

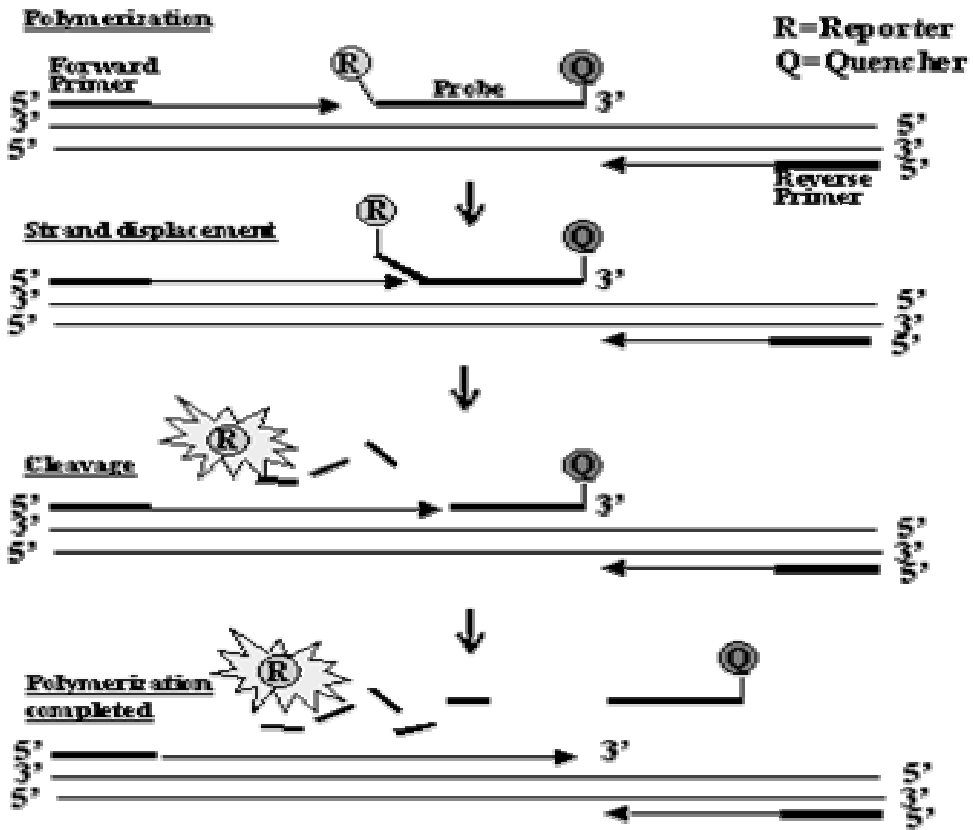
Real Time PCR

A useful new approach?

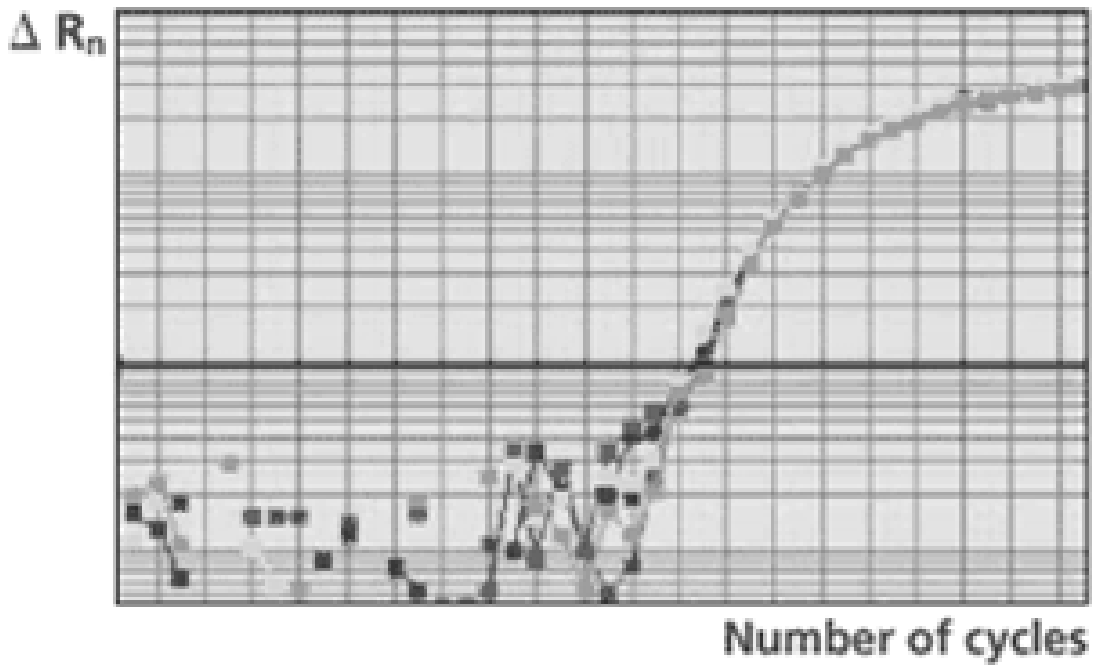
Statistical Problems?

