



QPCR in Biopharmaceuticals & Current Issues

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Summary



Biopharm lifecycle

- Candidate Plasmid Selection
- Cell Line Selection
- Cell Banking
- Process Support
- Potency/Stability testing
- Tox Studies
- Clinical Trials
- Post launch diagnostics

Issues

A bit about me...



- 1996 CBD Porton Down
Scientific Officer
Chemical & Biological Defence
Gene Probes- Detection
- 1999 GlaxoSmithKline
Principal Scientist
BPCEDD (Biopharmaceutical Center of
Excellence Drug Discovery)
- 2008 NDA Analytics
Head of PCR Services

Candidate plasmid selection

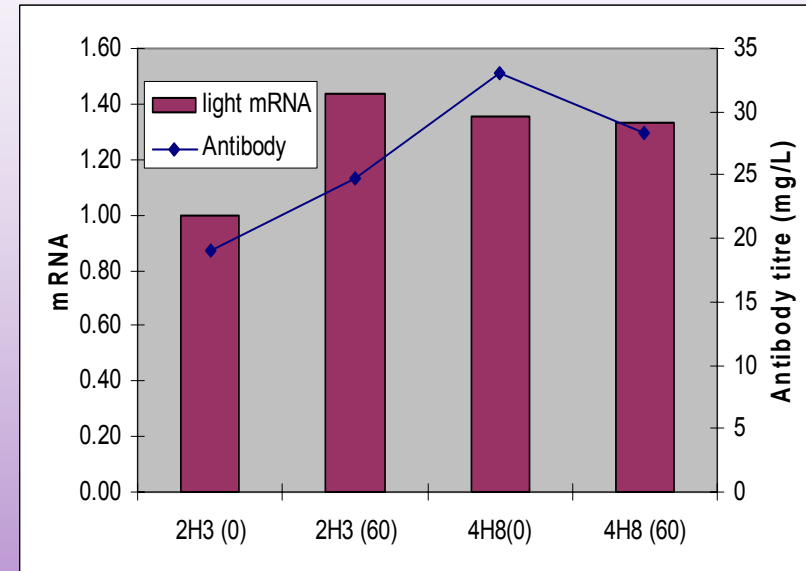
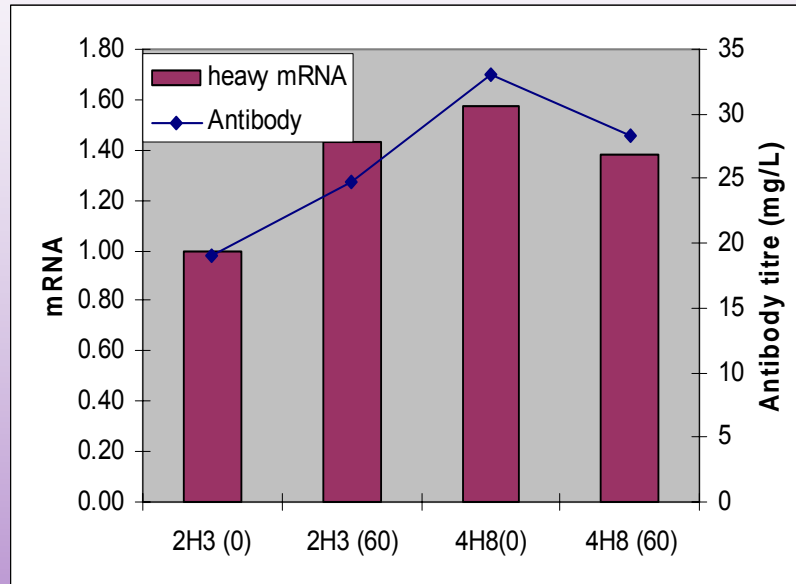
DNA vaccines, Proteins (mAbs, dAbs, fAbs...)



- Promoter
- Humanisation
- Codon optimisation

Selection using RT-QPCR based on the highest expressors (mRNA transcripts)

Cell line selection



Choosing the right target, and transcript can correlate with translated protein

Cell Banking

- Virus testing



Test	Method
Adventitious viruses	28 day 4-cell line in vitro assay (incorporates 324K cells for detection of Minute Mouse Virus)
	<i>In vivo</i> assays (adult and suckling mice, guinea pigs and embryonated eggs)
Retroviruses	Extended S+L- focus assay on mink lung cells
	Reverse Transcriptase, QFPERT
	TEM
	Co-cultivation with a human cell line or other species
Bovine Viruses	<i>In vitro</i> assay for bovine viruses
	QPCR and/or <i>in vitro</i> assay for bovine polyomavirus
Porcine Viruses	<i>In vitro</i> assay for porcine viruses
Species Specific Viruses	Hamster Antibody Production (HAP) test
	Lymphocytic choriomeningitis Virus (LCMV) challenge test

Process Validation

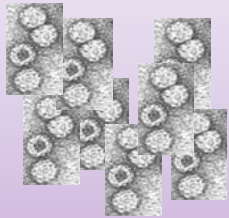
Virus Clearance Validation



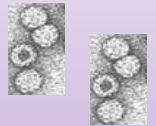
Virus spike

Orthogonal Process Steps

Quantify Output



- Partitioning by selective absorption
- Inactivation
- Size Exclusion + Mechanical inactivation



Robust Clearance (LRV > 4 log TCID₅₀/ml):

Low pH (inactivation of acid labile model virus)

