Detection of defective PCR samples with module Outlier of Kineret software

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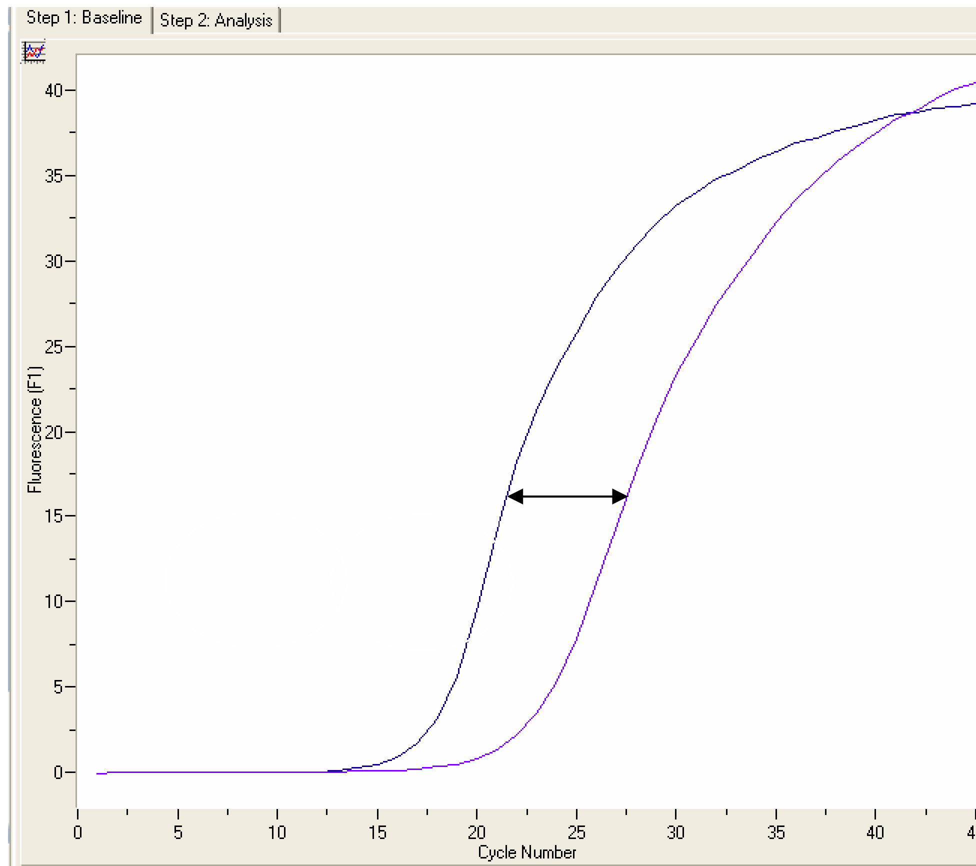
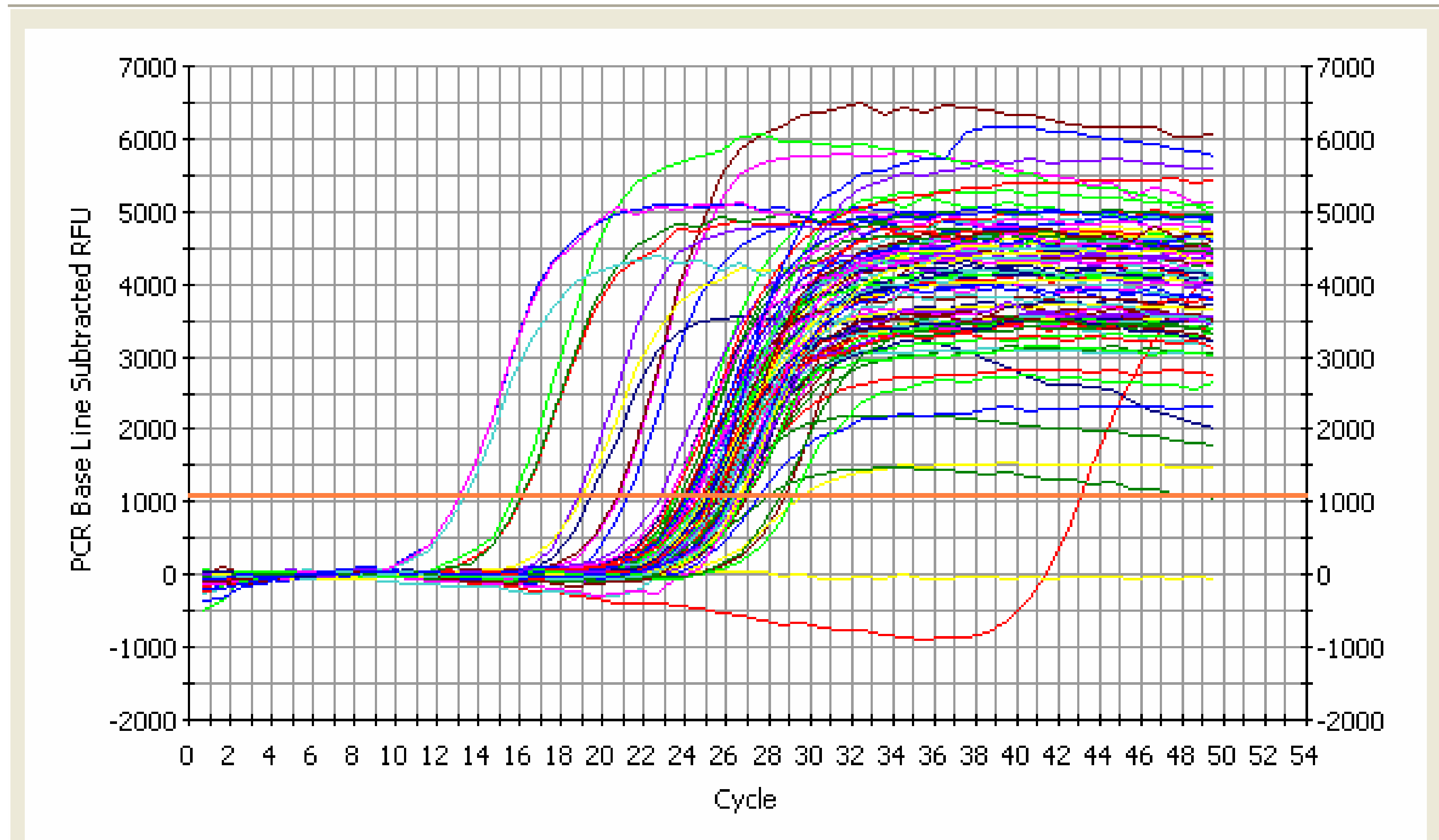
Would you exclude one of these curves from your data analysis?

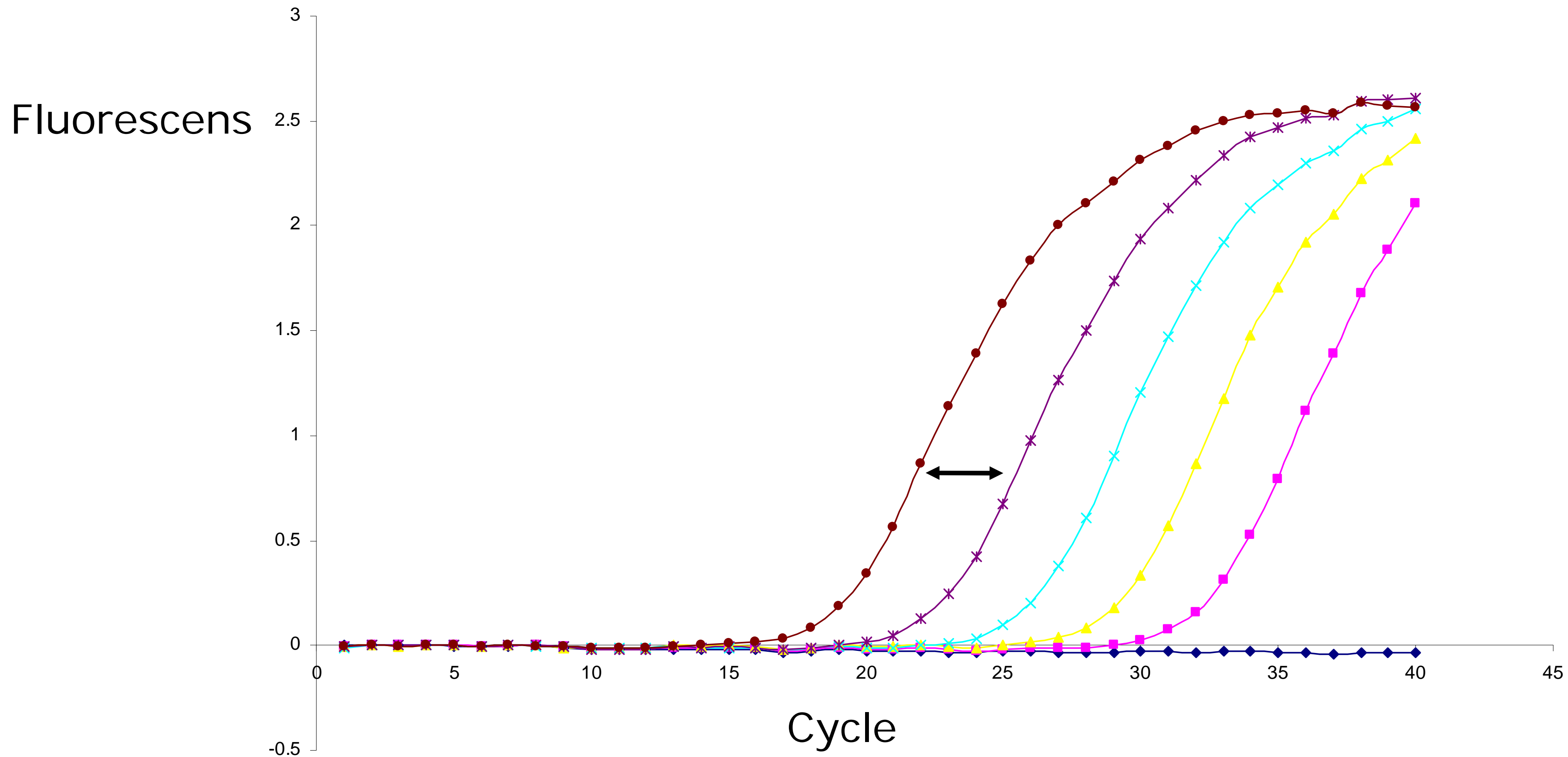


Which curves would you exclude here?



