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Efficient non-linear analysis of kinetic amplification for quantification and automated results calling.

Lee, M A, & Webster, B. BioGene Ltd, BioGene House, Harvard Way, Kimbolton, Cambs PE28 ONJ.

m.lee@biogeneresearch.co.uk

martinalanlee@aol.com

ABSTRACT

The analysis of real-time amplification curves for quantification usually involves determination of a cycle number value (Ct) at a set threshold. This threshold is determined either by an automated function related to the fluorescence yield with respect to background (automated fit), or is set manually by the user (threshold bar). In either case the method may be used to generate a standard curve for the determination of unknowns by interpolation. A linear correlation coefficient (r) for the Ct value with respect to the concentration of standards is often promulgated as a good indication of data quality. However, a linear fit for quantification is on the whole, a poor one given the variable efficiency of reactions across the broad dynamic range of possible target concentration, and the large differences in fluorescence yield that may occur in some samples. Here we discuss an overview of the current approaches and their limitations. A new kinetic approach is demonstrated for applications including quantification and automated results calling.



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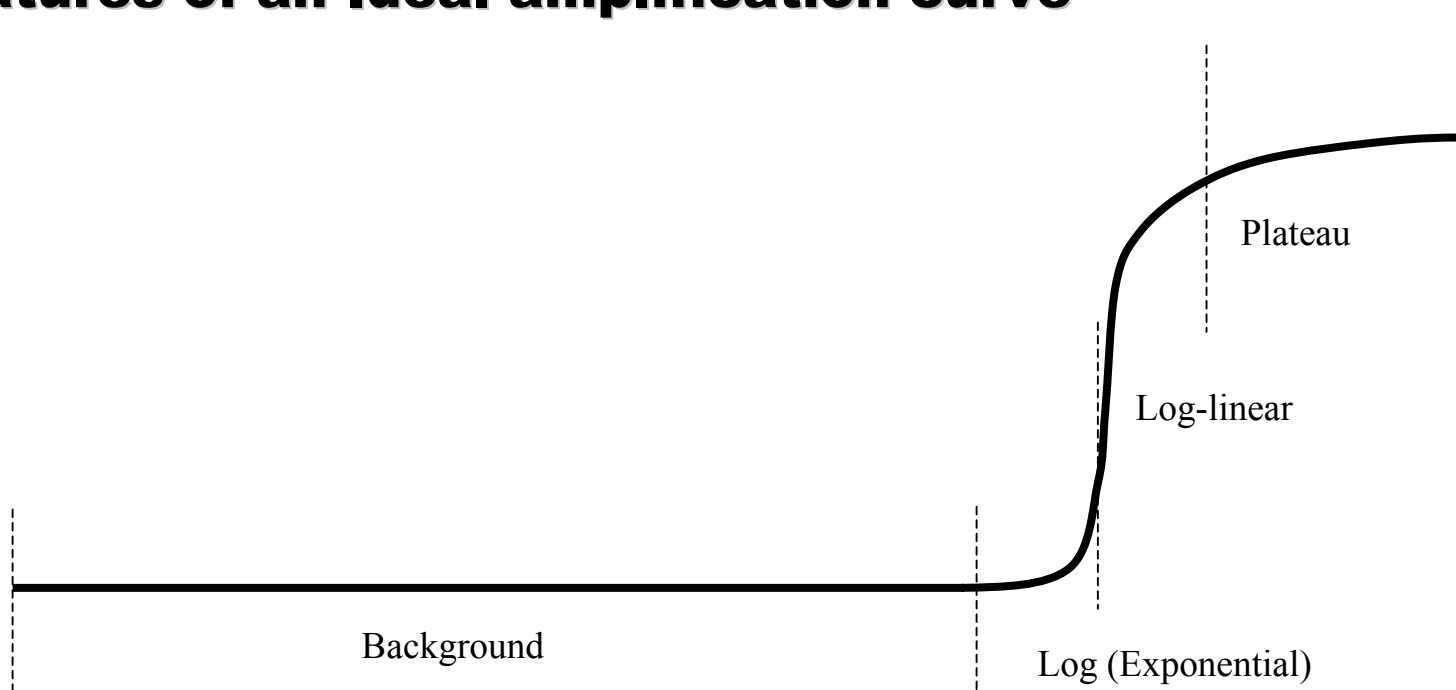
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“Not making straight-sense of curved lines”

- 💧 What do machines and software do to turn fluorescence into raw data?
- 💧 What do we do with data to turn it into information?
- 💧 Why are these things sometimes flawed?
- 💧 Could we do this better?

Amplification signals 1.

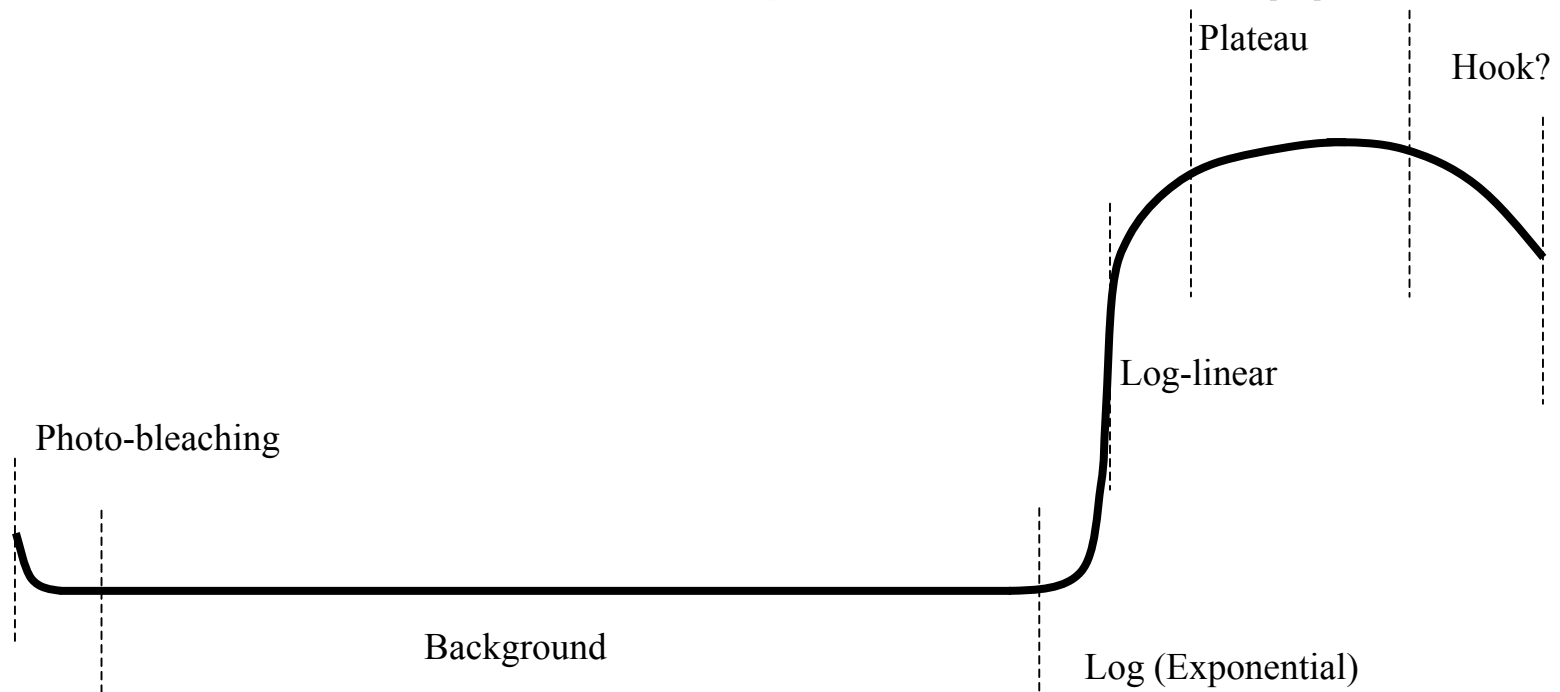
Features of an Ideal amplification curve