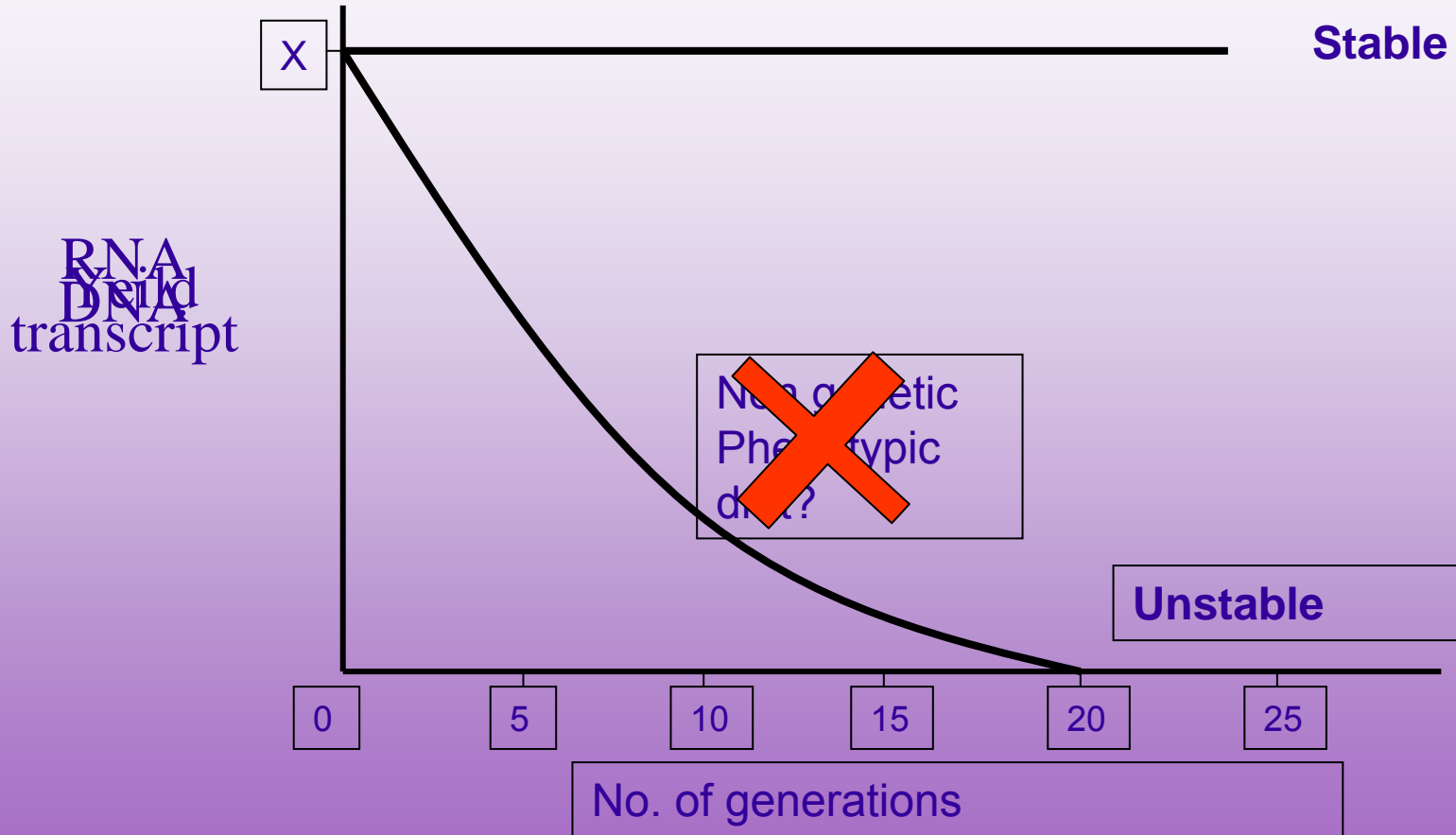
Nanofiltration (removal of small non- enveloped viruses)

Additional Clearance (LRV > 1 log TCID₅₀/ml):

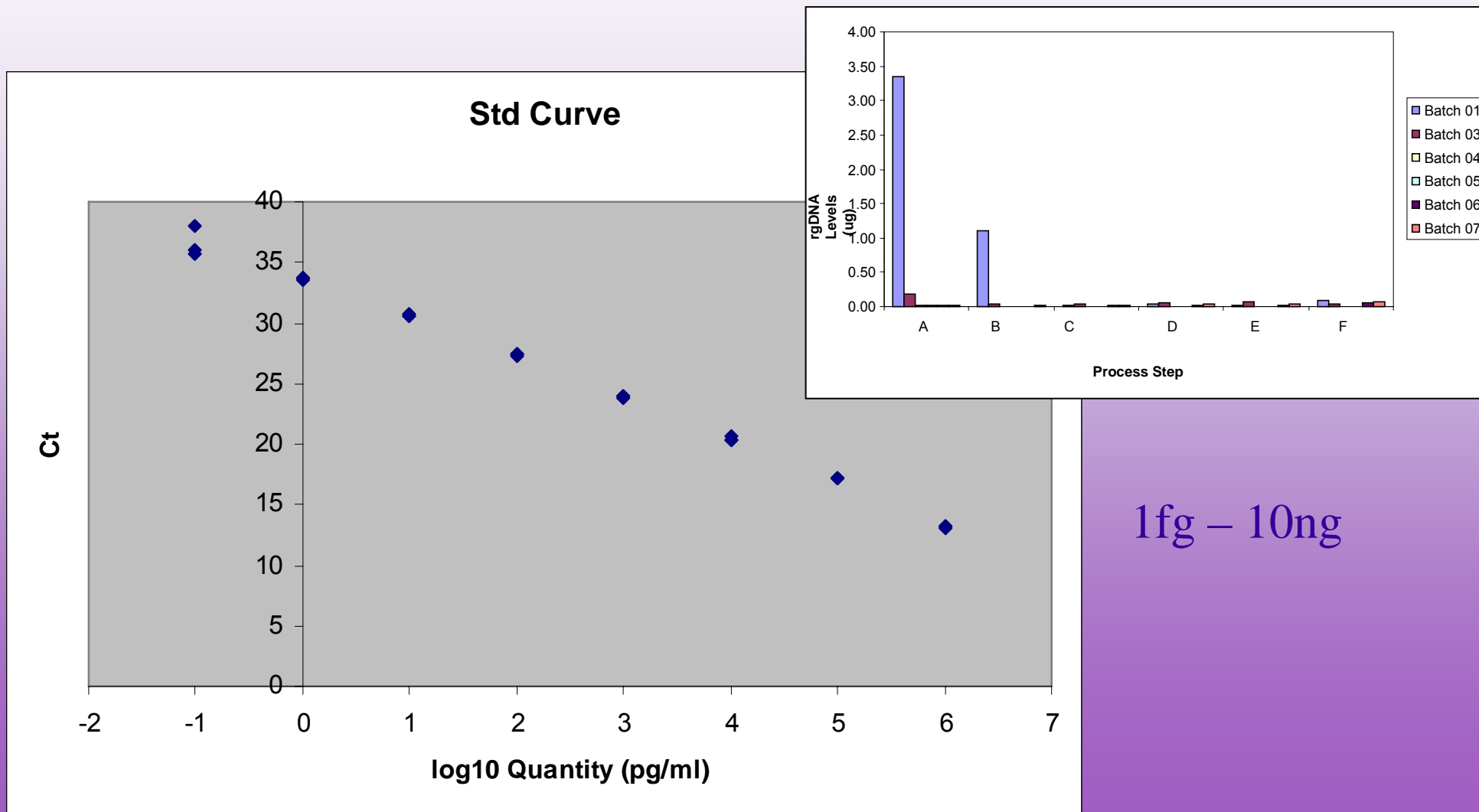
Column's (partitioning based on charge or functional ligand)

