

# Estimation of sample specific efficiency methods and applications

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# Outline

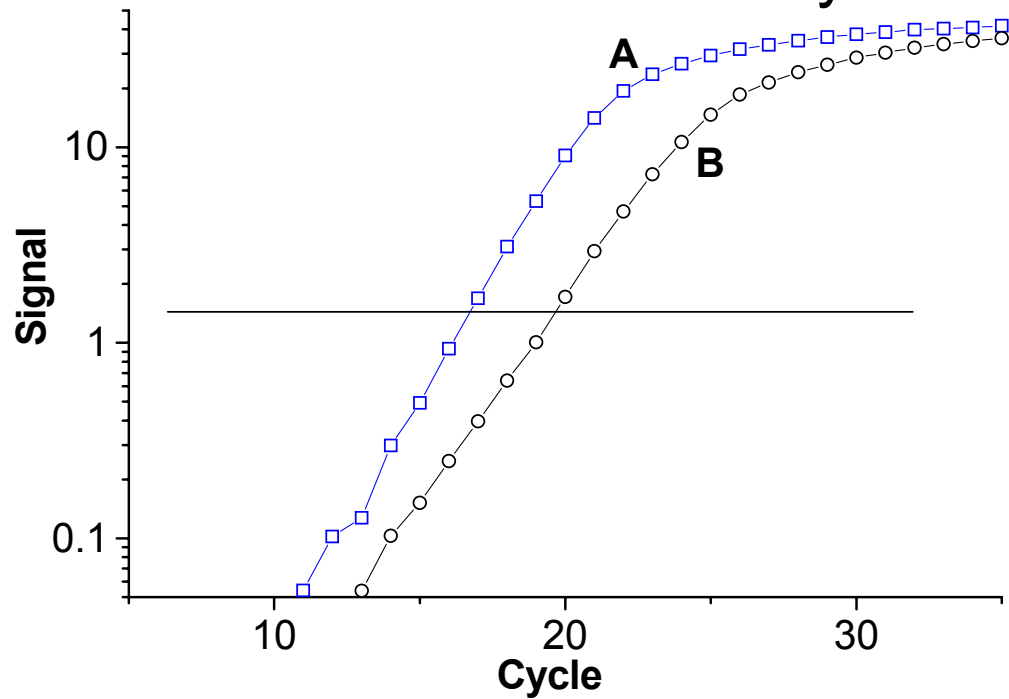
- The amplification efficiency
- Why to estimate amplification efficiencies?
- Amplification efficiency estimation
- Comparison of different efficiency estimation methods
- Applications
- Conclusion

# PCR efficiencies of the same gene in compared samples must be similar



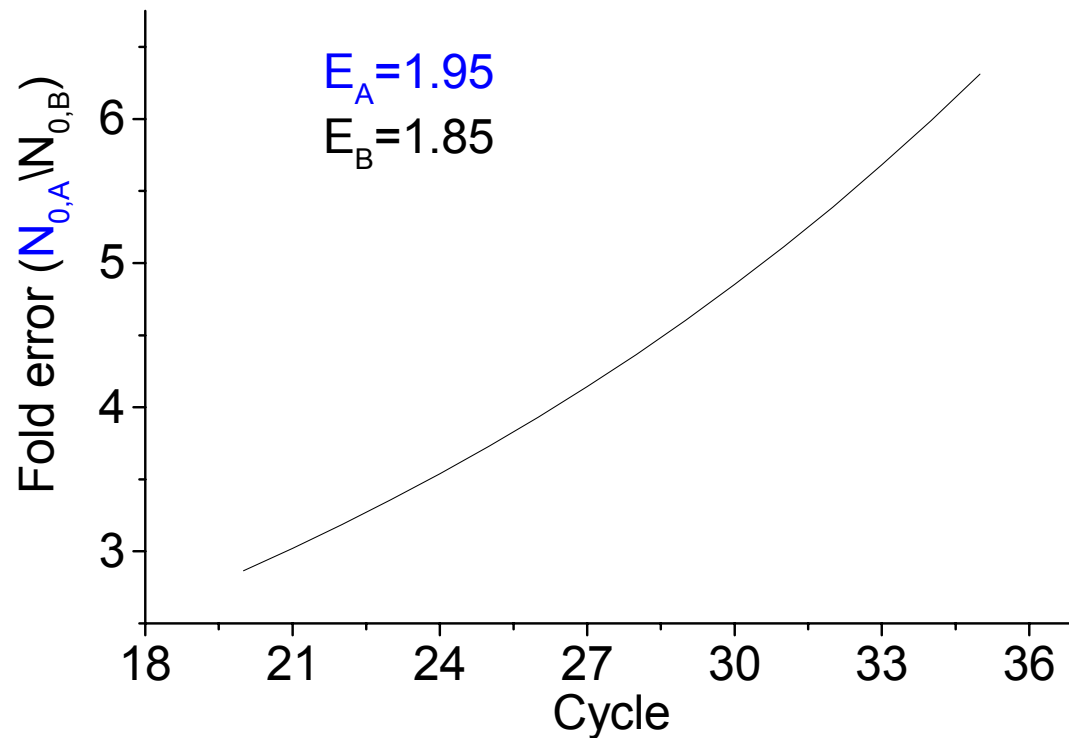
# What if the efficiencies are **NOT** similar?

Equal initial copy number  
Different PCR efficiency



# Estimation of error due to efficiency dissimilarity

constant CT, different efficiencies





# Do reference genes compensate for different efficiencies between samples?

- Not always
- Target and reference gene might have different amplification efficiencies  $E_{\text{target gene}} \neq E_{\text{ref. gene}}$
- Difference in  $E$  between target and reference gene ( $\Delta E$ ) doesn't stay constant but differs from sample to sample – interaction.

$$\Delta E = E_{\text{target gene}} - E_{\text{ref. Gene}}$$

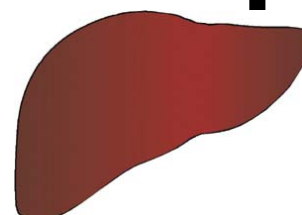
$$\Delta E_{\text{sample 1}} \neq \Delta E_{\text{sample 2}}$$

- i.e. **Interaction** *Sample\*Sequence*

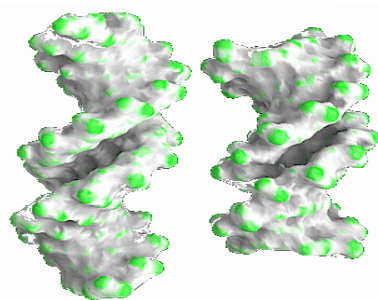
# Interaction Sample\*Sequence



SAMPLE 1

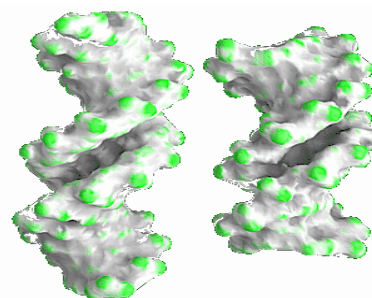


SAMPLE 2



Target

Reference



Target

Reference

n=10	n=10	n=10	n=10
E= 1.85 > E=1.79 <b><math>\Delta E=0.06</math></b>		E=1.80 < E=1.81 <b><math>\Delta E=-0.01</math></b>	



*versus*



*versus*



*versus*



## MINIREVIEW

### Inhibition and Facilitation of Nucleic Acid Amplification

IAN G. WILSON\*

*Northern Ireland Public Health Laboratory, Bacteriology Department, Belfast City Hospital, Belfast BT9 7AD,  
United Kingdom*

Factors that inhibit the amplification of nucleic acids by PCR are present with target DNAs from many sources. The inhib-

sensitivity, specificity, and reproducibility have been reported (16, 82, 86, 120, 122, 124). These may also be potentially

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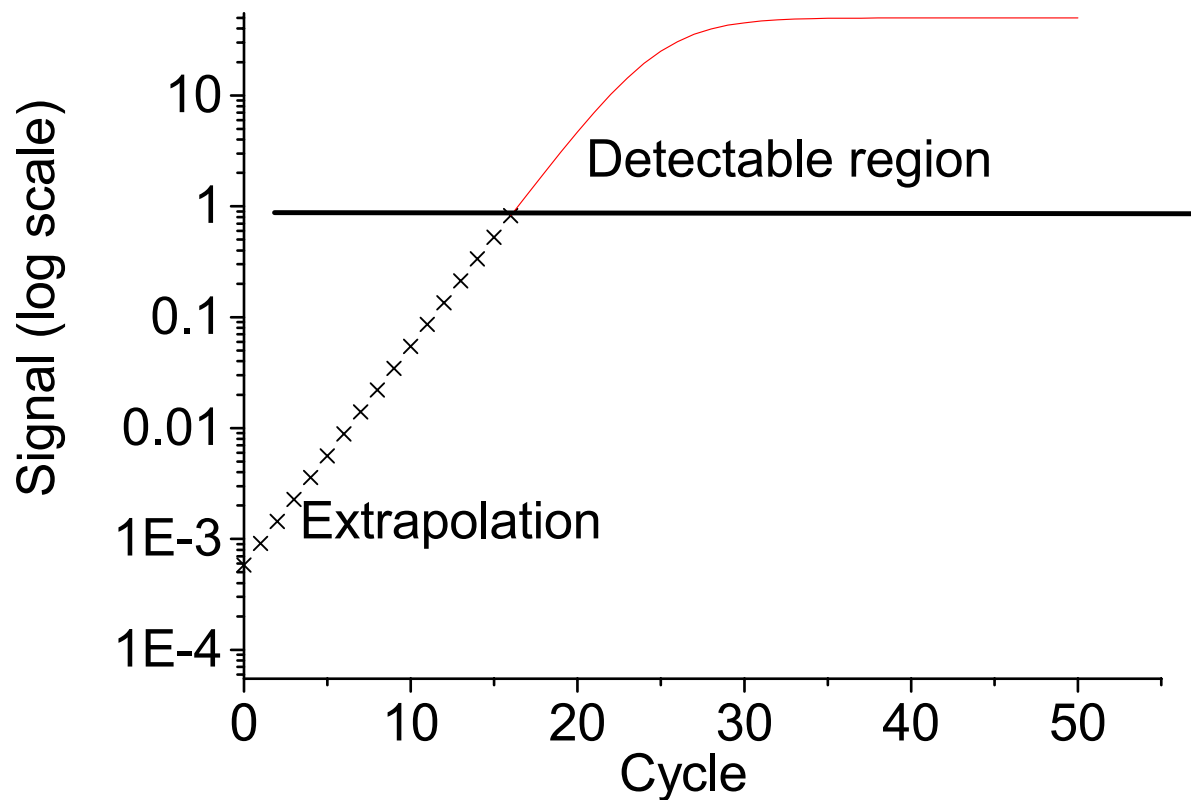
MINIREVIEW 3751

- 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.
139. Yoon, C., R. L. Burghoff, L. G. Keim, V. Minak-Bernero, J. R. Lute, and D. A. Glawe. 1993. Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. *Appl. Environ. Microbiol.* **59**:1972-1974.



