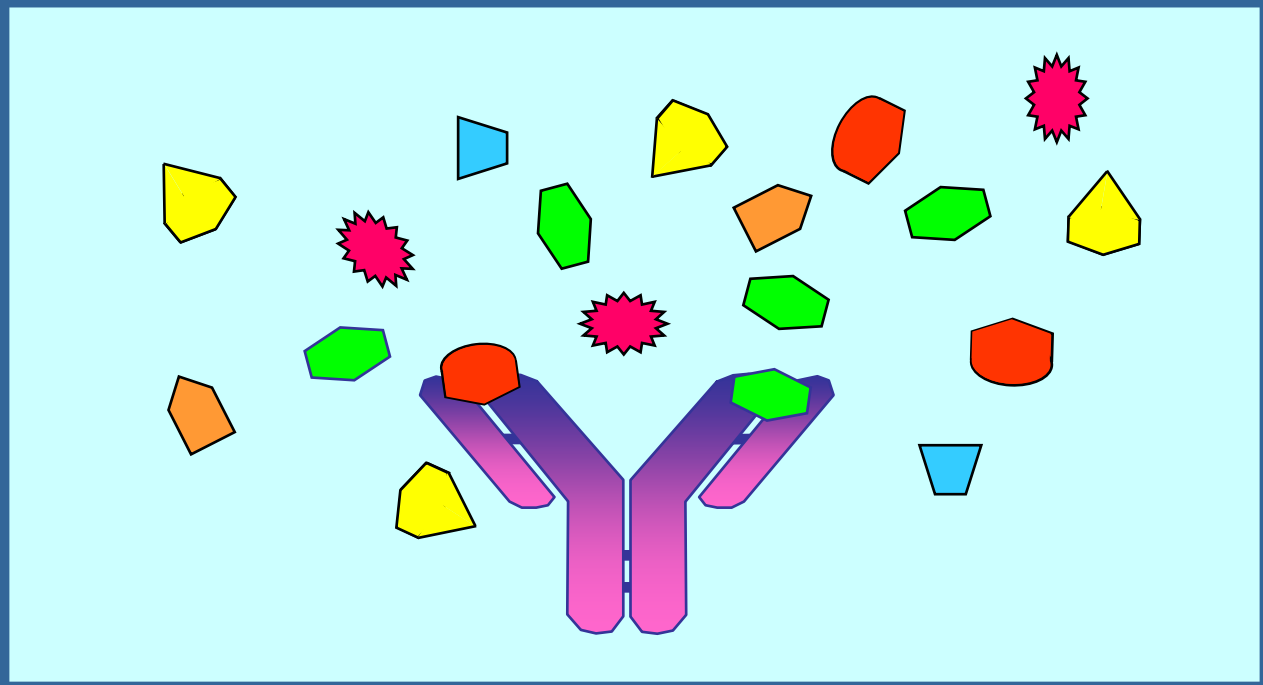
# Sensitivity

- **Change in the response divided by the corresponding change in the concentration of a standard (calibration) curve; i.e. the slope of the analytical calibration curve**

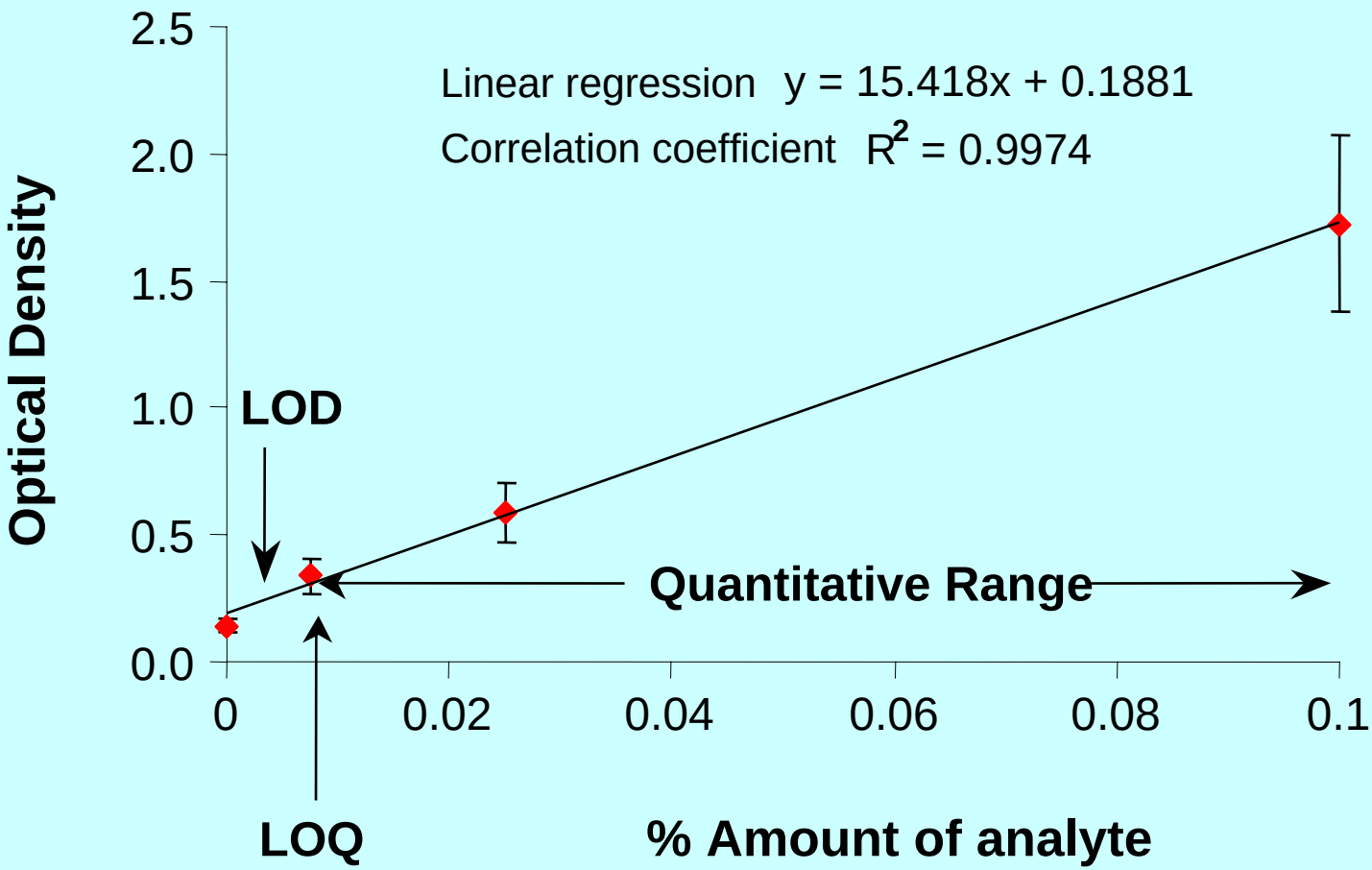
- **The minimum concentration of the target analyte (e.g. protein) that can be detected or quantified**
  - Limit of detection (LOD) – The concentration of protein which can just be detected by the assay (e.g. 50% of the time)
  - Limit of quantification (LOQ) – The concentration of protein that can be quantified with stated precision (e.g. ±10%)
- **Total method sensitivity must account for extraction efficiency**