The Problem:

Quantification is based on distance between curves assuming similar kinetics between compared curves



Kineret

Integrated solution for real-time PCR data warehousing and analysis

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0099-2240/97/$04.00+0
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MINIREVIEW

Inhibition and Facilitation of Nucleic Acid Amplification

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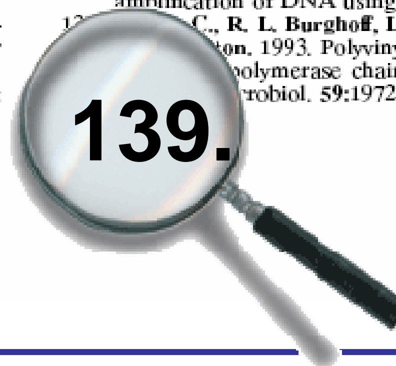
Factors that inhibit the amplification of nucleic acids by PCR are present with target DNAs from many sources. The inhibitors generally act at one or more of three essential points in sensitivity, specificity, and reproducibility have been reported (16, 82, 86, 129, 132, 134). There may also be potentially important effects in PCR typing reactions (121), and difficulties

Sussman (ed.), New techniques in food and beverage microbiology. Scientific Publishers, Blackwell Oxford, United Kingdom.
135. **Witham, P. K., C. T. Yamashiro, K. J. Livak, and C. A. Batt.** 1996. A PCR-based assay for the detection of *Escherichia coli* Shiga-like toxin genes in ground beef. Appl. Environ. Microbiol. **62:**1347–1353.
136. **Wu, P., S. Daniel-Issakani, K. LaMarco, and B. Strulovici.** 1997. An automated high throughput filtration assay: application to polymerase inhibitor identification. Anal. Biochem. **245:**226–230.
137. **Xia, J. Q., C. V. Yason, and F. S. B. Kibenge.** 1995. Comparison of dot blot hybridization, polymerase chain reaction, and virus isolation for detection of bovine herpesvirus-1 (BHV-1) in artificially infected bovine semen. Can. J. Vet. Res. **59:**102–109.
138. **Yoon, C., and D. A. Glawe.** 1993. Pretreatment with RNase to improve PCR amplification of DNA using 10-mer primers. BioTechniques **6:**908–910.
1[illegible] [illegible] C., R. L. Burghoff, L. G. Keim, V. Minak-Bernero, J. R. Lute, and [illegible]ton. 1993. Polyvinylpyrrolidone-agarose gel electrophoresis purification of [illegible]polymerase chain reaction-amplifiable DNA from soils. Appl. [illegible]robiol. **59:**1972–1974.



FROST & SULLIVAN

Strategic Analysis of Thermal Cycler Markets in Europe

Chennai , January 2007

FROST & SULLIVAN

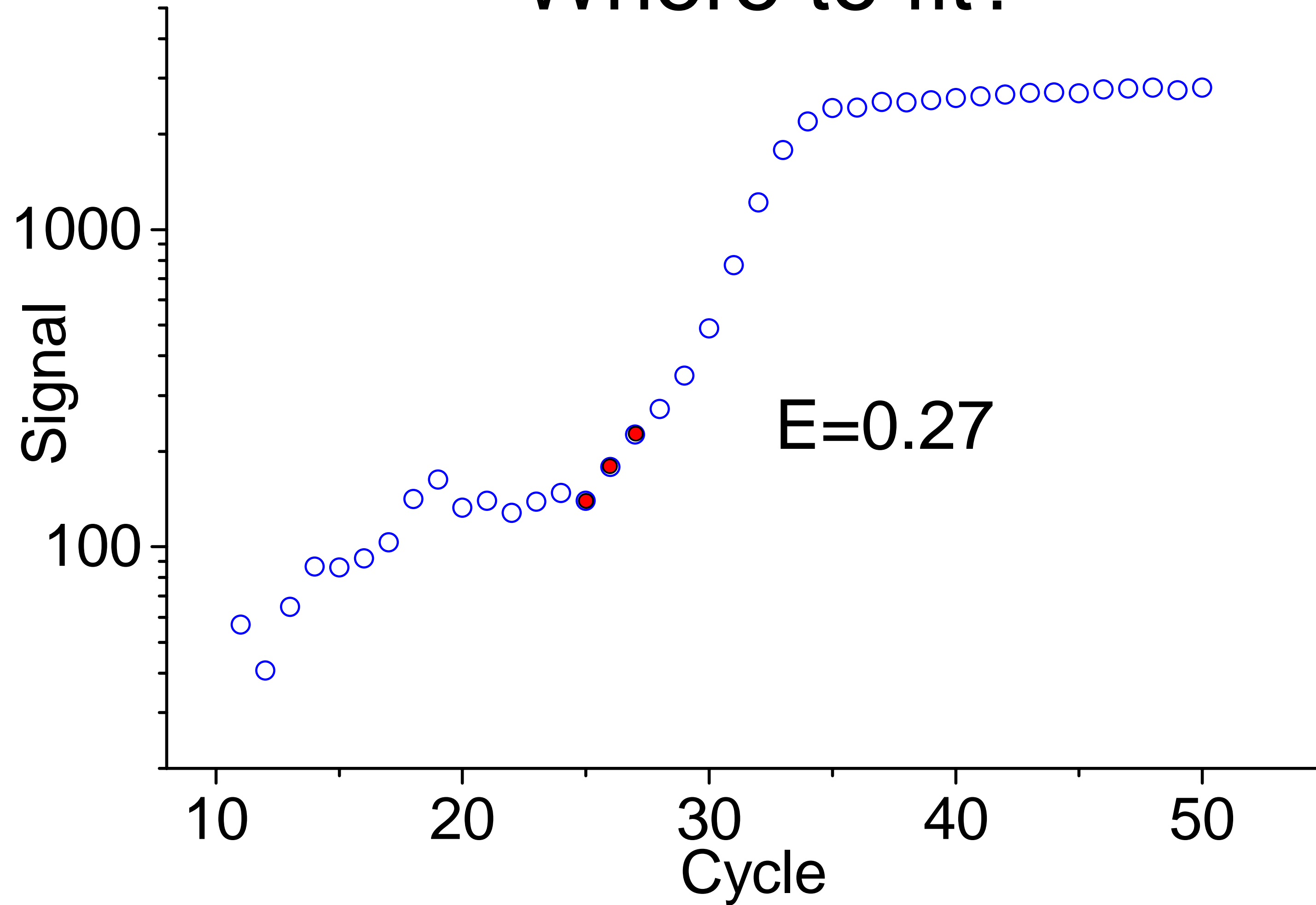
Strategic Recommendations

- PCR reactions are highly sensitive and hence maintaining stringent laboratory conditions is necessary to reap the maximum benefits out of the thermal cyclers. Each laboratory needs to ensure that they make quality as their policy.

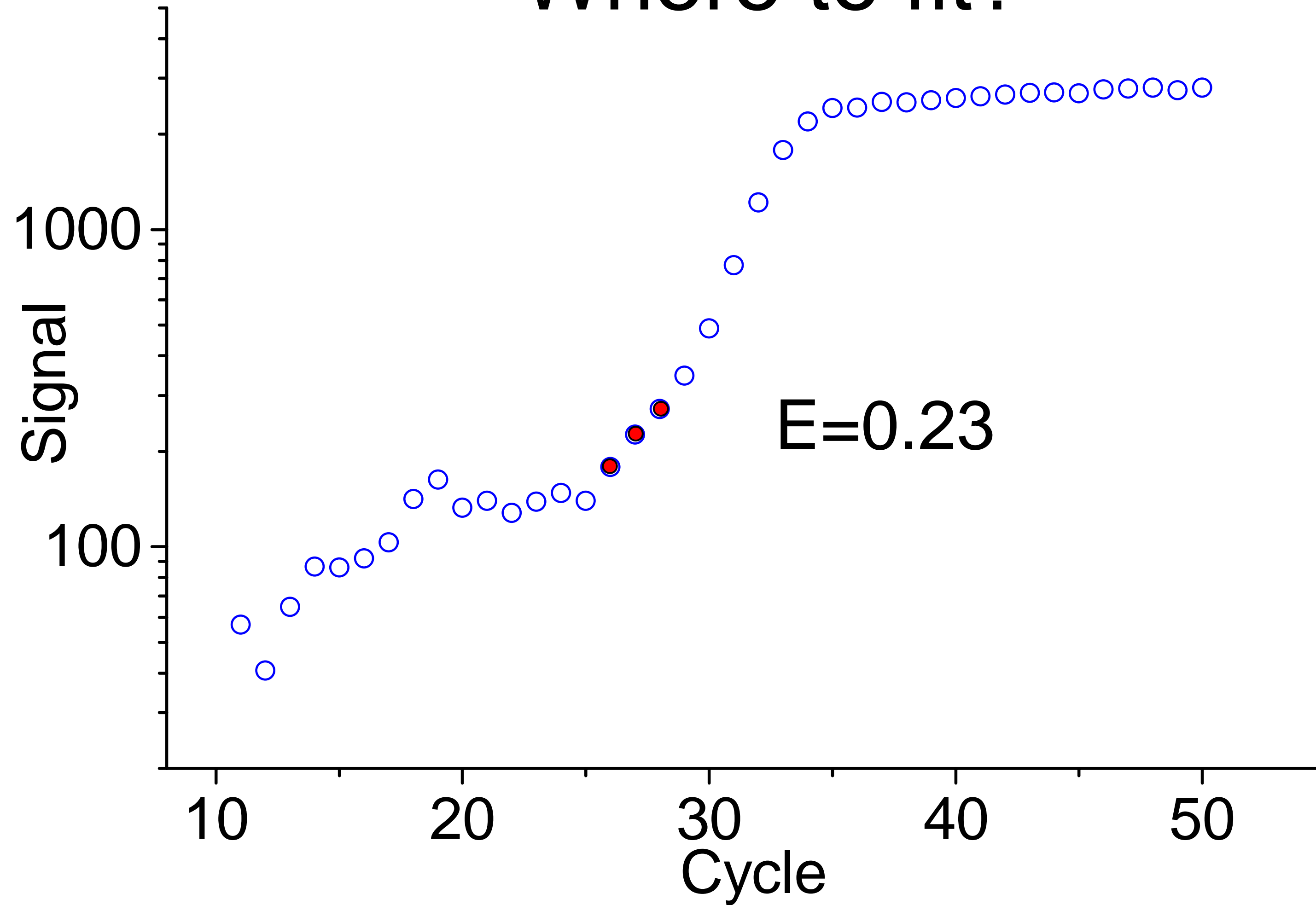
To see if the kinetics are similar, compare the slopes of the curves!

