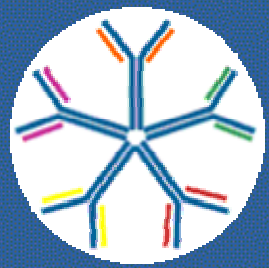
Methods for Detecting and Measuring Ag Biotech Products



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

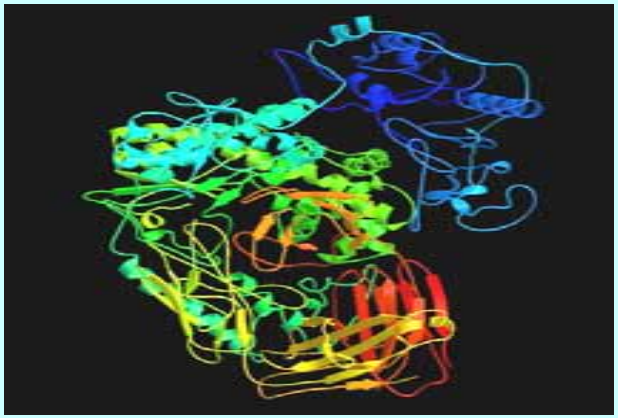
- **Consumer Choice**
- **Approved Events – Quantitative and threshold testing**
  - European Food Labeling Law
    - Labeling began April 10, 2000 and updated April 2004
    - Threshold adventitious < 0.9% - "genetically modified"
  - Japanese Food Labeling Law
    - Labeling began April, 2001
    -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

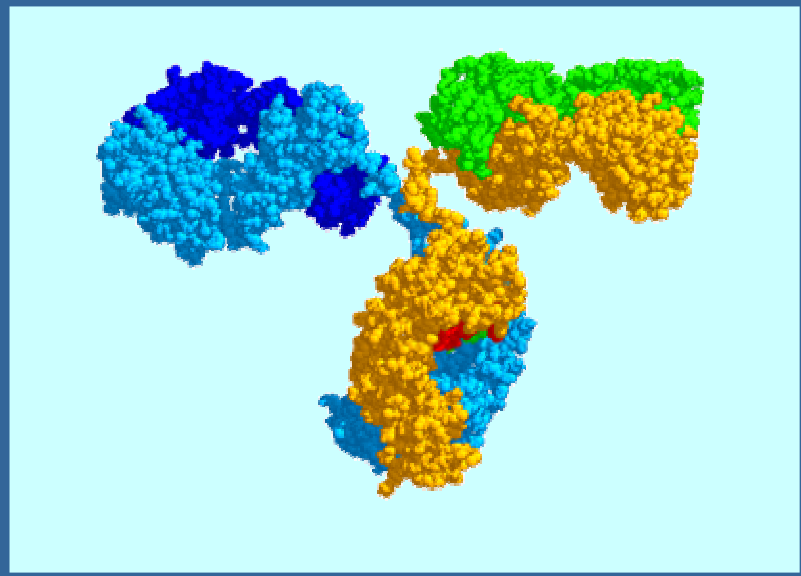
- **Results are reported in terms of % Ag Biotech**
  e.g. 1 Biotech corn kernel in 99 negative = 1%
- **Decisions are based on regulated thresholds (given in weight %)**
- **Testing is based on detection/quantitation of novel DNA or protein**
- **Ag Biotech concentrations are estimated from protein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