# The ideal solution: quantification by sample specific amplification



The signal from the detectable region is extrapolated and used to draw conclusions about the initial amount of DNA.



# Efficiency estimation methods:

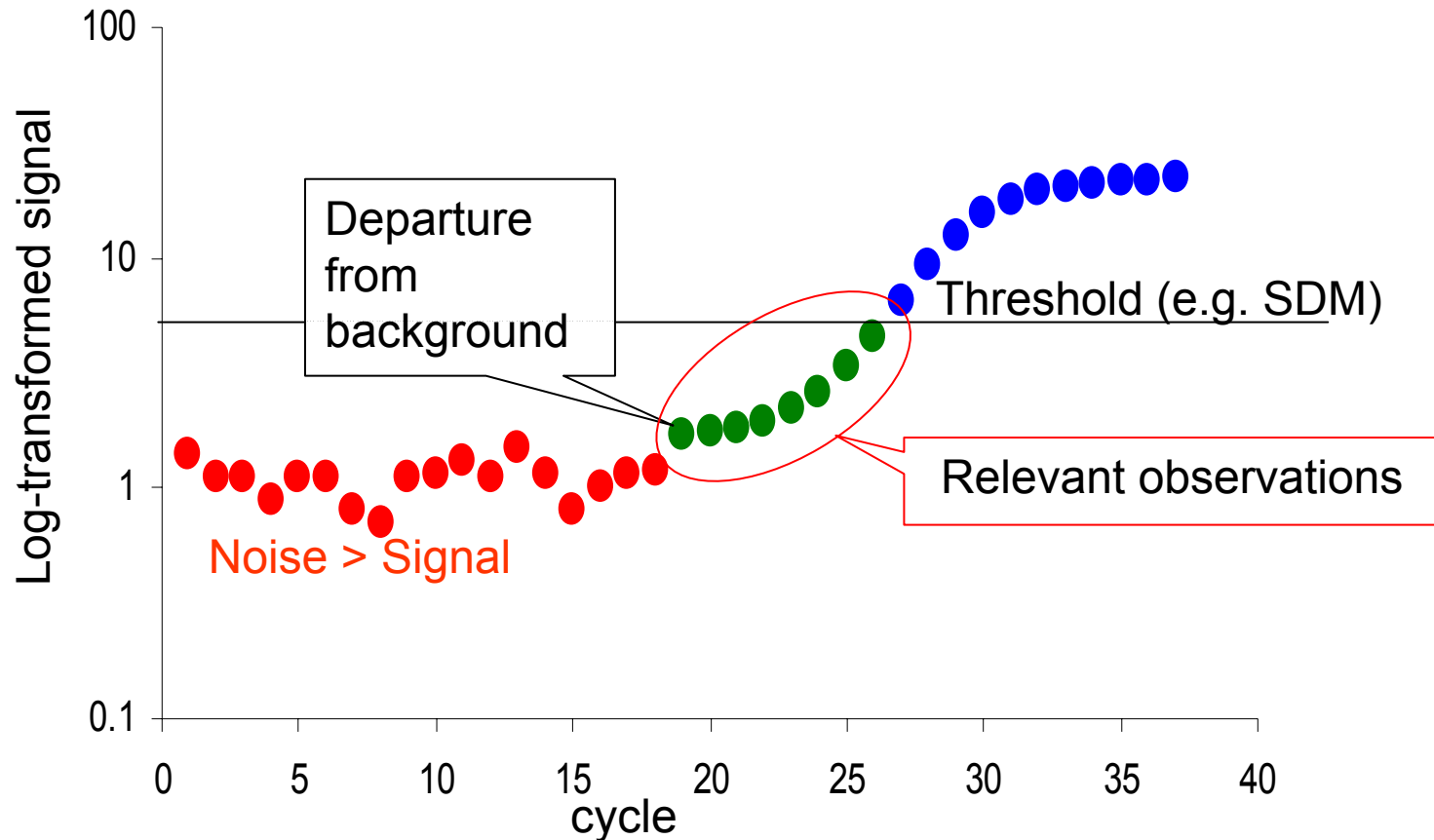
- Bar T & Muszta A 2005 BioTechniques 39: 333-340
- Liu W & Saint DA 2002a Anal Biochem 302
- Liu W & Saint DA 2002b Biochem Biophys Res Commun 294
- Marino JH et al. 2003 J Immunol Methods 283
- Peccoud J & Jacob C 1998 In Gene quantification (ed. F. Ferre)
- Peirson SN et al. 2003 Nucleic Acids Res 31
- Ramakers C et al. 2003 Neurosci Lett 339
- Rutledge RG 2004 Nucleic Acids Res 32
- Tichopad A et al. 2003 Nucleic Acids Res 31
- Wilhelm JA et al. 2003 Biotechniques 34



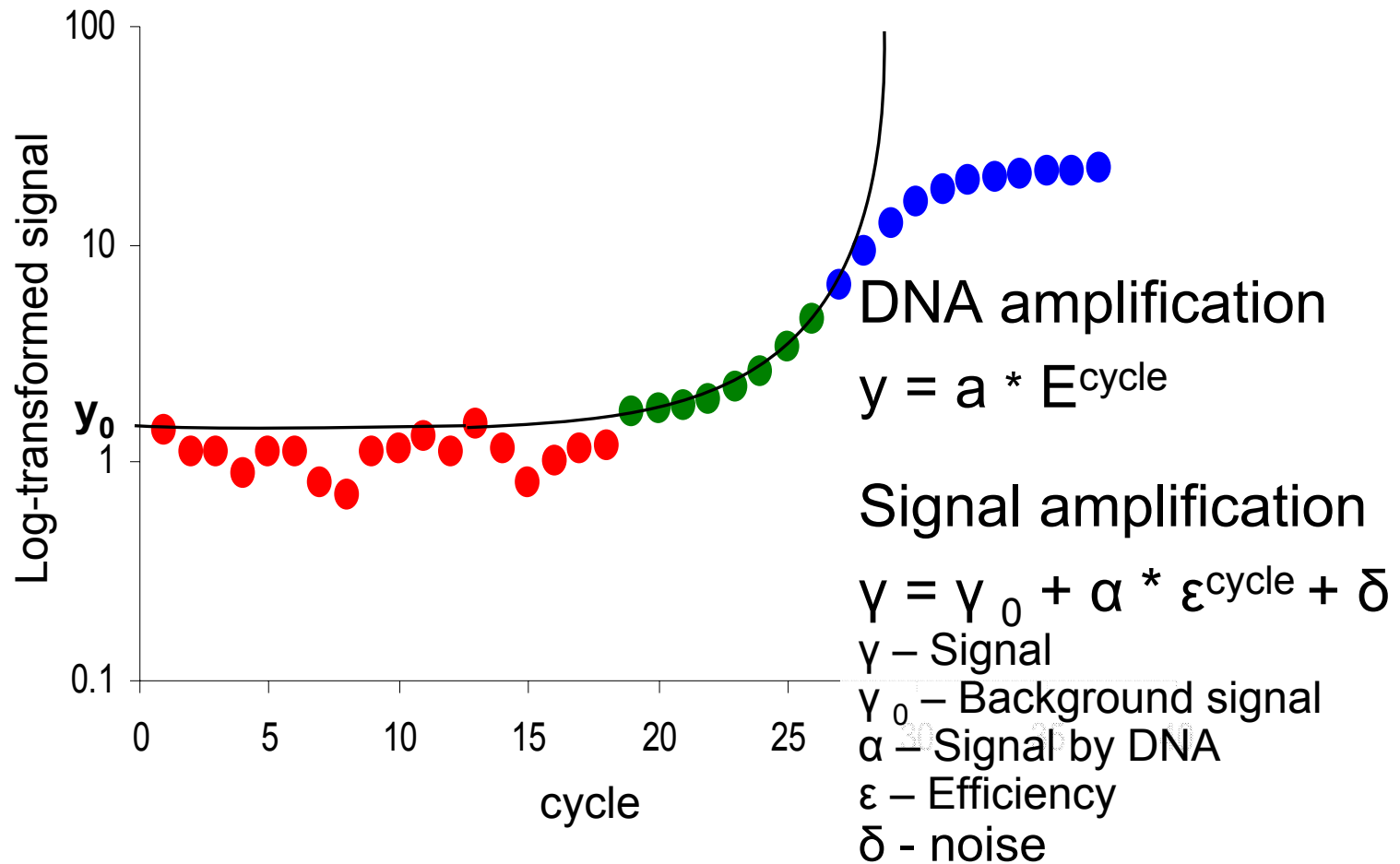
# Where is the problem in efficiency estimation?