Reverse transcription followed by Polymerase Chain Reaction

- Considered to be the most sensitive method for the detection and quantification of gene expression levels.
- Used as a follow-up when a particular gene is suggested in micro-array studies.
- Potential problems with sensitivity, specificity and reproducibility.



Fluorescence trajectory



Plot of sigmoid fluorescence trajectory

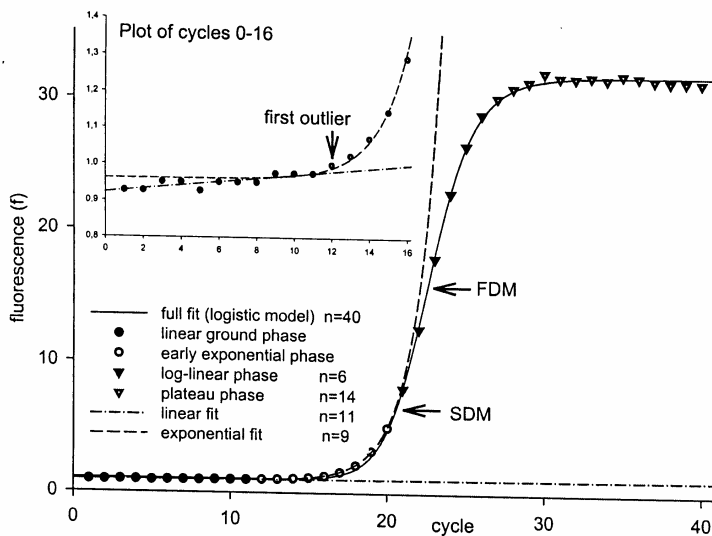


Figure 1. Plot of fluorescence observations from LightCycler (Roche Diagnostics). Forty observations give a sigmoid trajectory that can be described by a full data fit (FPLM). The ground phase can be linearly regressed (inlay). The following data of $n > 7$ are considered to behave exponentially and can be fitted using an exponential model. Various model fits are described in the legend within the figure. FDM and SDM denote the position of the FDM and SDM within the full data fit.

- Accumulation of fluorescence is proportional to the accumulation of amplification products.
- $C_n = C_0 (E)^n = k R_n = k R_0 (E)^n$
where C_0 is the initial concentration
 C_n is the concentration at cycle n ,
 E is the amplification efficiency,
and R_0 and R_n are equivalent
measures of fluorescence.

- The normal practice is to record the cycle number where the fluorescence rises appreciably above the background fluorescence.
- The commonly used value (CP) is the second derivative maximum value (SDM). This is measured in triplicate for each sample.

Absolute versus Relative Measurement

- In principle we can produce an absolute measurement by use of an external standard.
- However there are various practical difficulties with this and it is much easier to compare the concentration in a test sample against a control. Then the proportionality constant cancels out .

Expression ratio

- Expression ratio = $\frac{C_{0\text{test}}}{C_{0\text{cont}}}$
= $E^{(CP_{\text{cont}} - CP_{\text{test}})}$
- The CP values are averages of the triplicate readings.
- As all genes might change expression in the test sample, the expression ratio is usually calculated for the target gene relative to a reference gene.
- i.e. Relative Exp. Ratio = F
= Target Exp. Ratio/ Ref. Exp. Ratio.
(Pfaffl et al, 2002)

Reference Genes

- Initially housekeeping genes were recommended, e.g. GAPDH, albumin, actin, etc.
- However a recent study (Radonic et al, 2003) has suggested that a transcription-related gene RPII is a useful general reference gene but that using several reference genes is desirable.

Amplification Efficiency

- E is a value between 1 (no amplification) and 2 (complete amplification). There is evidence that E varies between genes, experimental conditions, etc, necessitating constant estimation in each situation.
- Initially E was estimated by assaying serial dilutions of a gene sample and regressing mean CP against $\log_{10}\text{Conc.}$

Accuracy of estimated E

- Even when the correlation is close to -1 and the R^2 value close to 100%, it is important to calculate a standard error for the estimated amplification efficiency, E.
- This can easily be done using a Taylor's series approximation.