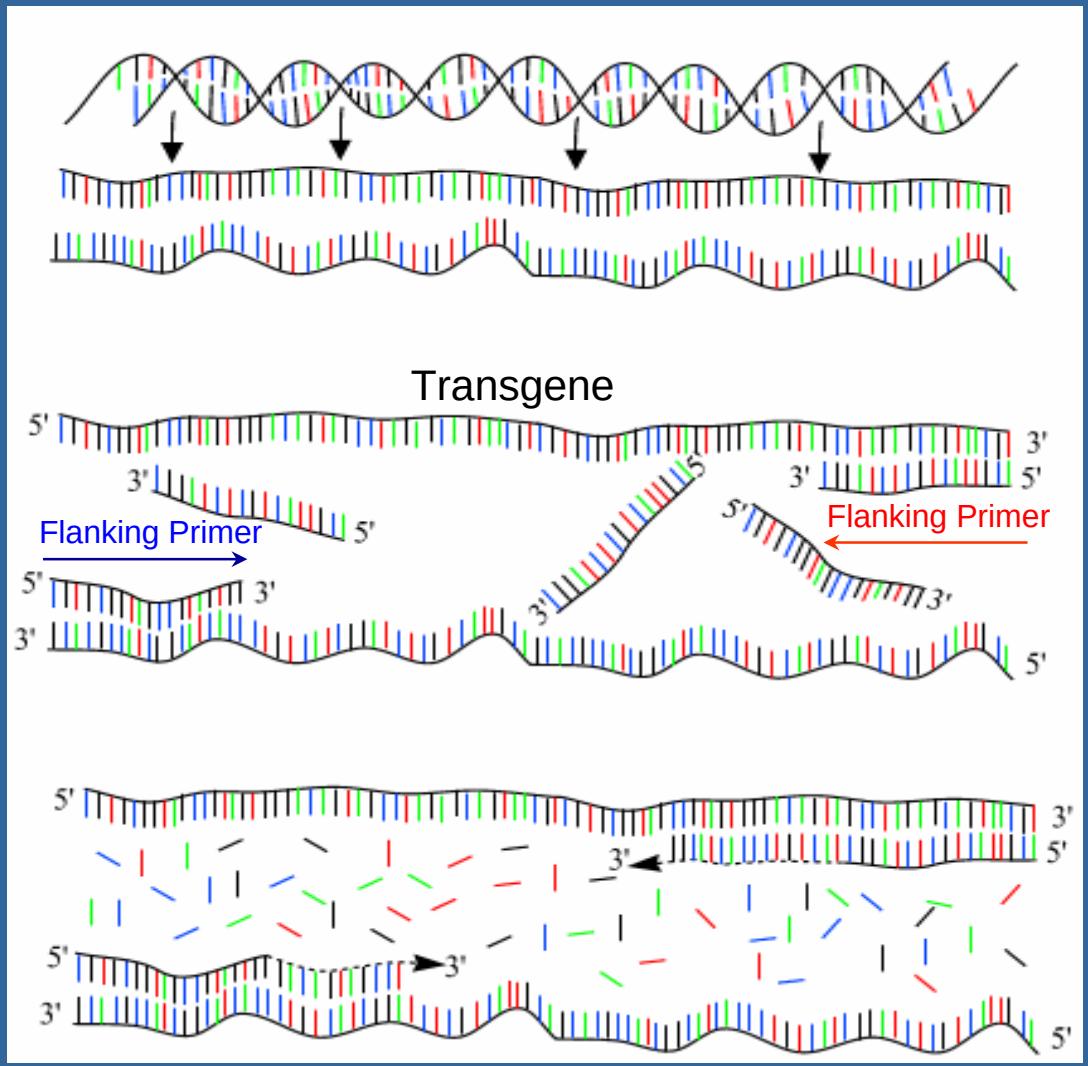
PCR – Uses

- 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)



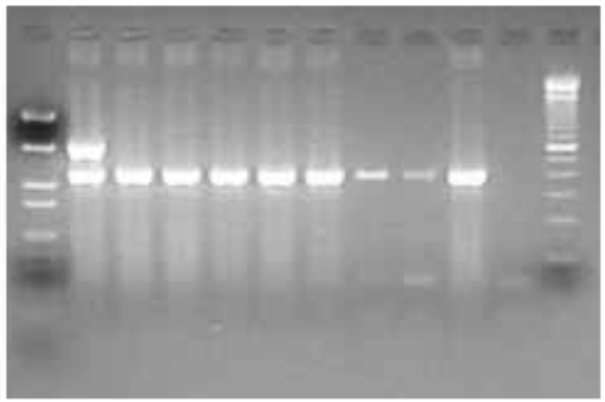
- **Denaturation Step**
  DNA denatures at high temperature (94C)
- **Anneal Step**
  Temperature lowered (50-65C)
  Primers anneal to target
- **Amplification Step**
  Temperature raised (72C)
  Taq polymerase extends annealed primers
  Target DNA is amplified