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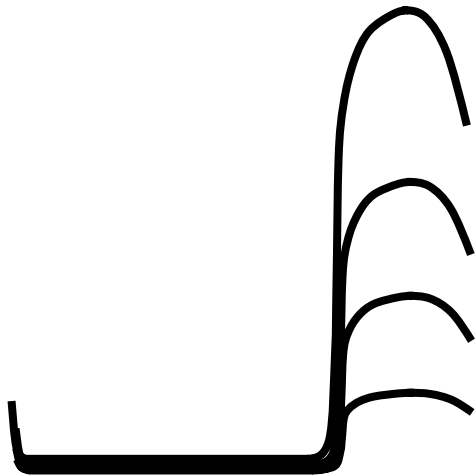
Amplification signals 2.

Addition features of real amplification curve (s)



Amplification signals 3.

“SIGNAL to NOISE”



Importance

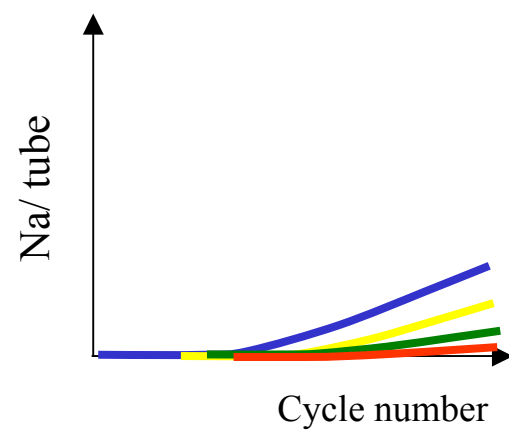
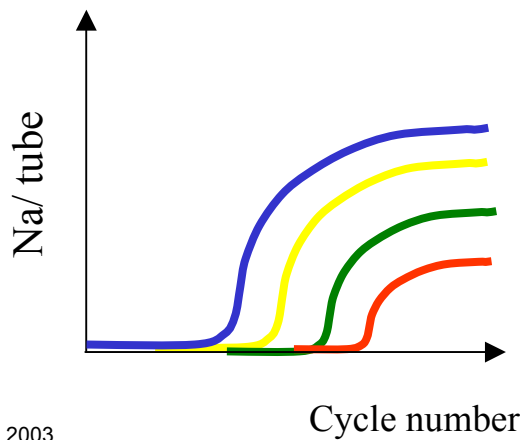
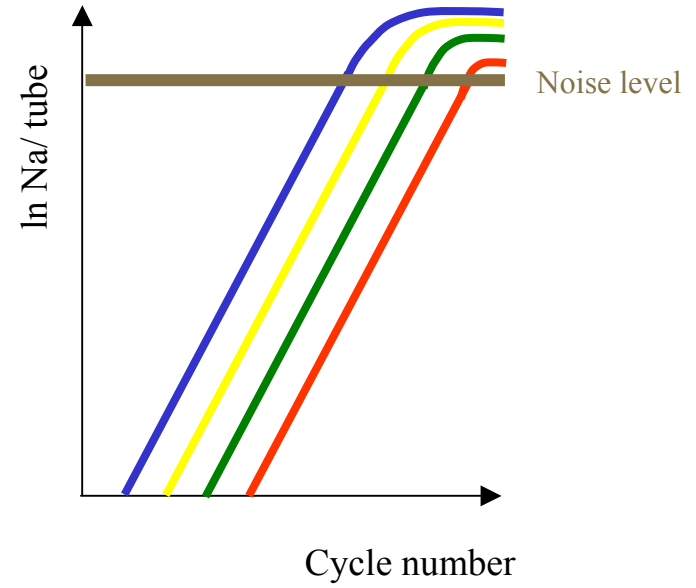
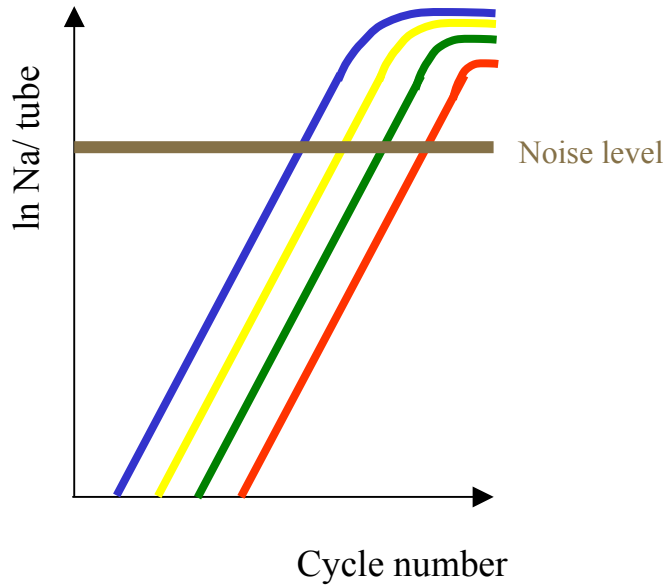
- Probe Type
- PCR Efficiency
 - Inhibition
- Probe efficiency
 - Hold times
 - Dye stoichiometry
- Background fluorescence
 - Temperature
- Instrument
 - Excitation source & detector
 - Plastic ware
- Mix formulation