Ion Exchange Filter

Stability



rgDNA Clearance

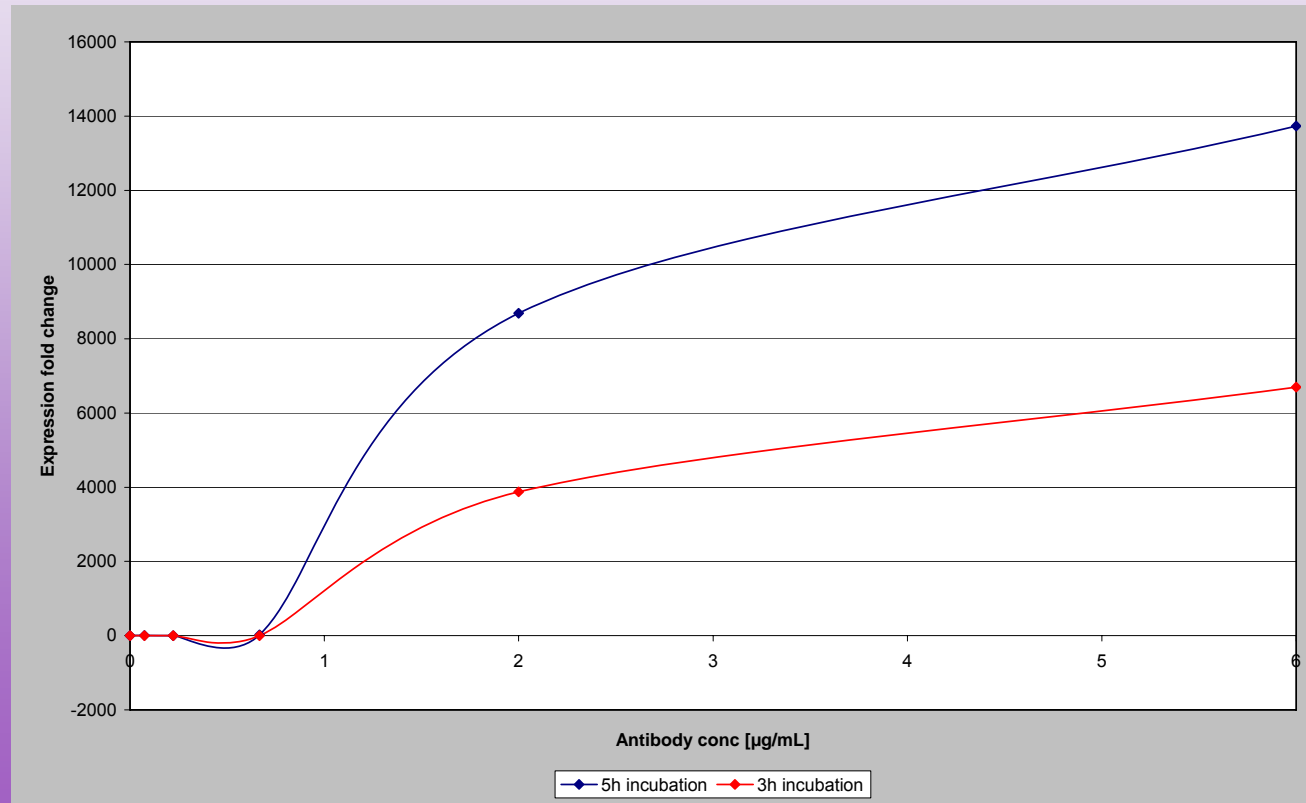


1fg – 10ng

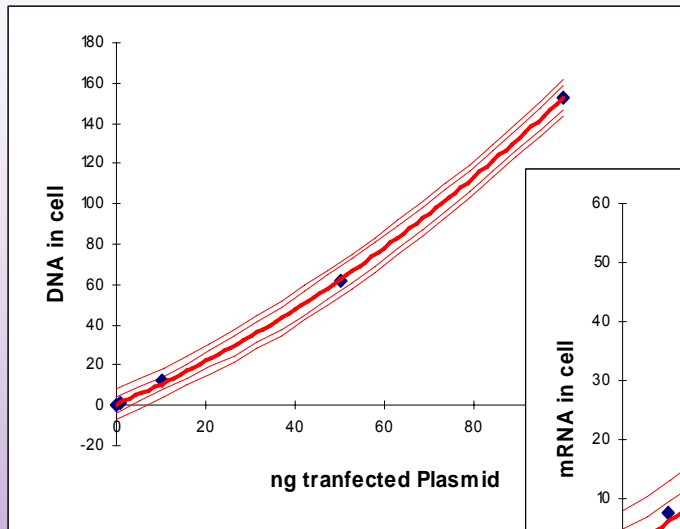
QPCR Bioassay for protein based products



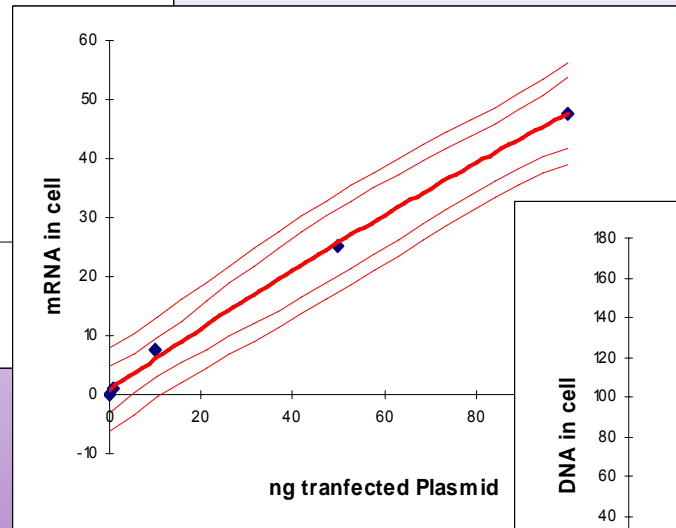
- Candidate Biomarkers involved in intracellular pathways can be identified using Arrays
- RT-QPCR subsequently used to validate and quantify expression of the biomarkers for potency and stability indicating assessment.



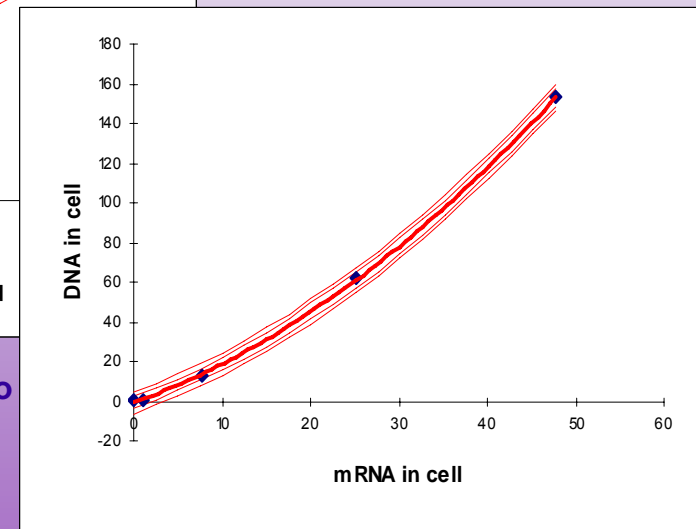
QPCR Bioassay for DNA Vaccine



A. Cell DNA compared to transfection load



B. mRNA compared to transfection load



C. mRNA compared to DNA.



Cell line Stability/Selection

- Quantitative Gene Expression
- DNA copy No.