- Early signal is hidden in the noise
- Modeling the background signal
- Where on the amplification curve the efficiency should be estimated?
- Variable Signal / DNA proportion

# Where to estimate efficiency by exponential model?

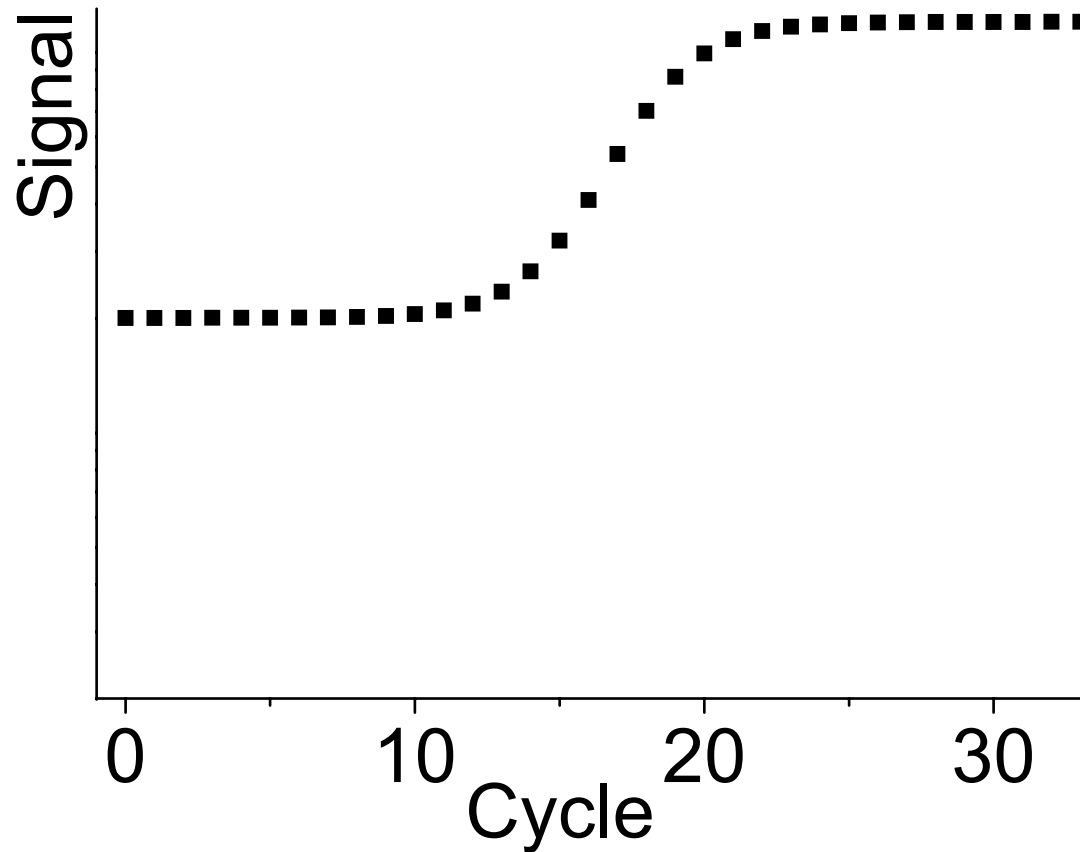


# What model to fit





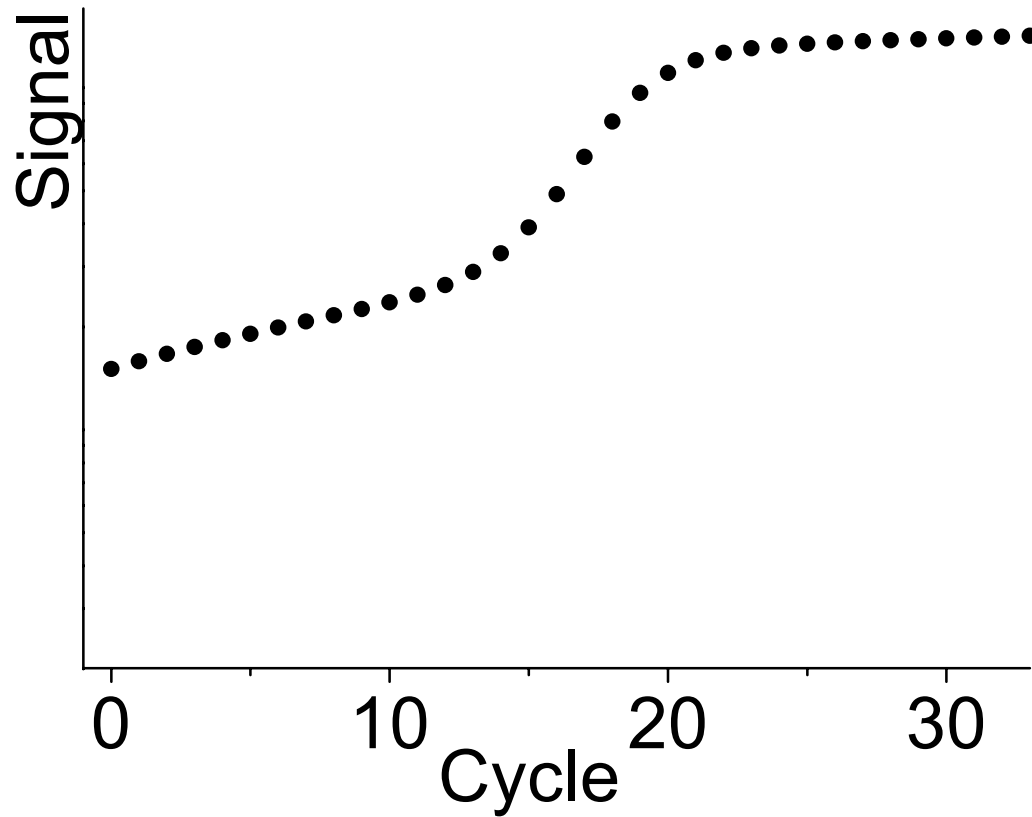
# Constant background

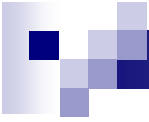




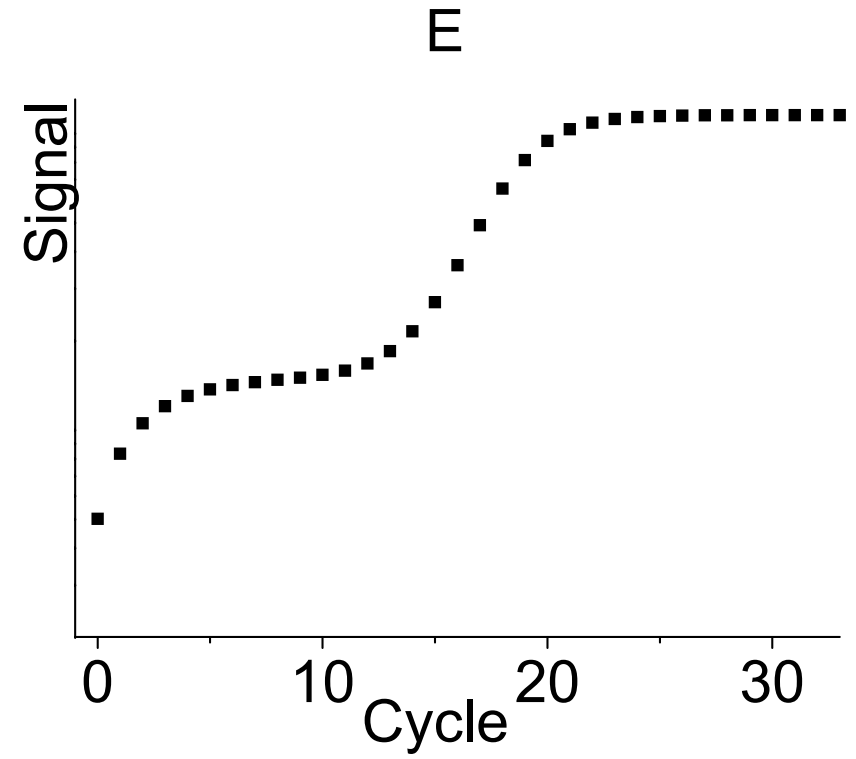
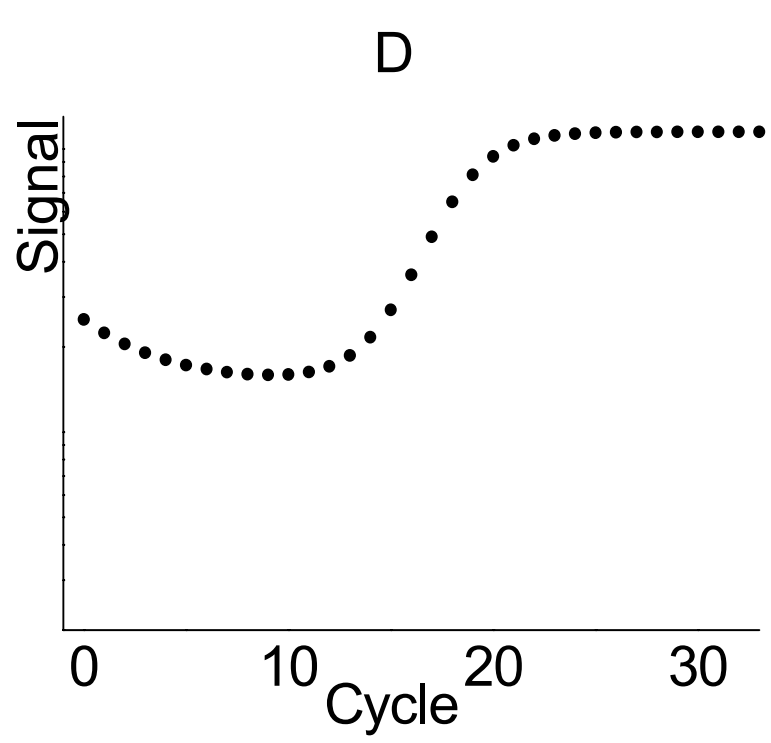
# Linear background