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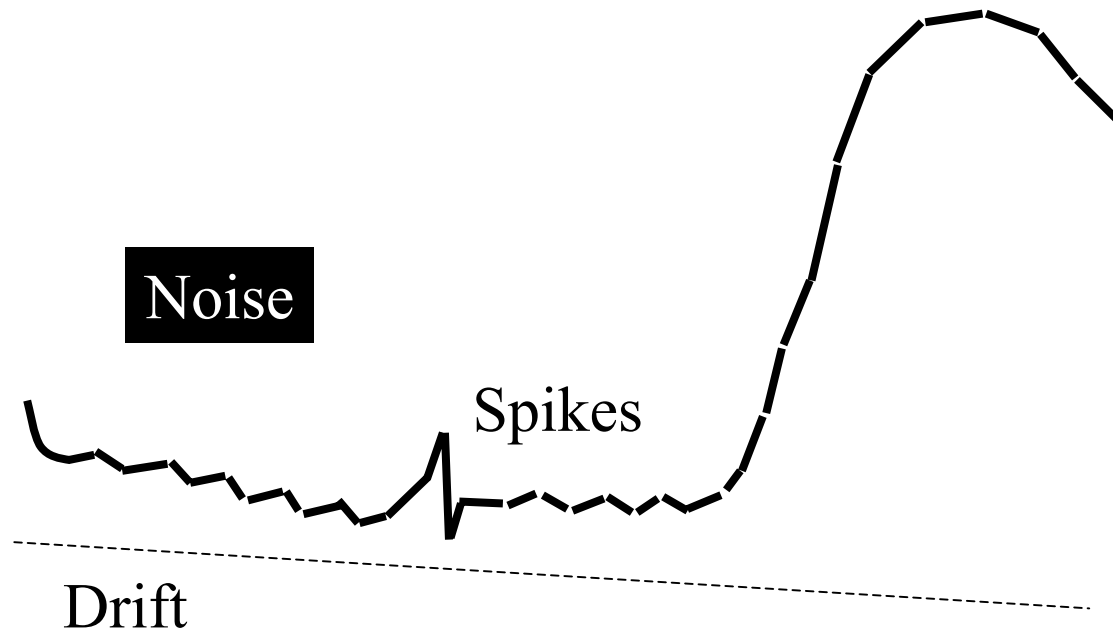
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Amplification signals 4.

