THE POLYMERASE CHAIN REACTION
--how it works and how to use it--

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February 8, 2008

Today's Agenda

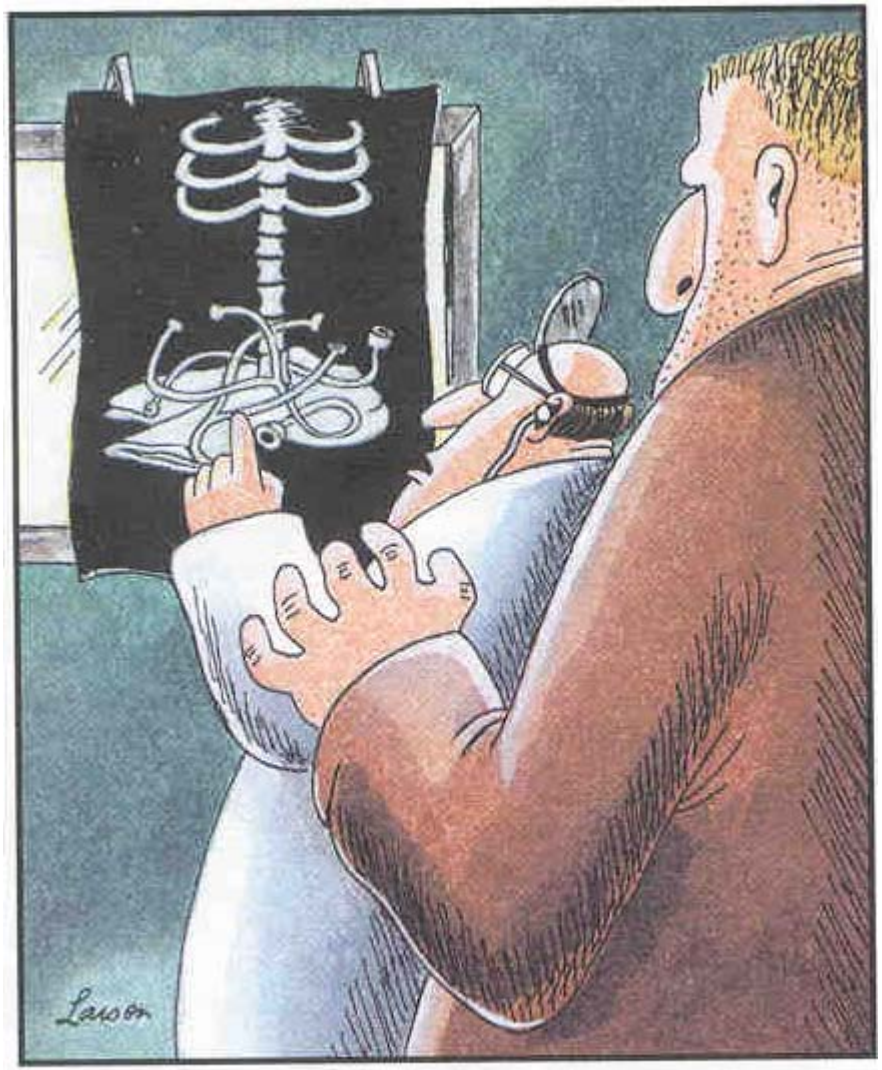
- PCR Basics
  - 8:30 – 10:30 Michael Thompson, BDI
- From Theory to Application
  - 10:45 – 12:00 Ryan Johnson, BDI
- Real Time vs. Traditional PCR
  - 1:00 – 3:00 William Kiffmeyer, ABI
- Group Questions and Discussion
  - 3:15-4:00 Panel

Focus Areas For Today's Presentation

- ***A brief history of PCR***
  - ✓ Not as old a technique as you might think
- ***PCR mechanics***
  - ✓ The central role of the 3'-OH
  - ✓ Process considerations
- ***Classes of assays for Ag Biotech***
  - ✓ Event
  - ✓ Gene
  - ✓ Construct

Modern View of Biotech Quality Control

PCR is the new X-ray



"My goodness, Mr. Osgood! ... Your X-ray reveals several stethoscopes, a smock, and ..."

A Brief History of PCR

--Newer than you might think--

1869 DNA first isolated

1985 Invitro amplification achieved --with *E.coli* DNA polymerase--

Saiki R, K.; Scharf S; Faloona F; Mullis K. B; Horn G. T; Erlich H. A.; Arnheim N., Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, Science, 1985 Dec 20, 230(4732):1350-4.

Mullis K. B; Faloona F. A; Scharf S; Saiki R. K; Horn G; Erlich H. A., Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symposia on Quantitative Biology, 1986

Scharf S. J; Horn G. T; Erlich H. A. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science, 1986 Sep 5, 233(4768):1076-8.

1988 Invitro amplification achieved --with *Taq* DNA polymerase--

Saiki R. K; Gelfand D. H; Stoffel S; Scharf S. J; Higuchi R; Horn G. T; Mullis K. B; Erlich HA. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 1988 Jan 29, 239(4839):487-91.

1989 *Taq* DNA polymerase cloned and expressed in *E.coli*

Lawyer F. C; Stoffel S; Saiki R. K; Myambo K; Drummond R; Gelfand D. H. Isolation, characterization, and expression in Escherichia coli of the DNA polymerase gene from Thermus aquaticus. Journal of Biological Chemistry, 1989 Apr 15, 264(11):6427-37.

PCR Achieves Fame and Fortune

--becomes standard in molecular biology tool box--

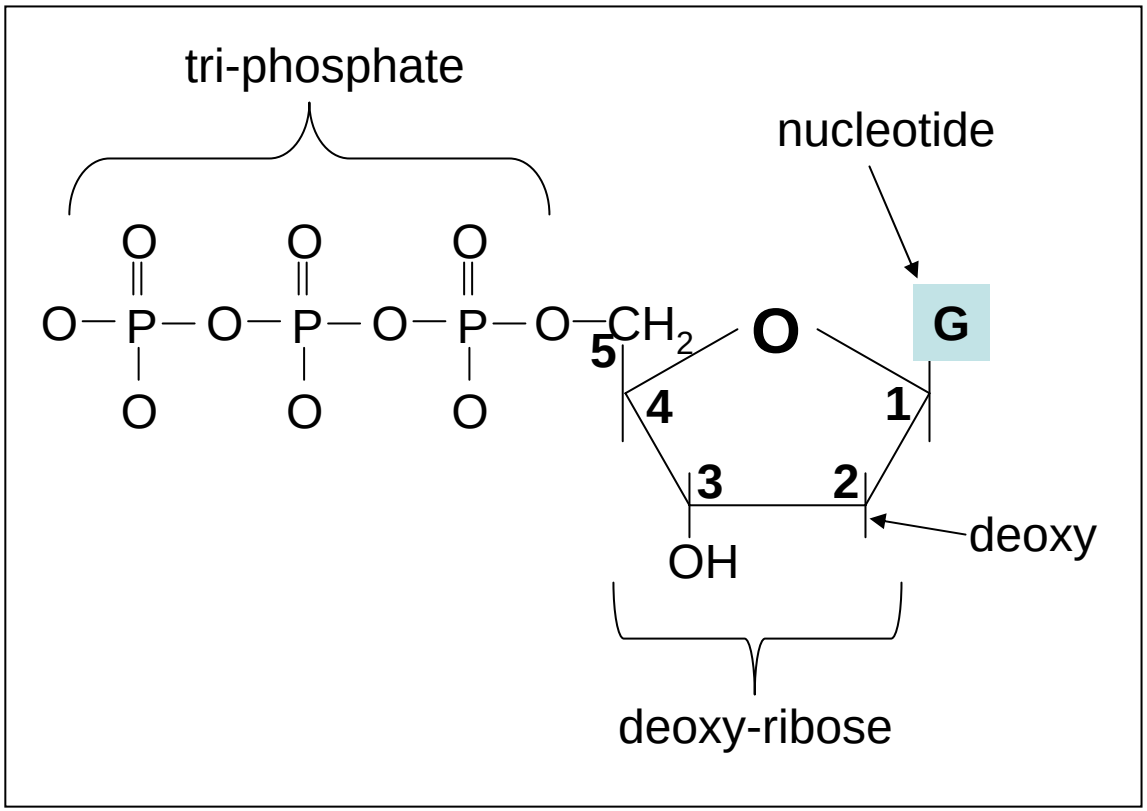
The Molecule of the Year

RUTH LEVY GUYER AND
DANIEL E. KOSHLAND, JR.

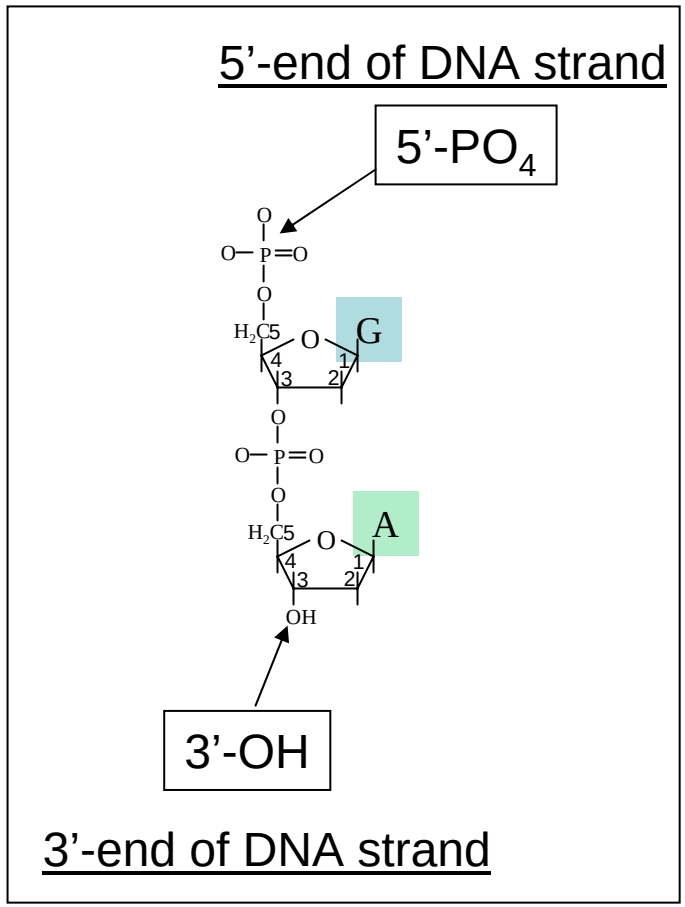
Science HAS SELECTED THE POLYMERASE CHAIN REACTION AS the major scientific development of 1989 and has chosen for its first "Molecule of the Year" the DNA polymerase molecule that drives the reaction. The list from which the polymerase chain reaction (PCR) was chosen included an impressive array of accomplishments in many areas of science and technology; additional kudos are therefore conferred below to 17 of the other big "stories" that made 1989 an exciting year for scientists and for followers and beneficiaries of science. Although the PCR procedure was introduced several years ago, use of the technique truly burgeoned in 1989; in much the same way, the full potentials of many of the interesting "runner-up" scientific achievements of this year are likely to be realized sometime in the years to come.

