Quantitative PCR for GMO Detection

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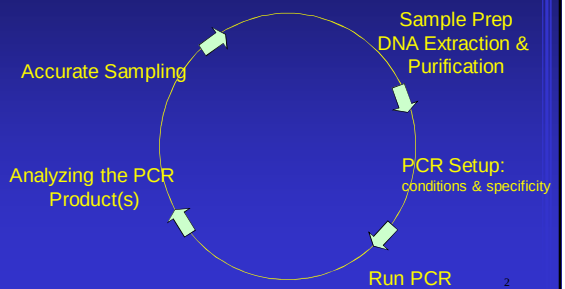
DNA-based Analytical Process



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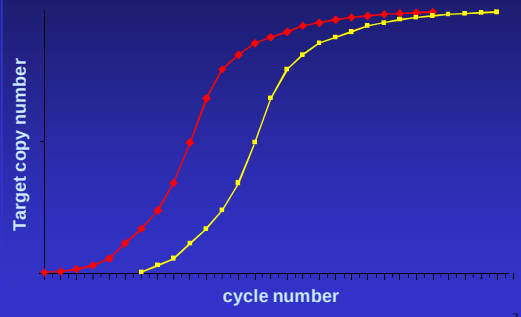
PCR Amplification Curve



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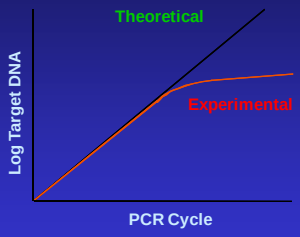
In practice:

- Efficiency is not 100%
- Depletion of PCR reagents

Therefore:

- Exponential increase is limited
- Linear increase follows exponential and plateau occurs



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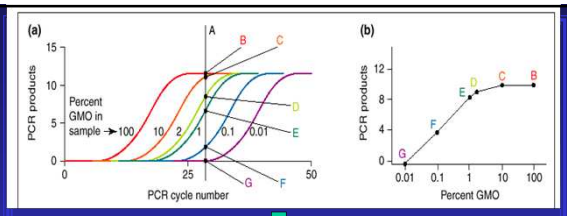

Proportionality between DNA concentration and PCR products is limited

Conventional end-point PCR is not suitable for DNA quantification

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What is Real-Time PCR?

A PCR system in which we can monitor the amplification reaction as it is occurring

Real-Time PCR incorporates the ability to directly measure and quantify the reaction while amplification is taking place

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Which detection strategies are available ?

Intercalating dyes

Hybridisation probes

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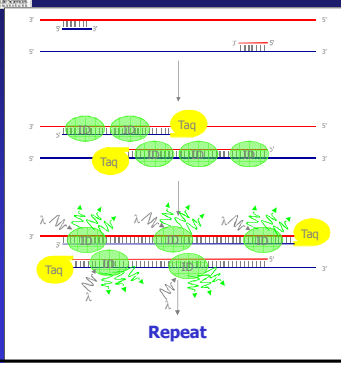
Intercalating Dyes - SYBR green



- Binds to double-stranded DNA (dsDNA).
- Fluoreseces only when bound to dsDNA.
- As PCR progresses [dsDNA] increases,
- fluorescent signal in sample increases.

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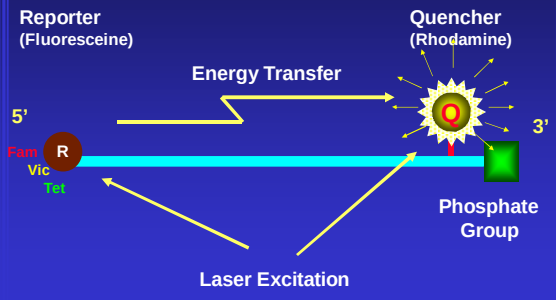
Hybridization Probes

TaqMan Fluorogenic Probe



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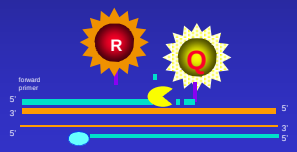
TaqMan PCR Chemistry

R = Reporter

Q = Quencher



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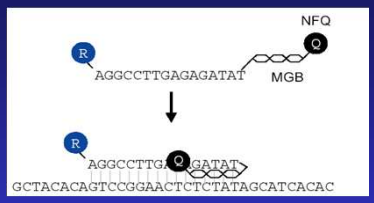
TaqMan Minor Groove Binder (MGB) probes