C



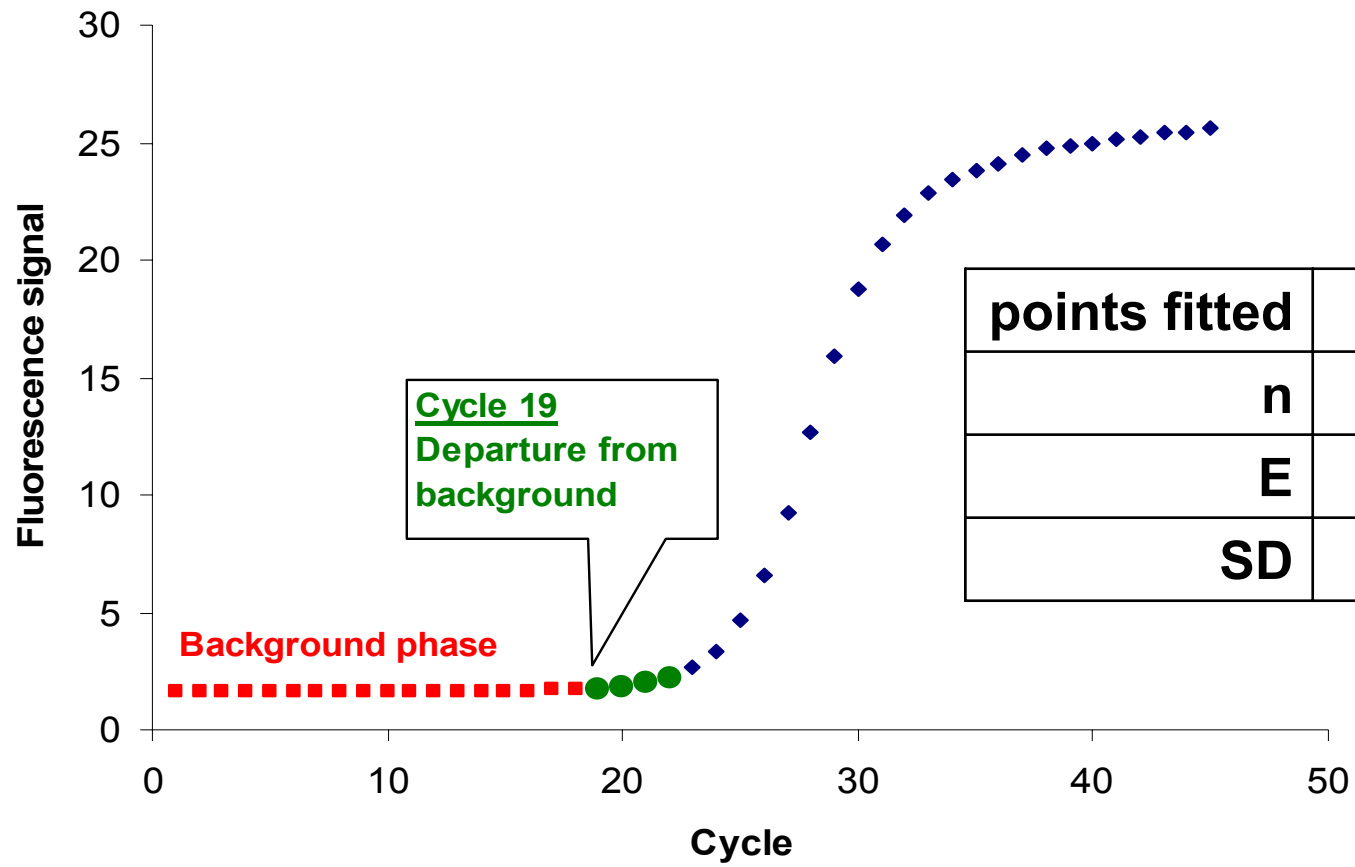


# Non-linear background

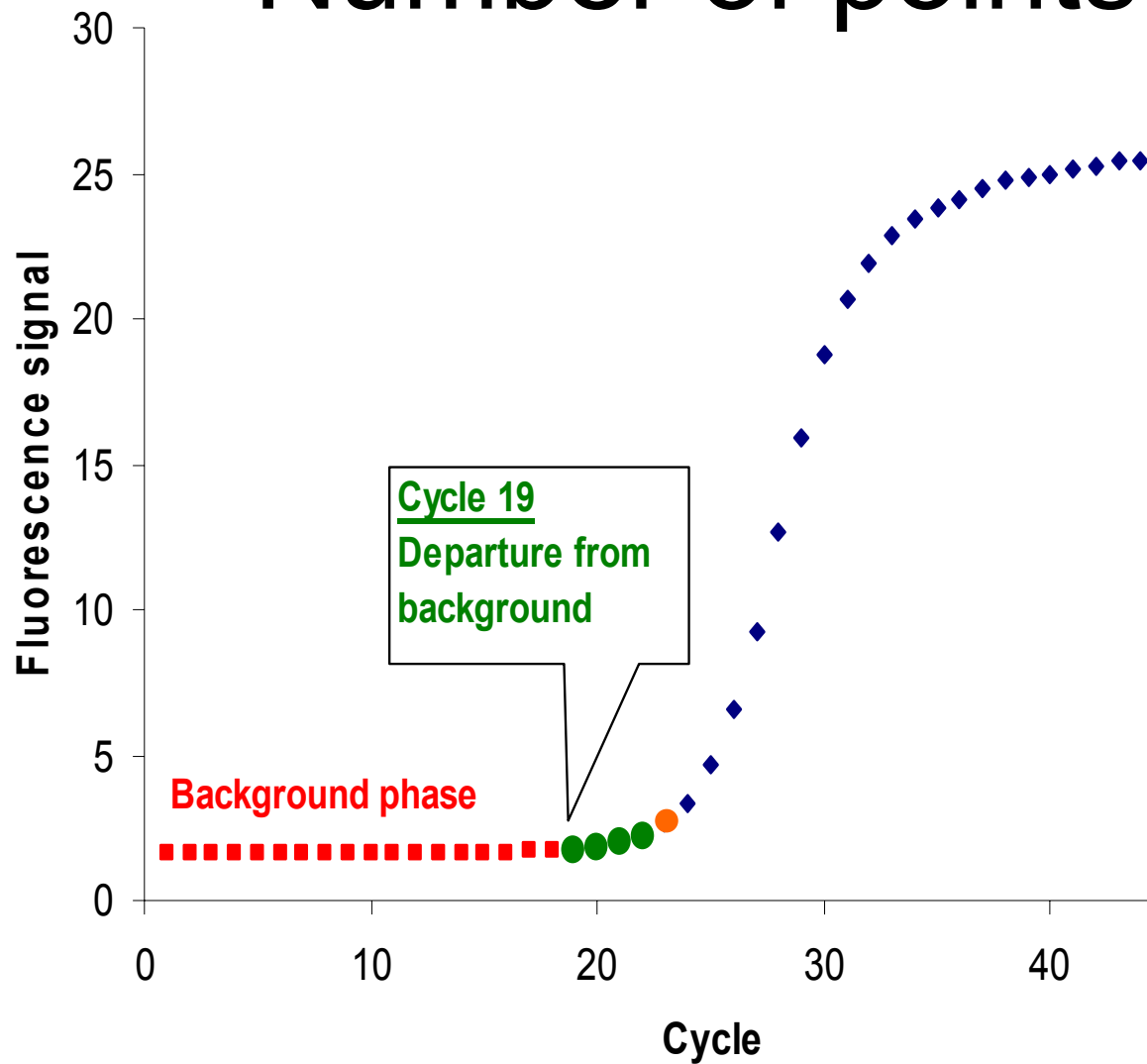


# Where to fit?

Number of points fitted



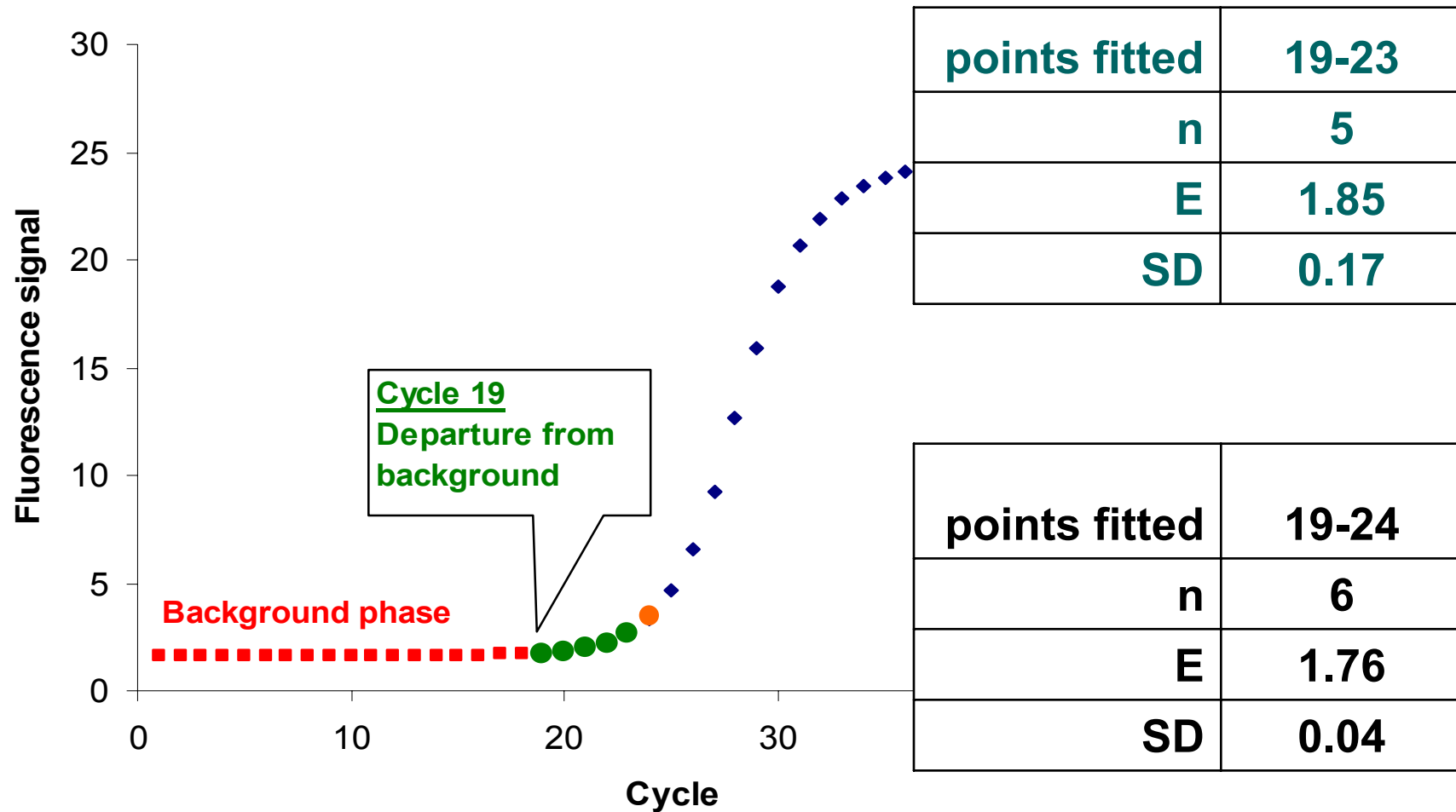
# Number of points fitted



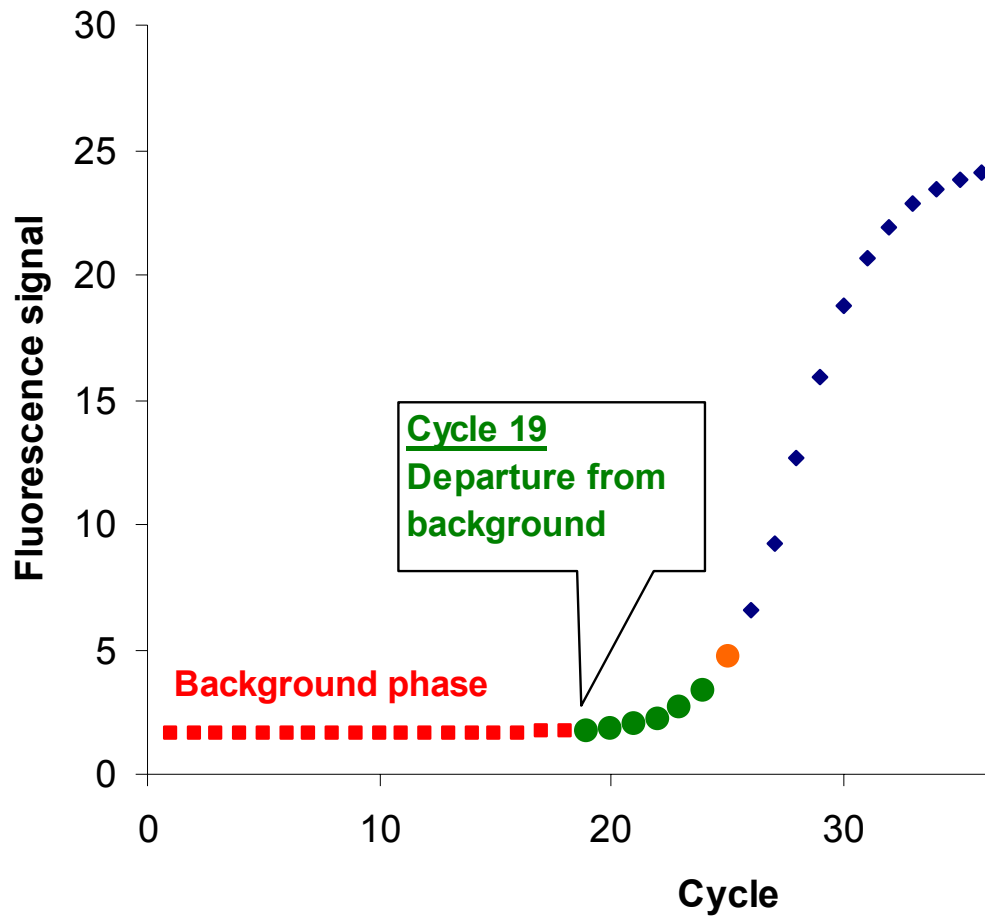
points fitted	19-22
n	4
E	1.86
SD	0.28

points fitted	19-23
n	5
E	1.85
SD	0.17