Defining the Key Molecules

dNTP
(Deoxy nucleotide triphosphate)



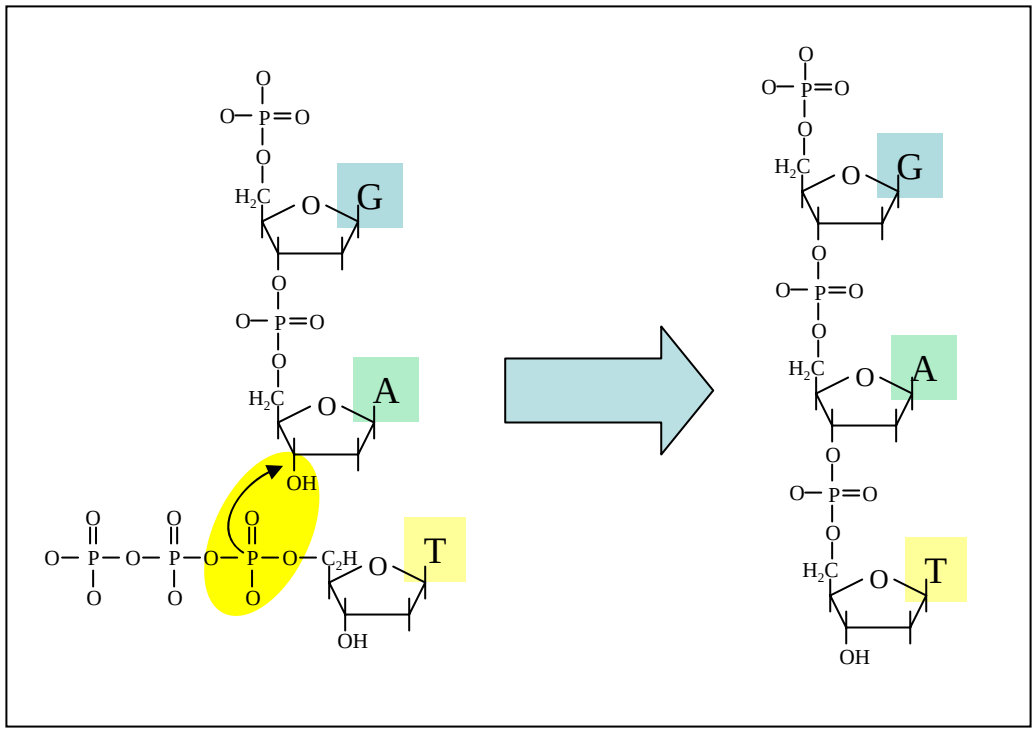
DNA
Deoxy ribonucleic acid



Everything Hinges on the 3'-OH

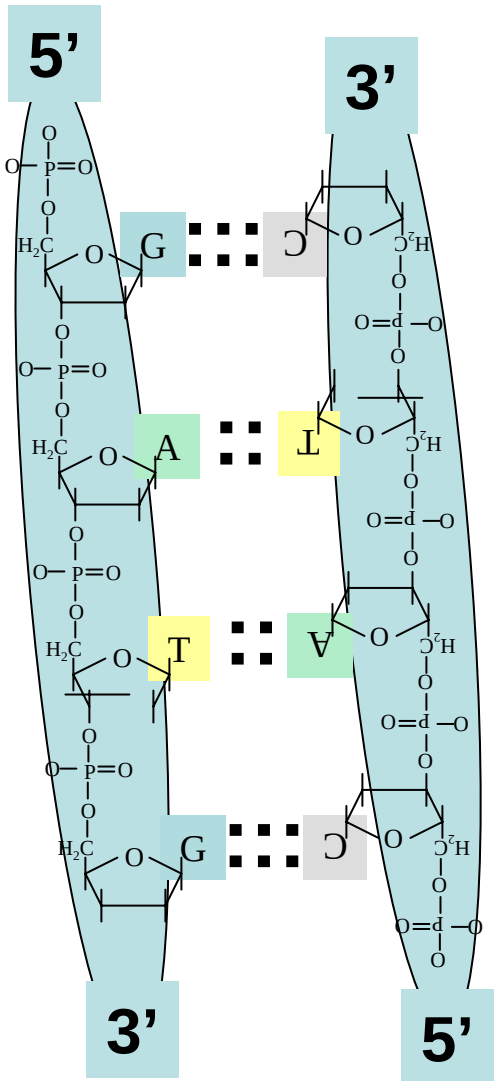
--DNA polymerase catalyzes addition of dNTPs to 3'-OH--

DNA-polymerase reaction



The DNA Backbone is Directional

--strands of a double helix are anti-parallel--

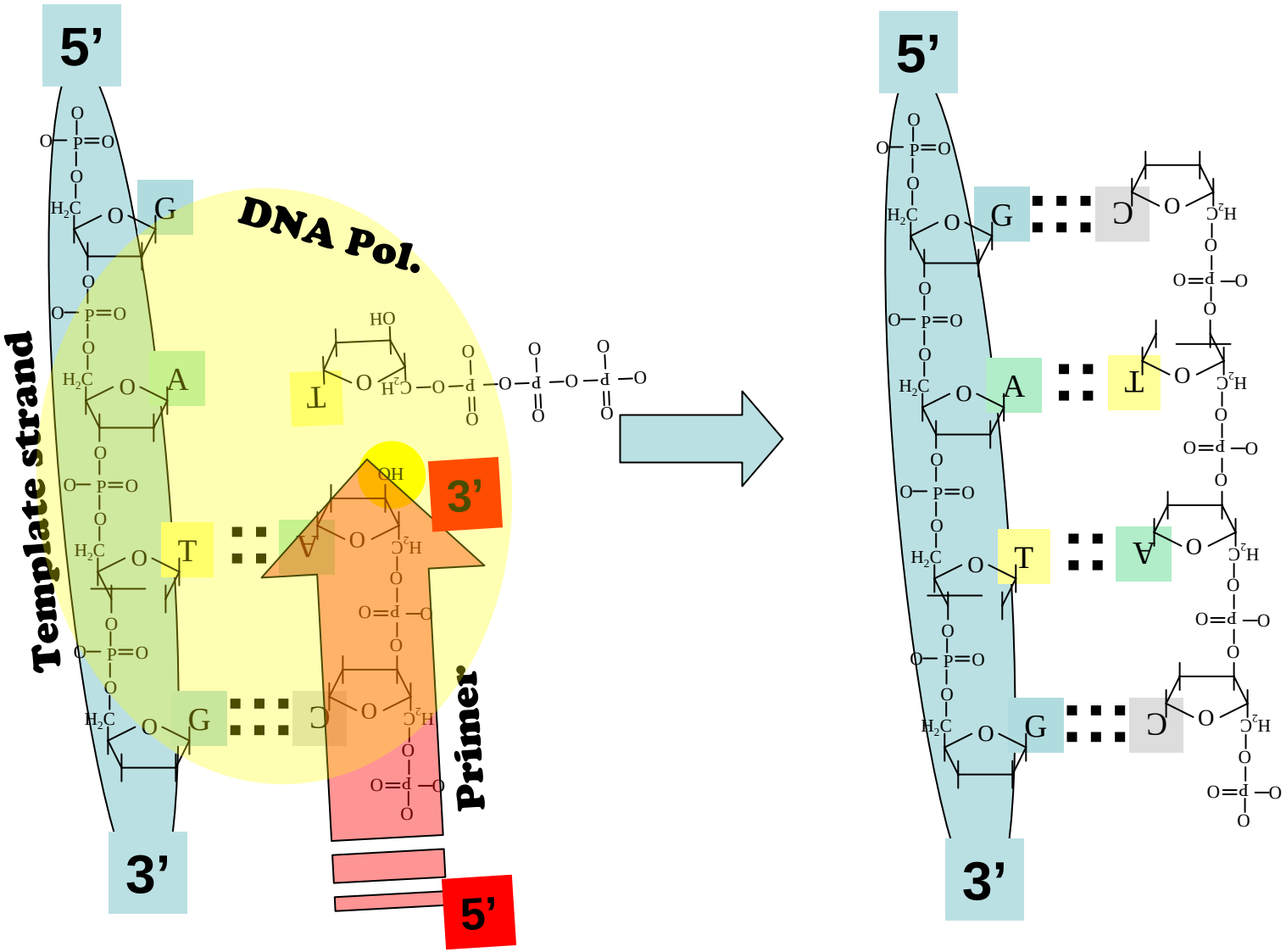


In what direction does strand elongation occur in DNA synthesis?