Imprecise

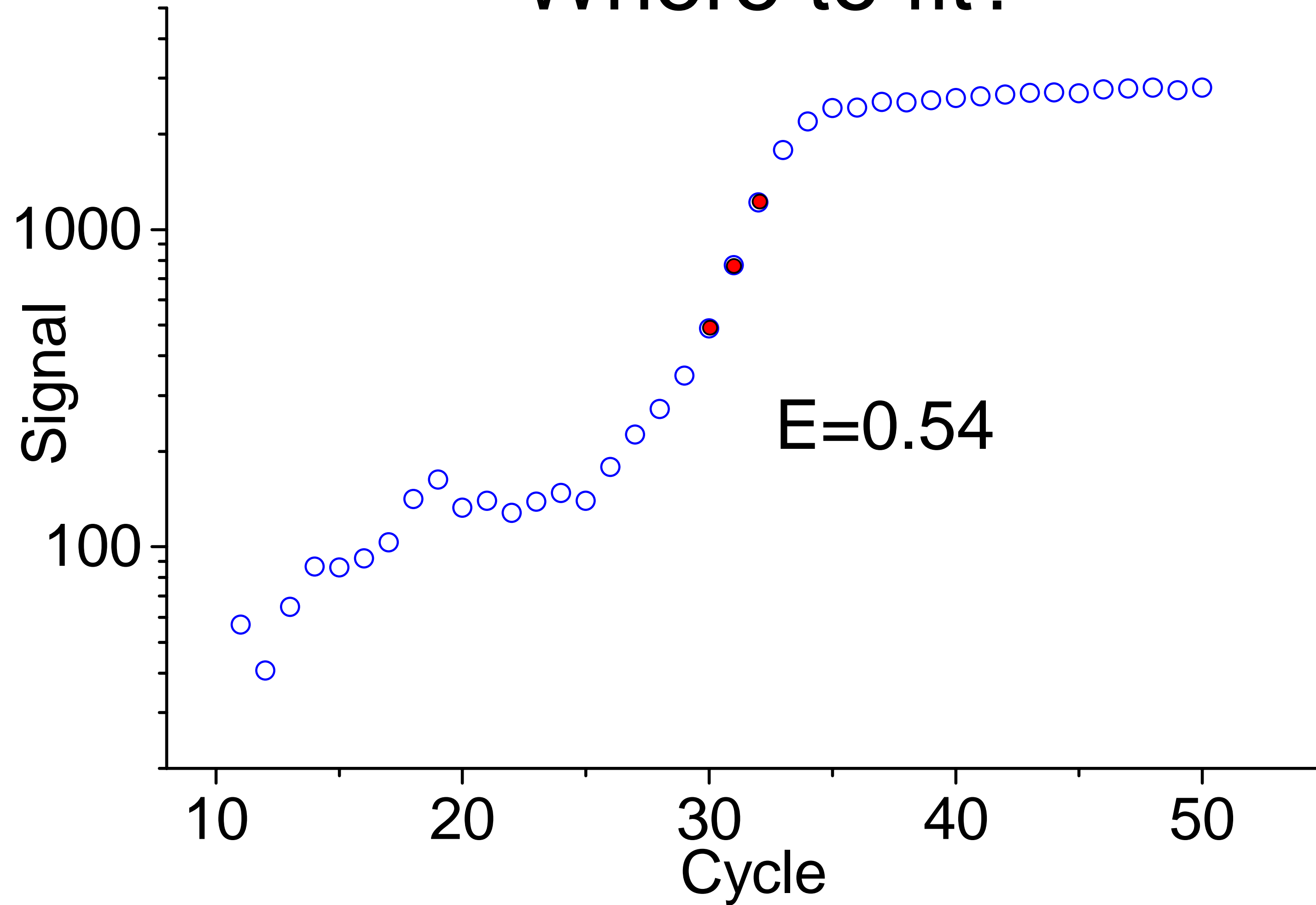
Where to fit?



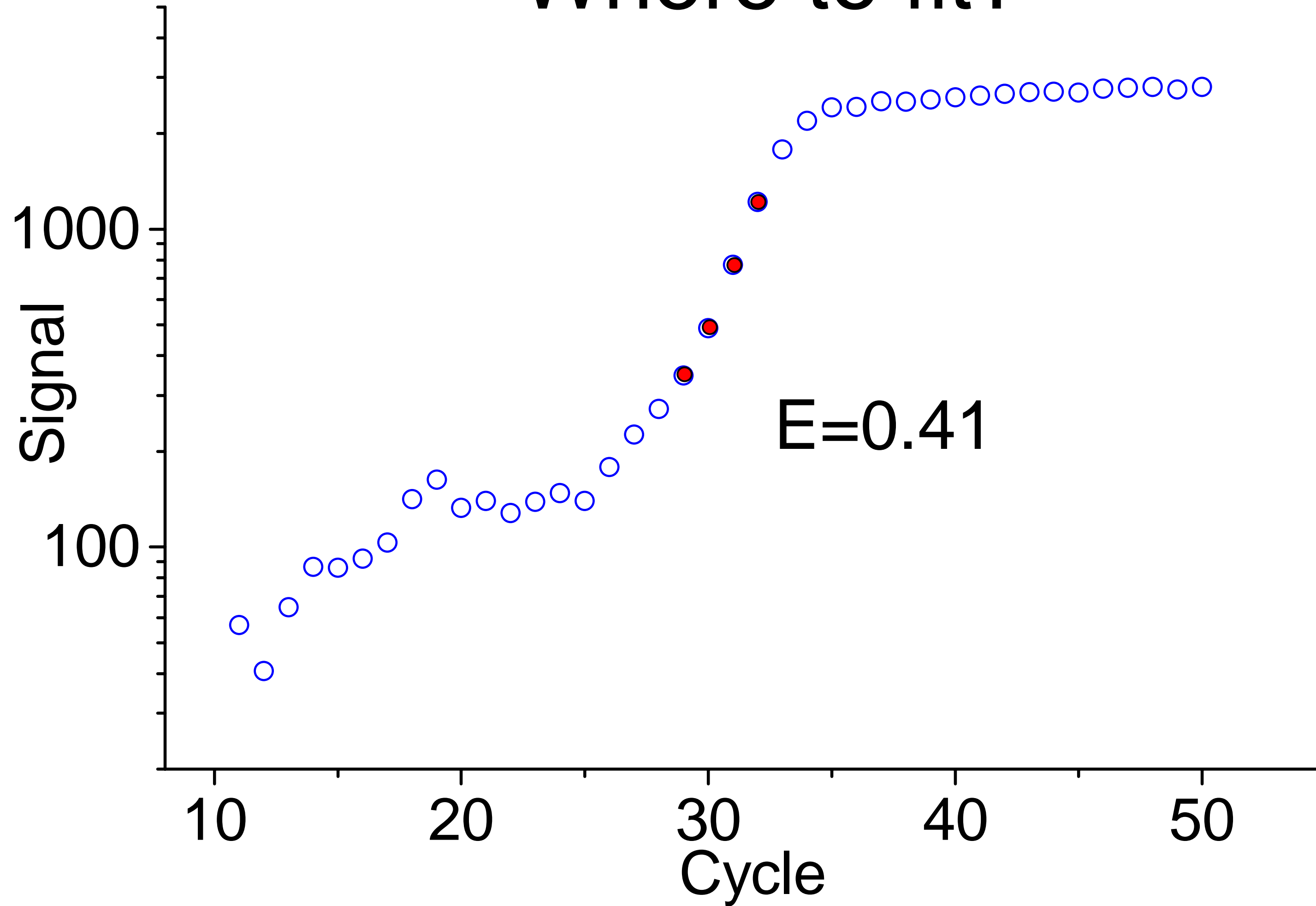
Where to fit?