Instrument features of real amplification curves

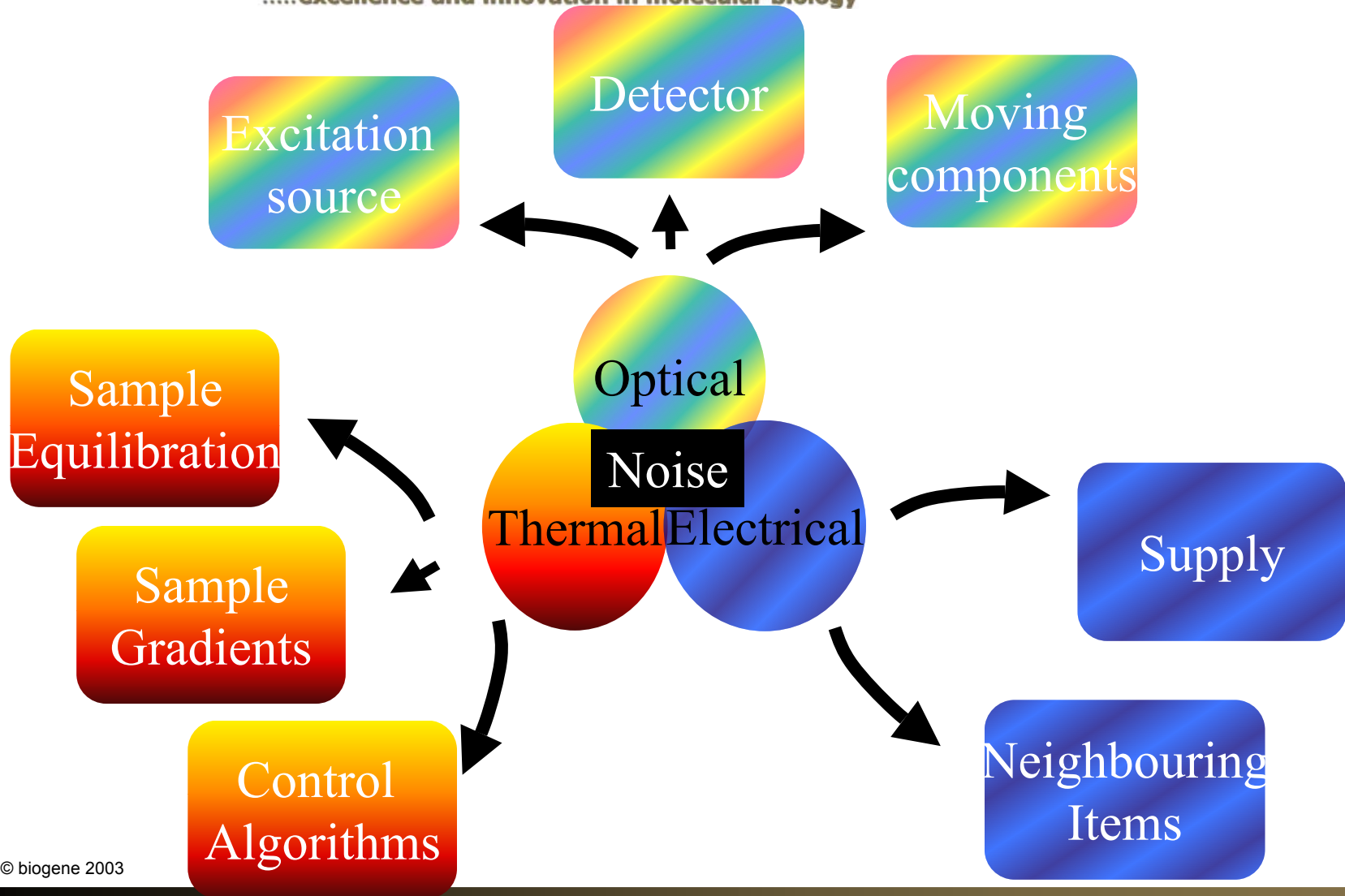




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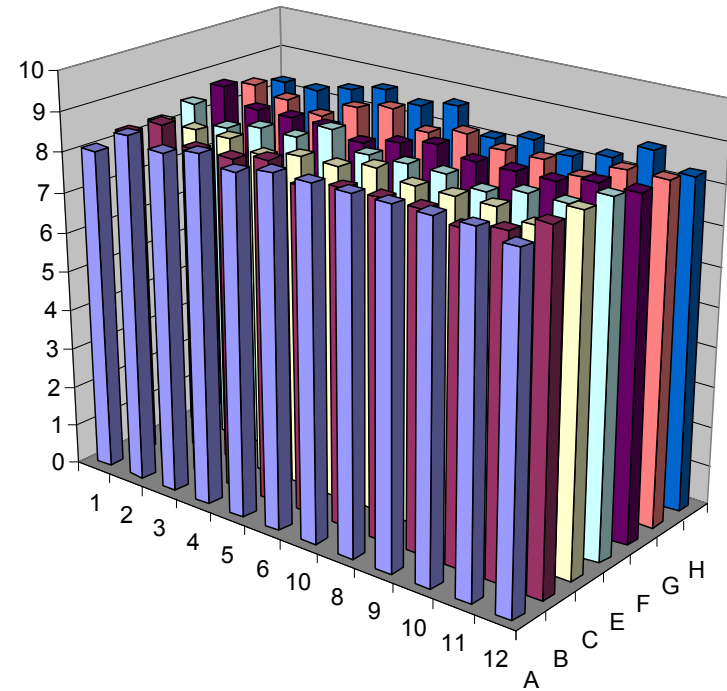
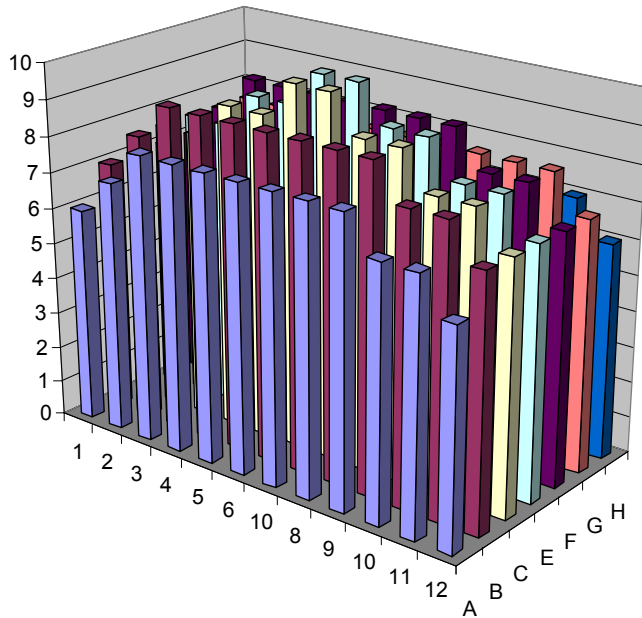
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DATA ANALYSIS 2.

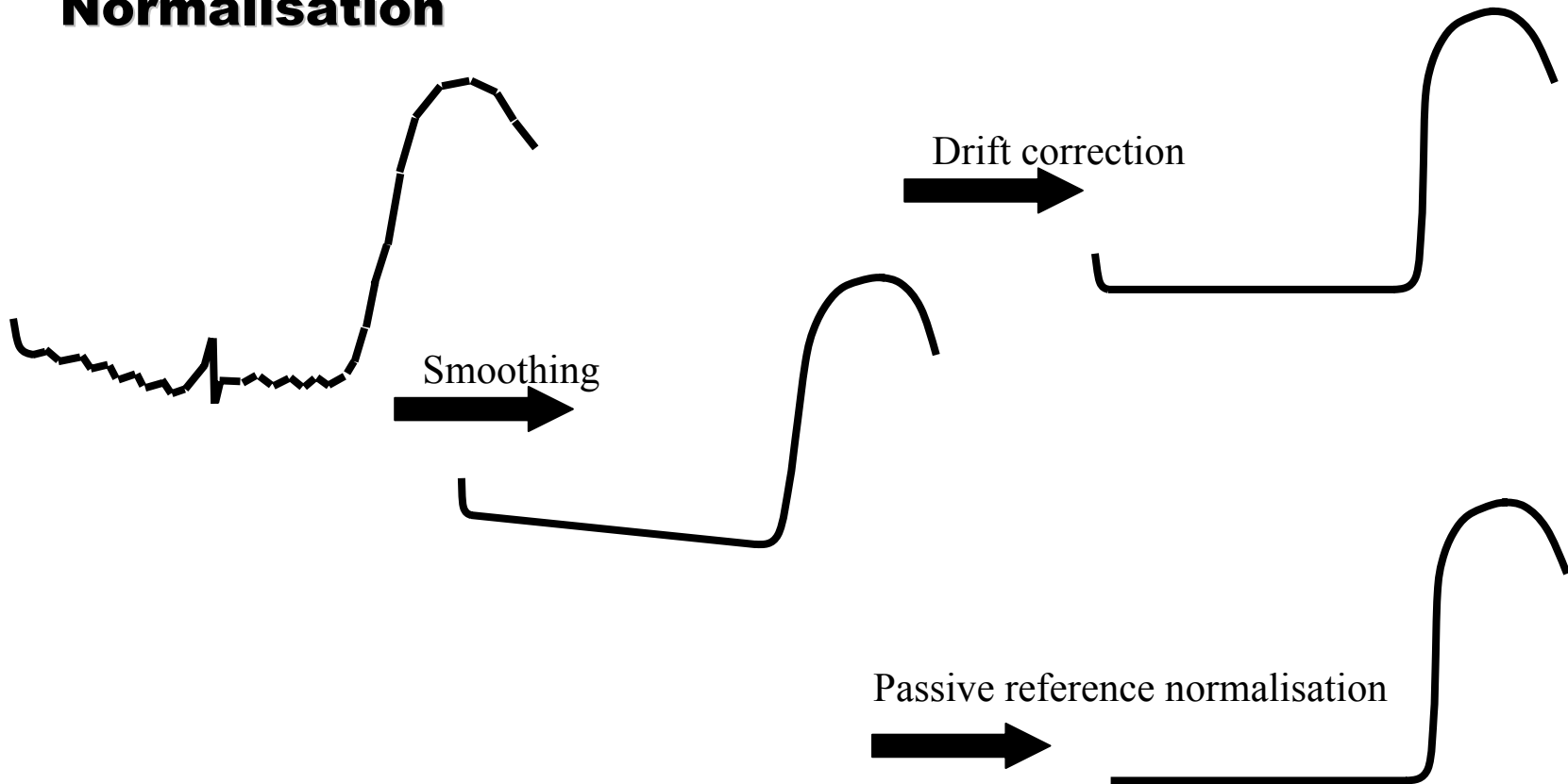
Plate or "optical" normalisation



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DATA ANALYSIS 1.