# Specificity/Crossreactivity

- **Specificity:** the property of a method to respond exclusively to the characteristic or analyte (e.g. protein) defined in the method
- **Crossreactivity:** antibody binding at the binding site with substances other than the analytes of interest



# Standard Curve



# Standards and Controls

- **Quantitative Methods**
  - A series of standards (calibrators) are used to construct a standard curve
    - Matrix-matched standards (e.g. corn flour)
      - Samples and standards are normalized for matrix effects and extraction efficiency
      - Must create standards for every matrix
    - Purified protein standards
    - Must establish that they give same response as protein in matrix
- **Qualitative methods**
  - Negative control
  - Positive control e.g. threshold concentration, need to determine LOD
  - May have multiple levels to group results in limited ranges (buckets)



# 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**

# 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

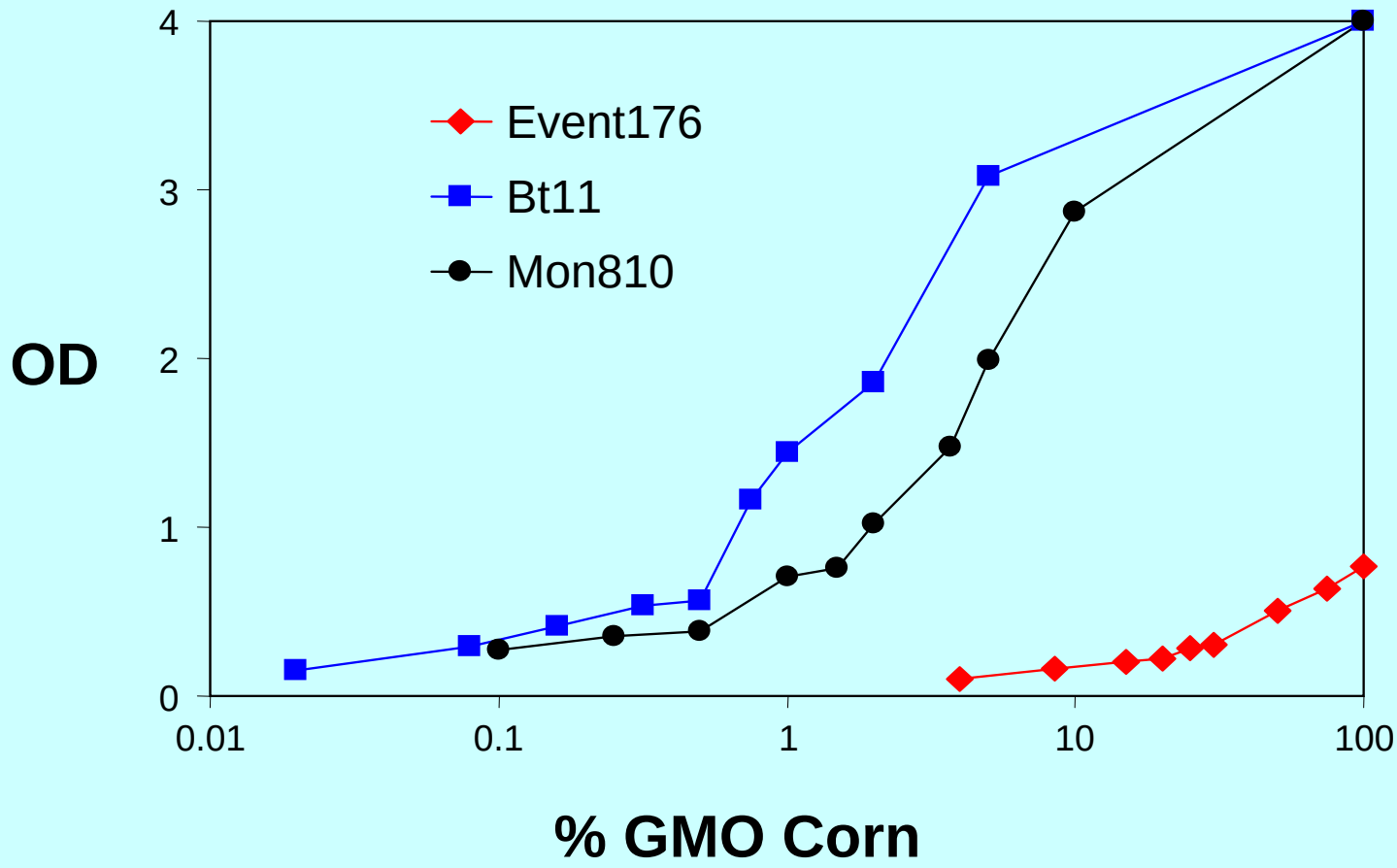
- No single method will detect all biotechnology-derived products (DNA or protein-based)
- Methods are trait specific and must be validated for each matrix (DNA and Protein-based)
- Limited to use of protein containing processed ingredients and final food products
- Some products may not express a detectable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

# Monoclonal vs. Polyclonal Antibodies

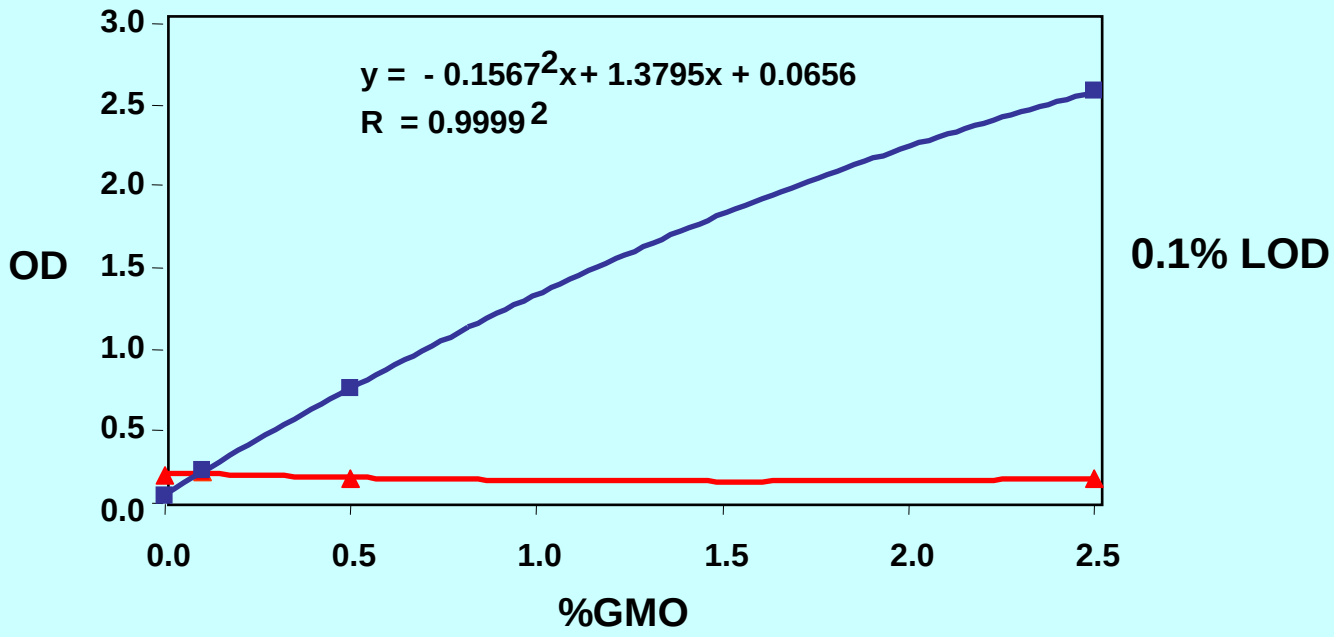
| Monoclonal | Polyclonal |
| --- | --- |
| • Lot-to-lot consistency | • Lot-to-lot variability |
| • Indefinite supply | • More broadly reactive |
| • Highly specific | • Often more sensitive |
| • Longer lead time | • Shorter lead times |
| • Higher initial costs | • Lower initial costs |