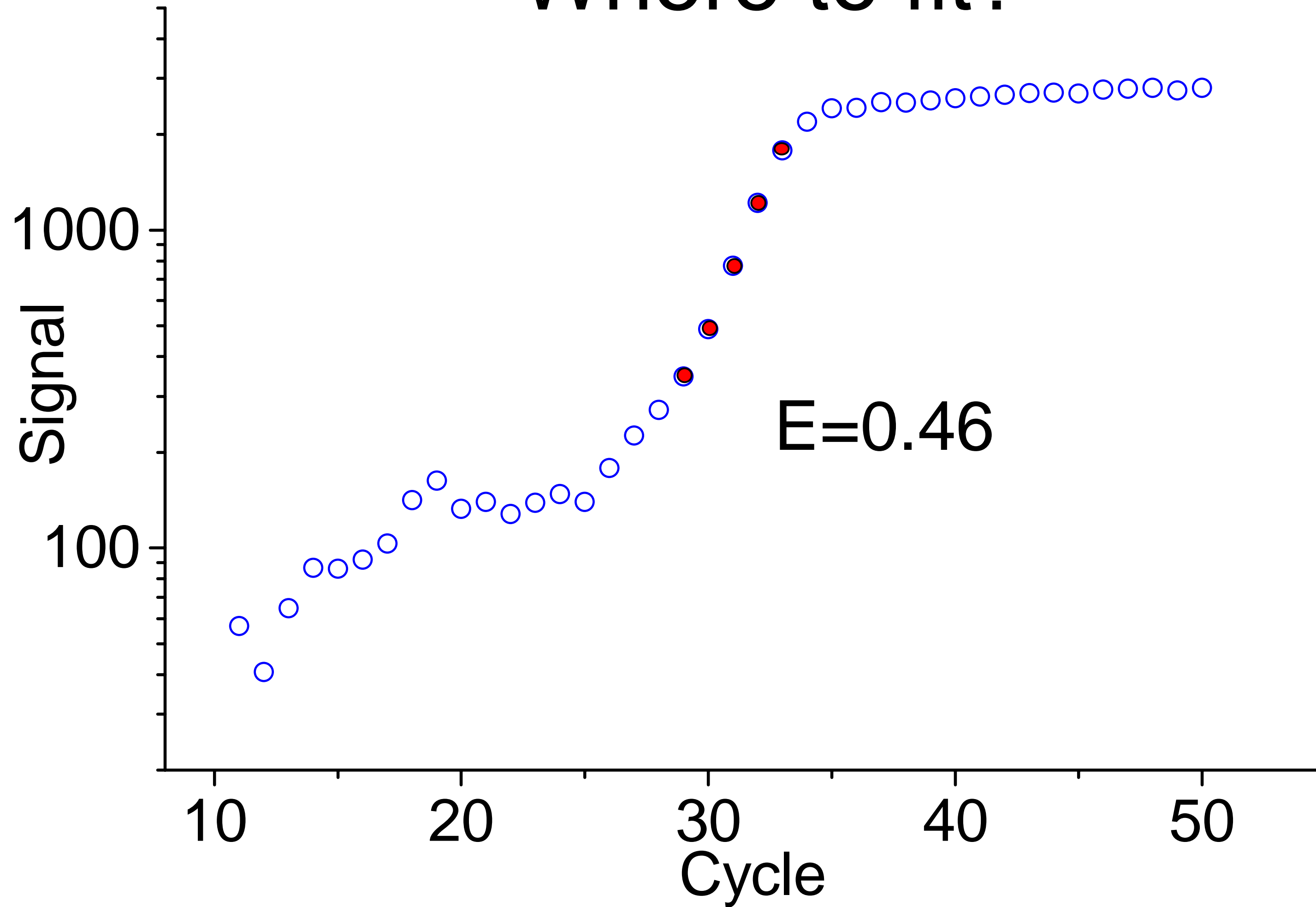
Where to fit?



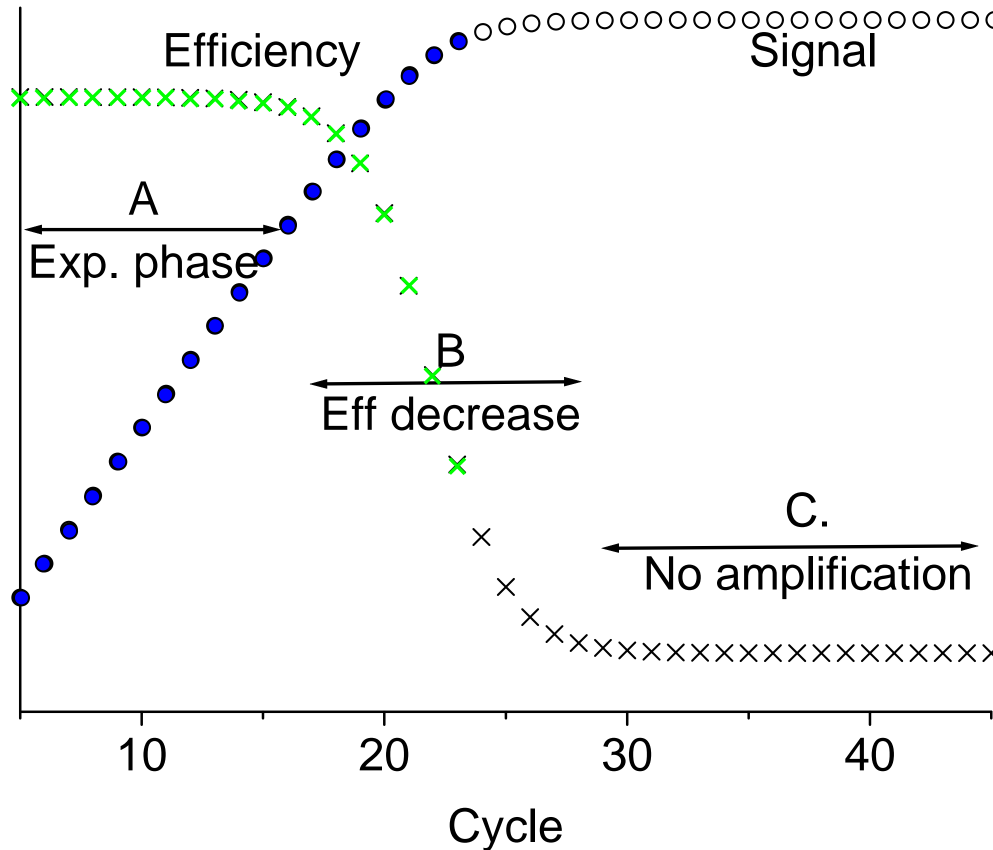
Where to fit?



Where to fit?



PCR kinetics



- Efficiency changes during the reaction.
- → variability in estimated efficiency

Precision of efficiency estimation

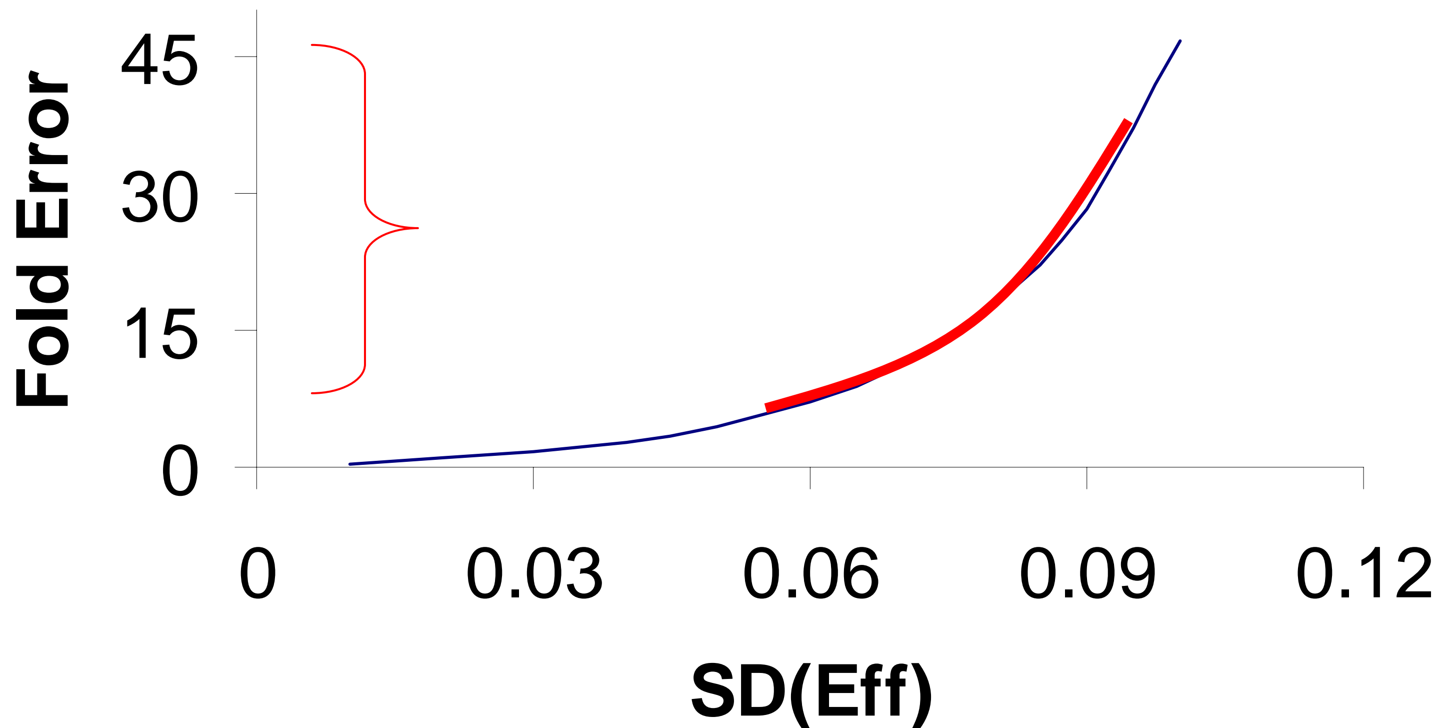
Median SD (E)

	Stand[1]	LinReg[2]	DART[3]	SoFAR[4]	4P[5]
Rotogene	0.076	0.102	0.118	0.076	0.071
LightCycler	0.036	0.043	0.034	0.055	0.044
iCycler	0.067	0.134	0.146	0.098	0.055
Total	0.062	0.101	0.11	0.081	0.058

[1]Tichopad et al., NAR **31**(20): e122
[2]Ramakers et al., *Neurosci. Lett.*, **339**, 62–66
[3]Peirson et al., NAR **31**(14): e73
[4]Wilhelm et al., BioTechniques **34**, 324-32
[5]Liu & Saint, Biochem. Biophys. Res. Commun. **294**(2), 347-53

High SD(Eff) is useless for kinetics outlier detection

Efficiency=0.9
Cycle of threshold= 29



Kineret

Integrated solution for real-time PCR data warehousing and analysis

Projects | Sessions | Runs | Sequances | Wells | Workshop | Reports | Class | Subject | Sample