Normalisation



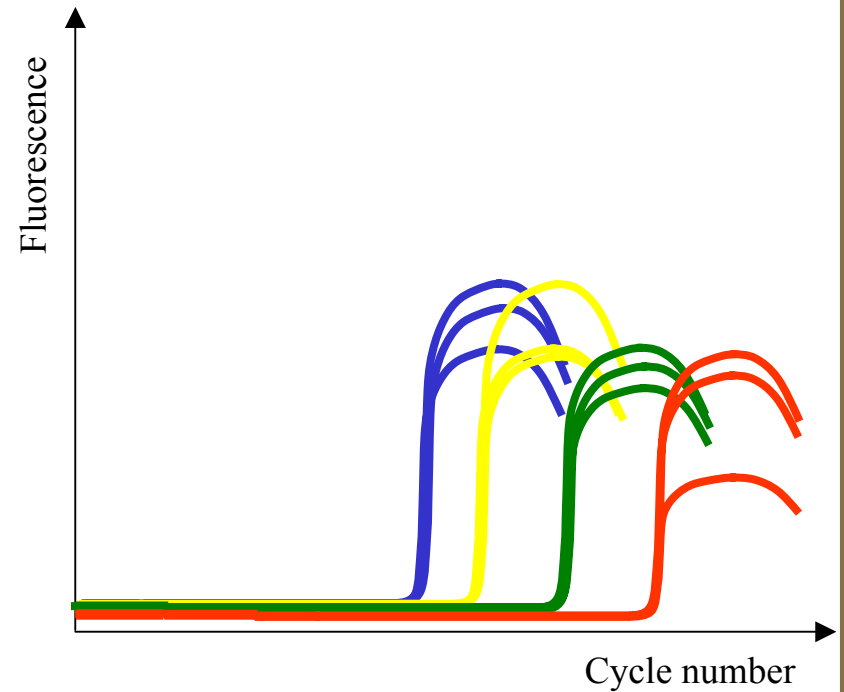
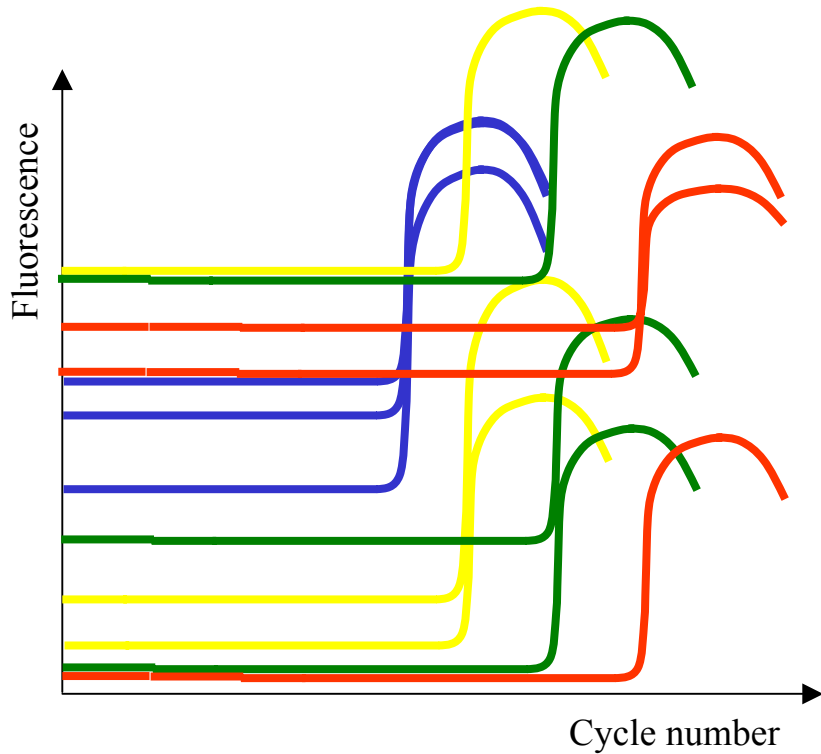


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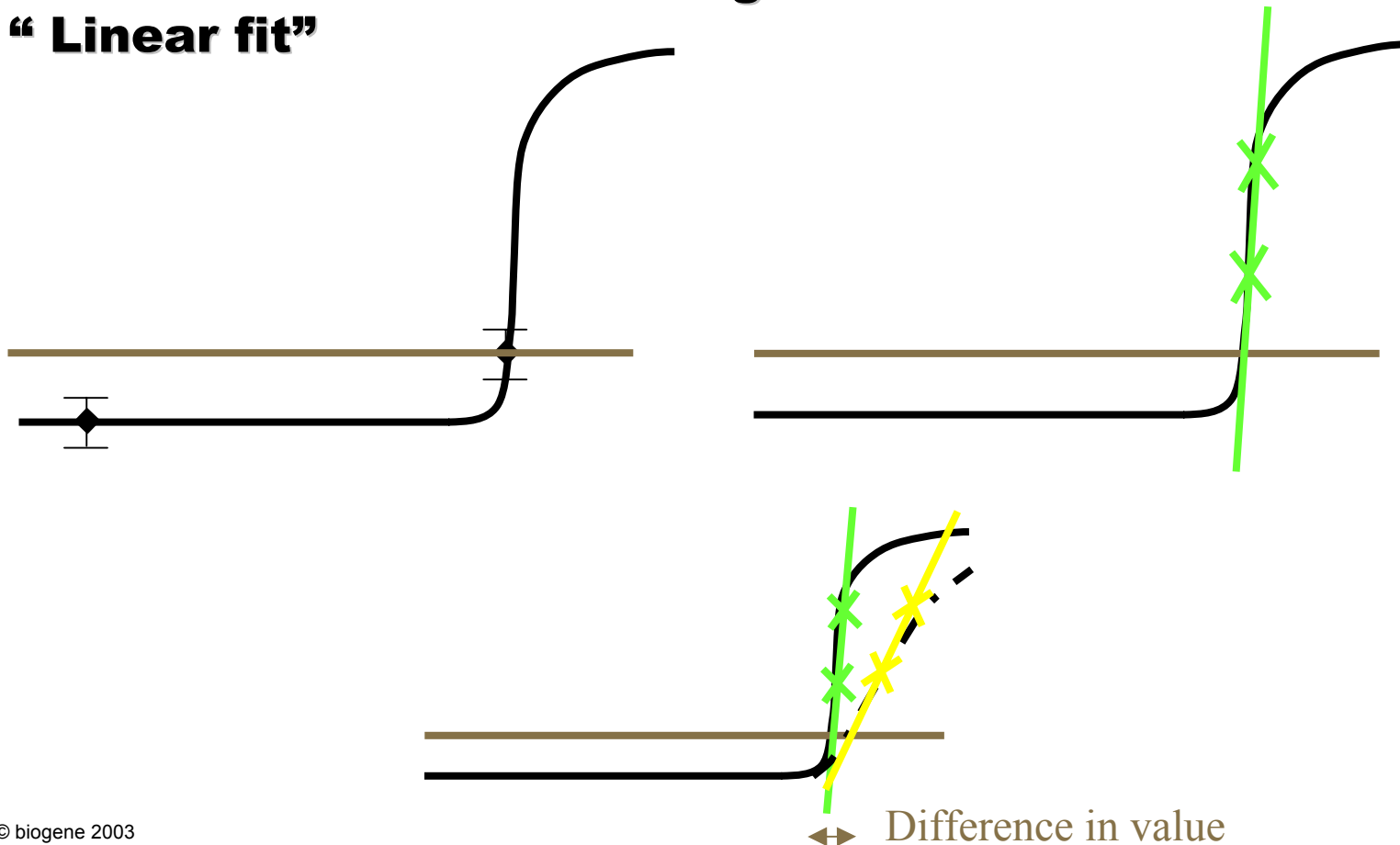
DATA ANALYSIS 3. Sample-to-sample normalisation



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DATA ANALYSIS 3.