Selection is based on application, time and money

# Reactivities of Different Varieties of Bt Corn in Cry1Ab ELISA



# Reactivity of 2 Different ELISA to RUR Toasted Soy Meal




Antibody detects toasted soymeal

Antibody not selective for toasted soymeal

**Soymeal was toasted for 60 min. at 100 ºC**

# Clinical Diagnostic Immunoassays

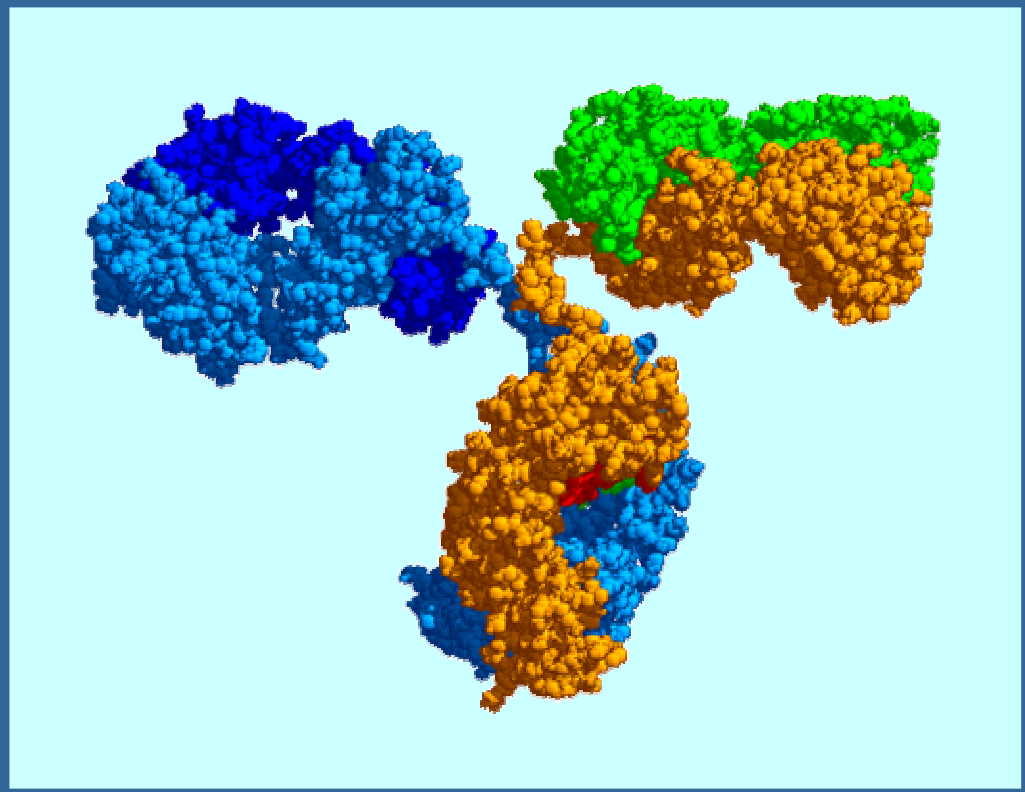
- **In Use >30 Years**
- **Basis for Critical Human Health Decisions**
  - Disease diagnosis (AIDS, Hepatitis, PSA)
  - Therapeutic drug monitoring
  - Drug abuse screening
  - Over 70 clinical analytes tested by immunoassay
  - Home pregnancy tests
- **Highly Reliable**

# Antibodies

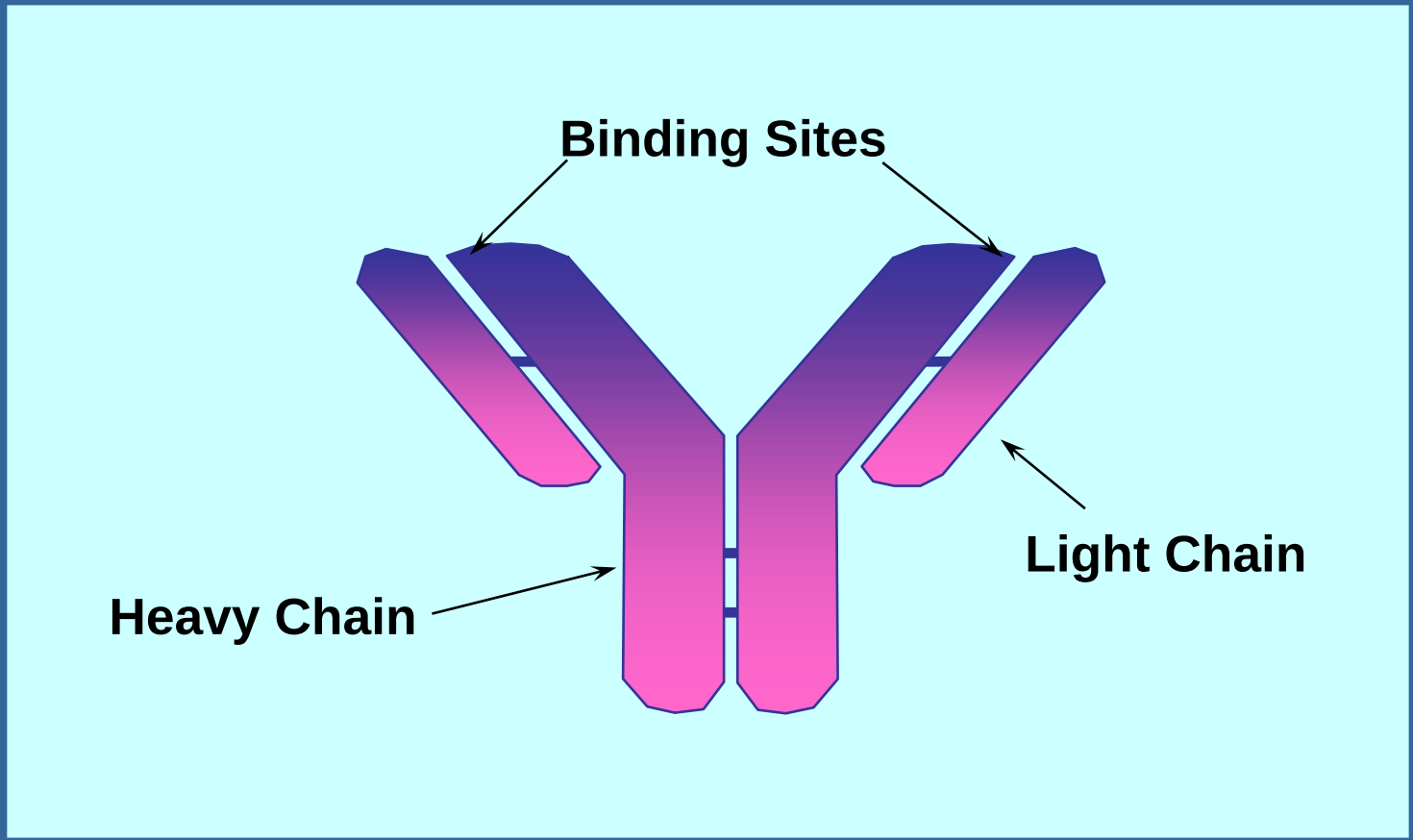
- **Key reagents in all immunoassays**
- **Proteins produced by immune system of higher animals**
  - Produced by specific white blood cells
  - In response to recognition of "foreign" substances
  - Examples:
    - Vaccinations
    - Response to natural infections (mumps, chicken pox)
- **Chemically 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**