# Number of points fitted



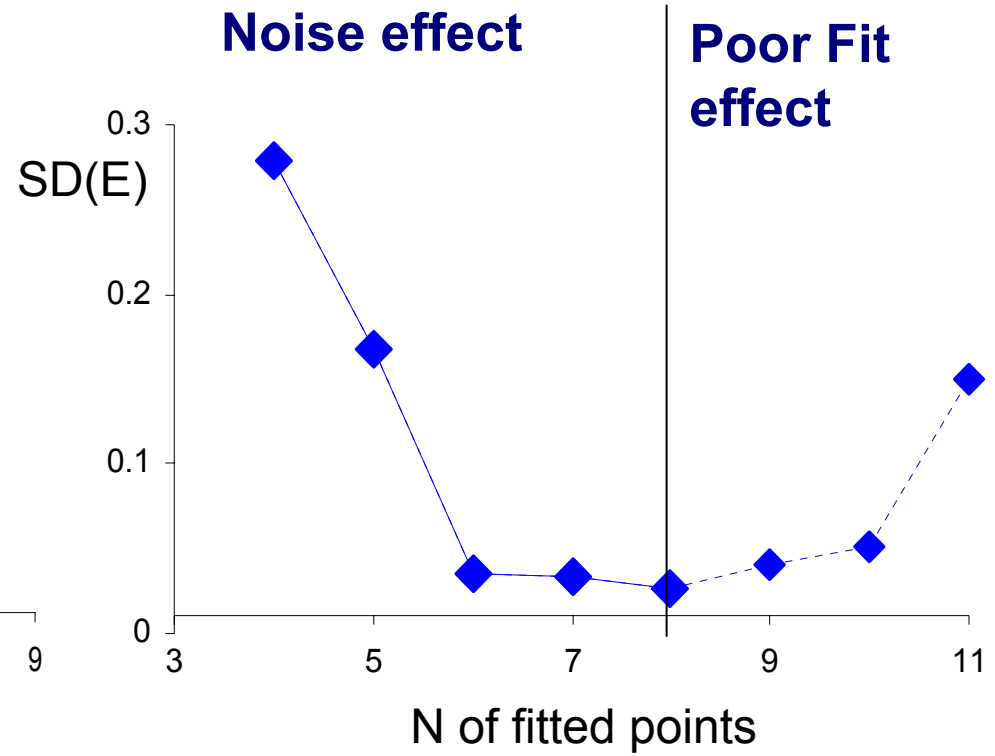
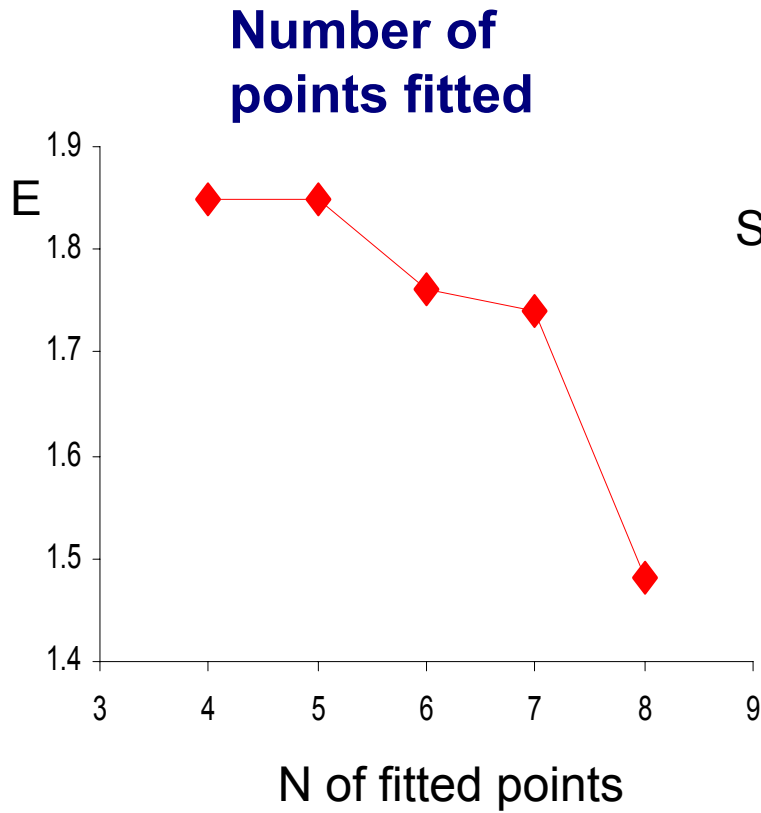
# Number of points fitted



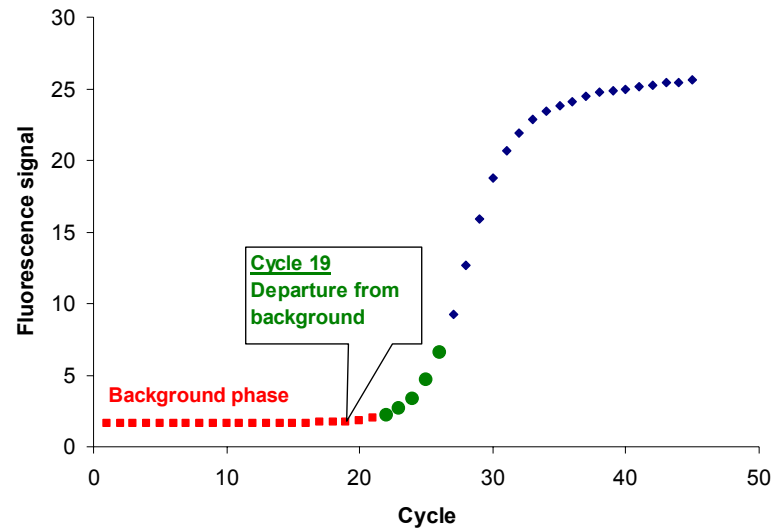
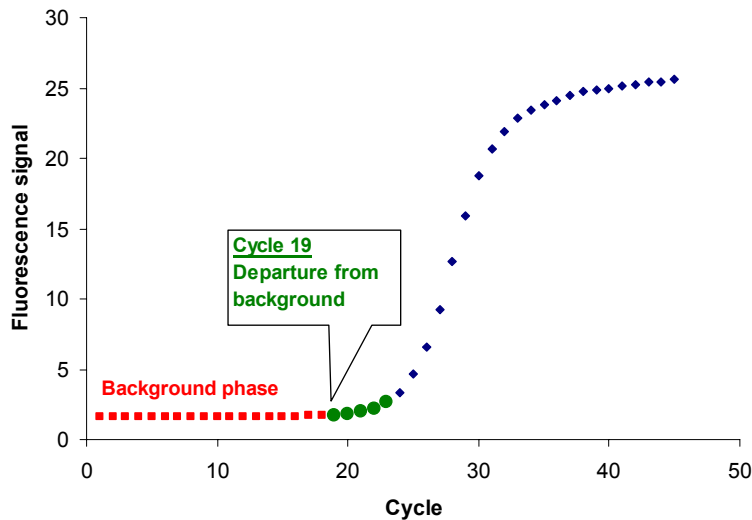
<b>points fitted</b>	<b>19-24</b>
<b>n</b>	<b>6</b>
<b>E</b>	<b>1.76</b>
<b>SD</b>	<b>0.036</b>

<b>points fitted</b>	<b>19-25</b>
<b>n</b>	<b>7</b>
<b>E</b>	<b>1.74</b>
<b>SD</b>	<b>0.033</b>