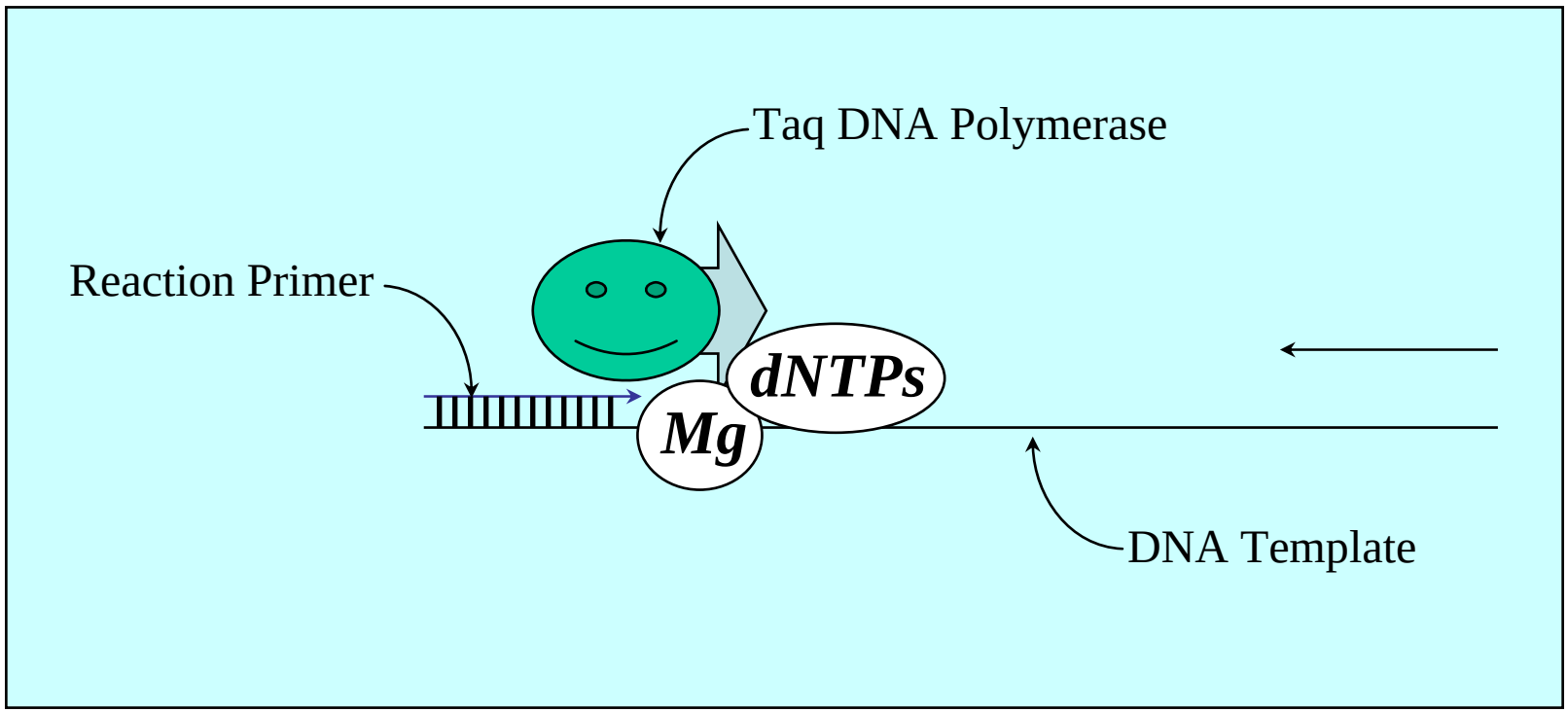
A. 5’ to 3’
B. 3’ to 5’
C. A and B
D. None of the above