Given that Beta hat is the estimated slope

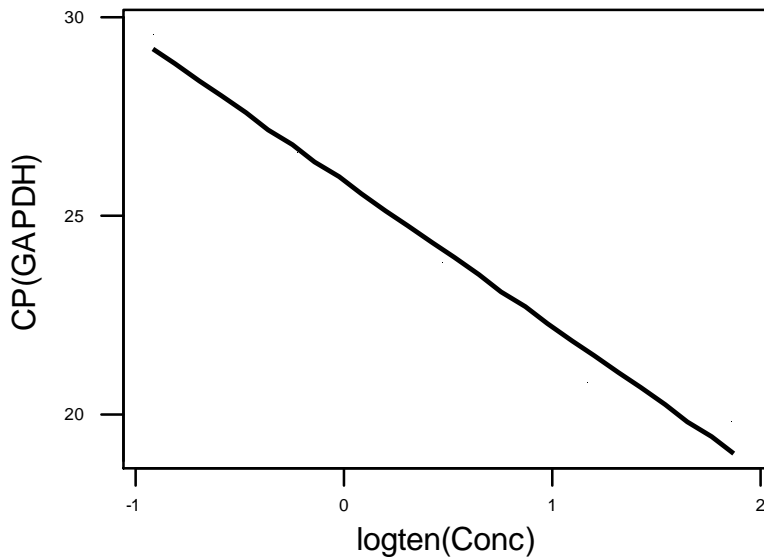
$$\hat{E} = 10^{-1/\hat{b}}$$

$$S.E.(\hat{E}) \cong \frac{(\hat{E} \log_e 10) S.E.(\hat{b})}{\hat{b}^2}$$

Regression Plot

$$CP(\text{GAPDH}) = 25.8691 - 3.63277 \log_{10}(\text{Conc})$$

S = 0.687476 R-Sq = 97.8 % R-Sq(adj) = 97.1 %



- Standard error of estimated slope = 0.3110
- Estimated E = 1.8848
- Standard error of estimated E = 0.1023

Alternative Method

- E can also be estimated by regressing $\log_{10}(\text{fluorescence} - \text{background})$ against cycle number for the data in the exponential phase.
- There are methods for choosing which points are in the exponential phase (Tichopad et al, 2003)
- The estimated slope is minus the estimated slope from the previous method and the formula for the standard error is unchanged.
- The two methods seem to give very similar estimates for E.

Sources of Error

- In order to calculate the standard error of the relative expression ratio, F , we must allow for variability in the four CP values and two E values.
- Any between run variability can be ignored because we are looking at differences between test and control.

Again using Taylor's Series

$$\begin{aligned} S.E.(\hat{\mathbf{j}}) = & \mathbf{j} \left\{ \frac{(CP_{\text{target,cont}} - CP_{\text{target,test}})^2}{\hat{E}_{\text{target}}^2} SE^2(\hat{E}_{\text{target}}) \right. \\ & + \frac{(CP_{\text{ref,cont}} - CP_{\text{ref,test}})^2}{\hat{E}_{\text{ref}}^2} SE^2(\hat{E}_{\text{ref}}) \\ & + (\log_e E_{\text{target}})^2 (SE^2(CP_{\text{target,cont}}) + SE^2(CP_{\text{target,test}})) \\ & \left. + (\log_e E_{\text{ref}})^2 (SE^2(CP_{\text{ref,cont}}) + SE^2(CP_{\text{ref,test}})) \right\}^{0.5} \end{aligned}$$

Illustrative Example

- Let us take a case of down-regulation where we look at $1/F$. The formula for the standard error is as above but with F replaced by $1/F$.
- $CP_{\text{target,test}} = 32.61$; $CP_{\text{target,control}} = 25.88$;
- $CP_{\text{ref,test}} = 22.35$; $CP_{\text{ref,control}} = 22.53$;
- $E_{\text{target}} = 1.670$ and $E_{\text{ref}} = 1.885$.
- This gives $1/F = 1.12/0.032 = 35.35$.
- $SE(E_{\text{target}}) = 0.036$ and $SE(E_{\text{ref}}) = 0.102$

- If we take the standard errors of the CP means to be 0.2 which given the literature seems to be a fair estimate,
- then we find that the standard error of the estimate of $1/F$ is 9.64. Thus the sampling error on our estimate of 35.35 is large; Two standard errors being 19.28.

Potential ways to reduce variability

- If E only varies between genes and can be accurately determined as a reference this could reduce S.E. (E). Acceptable assumption?
- Taking more than three CP readings would reduce the S.E. (CP).
- Do we need to look relative to a reference gene?

Conclusion

- This seems potentially a very useful technique but it is important that a standard error is put on the expression ratio obtained and that efforts are made to reduce sampling error.