Candidate Selection

- Potency of candidate vectors following optimisation

Master Cell Bank testing

- Virus
- Mycoplasma

Product issues

- Replication incompetent reversion
- Sterility

Product Stability

- Potency (RT-QPCR)

Real-time PCR in BPCEDD

Process Validation

- Host cell DNA/RNA clearance
- Virus clearance

Tox

- Bio distribution



Late stage and beyond

In the Clinic

- Transcriptomics to determine drug effects on gene regulation

After market

- Companion Diagnostics – genetic profile to determine suitability of a drug.
 - Health care costs will be driven down through improved prescribing and compliance, and reduction in ADRs. ie. DxS:K-Ras, mutations in VKORC1, and warfarin.



Issues

Standardisation



- Methods

- Sample preparation
- Targets used in normalisation for relative expression
- Absolute Quantitation- NO DNA Gold Standard!! OR Standardised means to quantify the QPCR Reference!

- Analysis

Data has to be comparable- it's a regulatory must!

- Subjective threshold vs 2nd derivative maxima algorithm
- Baseline normalisation
- Algorithms/methods for relative quantitation
- **Realtime kinetic efficiency.**

Sample preparation

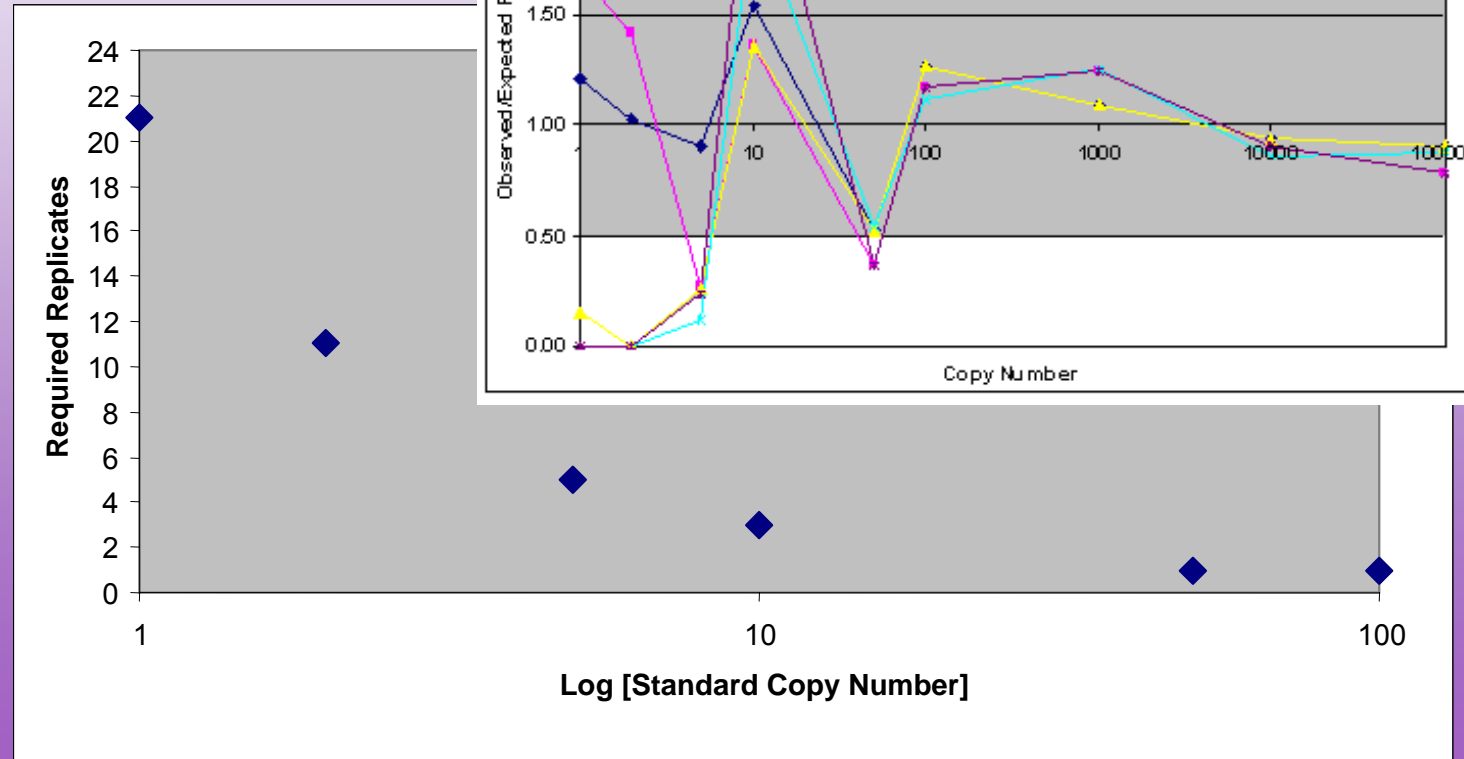


- Variable extraction efficiencies due to matrix
- Variable extraction efficiencies due to load
- NAT guidance 80% yield...relevance to QPCR?
- Extraction technologies to standardise on % Yield as a measure of comparison