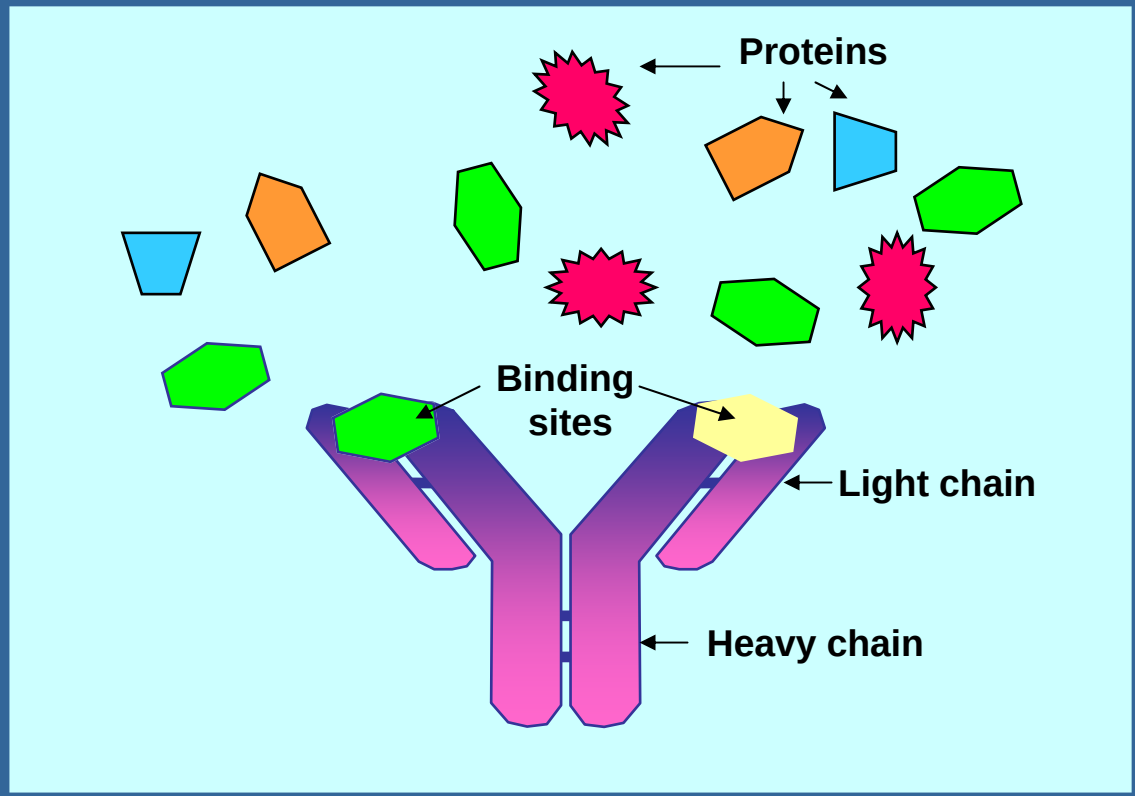
# Antibody Structure



# 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



# 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

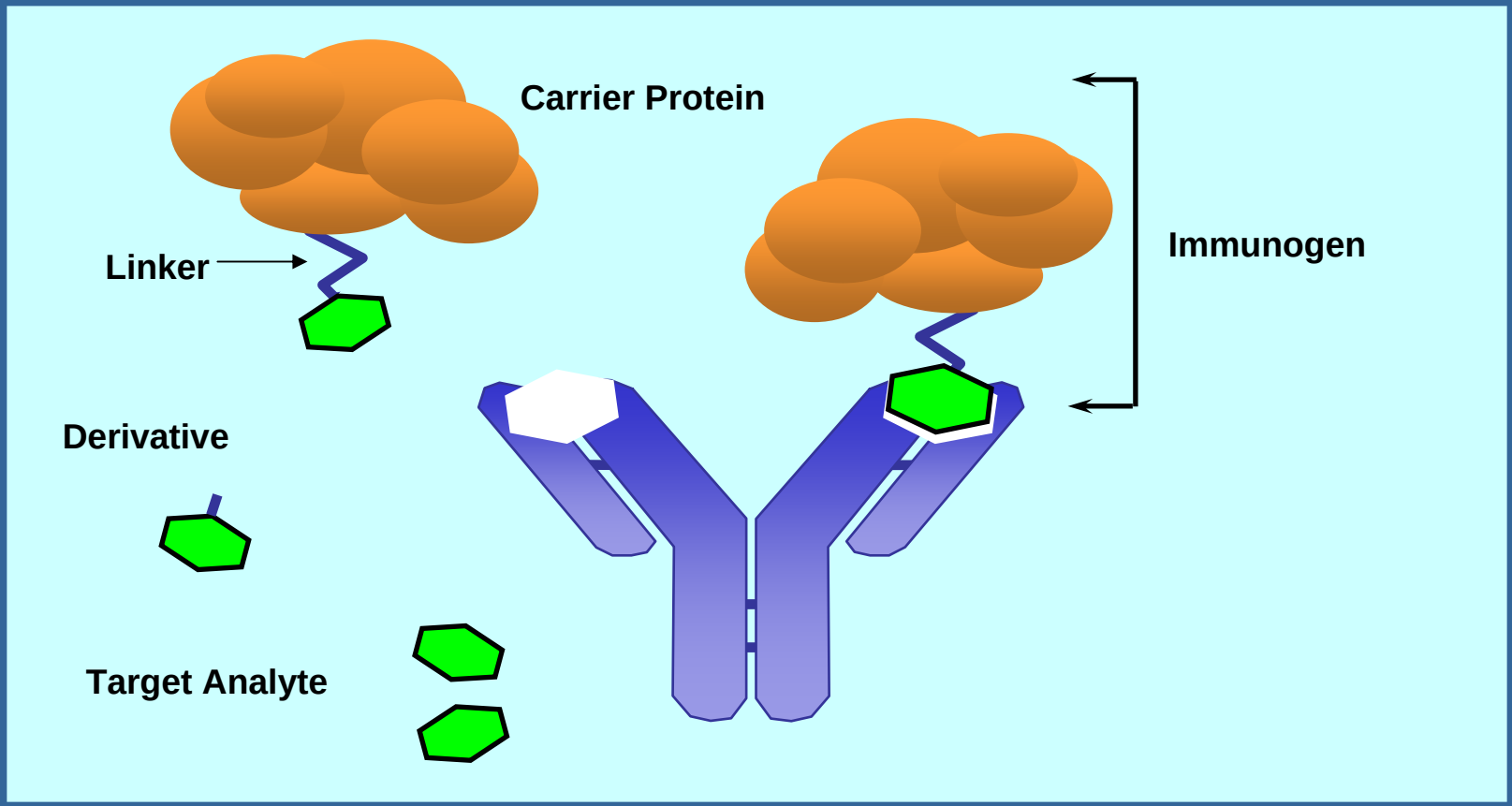


# Antibody Development

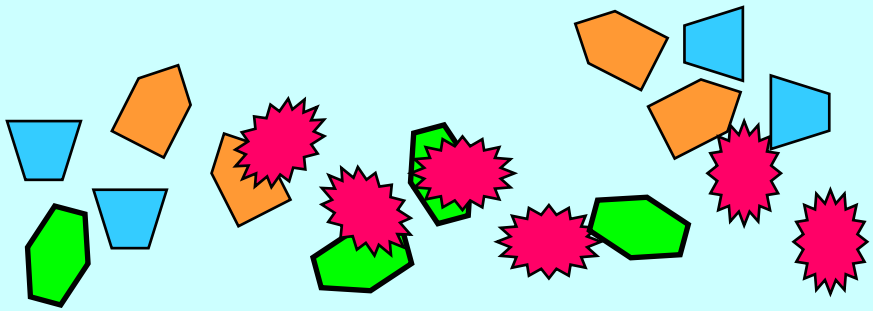
- **Immune system responds best to high molecular weight "Immunogens"**
  M.W. Typically > 10,000
- **Agrochemicals and environmental pollutants mostly small molecules**
  M.W. Typically < 1,000
- **Agrochemicals require preparation of suitable immunogen**
  Couple chemical to carrier protein

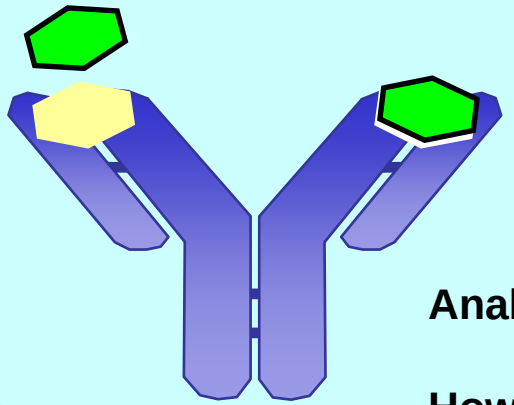


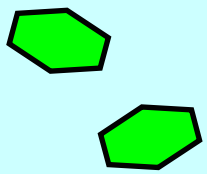
# Immunogens



# Immunoassay Visualization







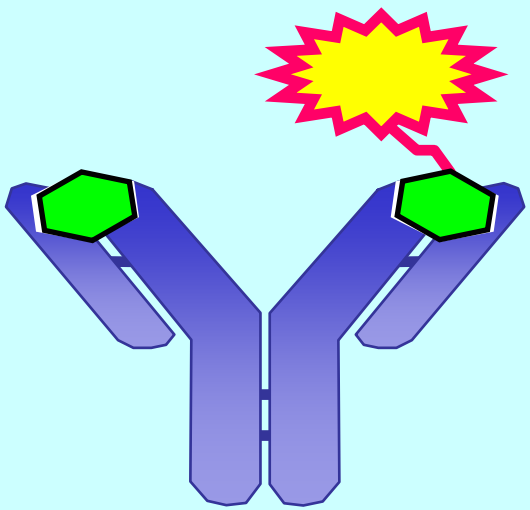
Target Analyte

Analyte bound by antibody

How to detect binding?