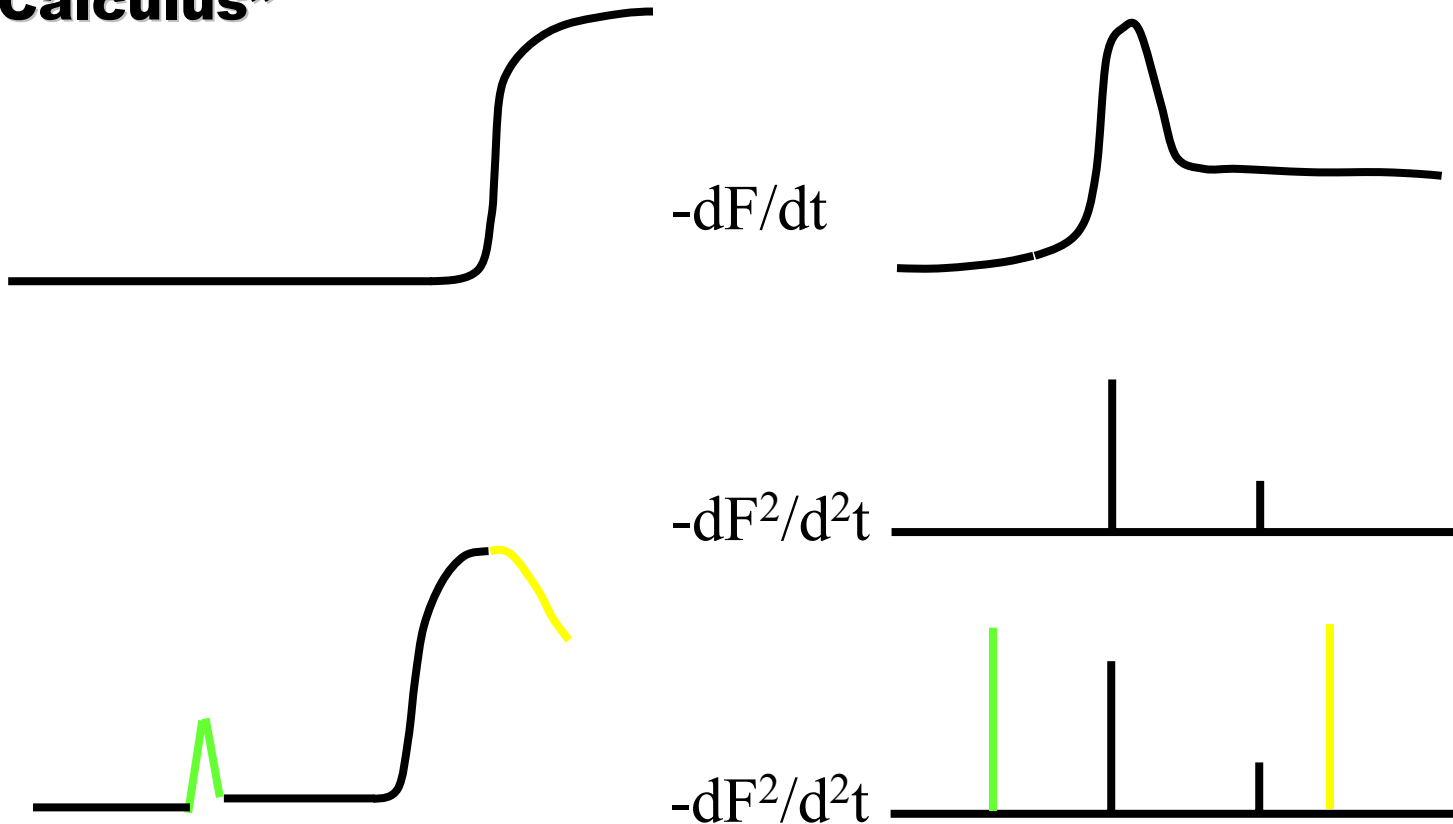
Common methods of setting threshold & determining Ct. “ Linear fit”



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DATA ANALYSIS 4.

Common methods of setting threshold & determining Ct "Calculus"





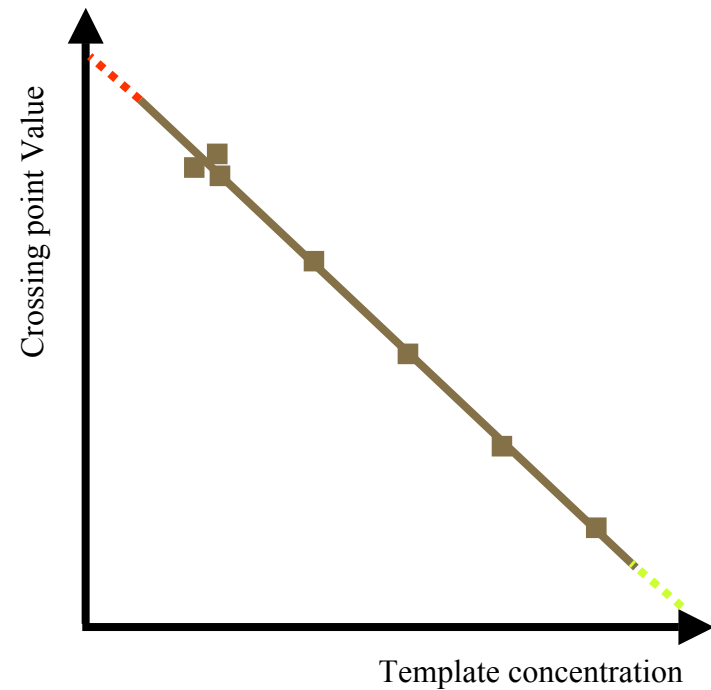
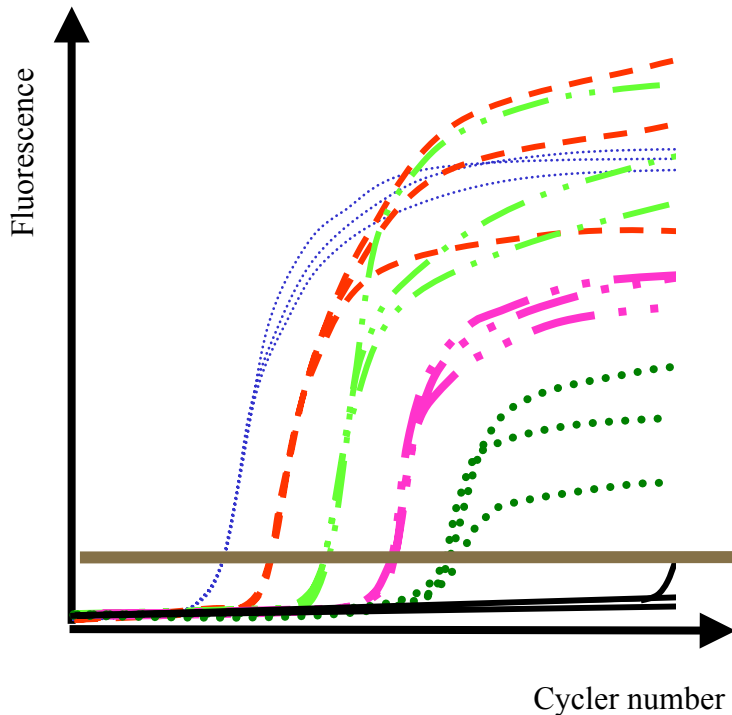
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Kinetic PCR