The Primer Supplies the 3'-OH for Elongation

--The template dictates which nucleotide is added next--

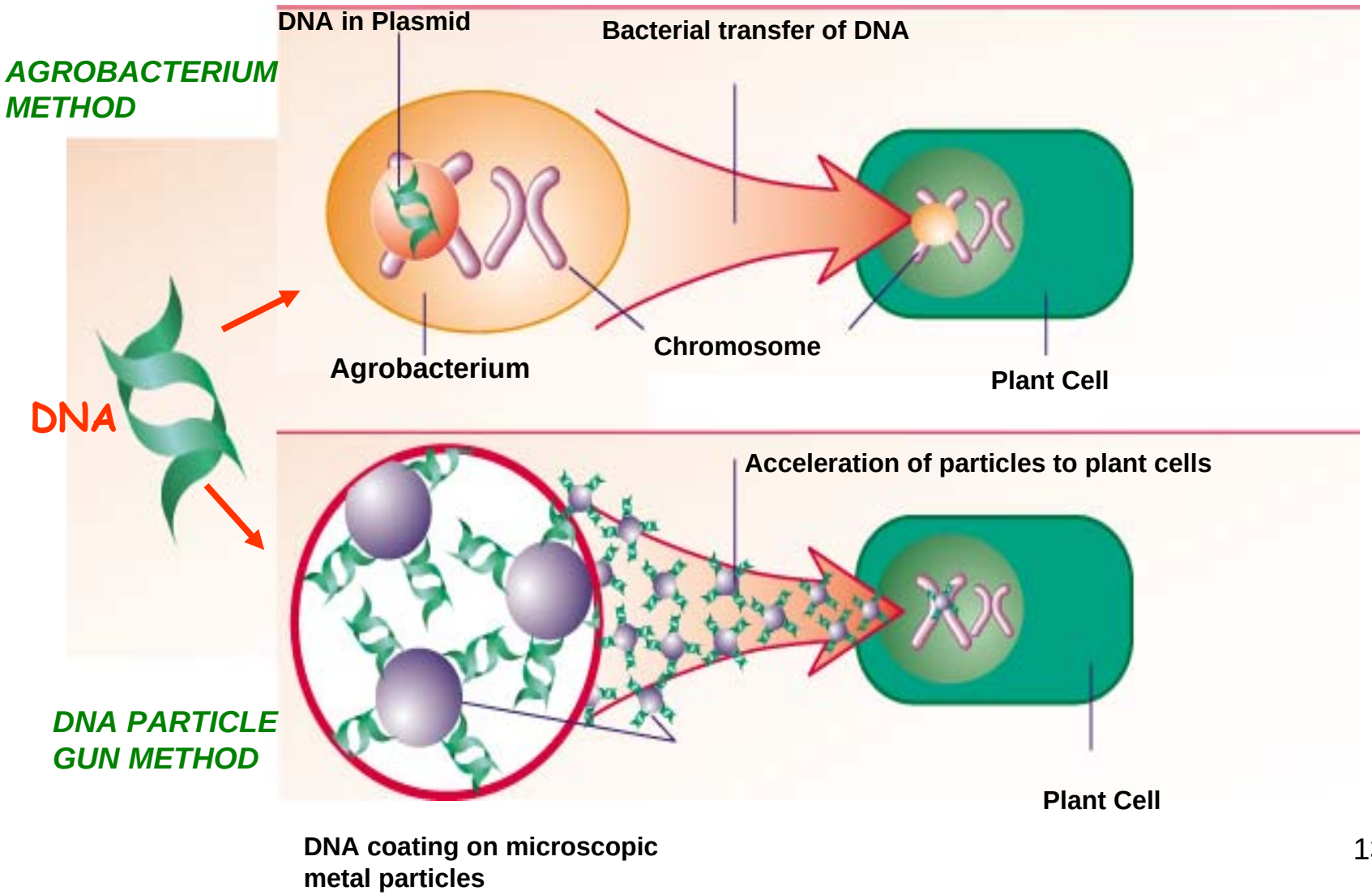


Five Components Comprise the Basic Framework for PCR

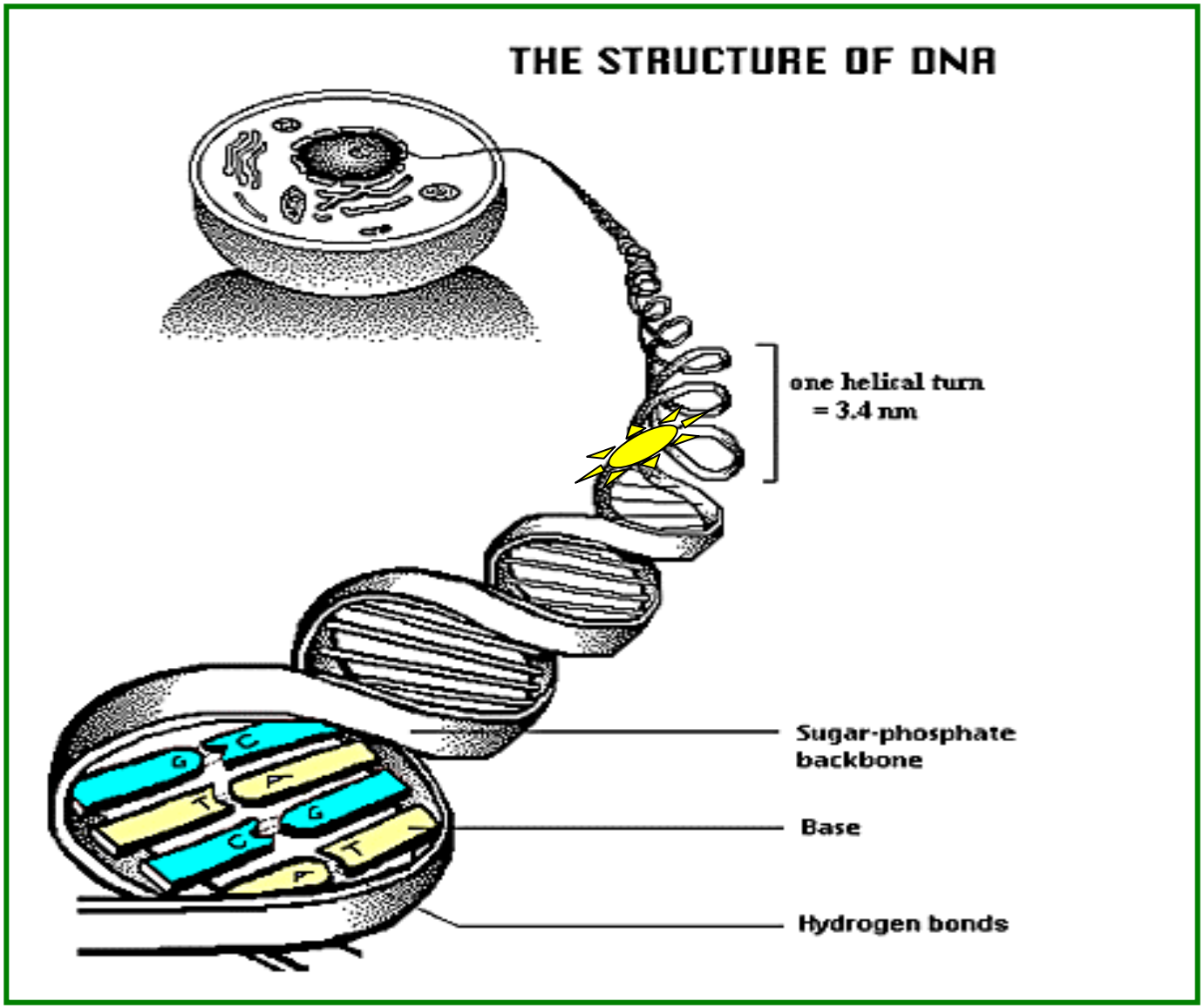


PCR ANIMATION

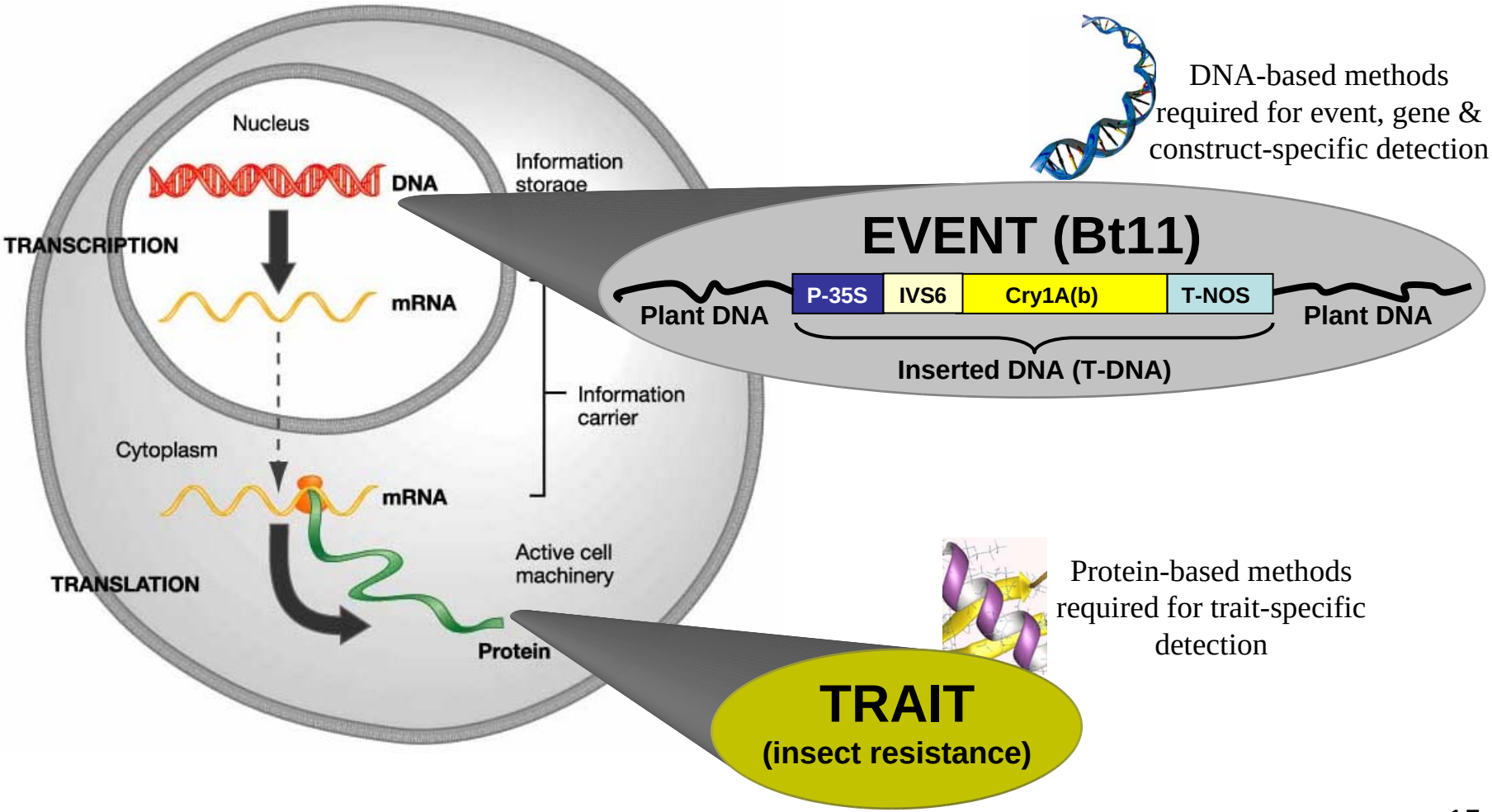
INSERTING GENES INTO PLANT CELLS



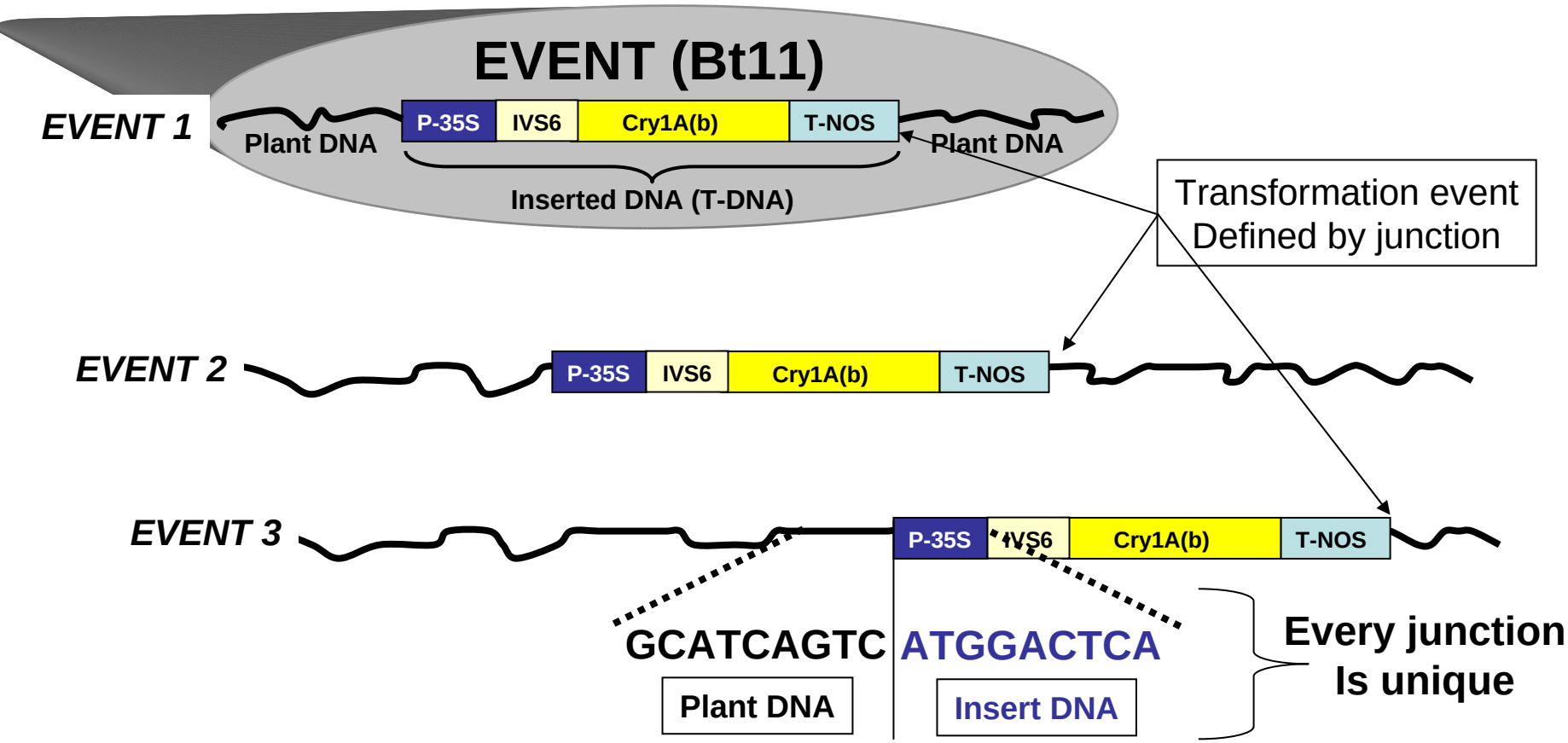
Structure of DNA



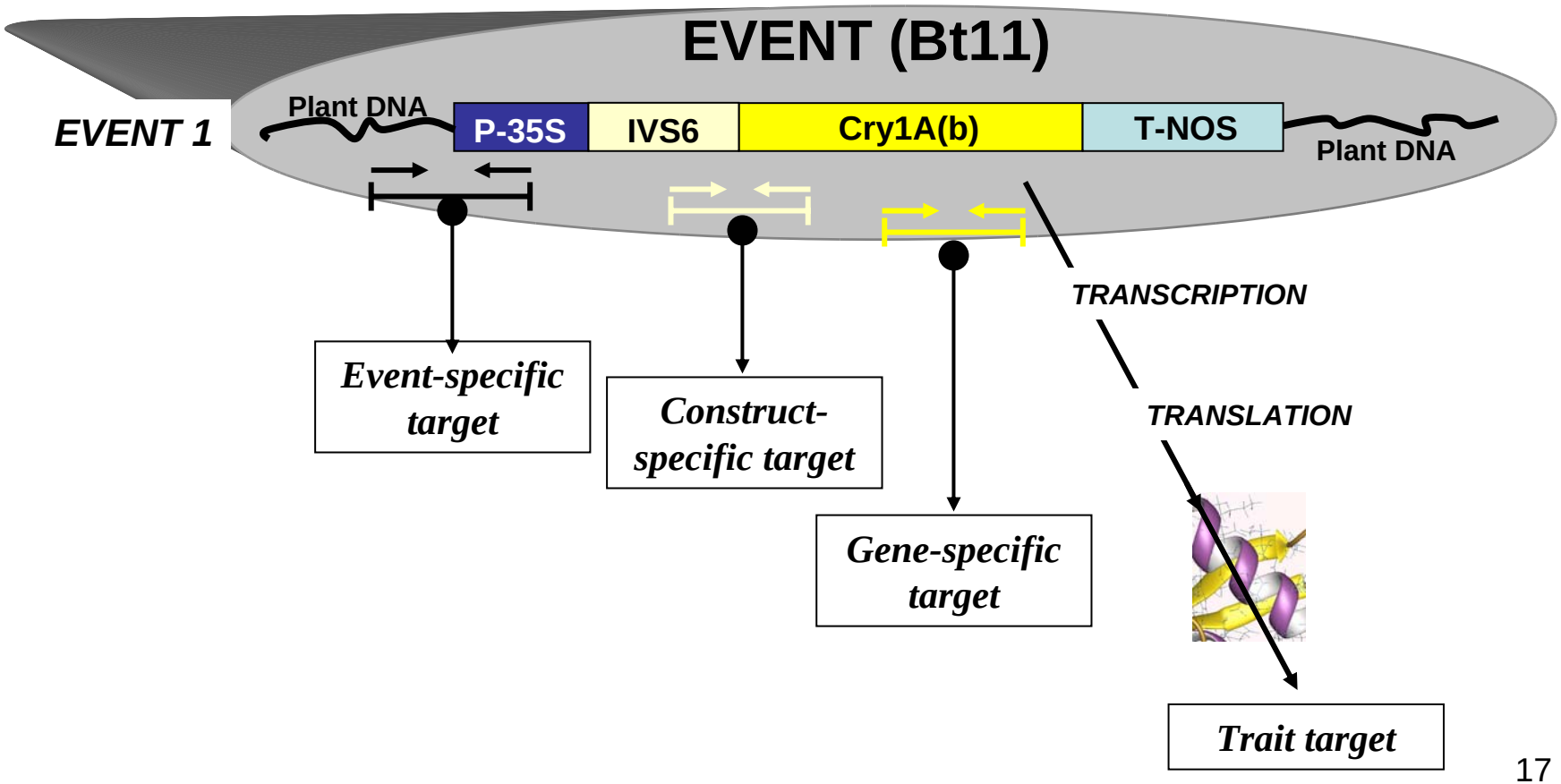
DNA-Based *"EVENTS"* Give Rise To Protein-Based *"TRAITS"*