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Home | Contact

Technical | Exp | Seq

Update tree | Item 1

115- plateTest
- 1- plateSes
  - 1- AnonClass
    - 1- AnonSubject
      - 1- AnonSample
        - 1458- 10^1
        - 1459- 10^1
        - 1460- 10^1
        - 1461- 10^1
        - 1462- 10^2
        - 1463- 10^2
        - 1464- 10^2
        - 1465- 10^2
        - 1466- 10^3
        - 1469- 10^3

Open From Project List | Create New Project | View Your Projects

Allocate to class | Class4 | Subject | InfoFile | Update tree

User name: Bar Tzachi
Project name: MATTest
Session name: MATSes
Run name: ABIInfoP
Sequance name: Beta actin

Report was generated on: 22/03/2007 7.26 PM

PCR location	PCR Name	Reference	Ct	Kinetics distance	Kinetics status
A1	10^7 [c]=0	true	9.39	0.01	Passed
A2	10^7 [c]=0	true	9.58	1.21	Passed
A3	10^7 [c]=0	true	9.73	1.87	Passed
A4	10^6 [c]=0	true	14.17	8.79	Failed (-)
A5	10^6 [c]=0	true	13.59	0.76	Passed
A6	10^6 [c]=0	true	13.55	1.73	Passed
A7	10^5 [c]=0	true	17.84	4.94	Passed
A8	10^5 [c]=0	true	17.6	2.53	Passed
A9	10^5 [c]=0	true	17.32	0.05	Passed
A10	10^4 [c]=0	true	21.05	2.69	Passed
A11	10^4 [c]=0	true	21.21	0.06	Passed
A12	10^4 [c]=0	true	21.38	3.19	Passed
B1	10^3 [c]=0	true	25.37	1.74	Passed
B2	10^3 [c]=0	true	26.08	6.1	Failed (-)
B3	10^3 [c]=0	true	19	5.24	Passed
B4	10^7 [c]=1	false	9.68	5.09	Passed
B5	10^7 [c]=1	false	9.46	5.39	Passed

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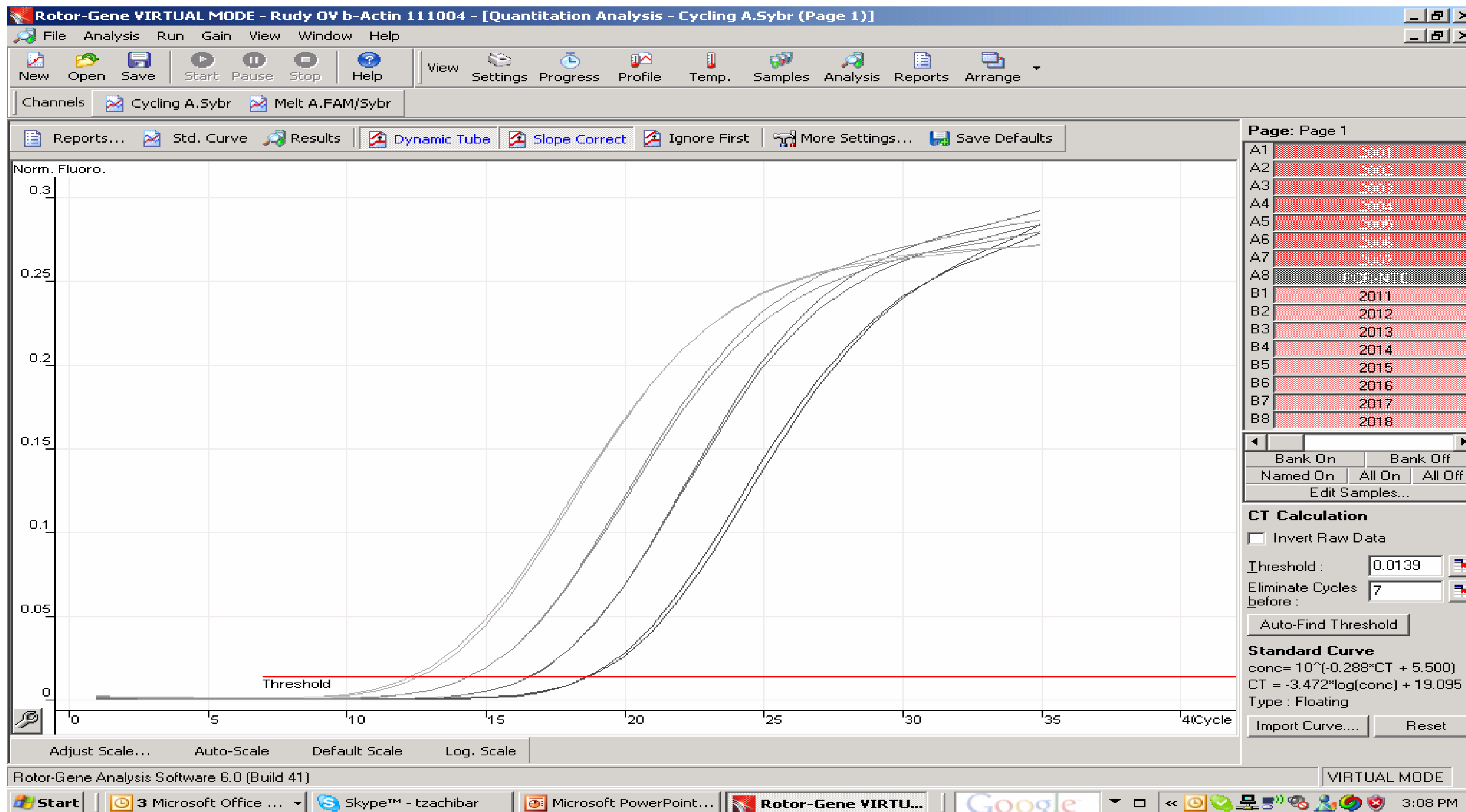
To identify outlying PCR

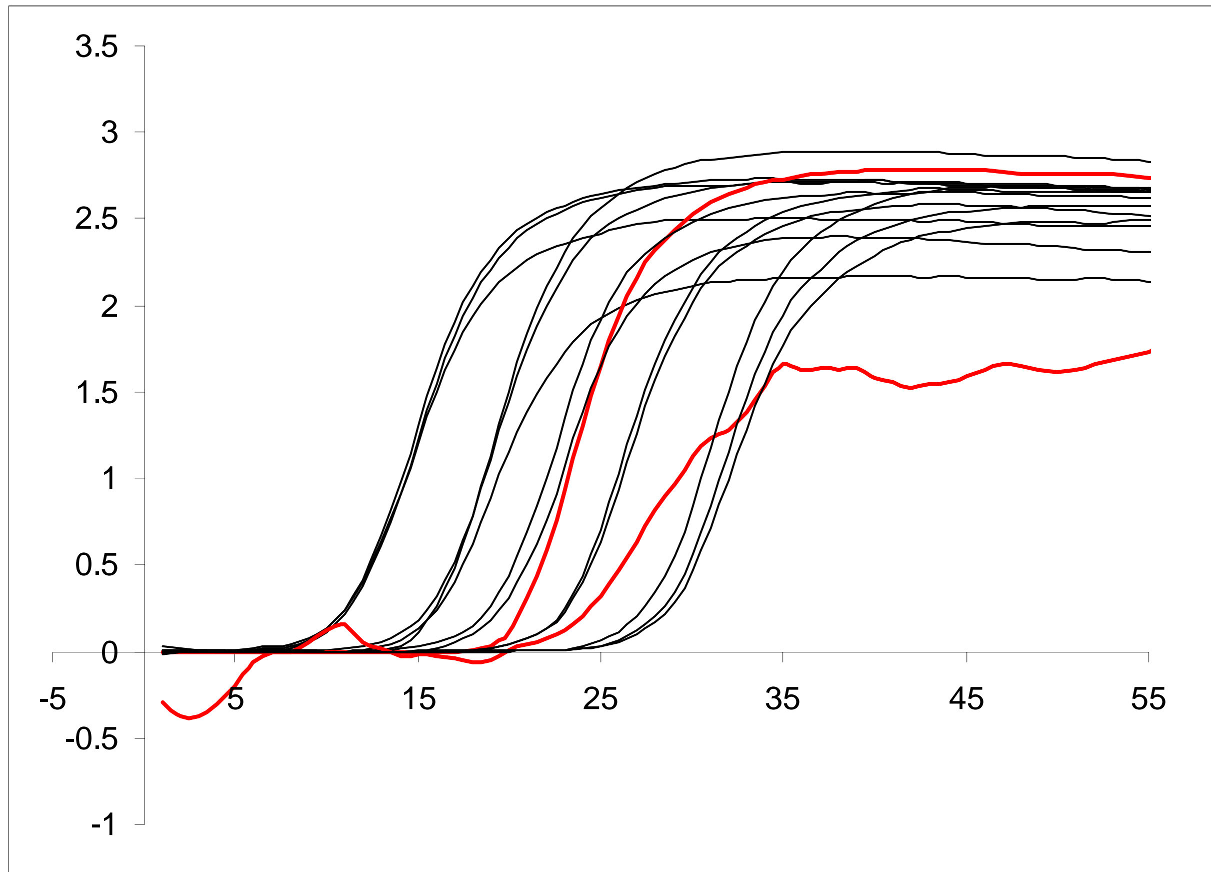
Step 1:

- Define a reference set consisting of well performing samples, usually the standard curve samples.