1. Theory





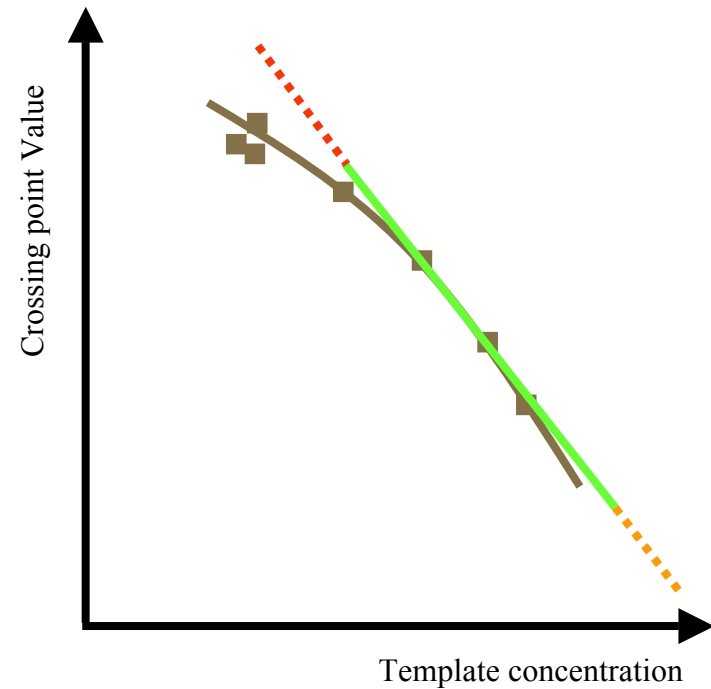
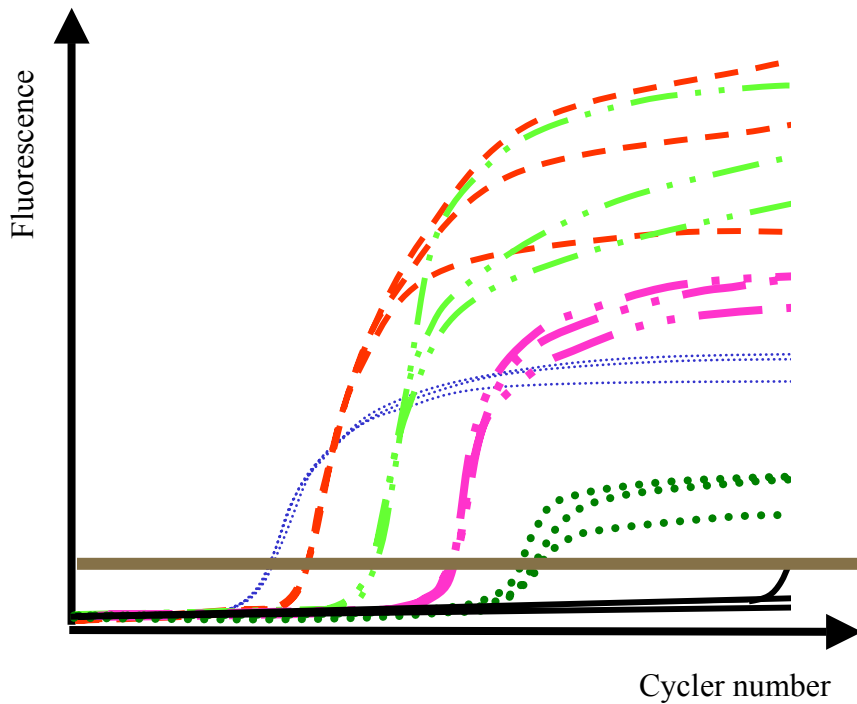
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Kinetic PCR

2. Reality



We real-time users tend to....

reject;

💧 Standards with low fluorescence and abnormalities

💧 Data sets with low values for linear correlation

analyse;

💧 Data that has a non-linear relationship using a linear fit

Such that....