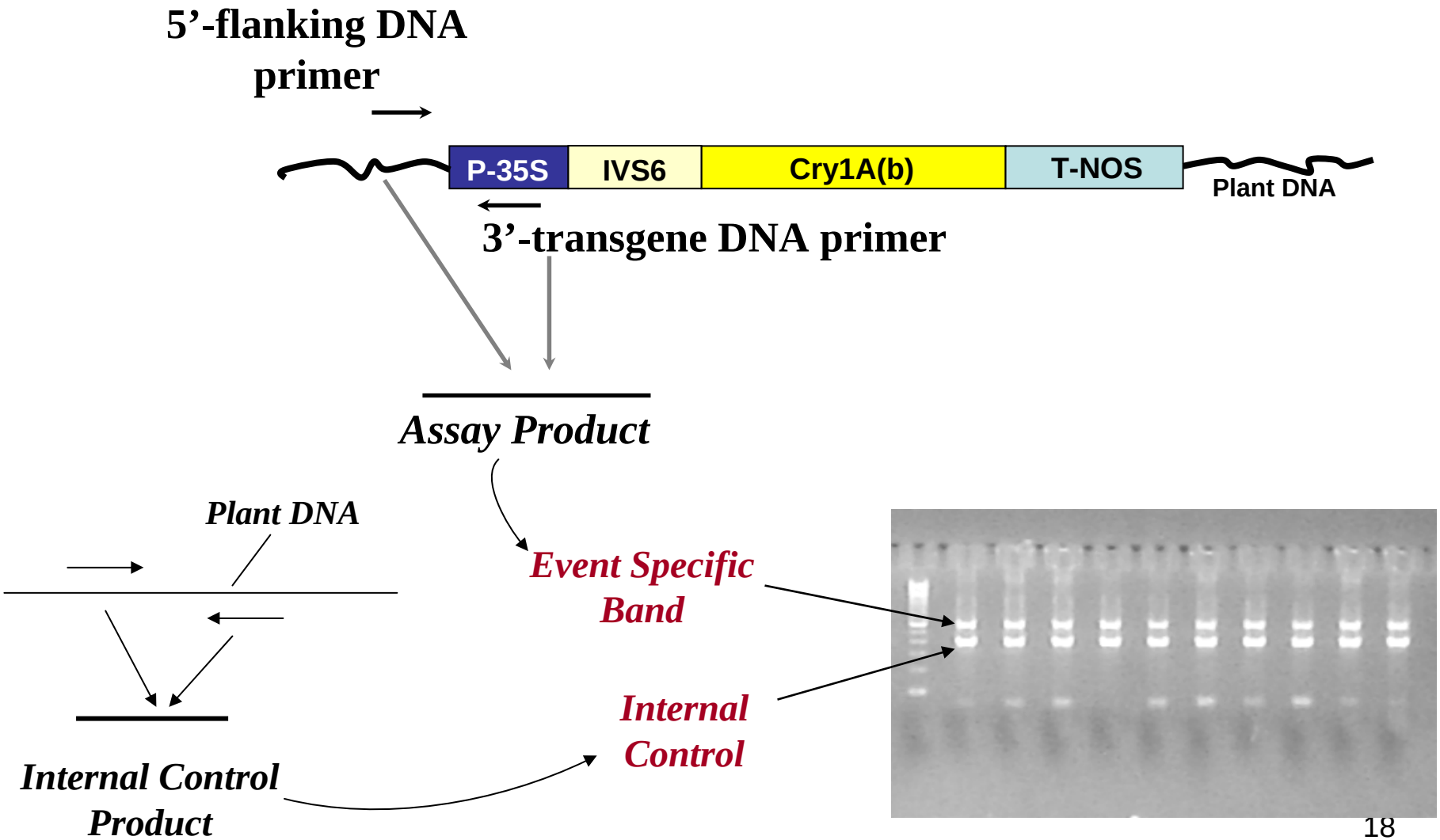
A Transformation Event is Defined by the Plant-DNA/T-DNA Junction



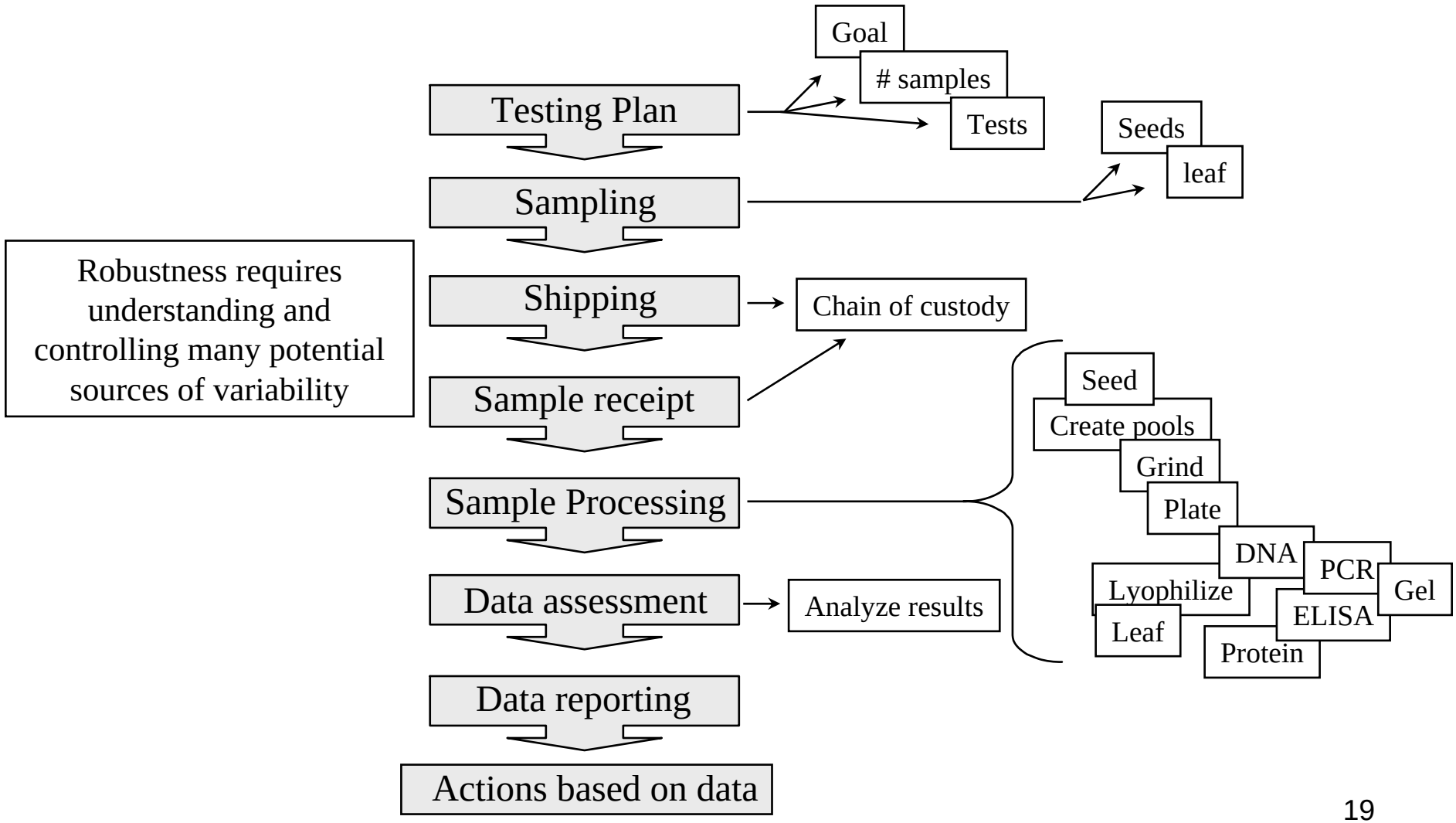
Event, Gene & Construct-Specific Detection Require DNA-based Methods
Trait Detection Requires Protein-based Methods….Today



One Example of an Event-Specific PCR Assay --with an internal control--



Making a Diagnostic Method Work is Straightforward --Robustness Is the Challenge— --how can you know your laboratory is accurate?--



Real World Scenario

Seed Producer: I am sending soybeans to Europe, can you test it to ensure there is no GMO in the lot?