1. **MGB** are peptides derived from natural antibiotics that bind DNA in the minor groove stabilizing duplex DNA/Probe
2. **N**o **F**luorescent **Q**uenchers (**NFQ**) lose excitation energy by emission of heat (instead of light as in the case of TAMRA).
3. Advantages: increased stability and specificity, lower background

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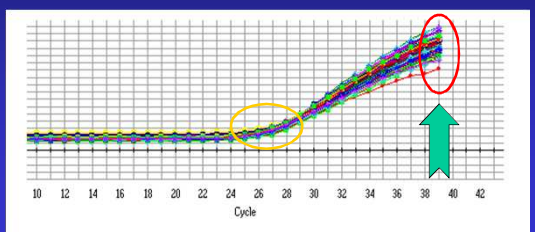
Which is the best phase to extract quantitative information?

96 PCR replicates of identical samples have very different individual efficiencies at the end of the reaction



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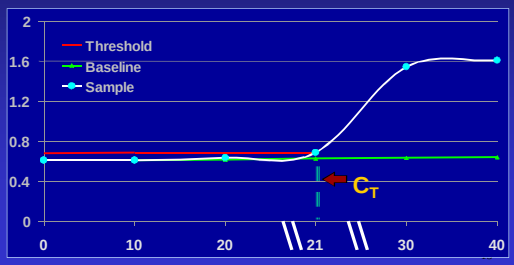
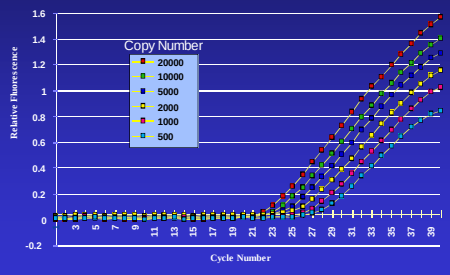
What is the Threshold Cycle (C_T)?

The Threshold Cycle (C_T) is the PCR cycle at which the fluorescence signal of the sample rises above the baseline signal



Relationship between template, copy number and Threshold Cycle



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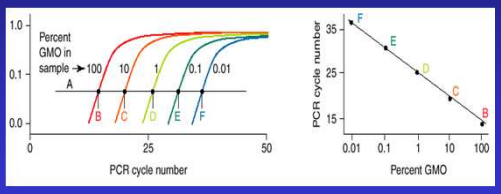
The Threshold Cycle, C_T

Correlates with the starting copy number

- If you have twice the template, you get to C_T one cycle earlier
- If you have half the template, you reach C_T one cycle later



The Threshold Cycle, Ct, is a reliable indicator of initial amount of template

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GMO quantitation by real-time PCR: what do we need ?

- Standard Curve prepared with material at known GM content, commonly Certified Reference Materials
- **Amplification of two targets:**
  - Transgene
  - Endogenous species-specific gene

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The quantitation of GMOs is a relative measurement

GM DNA QUANTITATION
(i.e. RR soybean, maize Bt-176 etc.)

SPECIES-SPECIFIC DNA QUANTITATION
(i.e lectin, invertase, zein, ADH)

% GMO = GM DNA/Species DNA

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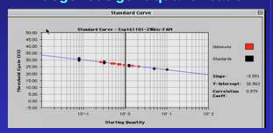
The "Standard Curve" method

Two separate Standard Curves: **transgene** and **endogenous gene**

GMO quantification

GM TARGET	Copy Number
Standard 1	2000
Standard 2	1000
Standard 3	500
Standard 4	250
Standard 5	125
Unknown 1	1853
Unknown 2	654
Unknown 3	312

Endogenous gene quantification



ENDOGENOUS	Copy Number
Standard 1	100000
Standard 2	50000
Standard 3	25000
Standard 4	12500
Standard 5	6250
Unknown 1	87500
Unknown 2	73280
Unknown 3	93989

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Percentage of Transgenic Material

The percentage of transgenic material (DNA) is determined as being the ratio of transgenic to total DNA quantities

$$\text{GM DNA \%} = \frac{\text{DNA Q transgenic system}}{\text{DNA Q endogenous system}} \times 100$$

Sample	Transgene Copy Number	Endogenous copy number	% GMO
Unknown 1	1853	87500	2.12
Unknown 2	654	73280	0.89
Unknown 3	312	93989	0.33

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Plate layout for GMO quantitation: example of "simplex" assay

S1	S1	S1	S2	S2	S2	S3	S3	S3	S4	S4	S4	GMO
A	A	A	A 1:10	A 1:10	A 1:10	B	B	B	B 1:10	B 1:10	B 1:10	
C+	C+	C+	C-	C-	C-	NTC	NTC	NTC				
S1	S1	S1	S2	S2	S2	S3	S3	S3	S4	S4	S4	ENDOGENOUS
A	A	A	A 1:10	A 1:10	A 1:10	B	B	B	B 1:10	B 1:10	B 1:10	
C+	C+	C+	C1	C1	C1	NTC	NTC	NTC				

C+ Positive DNA target control

C- Negative DNA target control

NTC Amplification reagent control

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What and how do we measure ?
(Some) Critical factors affecting quantitative measurements in GMO analysis.