# Immunoassay Conjugates Detecting Binding

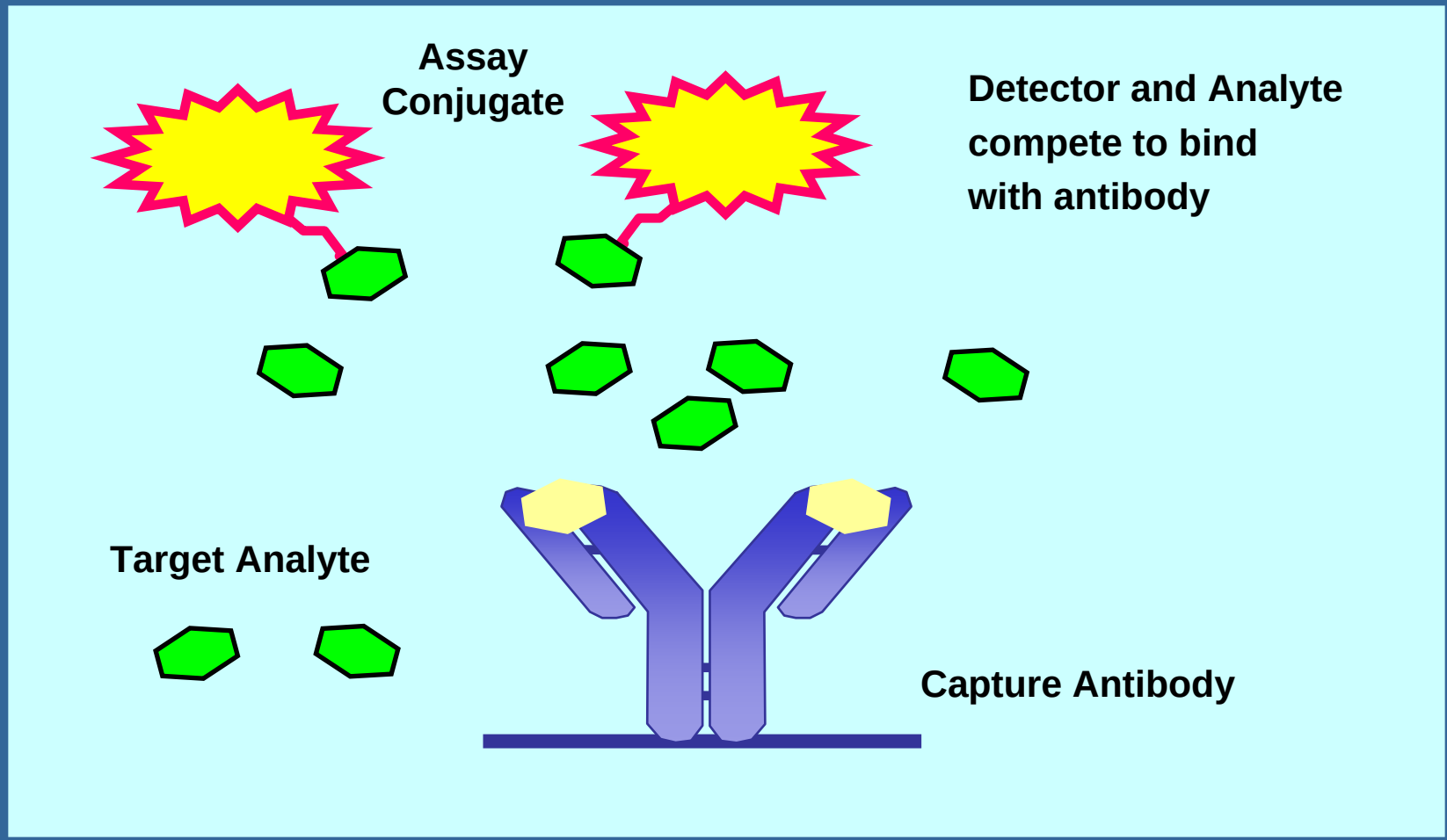
**Immunoassay Conjugate**



**Detectable Label**

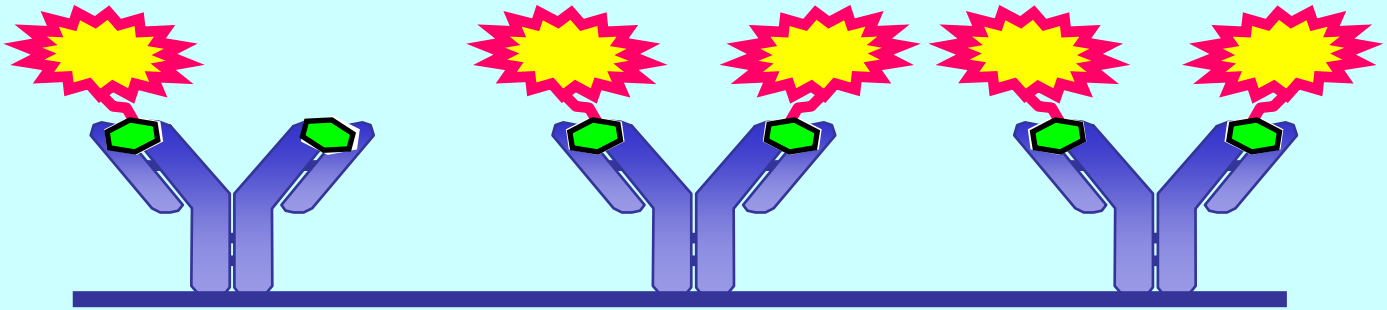
- Radiolabel (RIA)
- Enzyme (EIA)
- Fluorescence (FIA)
- Luminescence
- Electrochemical
- Visual
  - Colloidal gold
  - Colored latex

# Competitive Immunoassay

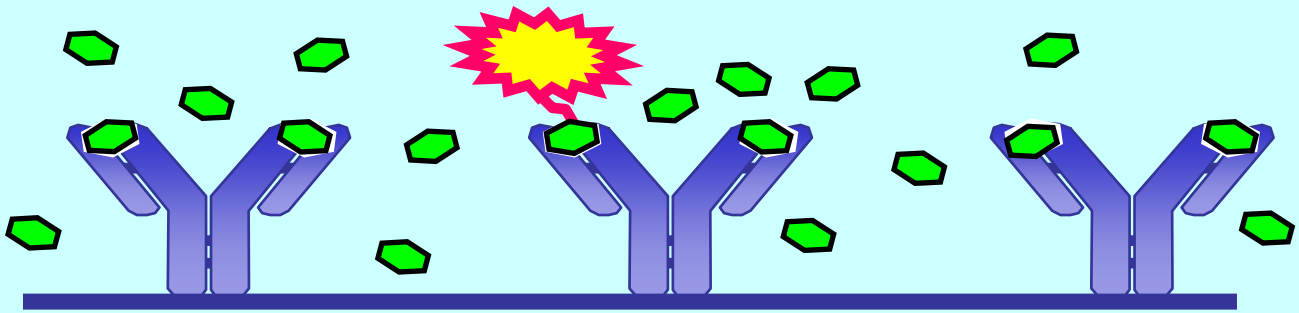


# Competitive Immunoassay

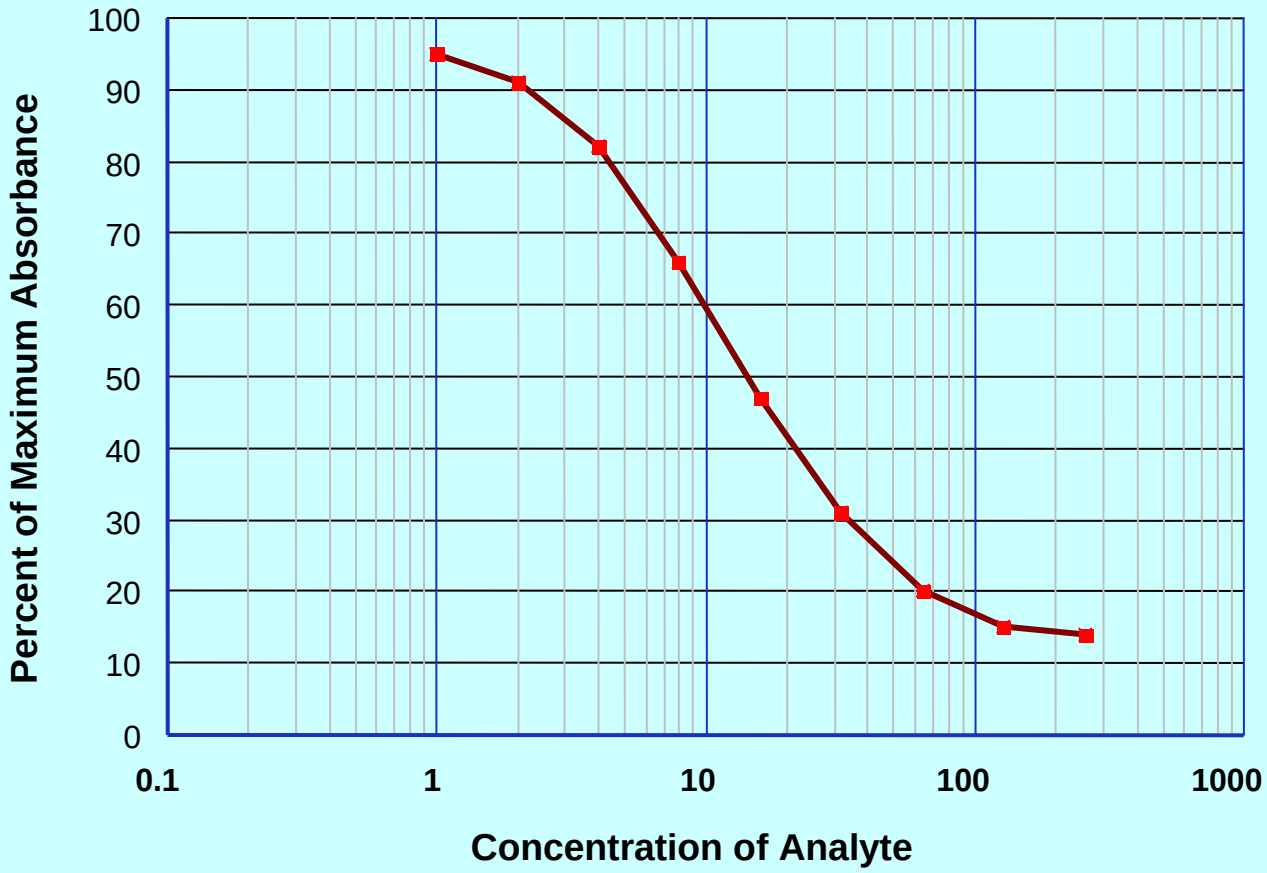
**I. No Analyte - high detection signal**