# Where to fit?



# Where to fit? Effect of noise



points fitted	19-23
n	5
E	1.85
SD	0.165

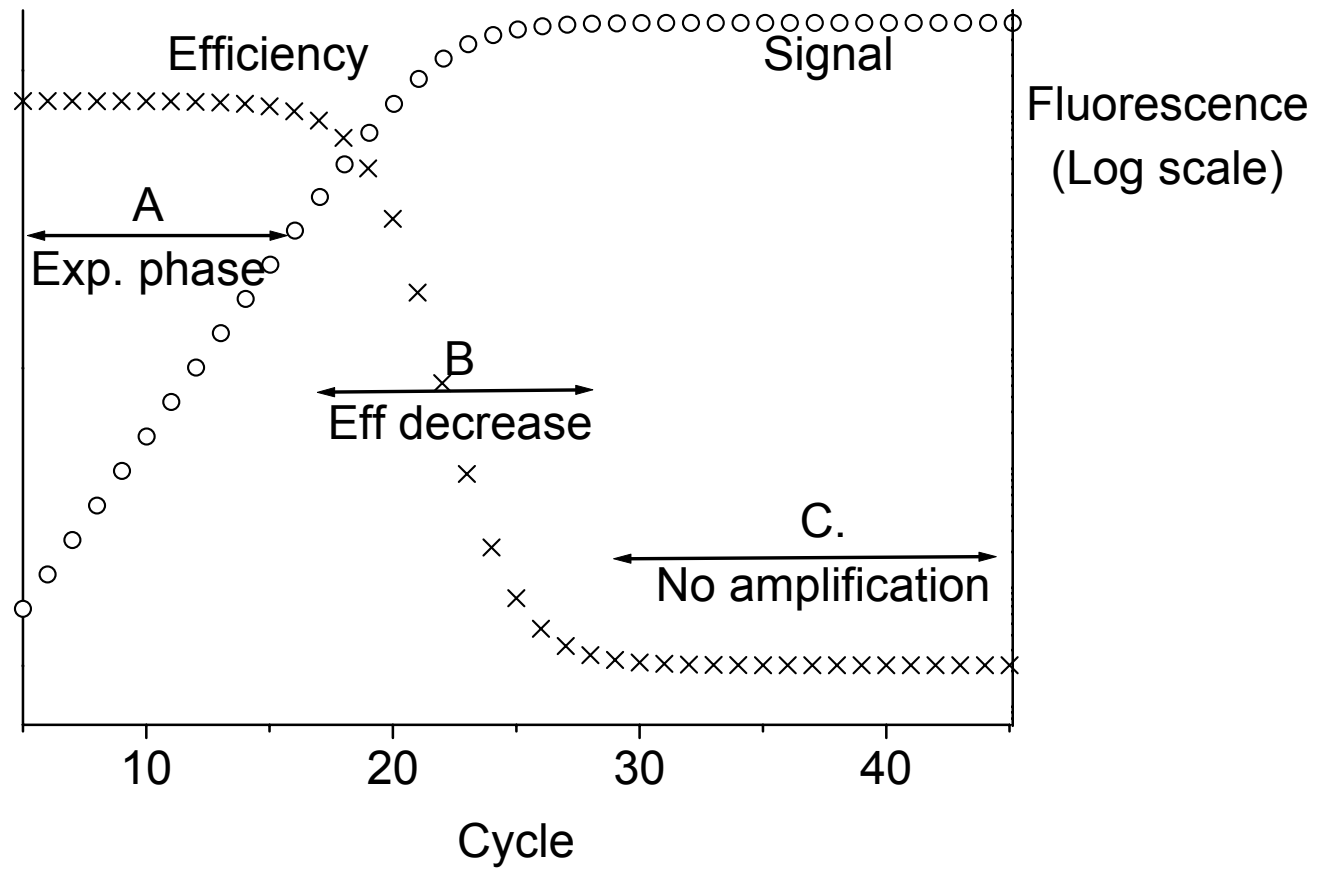
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points fitted	22-26
n	5
E	1.70
SD	0.027

# PCR kinetics

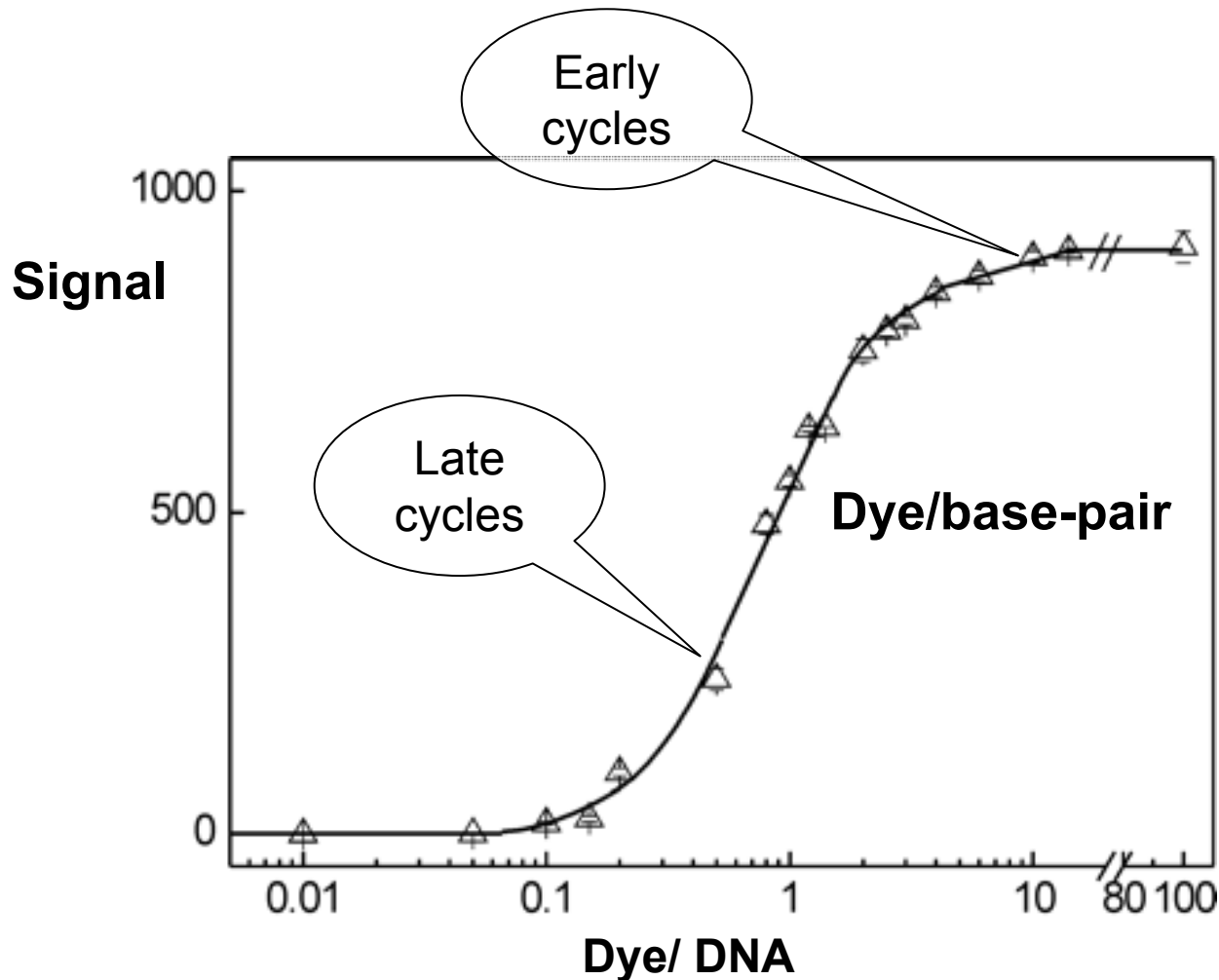





# Summary

- Efficiency decreases as the reaction progresses.
- Is PCR exponential or logistic process ?
- How to evaluate amplification efficiency in early cycles?

# SYBR Green Signal might not be proportional to DNA amount



...And might thus cause inaccurate efficiency estimation.




# Do the efficiency estimation methods deliver their promise?

Is the estimated efficiency precise enough?

How close is the estimated efficiency to the efficiency calculated from the standard curve?

What can we do with the estimated efficiency?



# Comparison of different efficiency estimation methods

- 53 standard curves, 8-21 samples each (mean=14.4, median=15)
- Total: 762 samples
- $R^2 > 0.99$
- Minimal span of dilution - 256 fold
- Most common span of dilution –  $10^4$  or larger



# Compared methods

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

# Precision

## Median SD(E)

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

\*Most precise efficiency estimation method on this machine  
Machine producing the most precise eff for a given method

<sup>1</sup>Tichopad et al., NAR **31**(20): e122

<sup>2</sup>Ramakers et al., *Neurosci. Lett.*, **339**, 62–66

<sup>3</sup>Peirson et al., NAR **31**(14): e73

<sup>4</sup>Wilhelm et al., *BioTechniques* **34**, 324-32

<sup>5</sup>Liu & Saint, *Biochem. Biophys. Res. Commun.* **294**(2), 347-53

# Closeness to standard curve value

## Distance from standard curve value

	Stand <sup>1</sup>	LinReg <sup>2</sup>	DART <sup>3</sup>	SoFAR <sup>4</sup>	4P <sup>5</sup>
Rotogene	-0.47	-0.26	-0.24*	-0.31	-0.33
LightCycler	-0.3	<u>-0.08</u>	<u>-0.06</u>	<u>-0.06*</u>	-0.26
iCycler	<u>-0.27</u>	-0.11	-0.11	-0.12	<u>0.03*</u>
Average	-0.33	-0.15	-0.14*	-0.17	-0.16