Target copy number	➔ Discrepancies in GM target copy number between standard and unknown samples may lead to at least 100% over/underestimation of the GM content
Stability of reference gene	➔ Reference gene should be tested for stability of amplifiable target copy number across species (i.e. allelic variation)
PCR efficiency/inhibition	➔ Sub-optimal PCR efficiency due to inhibition may greatly affect linearity of the system and thus correctness of measurements.

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Main Critical performance indicatorsfor quantitative PCR methods in GMO analysis

Specificity	>>>	Primer design and testing, choice of unique target sequences. Occurance of false positive results
Sensitivity	>>>	PCR design and optimisation, quality of DNA template (degree of DNA degradation, presence of inhibitors, etc.). LImit of Detection and false negative results.
Precision and trueness	>>>	Method optimisation, inter-laboratory transferibility. Limit of Quantification

What does '% GMO' mean ?
What is the Unit of Measurement ?

weight : weight ?
seed : seed ?
mass : mass ?
protein : protein ?
DNA : DNA ?
……. ??

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'GMO' Quantification by PCR - A Ratio, not an Absolute Amount

	Corn DNA*	'GMO' DNA*	Result on test report
Corn	80,000	800	1 %
Corn gluten	8,000	80	1 %
Corn starch	800	8 ?	Not quantifiable
Maltodextrin	80	0.8 ??	Not quantifiable
Glucose syrup	0 ?	???	Not quantifiable

*Approximate relative numbers of target DNA molecules that can typically be extracted from a sample and analyzed in quantitative PCR reactions

- Percentages represent the *relative* composition of the DNA solution extracted from a sample: amount of biotech DNA relative to the amount of the plant species DNA.
- This percentages is not a measure of the absolute amount of biotech DNA in a sample.

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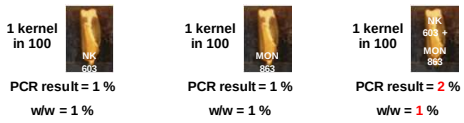
Testing for % weight / weight?

- Stacked events result in an analytical overestimate of the percentage of biotech corn as compared to the actual w/w percentage of biotech corn in the sample.



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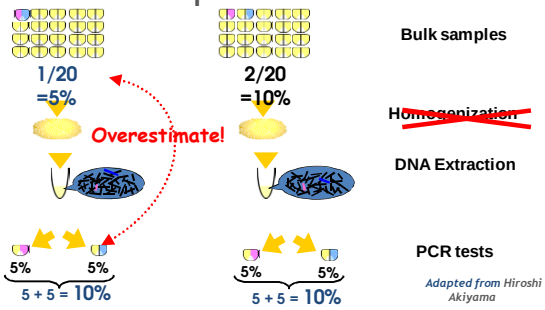
Adventitious presence of stacked events



Adapted from Hiroshi Akiyama NIHSS, Japan

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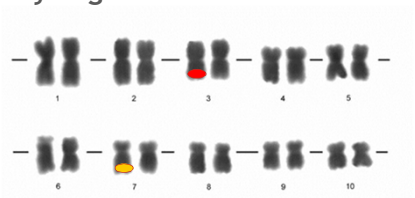
Analysing for combined events



– No PCR or other molecular approach can distinguish between the presence of a low percentage of stacked events in bulk grain and the presence of a mixture of the two or more individual events that comprise the stack.

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Analysing for combined events

– Analysis of Single seeds or sampling approaches have been proposed:
  - Akiyama et al. proposed testing individual grains
    – difficult and expensive
    – only feasible for high % GM
  - The International Seed Testing Association (ISTA) has proposed a statistical approach for seed lots
    – Only effective for seed, for limited numbers of stacks and at low concentrations

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Challenges & Technical Limitations of PCR

- Is sample representative
- DNA quality and abundance
- Genetic makeup of the sample (i.e. stacked events)

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