💧 We make our data fit a poor model rather than model our data

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Kinetic Quantification

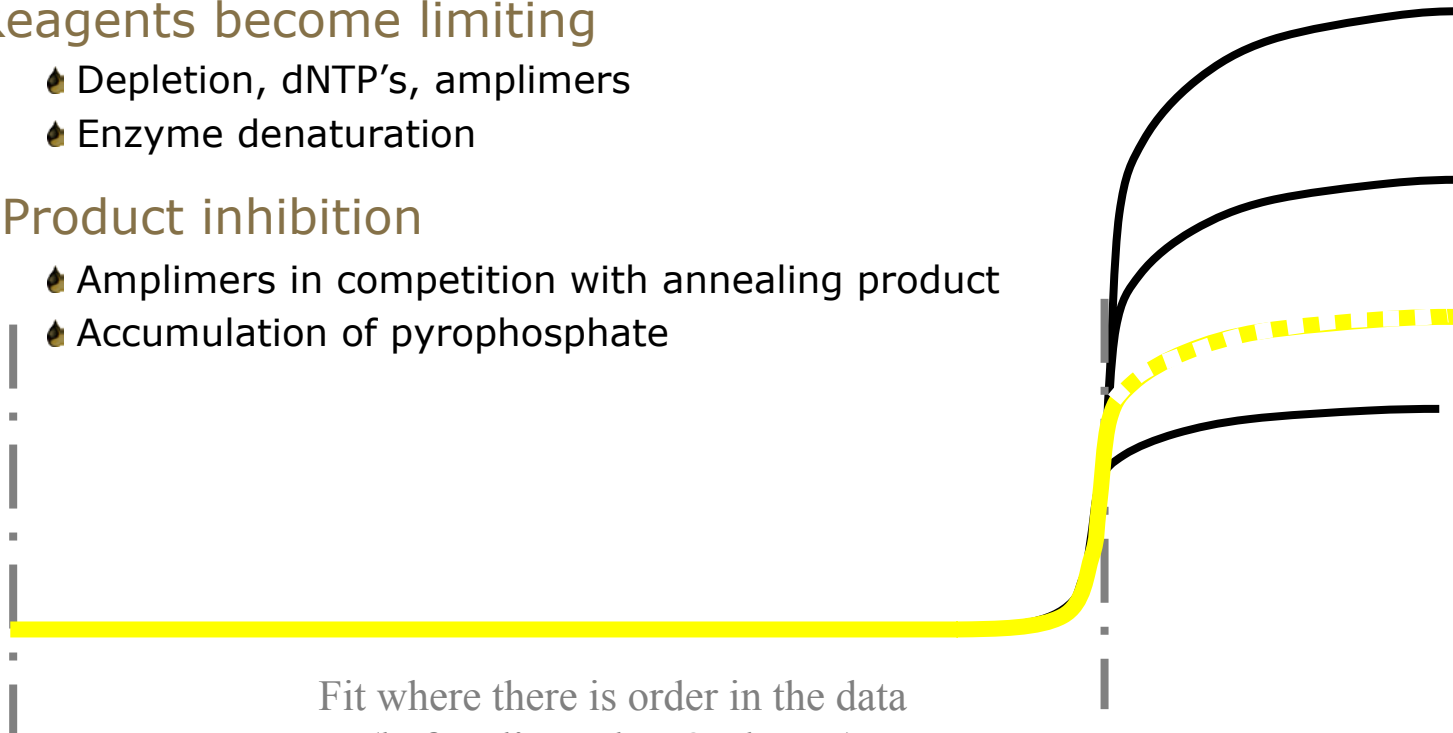
Factors influencing plateau formation

Reagents become limiting

- Depletion, dNTP's, amplimers
- Enzyme denaturation

Product inhibition

- Amplimers in competition with annealing product
- Accumulation of pyrophosphate



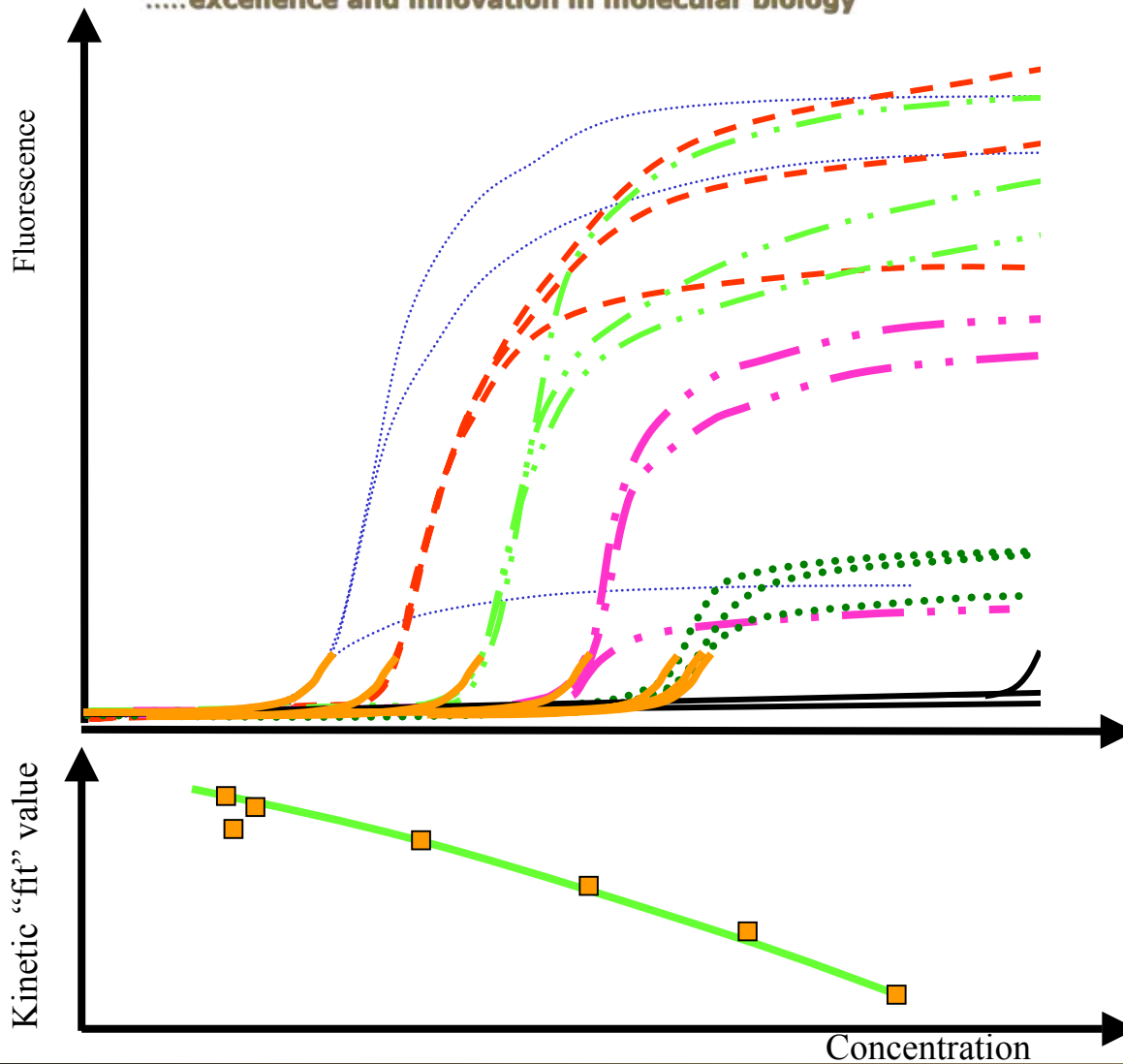
Fit where there is order in the data
(before linear-log & plateau)



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Benefits of kinetic PCR for quantification

- ◆ Uses a significant proportion of the data set (rather than one discrete point)
- ◆ Not dependent on uniform fluorescent yield
- ◆ Accommodates noise and fluctuations
- ◆ Provides for linear and polynomial fits
- ◆ Amplification efficiency determined from kinetic data not linear fit

Kinetic results calling



$$N_a = N_0 \times 2^{(E \times n)}$$

Where;

N_a	=	Number of amplicons
N_0	=	Number of starting templates
E	=	Efficiency
n	=	Number of cycles

Kinetic results calling

- Using this approach for a given reaction you can the
- Determine if a reaction is +/-
- Concentration of target without the need for standards *per se*
- Efficiency of amplification

Results Calling

- 💧 High confidence applications (control & accountability)
 - 💧 Genotyping
 - 💧 Pharmacogenomics
 - 💧 Diagnostics / Health monitoring
 - 💧 Theranostics
 - 💧 Environmental
 - 💧 Food testing
- 💧 Application orientated operators (Simplified operation)
 - 💧 Non-scientist
 - 💧 Clinician
- 💧 High throughput applications (Automation)
 - 💧 Genotyping
 - 💧 Research

Acknowledgements

Development team:

- ◆ *Ben Webster*
- ◆ James Howell
- ◆ Richard Skilbeck
- ◆ Steve Brown