Testing Lab: Yes. I need to get the following information

1. Is the seed lot intended to be GMO free?
2. How much confidence do you want in it's GMO freeness?

Seed Producer:

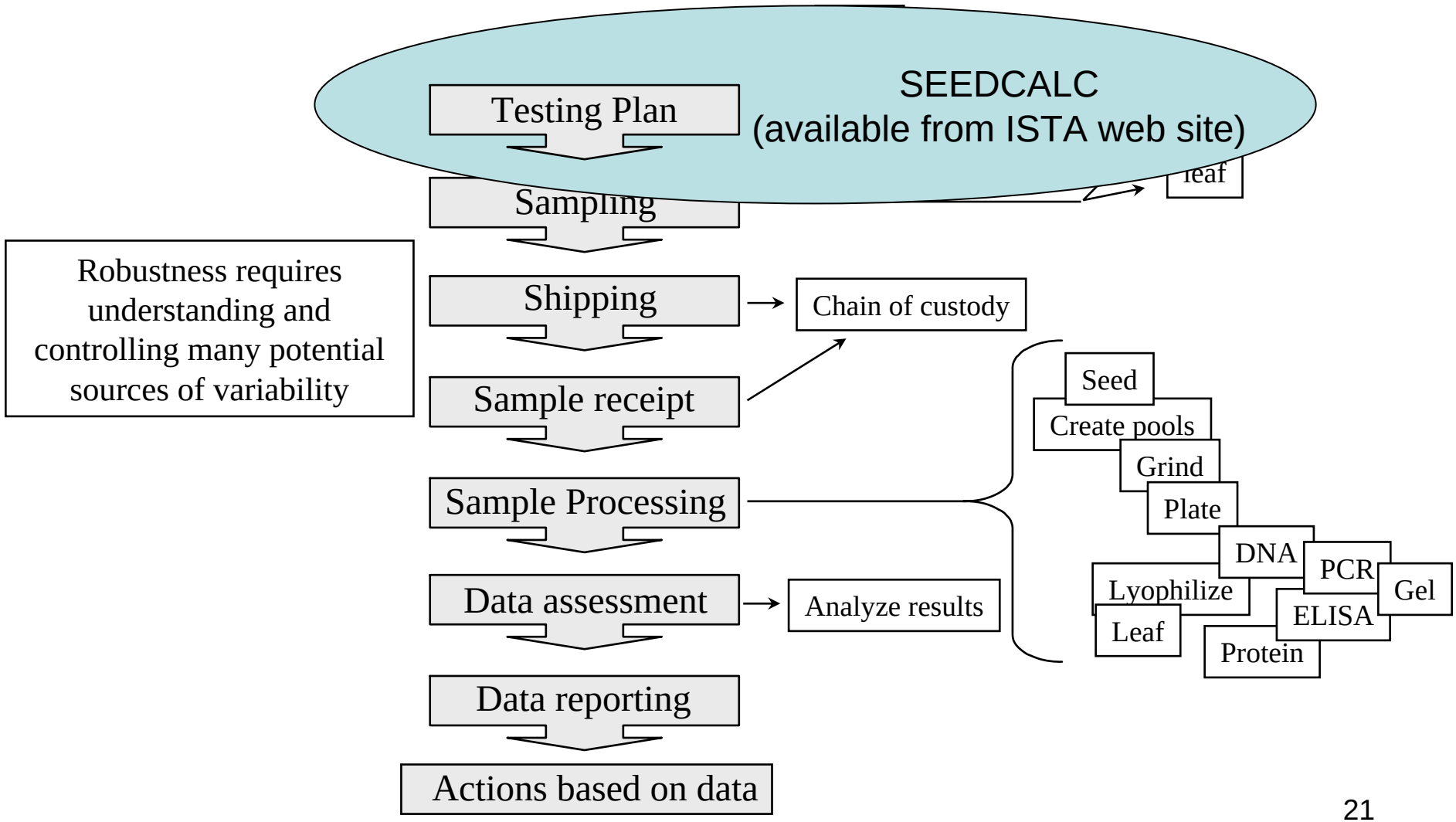
1. Yes. (what if it were RR, YG, Herc, etc?)
2. Guarantee that it is 100% GMO free.

Testing Lab: OK, but I will need to test every seed in the lot to make that gaurantee and it will cost you a lot of money. Did Europe give any guidelines about what they would do with the seed?

Seed Producer: Yes, they said they would test to ensure it was less than 0.9%

Testing Lab: OK, we will work out a test plan that gives you confidence your seed lot is below 0.9% GMO.

Road Map for Real World Scenario

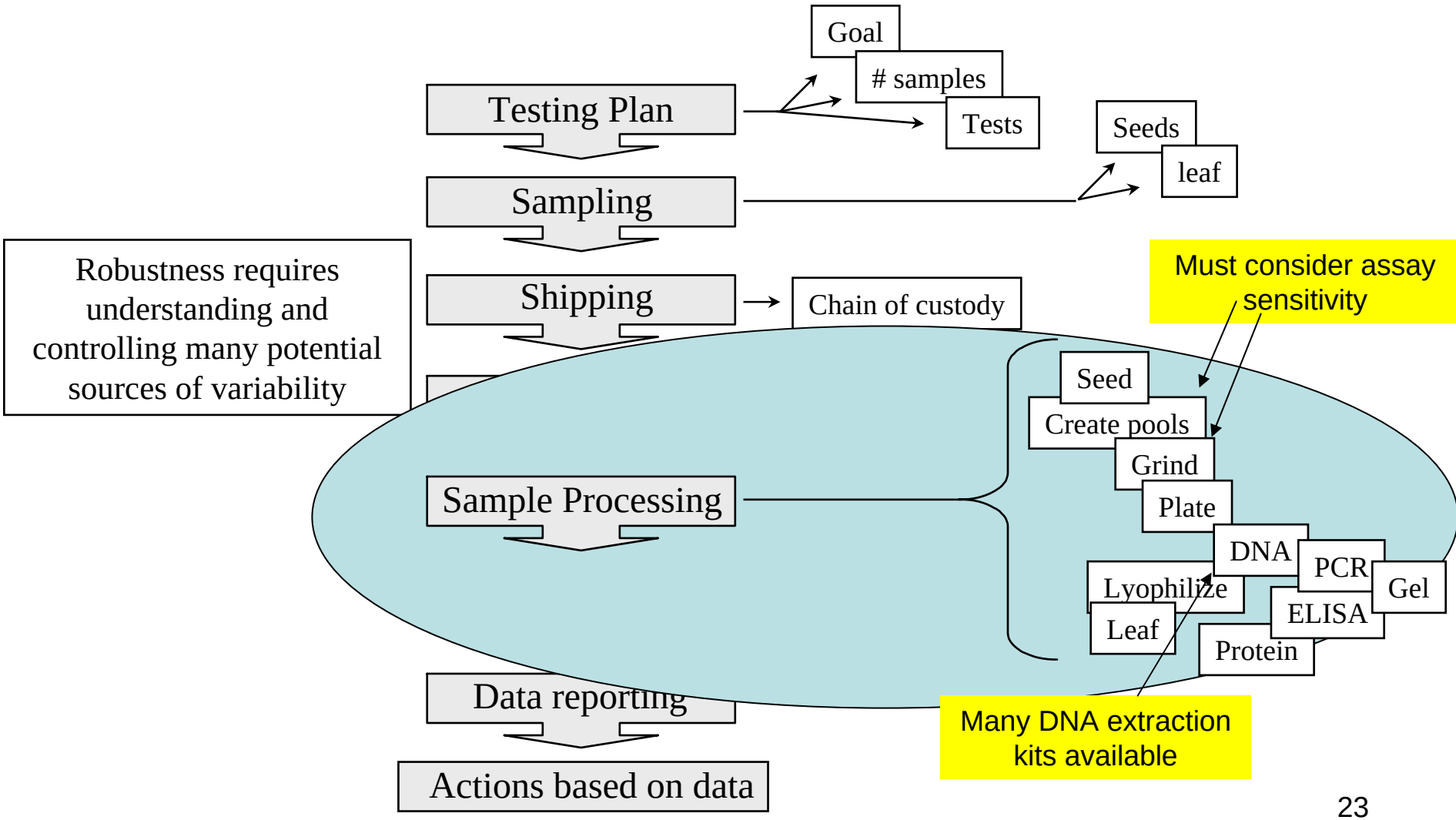


Sample Size by Tolerance Requirements
--for population sampling--

	# Seeds Required*	
Tolerance Range	95% Confidence	99% Confidence
0.0% - 1.0%	300	500
0.0% - 0.5%	600	1000
0.0% - 0.3%	1000	1600
0.0% - 0.2%	1500	2300
0.0% - 0.1%	3000	4600

*Based on SeedCalc

Road Map for Real World Scenario



Ready for PCR……But What Assays Should be Run?

1. Soybeans are straightforward, 35S picks up everything

2. Other crops not so straightforward

Partial List of Genetic Elements Across Soy Events

--from Agbios web site--

Trait	Company	Event/Line name	35s	Nos	Gus	Fad2	PAT
Herbicide resistance	Aventis Crop Science	A2704-12	+				+
Herbicide resistance	Aventis Crop Science	A2704-21	+				+
Herbicide resistance	Aventis Crop Science	A5547-35	+				+
Herbicide resistance	Bayer Crop Science (Aventis Crop Science; Agrevo)	A5547-127	+				+
High oleic acid	Dupont	G94-1	+	+	+	+	
High oleic acid	Dupont	G94-19	+	+	+	+	
High oleic acid	Dupont	G168	+	+	+	+	
Herbicide resistance	Monsanto	40-3-2	+	+			
Herbicide resistance	Bayer Crop Science (Aventis Crop Science; Agrevo)	GU262	+				+
Herbicide resistance	Bayer Crop Science (Aventis Crop Science; Agrevo)	W62	+		+		
Herbicide resistance	Bayer Crop Science (Aventis Crop Science; Agrevo)	W98	+		+		

A Typical PCR Reaction

--There are many variations on this theme--

Reaction mix

Taq polymerase	1U
PCR buffer	22mM Tris-HCl (pH8.4) 55mM KCl 1.65mM MgCl2
dGTP	220mM
dATP	220mM
dTTP	220mM
dCTP	220mM
Primer 1	200nM
Primer 2	200nM
DNA template	10-50 ng