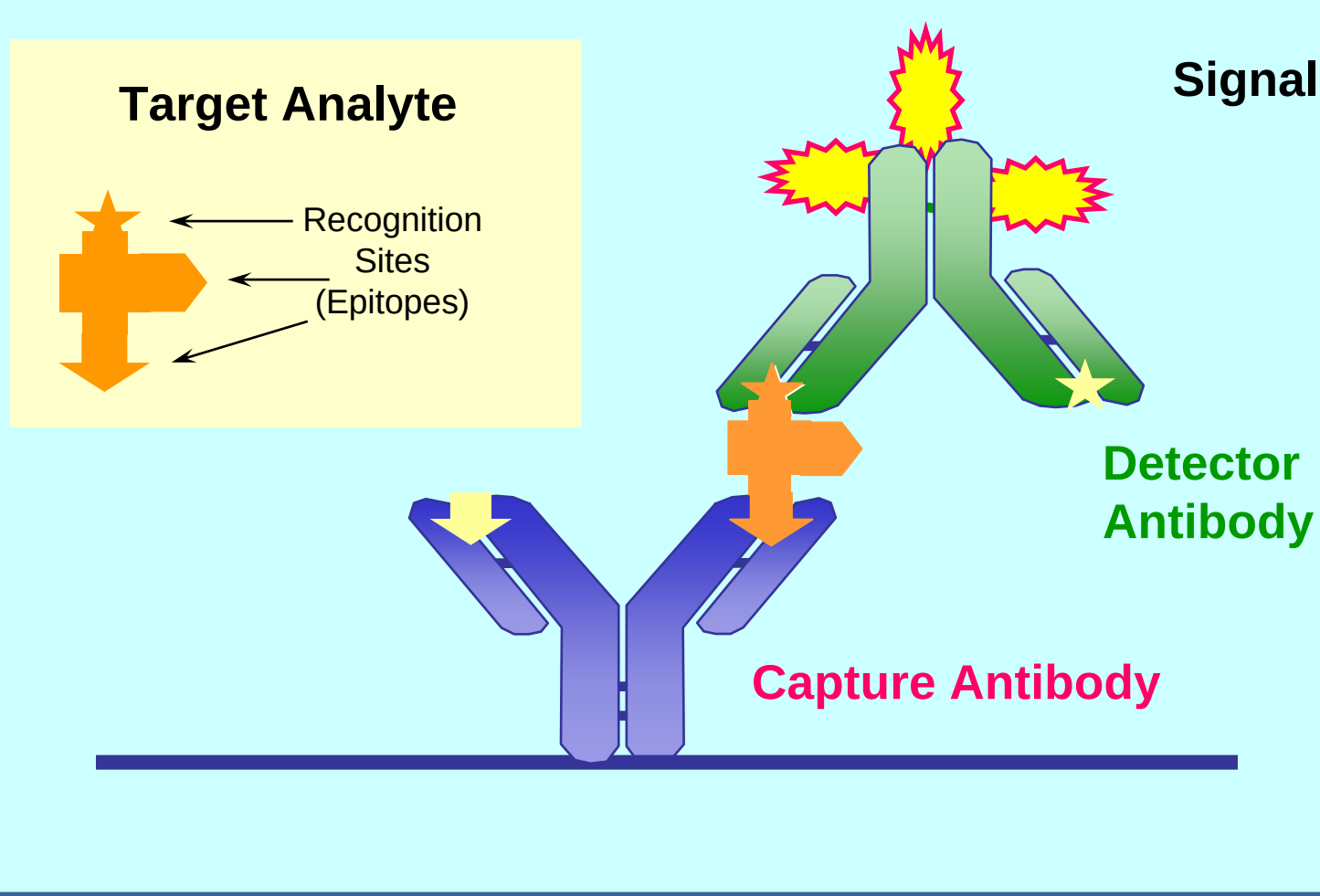
**II. Analyte present - detection signal reduced**