PCR Processing Steps

DNA Extraction

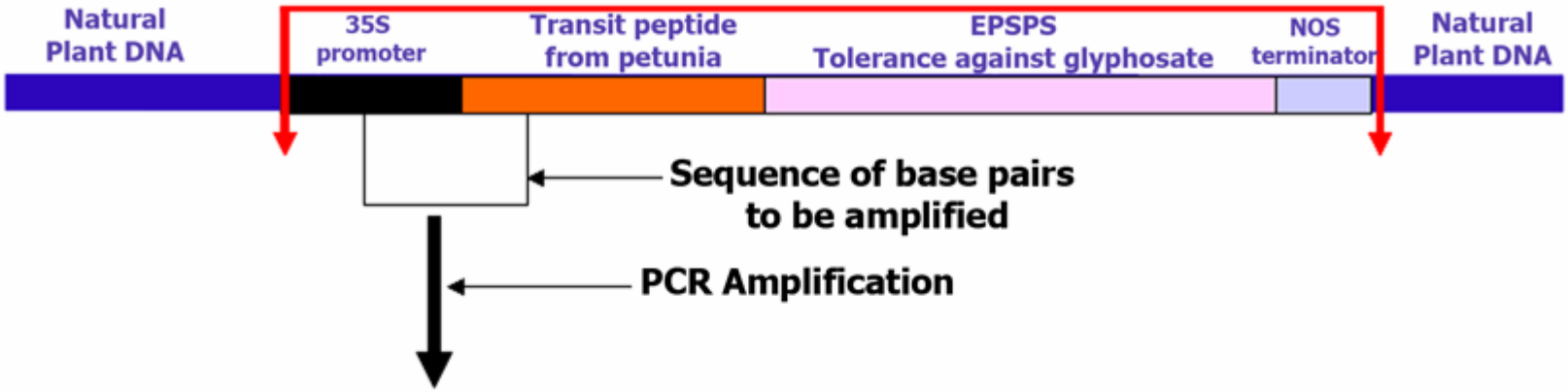
Thermal Cycle PCR

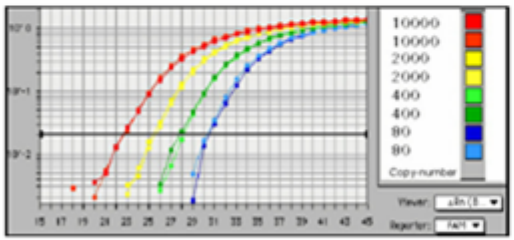
Qualitative or Quantitative Analysis
Detection e.g. by gel analysis



Has The DNA Been Modified?

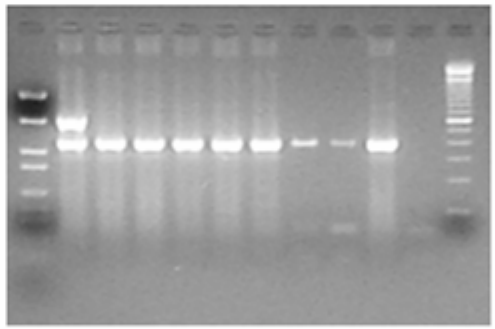
Modified Genetic Sequence (RoundUp Ready™) Inserted Into Soy DNA





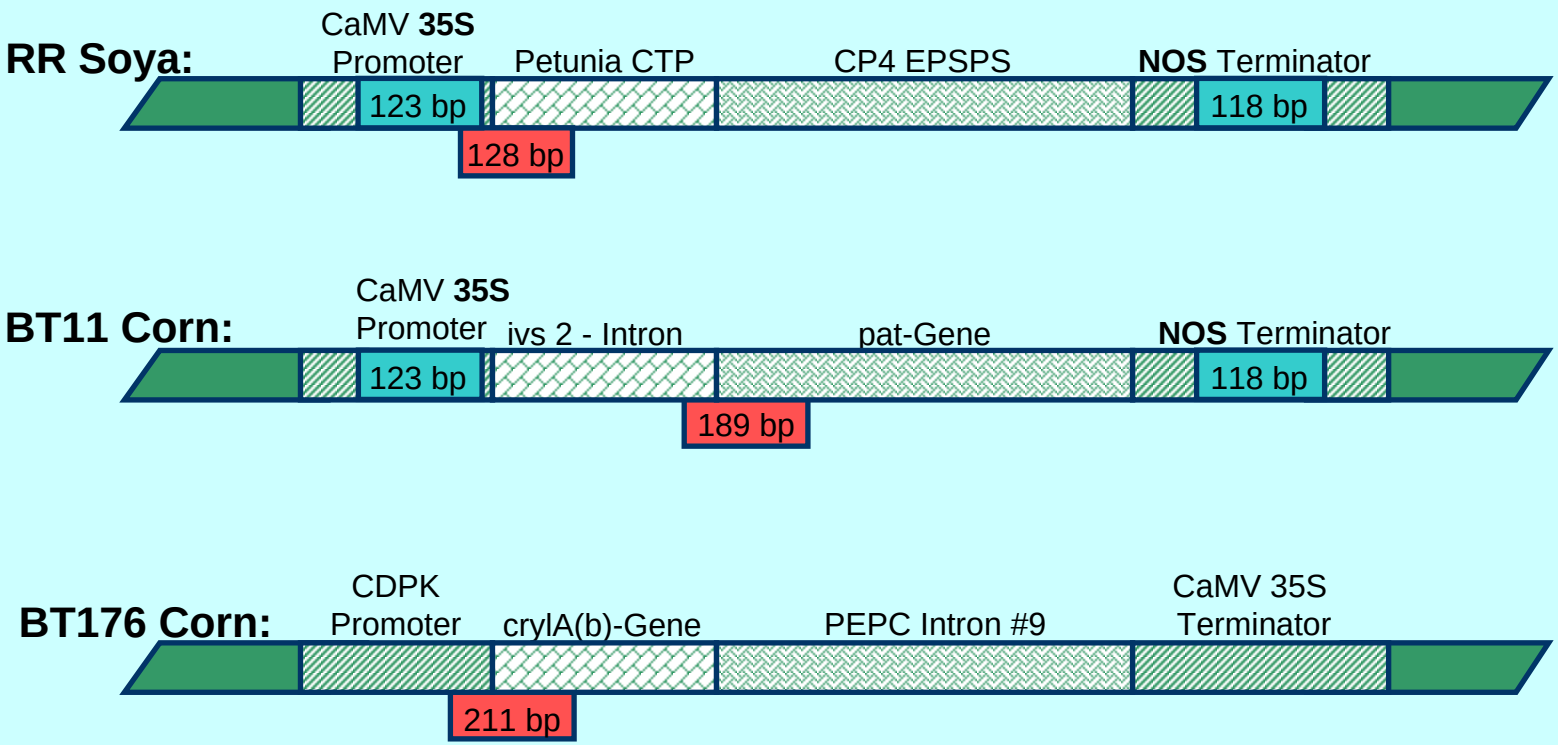
Quantification by fluorescence measurements (real-time PCR)