High reproducibility – CTs are similar



Low reproducibility – CTs are different

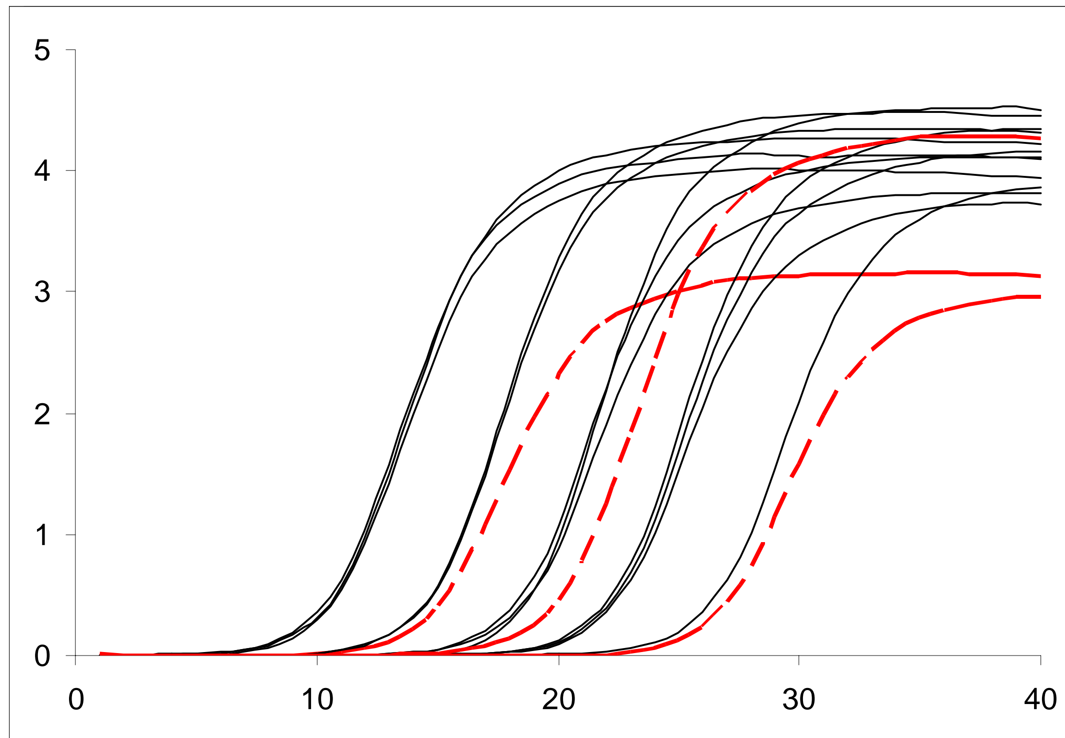


Step 2:

- Characterize the kinetics of each amplification curve by several parameters (automated).

Step 3

- Test the reference set for PCRs with outlying kinetics



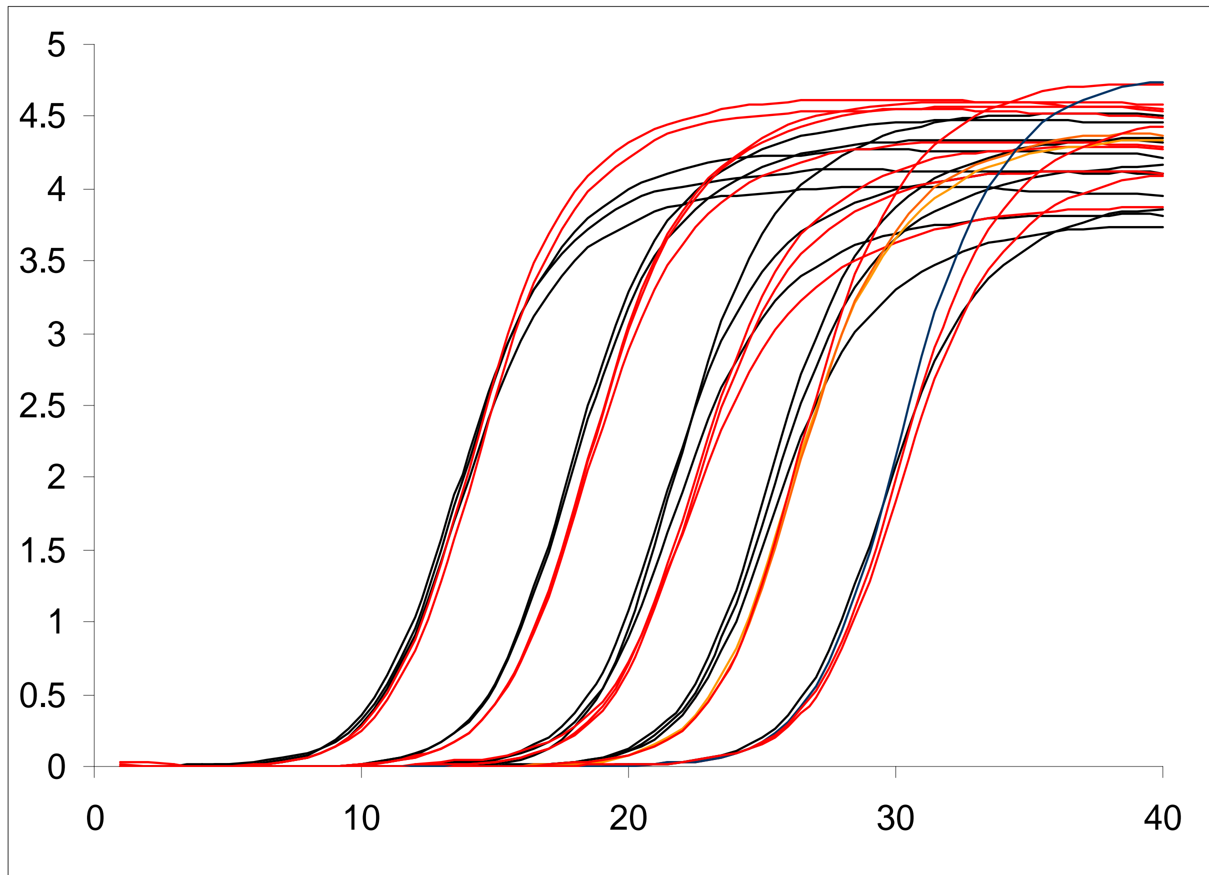
Step 4

- Test each unknown PCR vs. reference set

Experiment:
Detection of inhibited PCR

- Reference set – a standard curve consisting of 15 PCRs with no treatment
- Test samples – 5 sets of 15 PCRs each inhibited with elevated concentrations of competimers.
- Competimers – primers blocked at the 3’ end

Ability of Kineret to detect kinetics outliers



Black – reference, not inhibited

Red – test, inhibited

Kineret vs. other methods

n = 15	% of outliers detected					
Competimers [%]	**No inhibition**	**1**	**2**	**4**	**8**	**16**
Average deviation of CT	**0**	**-0.066**	**0.030**	**0.152**	**0.347**	**0.469***
Bar et al. (1)	**7**	**27**	**33**	**13**	**7**	**13**
Chervoneva et al. (2)	**0**	**7**	**0**	**14**	**7**	**0**
Kineret	**14**	**67**	**33**	**27**	**80**	**100**

* Significantly different Ct values (one-tailed t-test, alpha=0.05).

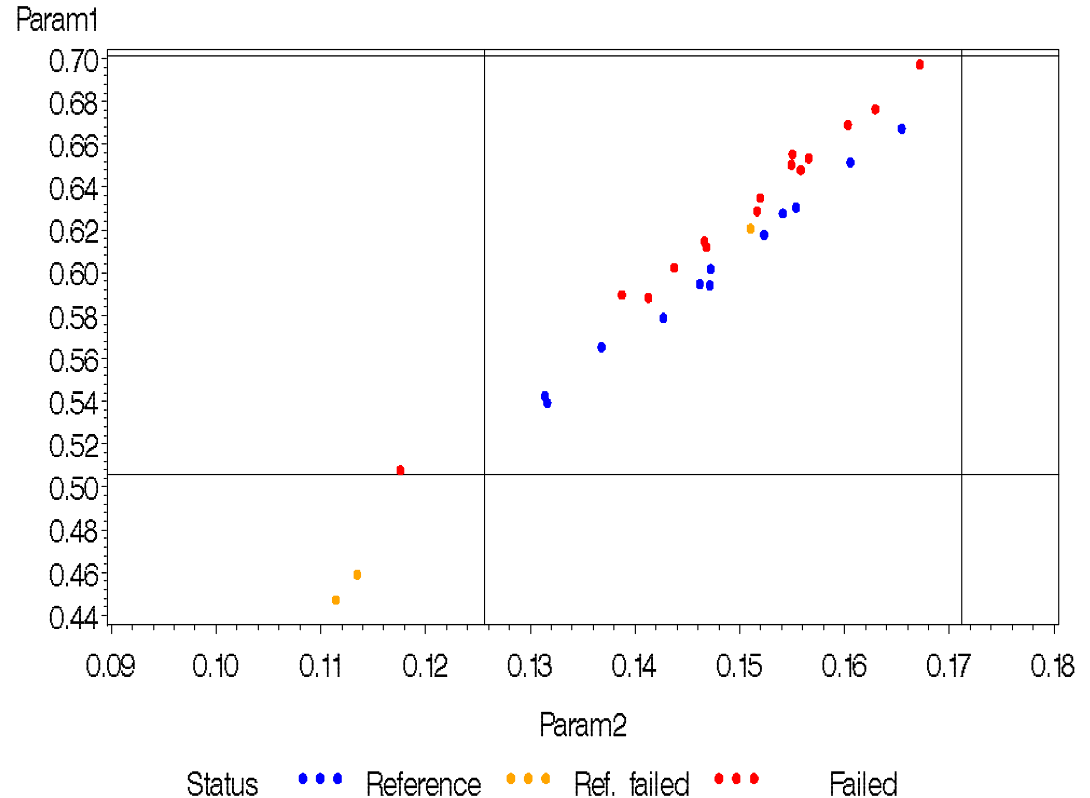
1. Bar T, Stahlberg A, Muszta A, Kubista M. (2003). Nucleic Acids Res. 31, e105

2. Chervoneva I, Hyslop T, Iglewicz B, Johns L, Wolfe HR, Schulz S, Leong E, Waldman S (2006). Anal Biochem. 348, 198-208.