Replicates



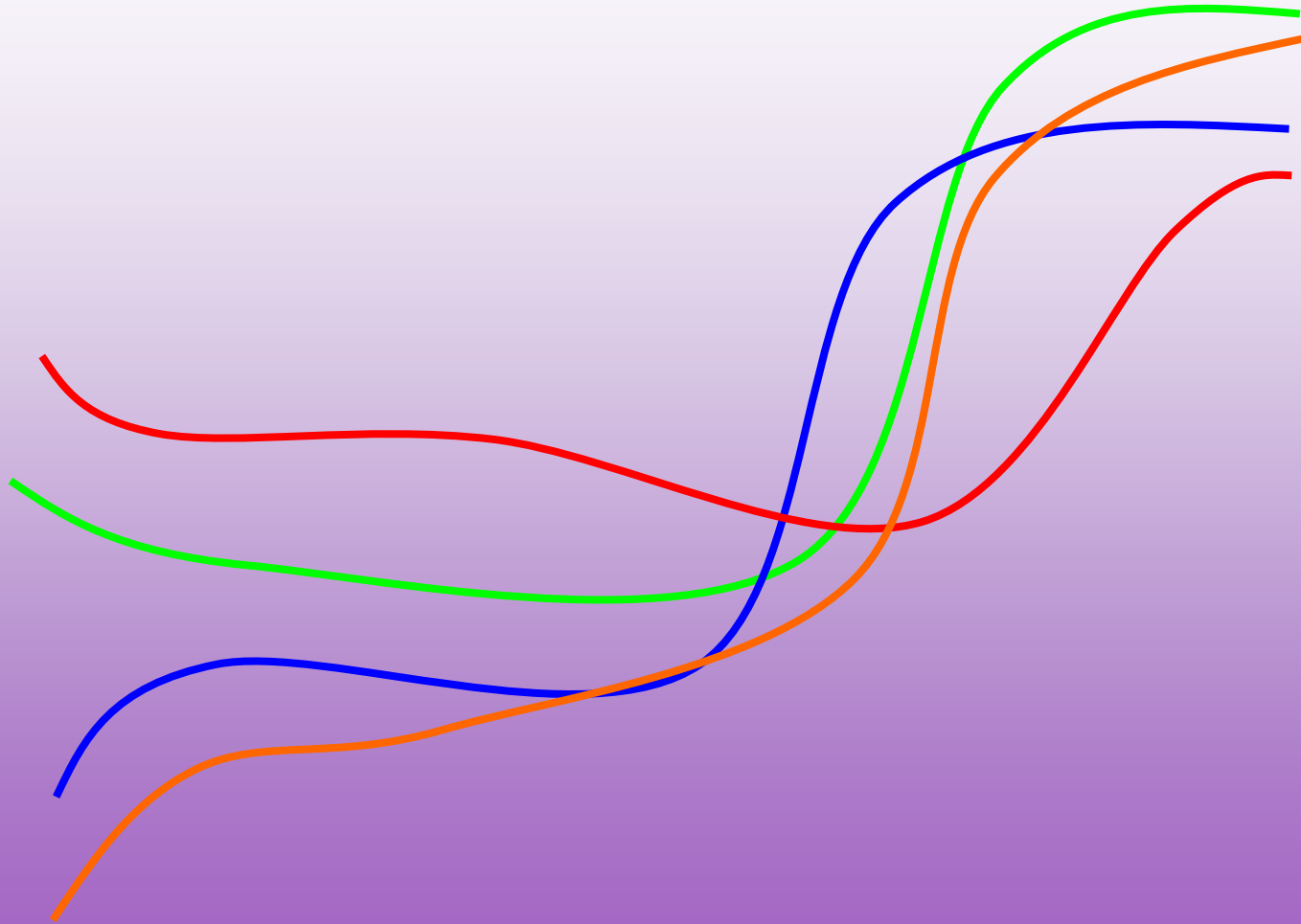
- Software ignore



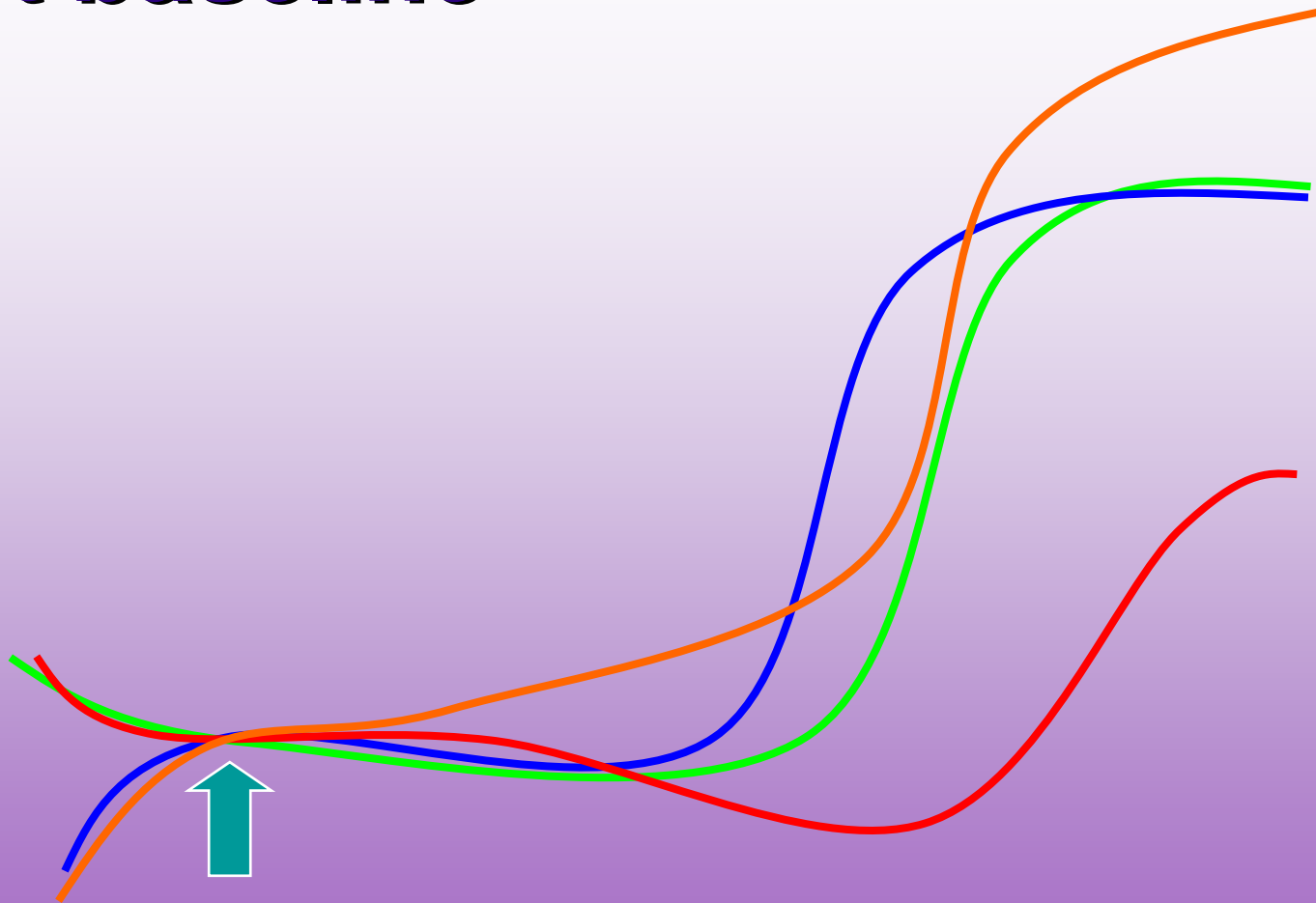
Poisson Distribution Model

Copy Number	Replicates Required
1	21
2	11
5	5
10	3
50	1