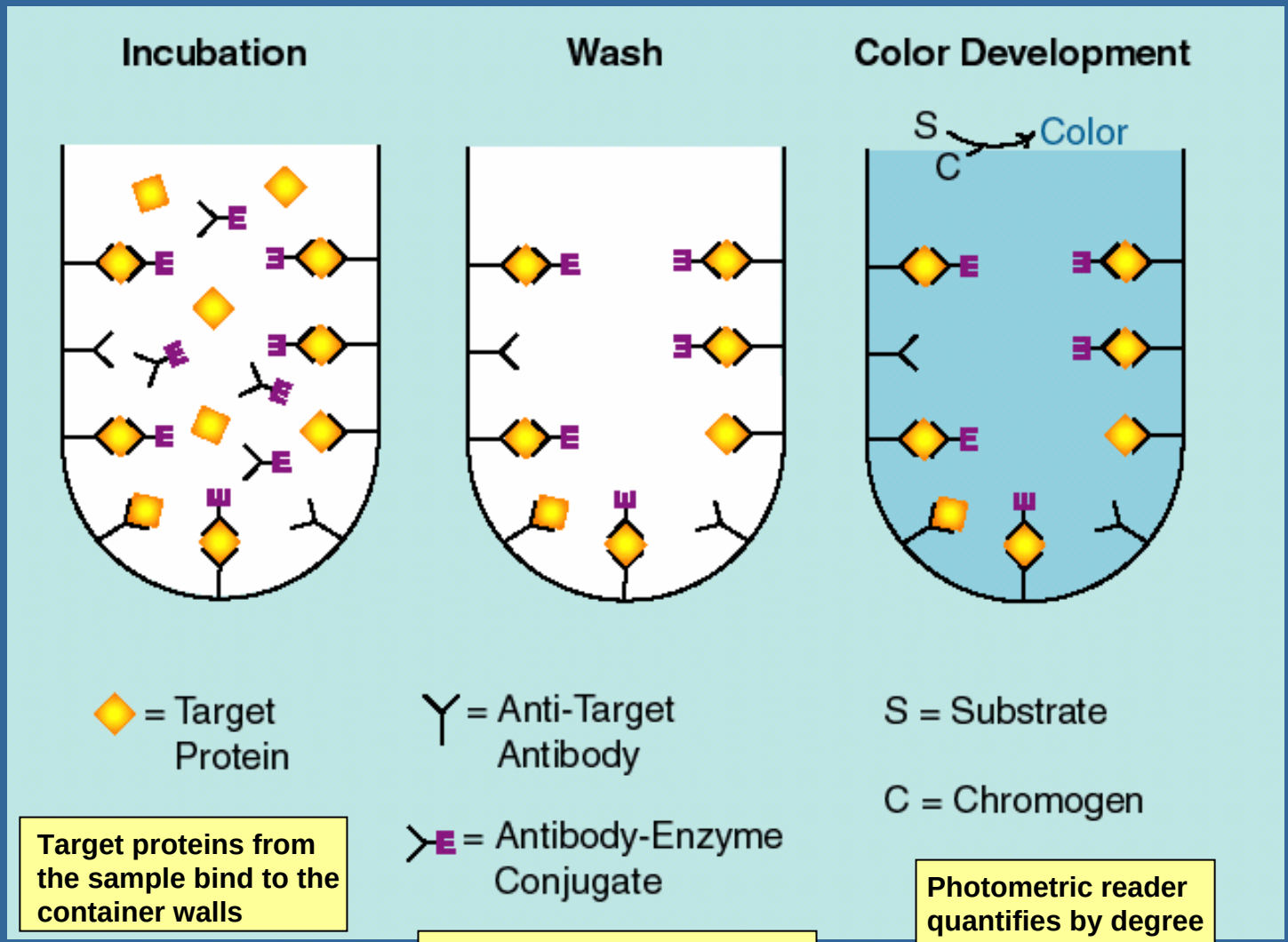
# Competitive Immunoassay Data Format



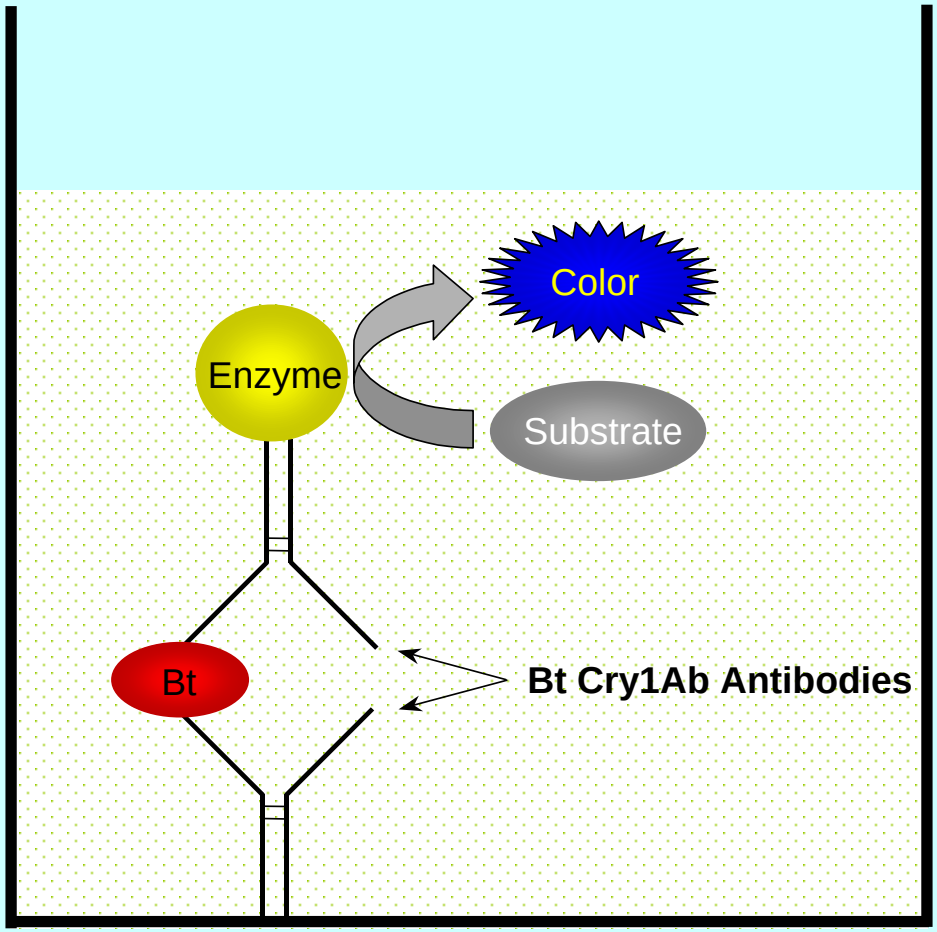
# Double Antibody Sandwich Immunoassay



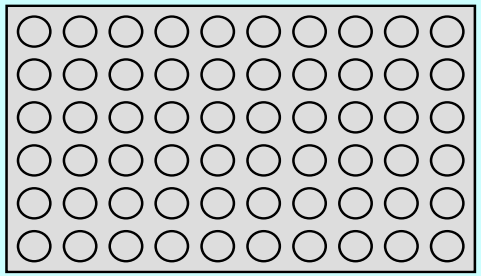
# Sandwich Immunoassay Principle



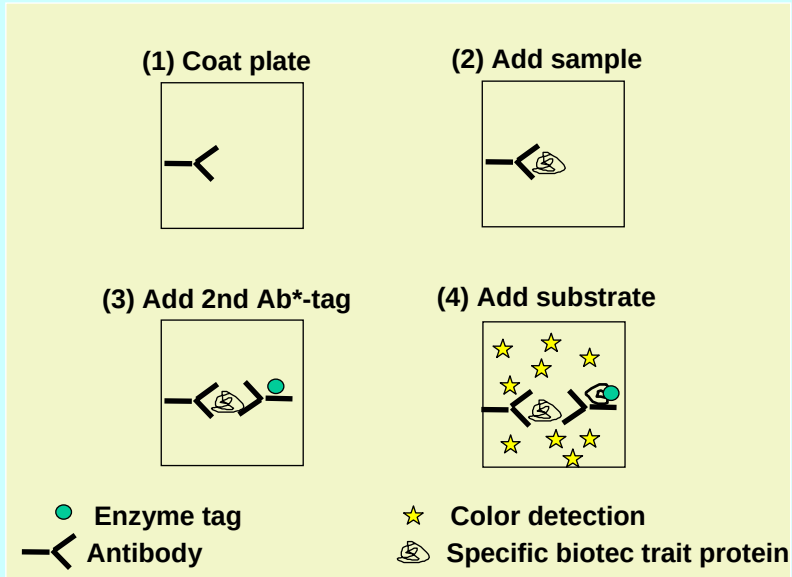
# Bt Cry1Ab Sandwich ELISA



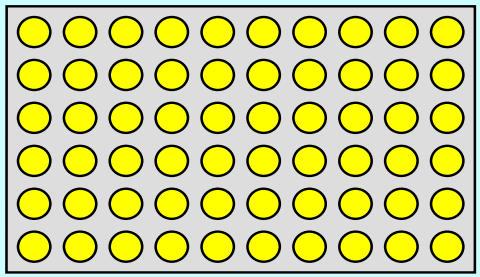
# Protein Based Detection by ELISA



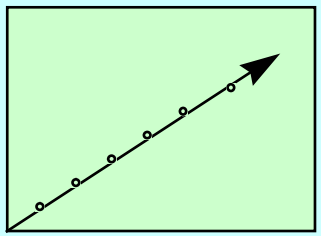
Within each well




Color detection



Determine unknown concentration from a standard curve



# Microwell Plate Format

- Quantitative
- 1-2 hours
- Plate reader required

# Coated Tube Format

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

# Comparison of PCR, ELISA and Strip Methods

| | PCR | ELISA | Strips |
|---|---|---|---|
| **Design** | DNA/Line specific | Protein specific | Protein specific |
| **Requirements** | Sophisticated | Moderate | Easy |
| **Assay time** | 3-14 days | 2-7 days | 5-10 min |
| **Sample cost** | $400-600 | $100+ | $7-10 |
| **Availability** | Published/Testing Co | Diagnostic Co. | Diagnostic Co. |
| **Validation** | JRC (qualitative) | JRC/USDA | USDA |
| **Application** | Qualitative/semi-quant | Qual/semi/quant | Qual/ test/ compliance with threshold |
| **Limitations** | Basic pH, heat, false pos, false neg dynamic sys | heat, extraction, reference materials Protein dependent | Same as ELISA pro-zone, 1 sample/test |
| **Sensitivity** | ~>0.1% GMO | ~>0.3% GMO | ~1% GMO |

# Other Immunoassay Markets

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

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

# Clinical vs. Environmental Immunoassay

## The Sample

- Clinical
  - Urine, blood, saliva
- Environmental, Agriculture (more diverse matrices)
  - Water
  - Soil extracts
  - Plant extracts
  - Animal products/tissues - blood, urine, milk, meat
  - Food
  - Industrial processes and effluents