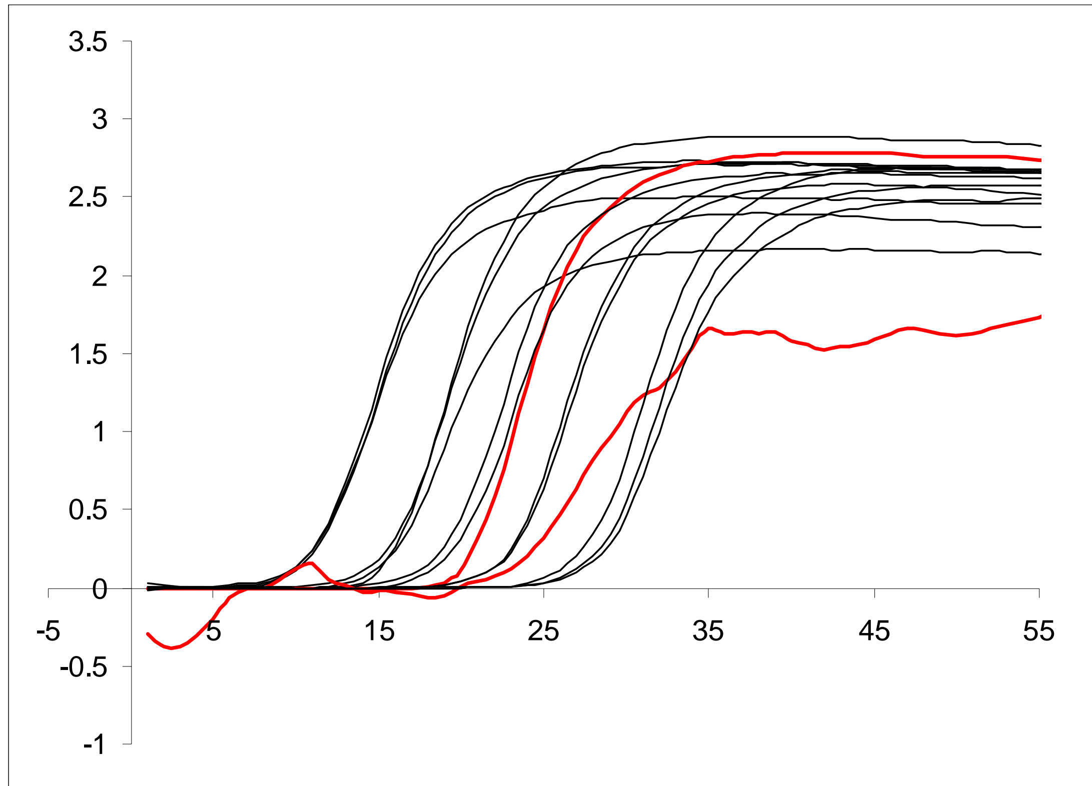
Multivariate vs. univariate outlier detection

Upper/Lower Fence=upper/Lower Quartile + 3 * Interquartile Rang

Interquartile Range = Upper quartile - Lower Quartile



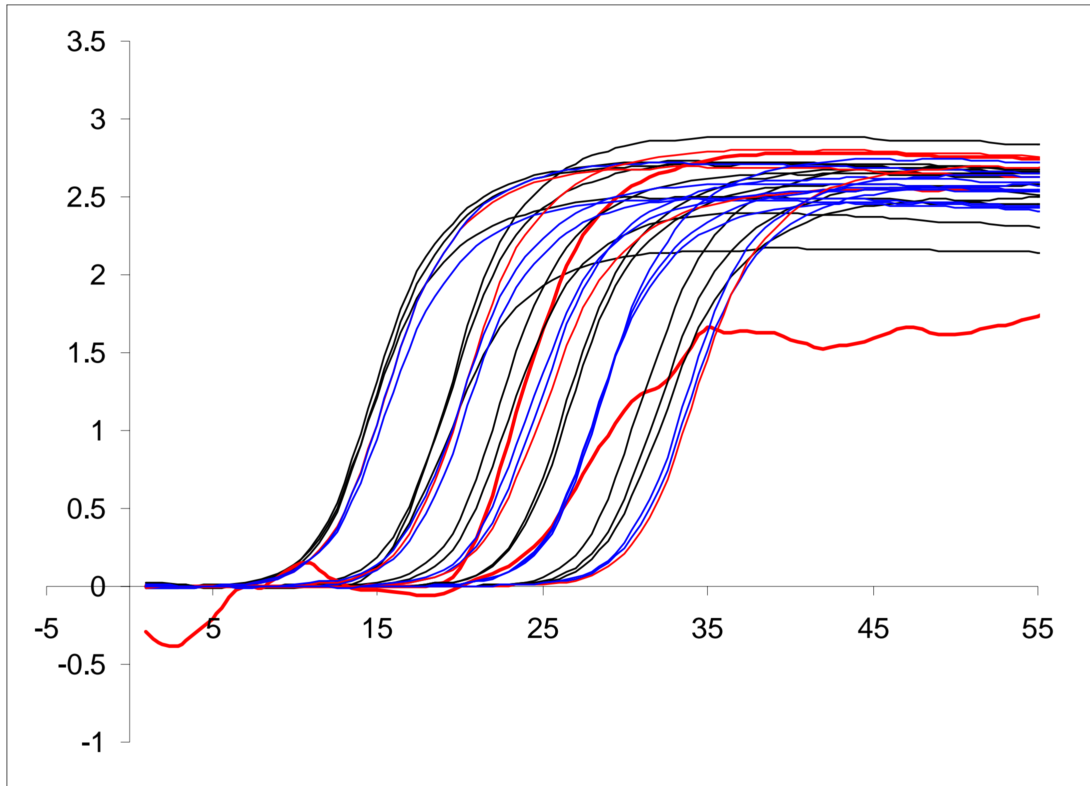
Example of poor reference set



Black – reference, not inhibited

Red – kinetics outliers

Garbage in… garbage out

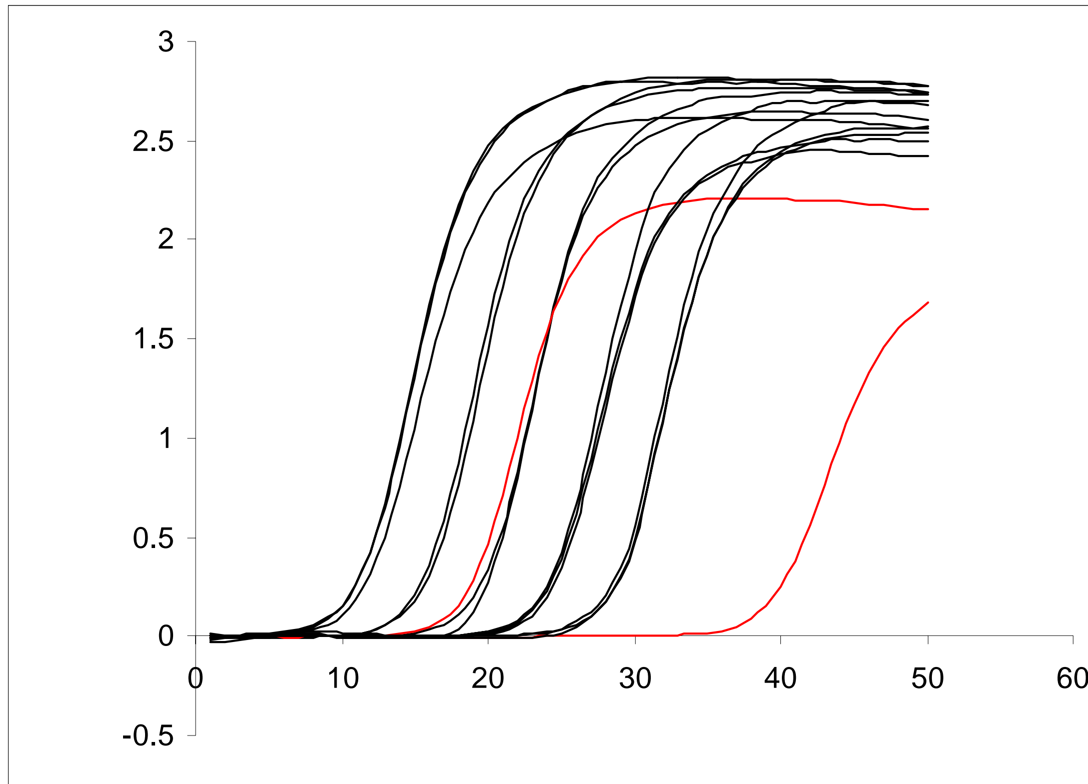


Black – reference, not inhibited

Red – test samples, kinetics outliers

Blue – test samples, undetected

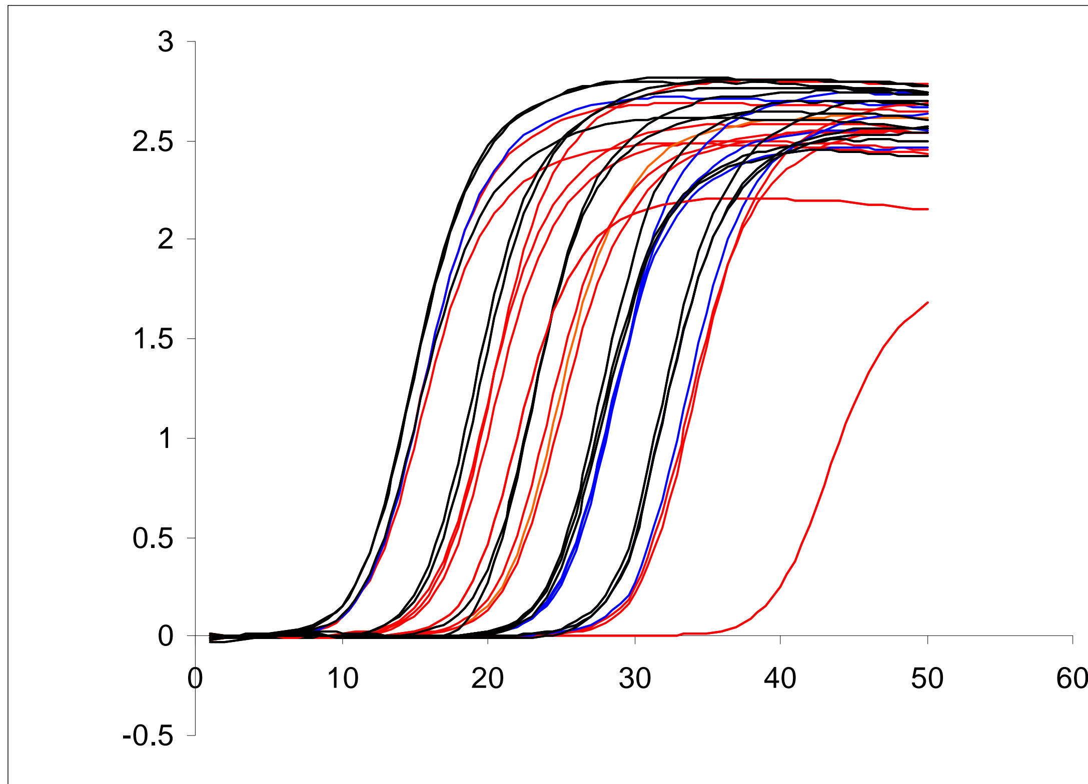
Changing the reference set (same experiment)