100	1

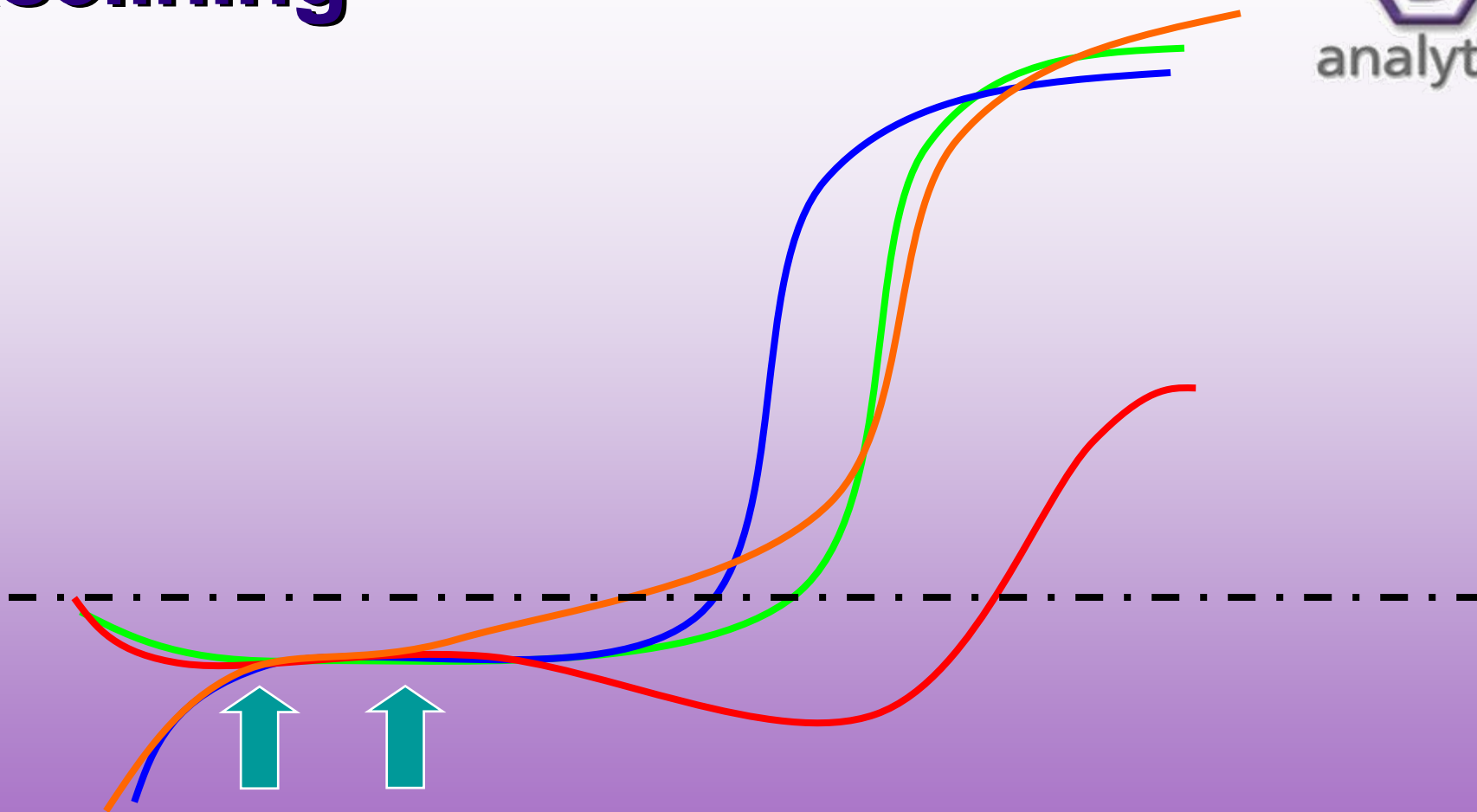
Raw data



Start baseline



Baselining



Baselining

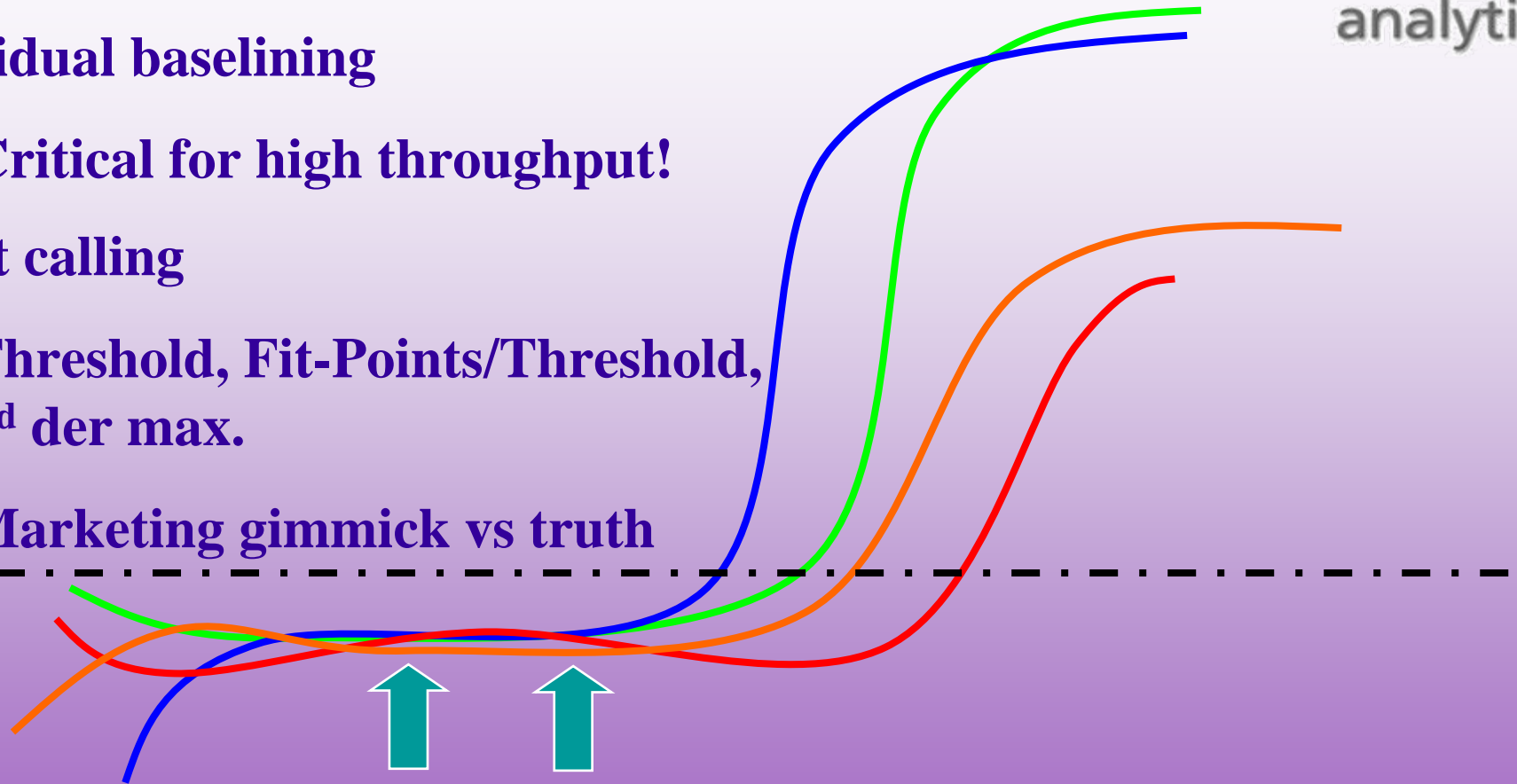


Individual baselining

- Critical for high throughput!