or



Qualitative results via gel electrophoresis

Some Synthetic Sequences



Applications of PCR

Qualitative

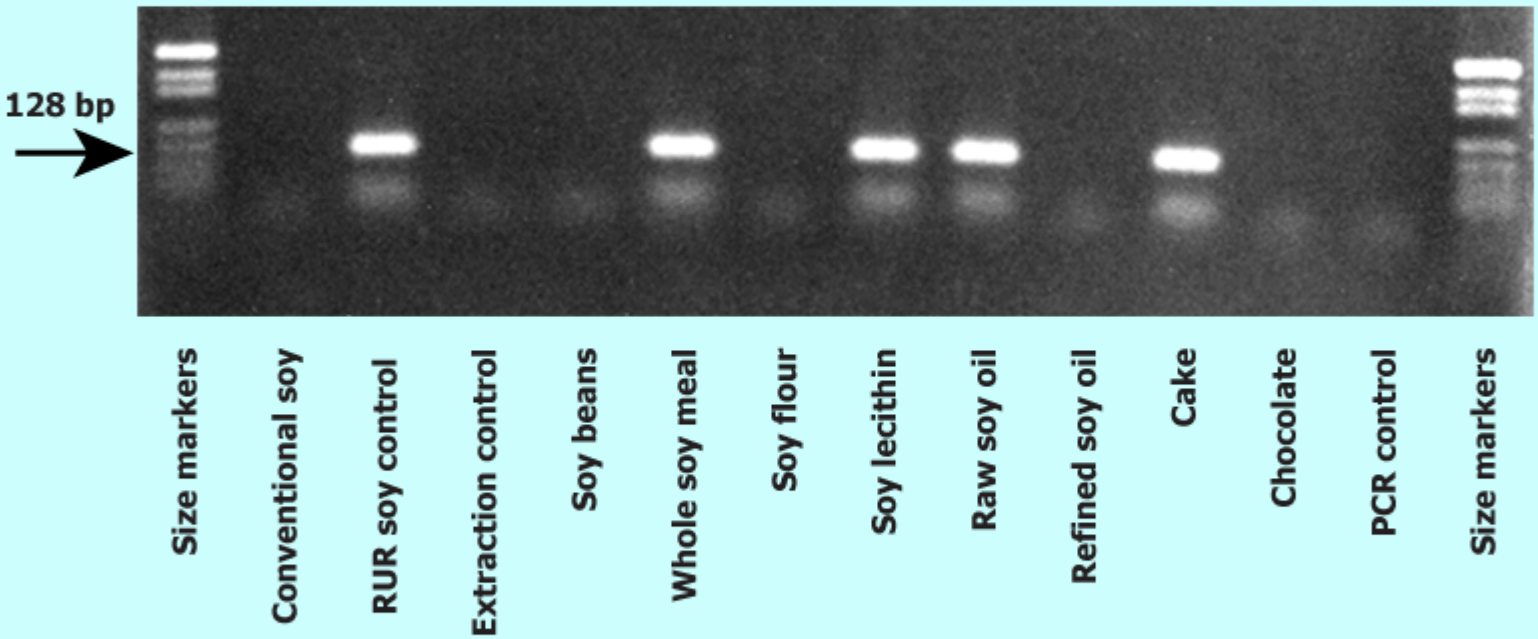
- A "YES" or "NO" answer
- Can look for specific DNA event such as RUR, MON810, etc. or
- Can look for generic elements such as NOS, 35S
- Applicable to a zero tolerance situation