Black – reference, not inhibited

Red – kinetics outliers

Improves the kinetics outlier detection



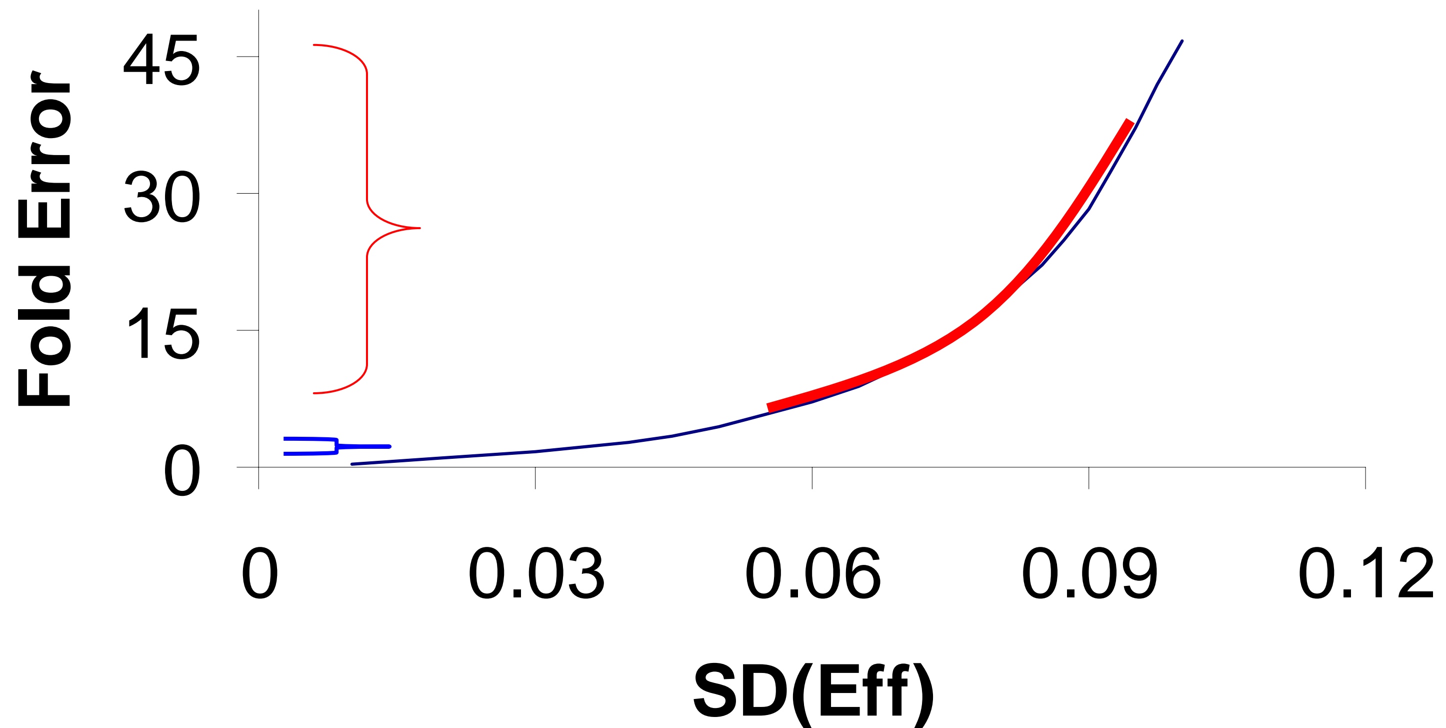
Black – reference, not inhibited

Red – test samples, kinetics outliers

Blue – test samples, undetected

Kineret makes kinetics outlier detection a useful tool

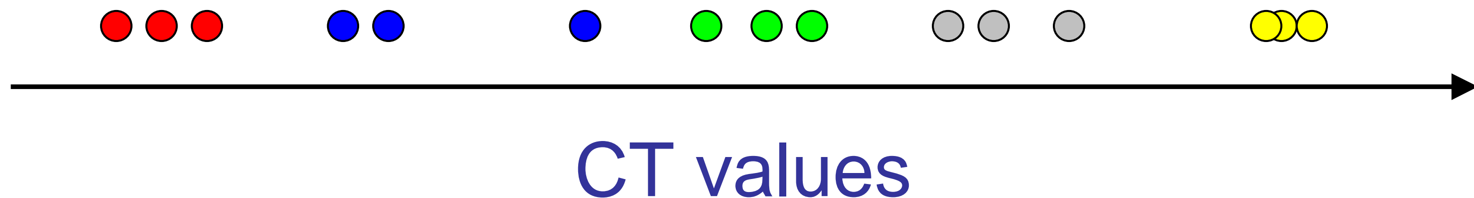
Efficiency=0.9
Cycle of threshold= 29



CT outlier detection

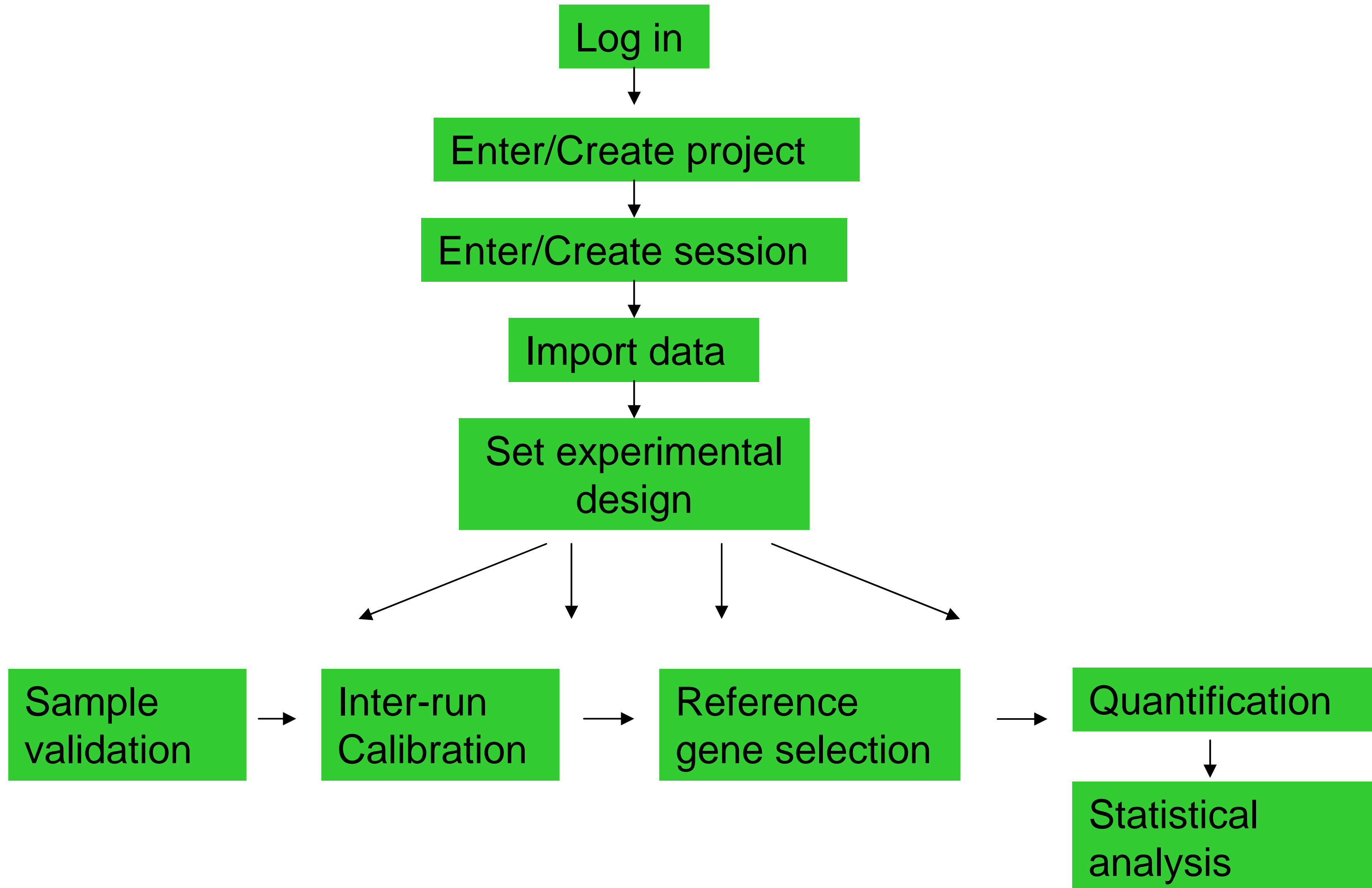
- Use experimental design to recognize outlying replicates.

  Example: replicates are marked with same color.



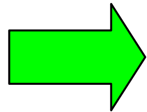
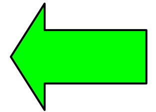
Intersect Kinetics Outlier Detection with CT outlier detection

Kineret work flow



Summary

- Analysis of PCR data should start with validation of individual samples
- Validation should consider both kinetics and CT


High quality standardized real-time PCR job


Labonnet team

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- Eliad Dahan