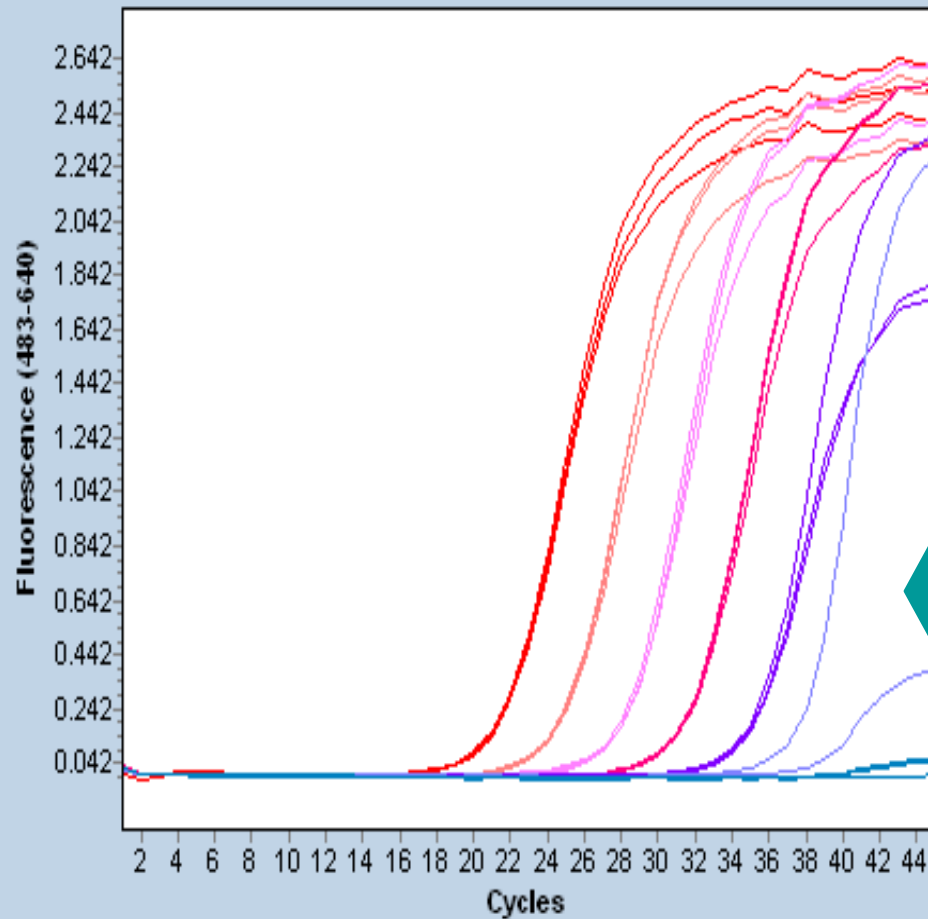
Cp/Ct calling

- Threshold, Fit-Points/Threshold, 2nd der max.
- Marketing gimmick vs truth



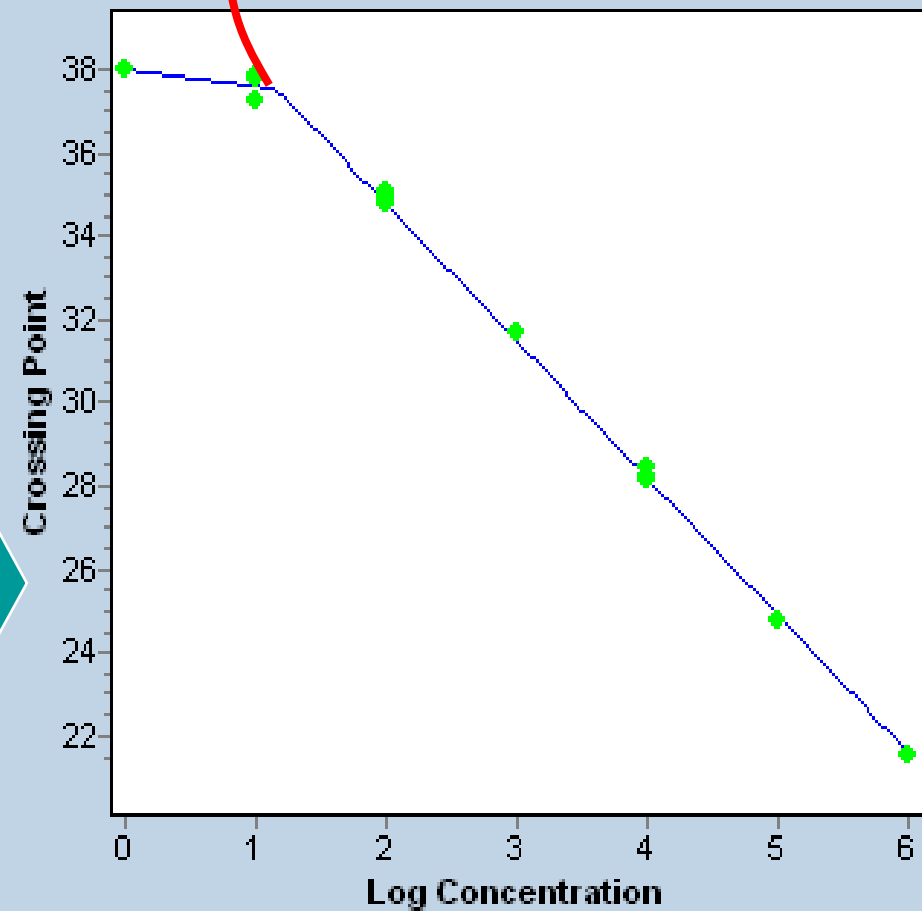
Efficiency correction?