Thermal cycling parameters

25 – 35 cycles

94^{o}C	15-30 sec.
55^{o}C	15-30 sec.
72^{o}C	1min. Per kb

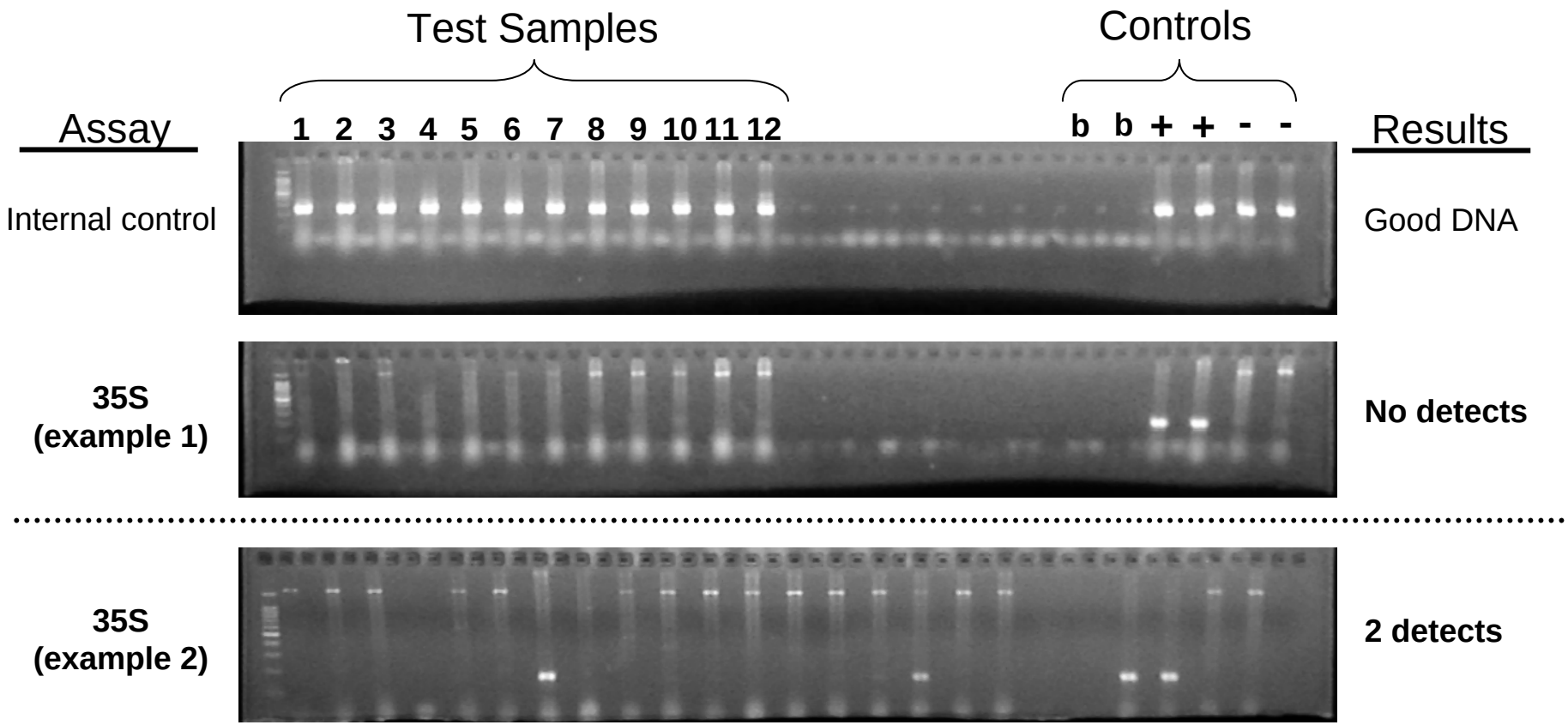
Hold at 4^{o}C

There are Many Taq Polymerase Options

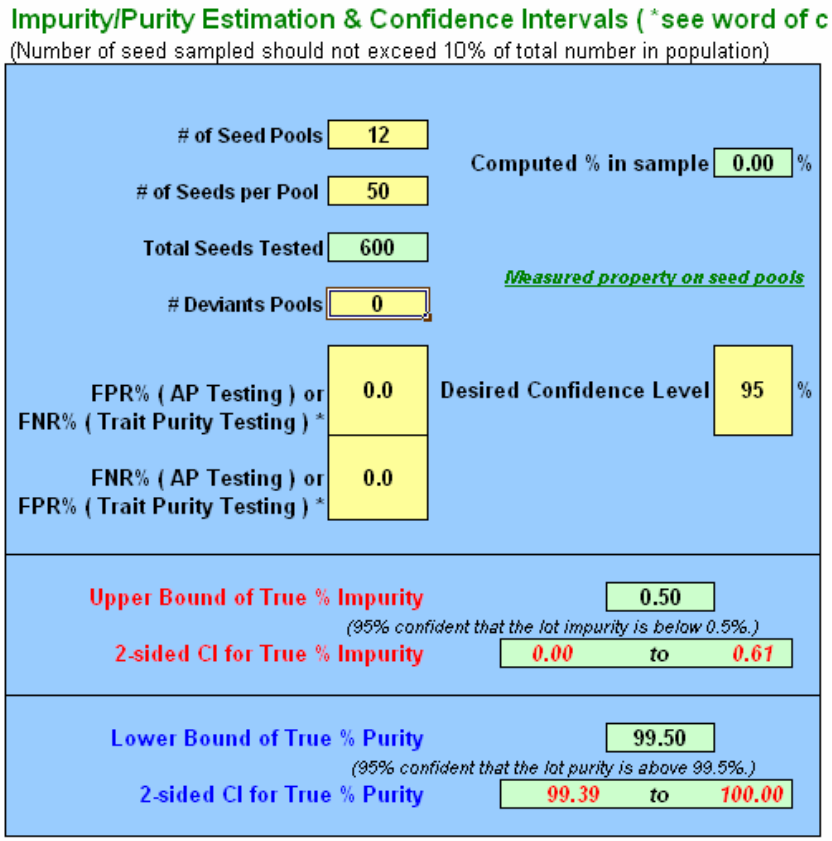
SurePrime	recombinant	hot-start	Q-biogen
Surestart	recombinant	hot-start	Stratagene
Eppendorph taq	recombinant	un-modified	Eppendorph
Super-hot taq	recombinant	hot-start	Genaxxon
Platinum taq	recombinant	hot-start	Invitrogen
Taq gold	recombinant	hot-start	ABI
Taq	recombinant	un-modified	Fermentas
Hot-start taq	recombinant	hot-start	Fermentas
Hot Gold-star	recombinant	hot-start	Eurogentec
Hot-Start-It	recombinant	un-modified	USB corp.
Red-Taq	recombinant	un-modified	Sigma
Jump Start	recombinant	hot-start	Sigma

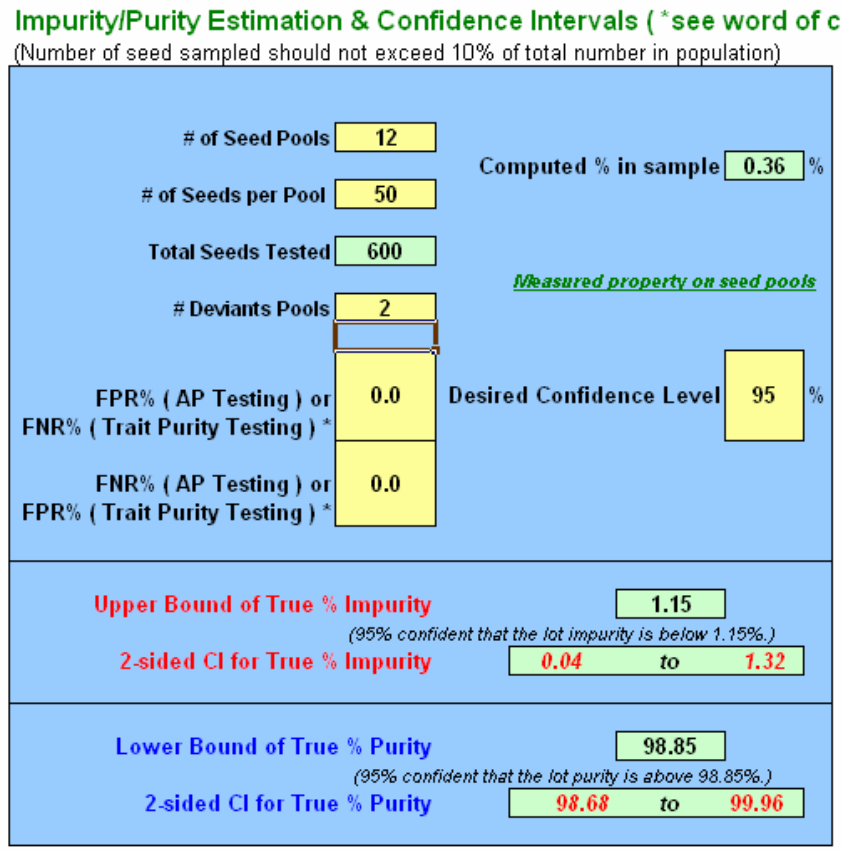
Modifications can be chemical or anti-body based

Agarose-Gel-Based Results



Results Analysis Based on Seedcalc





PCR
---VIRTUES & LIMITATIONS--

VIRTUES

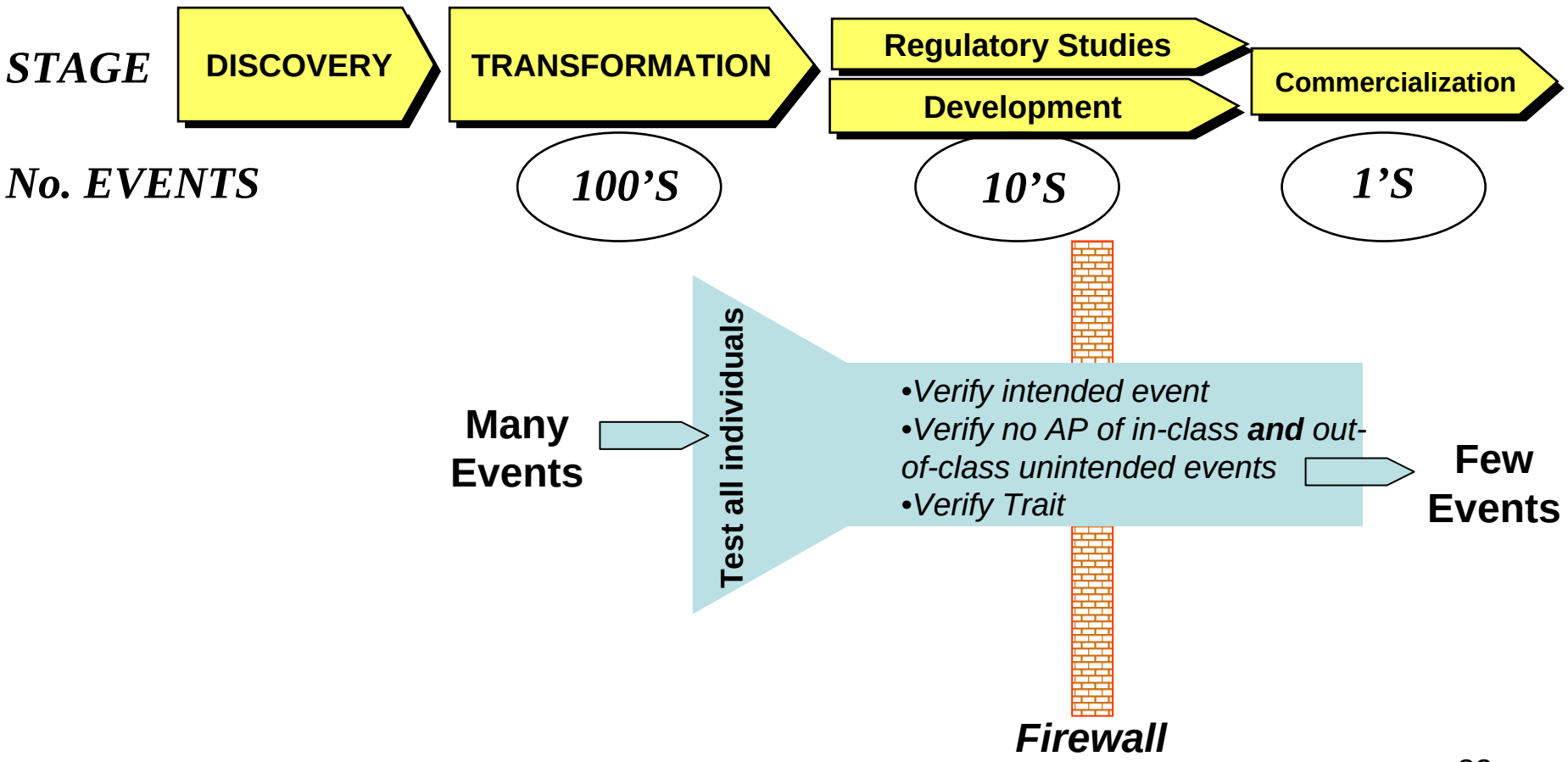
- High sensitivity – in theory
- Can detect & quantify specific events
- Enhanced flexibility in test plan design with capability of detecting classes of events/constructs
- Higher stability of DNA (than protein) permits analysis of food samples.
- Quantitative or Qualitative