\*Closest efficiency estimation method on this machine

Machine producing the closest efficiency for a given eff estimation method

<sup>1</sup>Tichopad et al., NAR **31**(20): e122

<sup>2</sup>Ramakers et al., *Neurosci. Lett.*, **339**, 62–66

<sup>3</sup>Peirson et al., NAR **31**(14): e73

<sup>4</sup>Wilhelm et al., *BioTechniques* **34**, 324-32

<sup>5</sup>Liu & Saint, *Biochem. Biophys. Res. Commun.* **294**(2), 347-53

# Application 1: Quantification

$N_0$  = No. of molecules at cycle 0

$N_{CT}$  = No. of molecules at threshold =  $10^9$

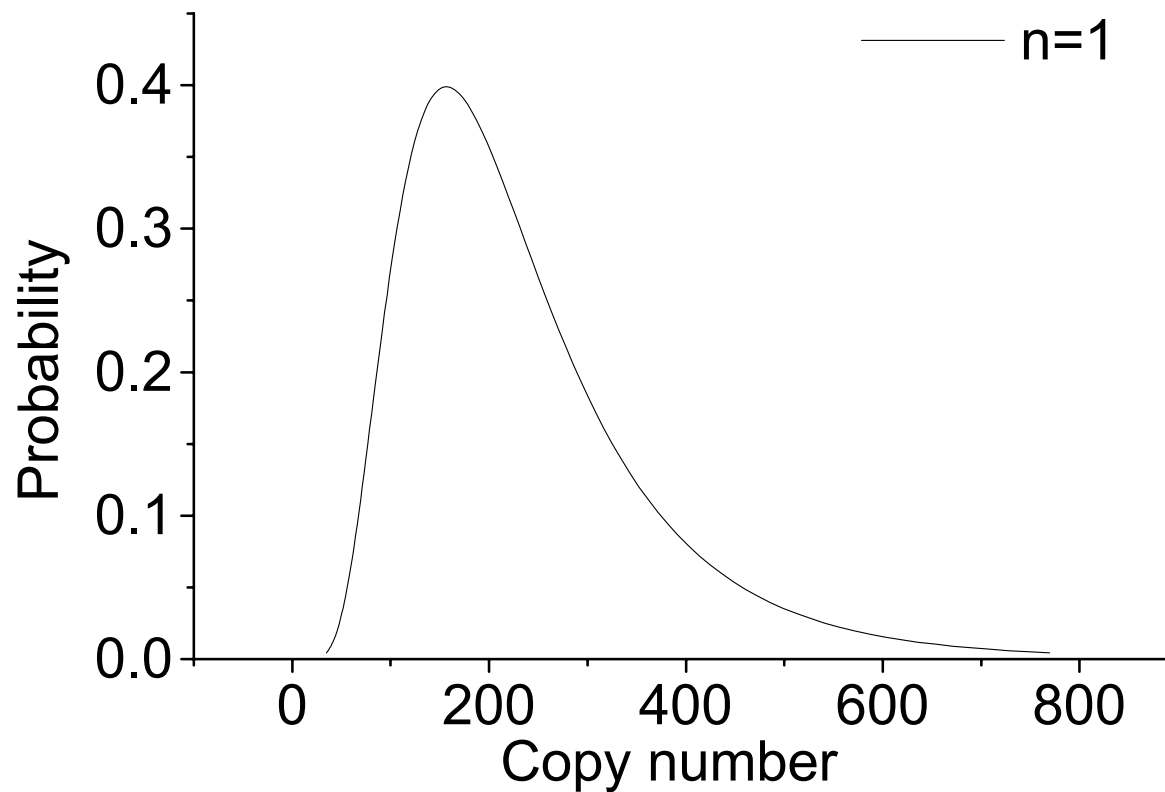
SD = Standard Deviation

$$N_0 \equiv \frac{N_{CT} N_{CT}}{\left( \left( 1 + \frac{E}{3} \right)^{CT} * SD(E) \right)^{CT}}$$