Amplification Curves

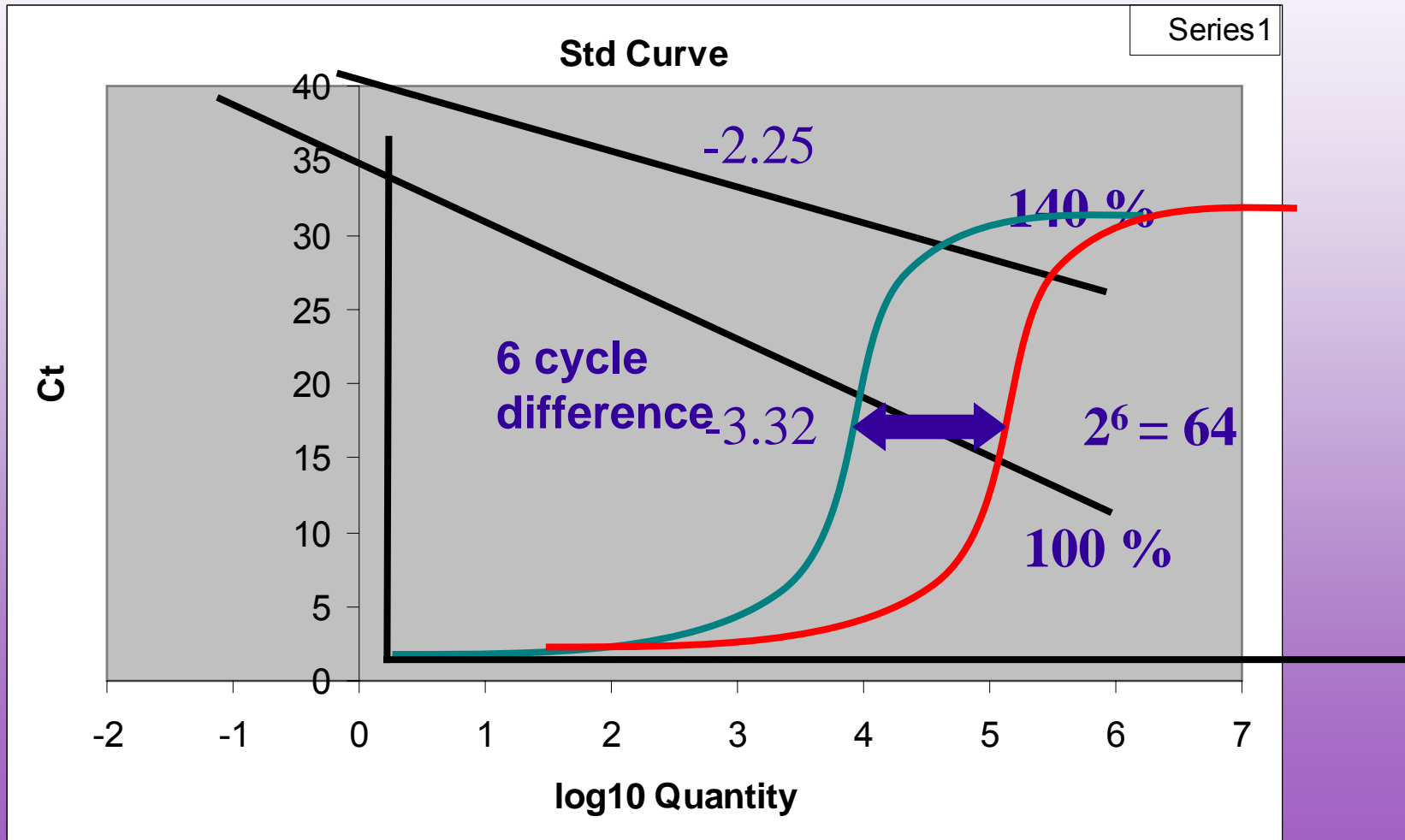


?

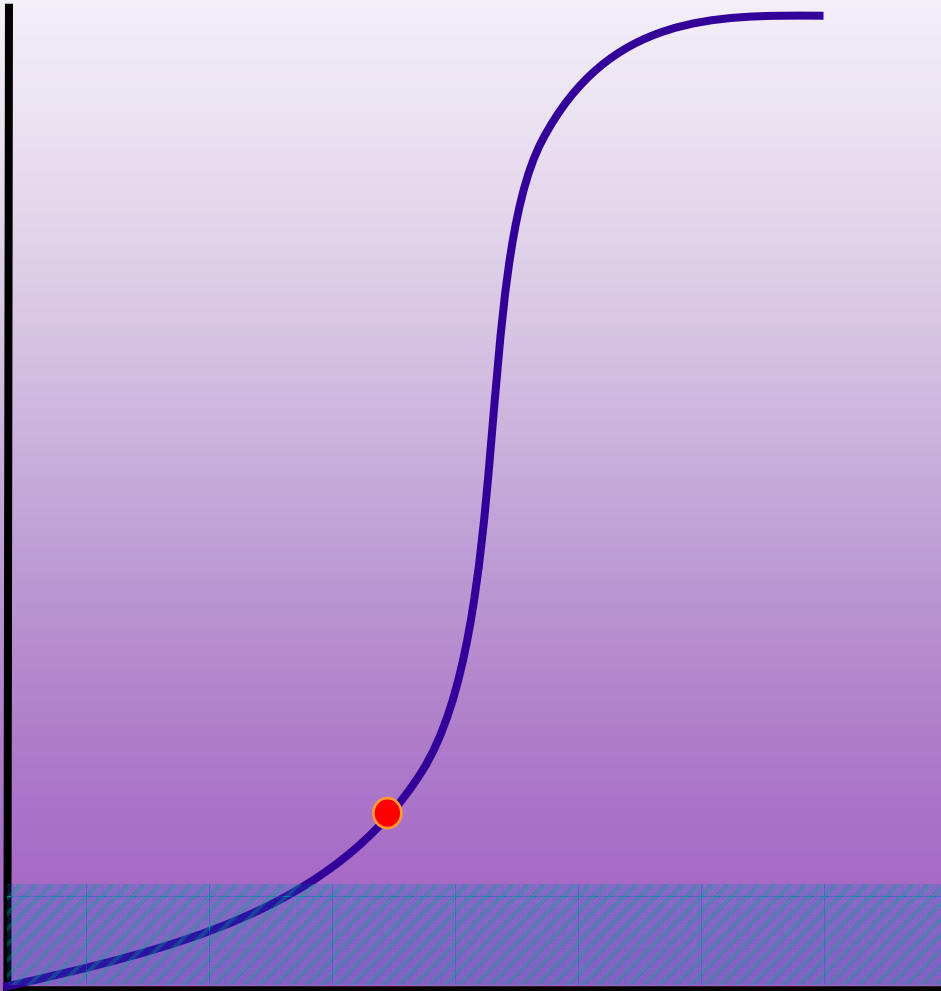
Standard Curve



Training



What's missing?



- Currently most of the available data is ignored in the final analysis
- Currently most base PCR efficiencies according to separate reactions
- A lot of potential data is lost due to fluorescent noise.
- More investment into realtime kinetic algorithms for both efficiency determination, QC criteria, and non subjective Cycle calling.
- Base lining algorithms.
- Investment into gated detection and time resolved chemistries.

Please help!



Put individual baselining & truthful
cycle calling at the top of the list

Acknowledgments



NDA Analytics

- Gerry Maxwell

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- Jean Engela
- Anja Grohnert
- Phil Henwood
- Mike Aylott

University of Sussex

- Kyle Morris

Enigma Diagnostics

- Martin Lee

Backup



Technical Innovation



- Reagents

- Enzymes etc- speed and utility are constantly improving.
- Time delay chemistries

- Instrumentation

- Speed + combined sample prep. (<30min from raw sample)
- Gated/time resolved detection
- Non-subjective software algorithms

Experimental vs Poisson



- Both models agree that as copy number per volume is decreased, so variation is increased. At the low end due to poisson distribution.
- Increasing replicates at the lower end brings the observed average values closer to the expected values

What I'm trying to get at...



Applicable well beyond the realms of real time – any assay using a standard curve for absolute quant.

Replication of standard curve points
(and where possible samples)
should be increased at lower copy numbers.

Reliability of abs quant below 100 copies should be questioned.