LIMITATIONS

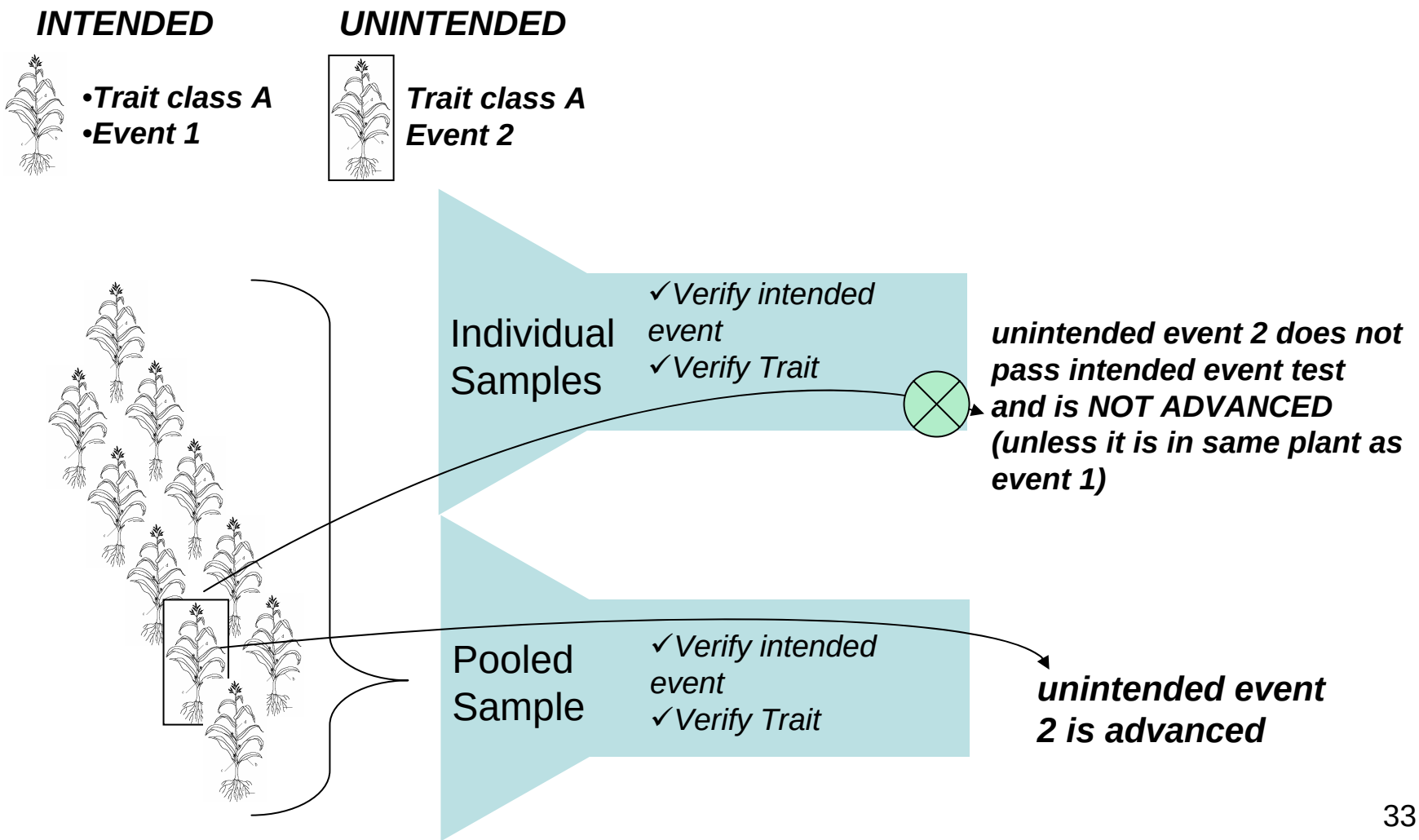
- Generally costs more than protein-based testing
- Can be very sensitive to low levels of contaminating genomic DNA or amplicon
- Can be sensitive to inhibitors in DNA preparation
- Best approach to standardizing still being debated
- Generally more time consuming than ELISA

Extra slides

Census Testing a Nursery Ensures Nothing Unintended is Advanced



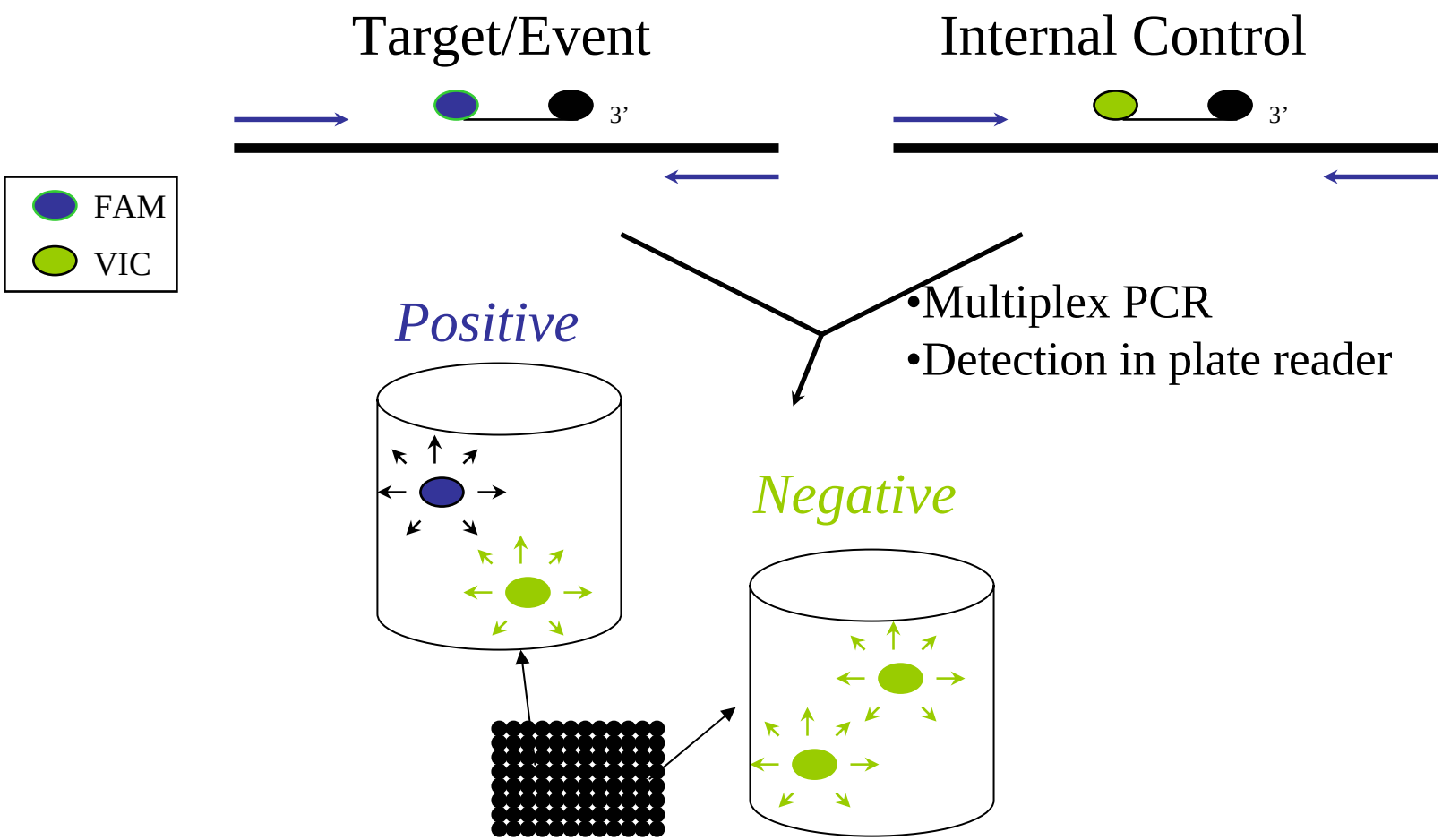
Pooled Testing For Only the Trait and Intended Event Can Result in Advancement of Unintended Events



Two Examples of Methods for PCR-Product Detection

- *Agarose Gel Electrophoresis*
- *Fluoresence*

Discriminating a Negative From a Positive Using Fluorescence-based Detection --qualitative--



Reaction Efficiency is Important in PCR

Efficiency Equation

$$y = x(1 + e)^n$$

y = yield
x = starting quantity
n = # cycles
e = efficiency

Keys to Robustness
- Well designed assays
- Pure DNA
- Controlled Processes

Yield
(copies)

Starting Quantity (copies)	*Reaction Efficiency* 0.8	1.12X	0.9
100	4.6×10^9	5X	2.3×10^{10}
110	5.0×10^9	0.1X	2.5×10^{10}
1000	4.6×10^{10}		2.3×10^{11}