$$0 \leq E \leq 1$$

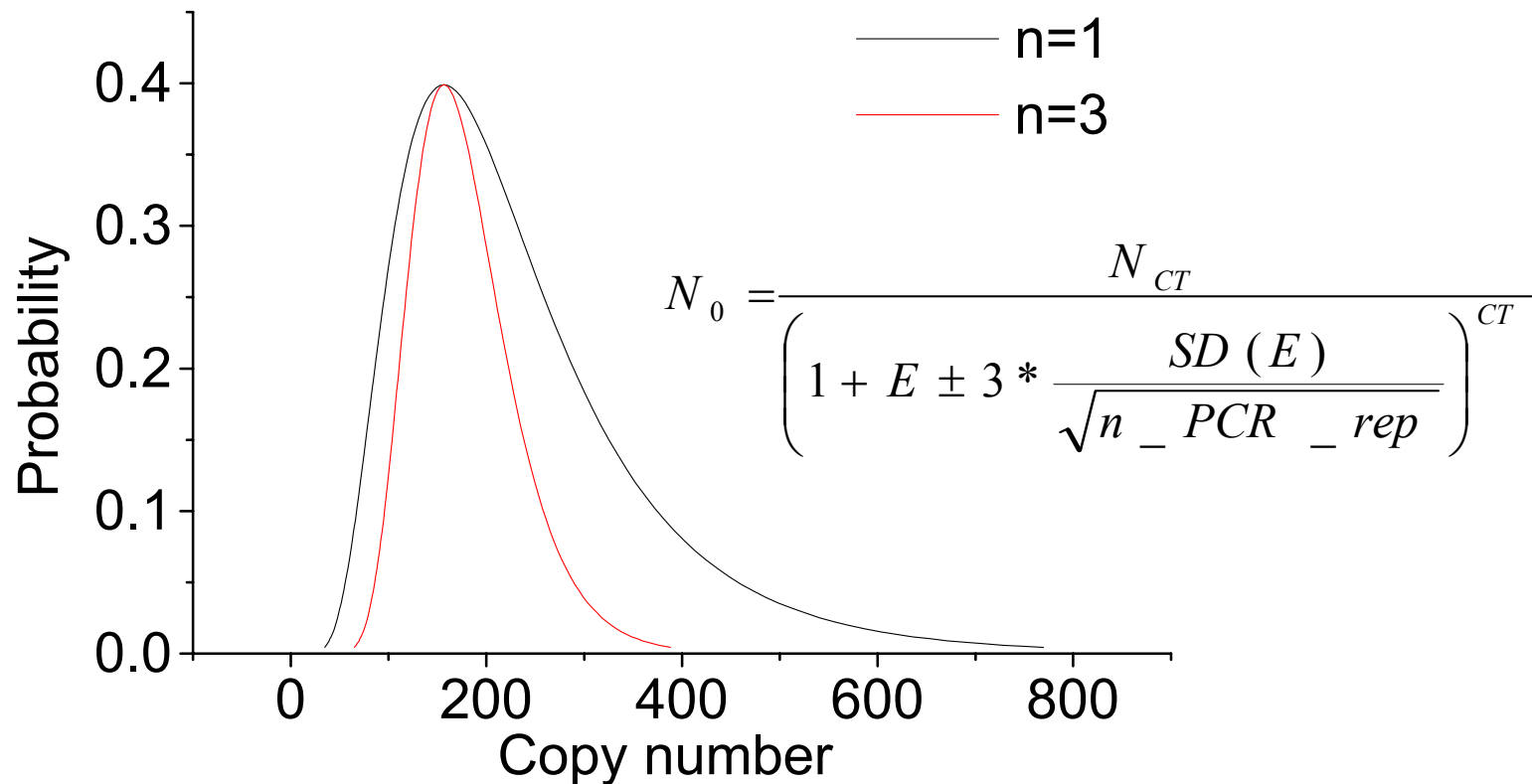
# $N_0$ Distribution

CT=28,  $N_{CT}=10^9$ ,  $E=0.95$ ,  $SD(E)=0.035$



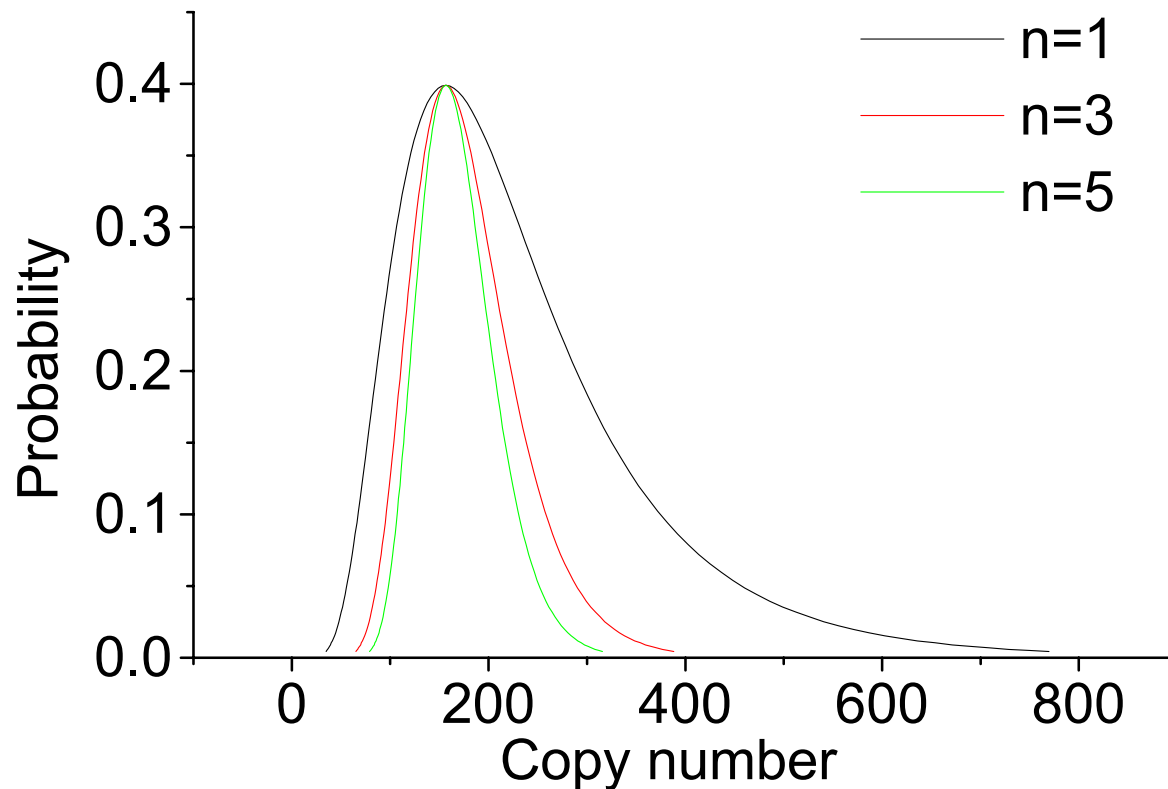
# $N_0$ Distribution

CT=28,  $N_{CT}=10^9$ ,  $E=0.95$ ,  $SD(E)=0.035$



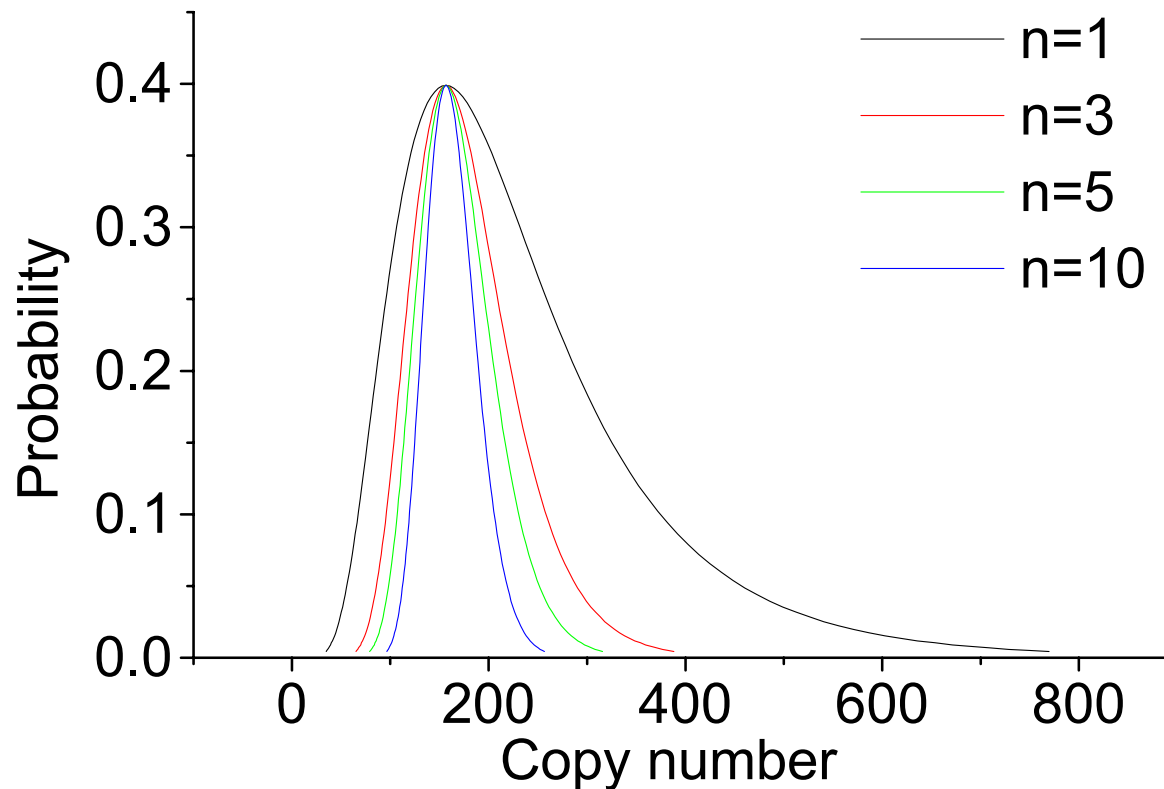
# $N_0$ Distribution


CT=28,  $N_{CT}=10^9$ ,  $E=0.95$ ,  $SD(E)=0.035$



# $N_0$ Distribution

CT=28,  $N_{CT}=10^9$ ,  $E=0.95$ ,  $SD(E)=0.035$





Precise quantification  
based on sample specific  
efficiency requires at least  
3 PCR replicates



# Replacement of standard curve value

- Gentle et al. 2001
- Peirson et al. 2003
- Tichopad et al. 2003
- Peyton et al. 2004

# Replacement of standard curve value

Distance from standard curve value

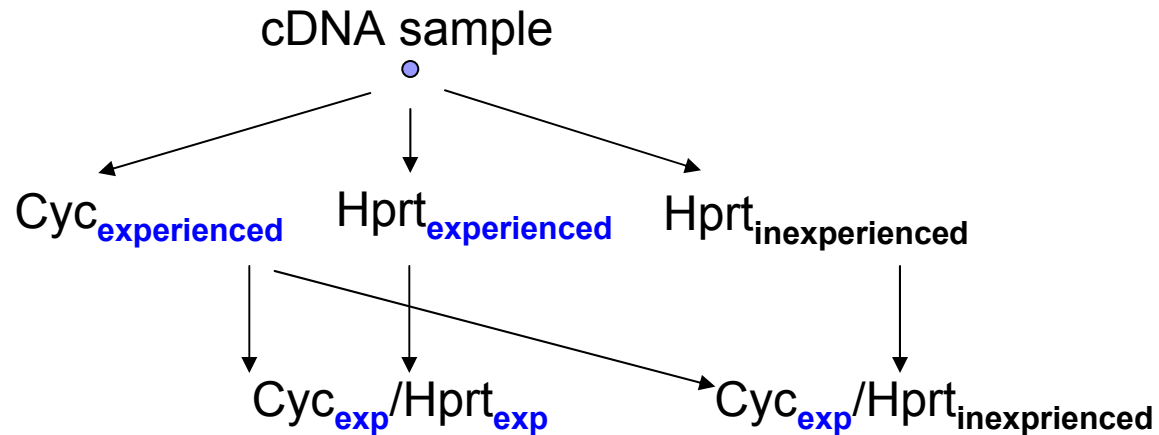
	Stand <sup>1</sup>	LinReg <sup>2</sup>	DART <sup>3</sup>	SoFAR <sup>4</sup>	4P <sup>5</sup>
Average	-0.33	-0.15	-0.14*	-0.17	-0.16

$$\text{Ratio} = \frac{E_{\text{target}}^{\Delta CT_{\text{target}} (\text{control-test})}}{E_{\text{reference}}^{\Delta CT_{\text{reference}} (\text{control-test})}}$$

**Using inaccurate efficiency affects the accuracy of quantification.**

# Kinetics Quality Assessment: Experimental design

15 sets of 5 replicate animals  
(total of 75 cDNA samples)



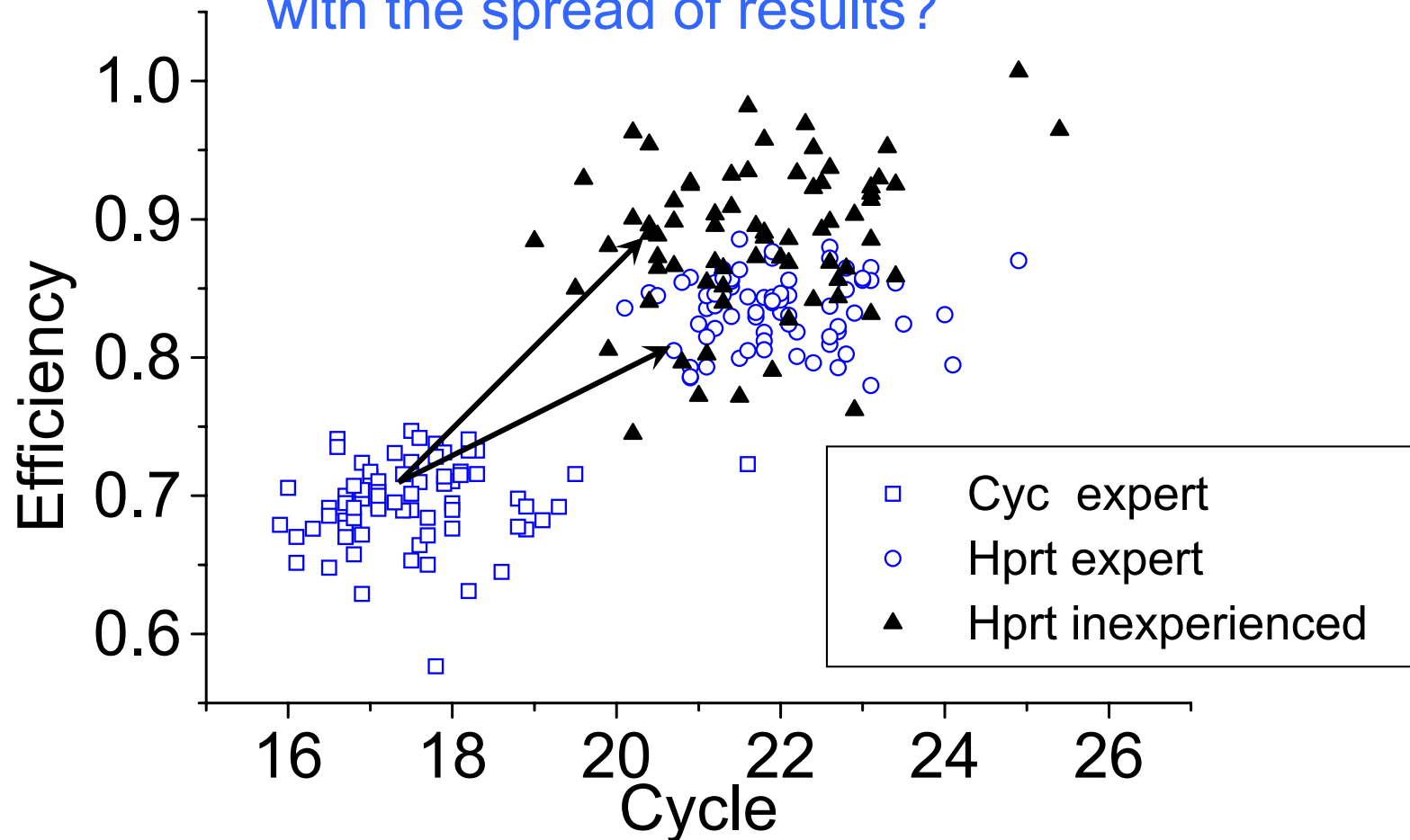
Spread of results

15 sets of 5 + 15 set of 5 = 30 sets of 5


\*Bar and Muszta, Biotechniques, 39(3), 2005, 333-340

# Efficiency spread of samples

Will the spread of efficiencies be associated with the spread of results?







# Do low quality sets contain more outliers?

% of outliers in <b>high quality</b> sets (similar efficiencies)	% of outliers in <b>low quality</b> sets (dissimilar efficiencies)
4.9% (*16%)	38% (*43%)

\*In parenthesis – results obtained with average efficiency of the standard curve



# Conclusions:

Sample specific efficiencies may be used:

1. For quantification, but need adjustment and use of replicates.
2. To replace standard curve efficiency after adjustment.
3. For kinetics QA.



# Acknowledgments:

- Stuart Peirson for help with DART
- Jochen Wilhelm for help with SoFAR
- Mikael Kubista and Michael Pfaffl for insightful discussions and comments



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