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



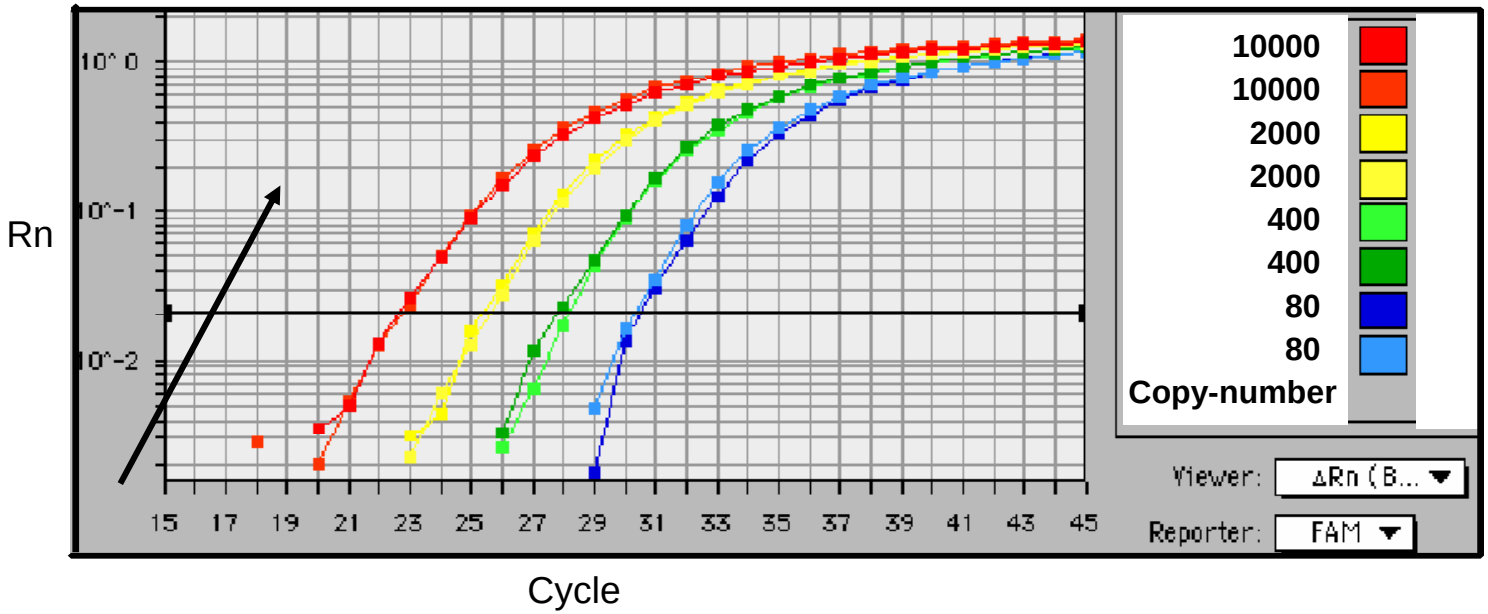
Detection of Roundup Ready® soy-DNA (128 bp amplicon). The samples RUR soy. whole meal, lecithin, raw oil, and cakes contain genetically modified DNA

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:** ▪ **Search data base for potential unwanted (homologous) annealing sites** ▪ **Test new primer system on a wide range of DNA types that appear in food samples** ▪ **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:** ▪ **Follow strict rules how to work cleanly in a molecular biology lab** ▪ **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** ▪ **Use duplicate analysis of each sample** ▪ **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: ▪ Use computer aided primer design ▪ Test the sensitivity of new primer systems for the intended concentration range
Inhibition of PCR reaction	During validation: ▪ Test primer system on sample DNA solutions known to contain inhibitory compounds During routine analysis: ▪ Test each PCR reaction for inhibition by an individual positive control (a spiked counterpart) ▪ Use duplicate analysis of each sample
DNA extraction failed	During design and validation: ▪ Choose appropriate extraction protocol for sample type ▪ Run controls to monitor efficiency of each extraction series