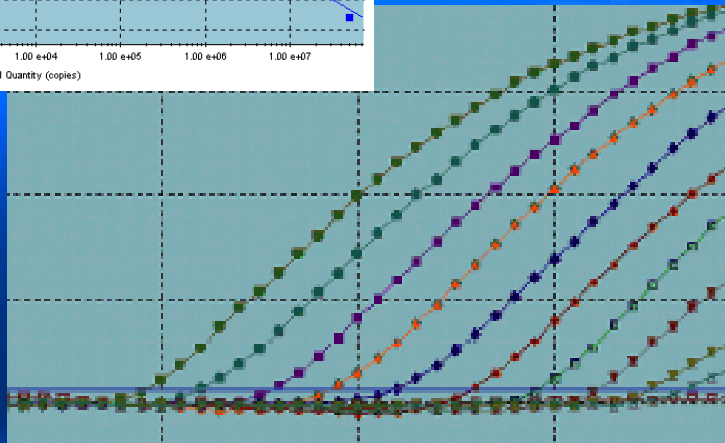
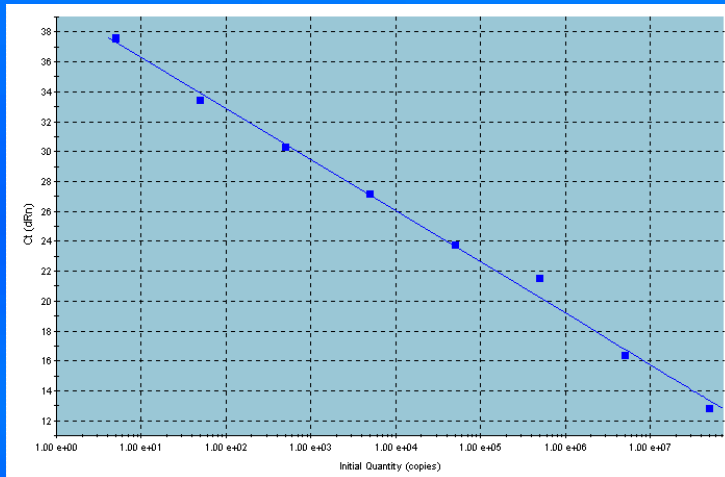


# A “Housekeeping Gene” Free Zone For Normalisation



Tania Nolan, Ph.D.

Senior Q-PCR Applications Scientist



[tania.nolan@stratagene.com](mailto:tania.nolan@stratagene.com)

Mx3000<sup>P</sup>



# A Common Protocol for Gene Expression Profiling

- Collect tissues samples
- Extract total RNA (usually “small amount”)
- Use eg 1 $\mu$ l total RNA into a cDNA reaction (random primers or oligo dT)
- Use eg 1 $\mu$ l RT reaction in QPCR
- Normalise gene of interest to housekeeping gene quantity

# Is QRT-PCR the same as QPCR with an irritating first step?

- QPCR of DNA is linear over a wide dynamic range
- QPCR of DNA is reproducible from day to day, machine to machine, template to template, operator to operator.....
- What is the effect of adding the RT step?

# First questions first.....

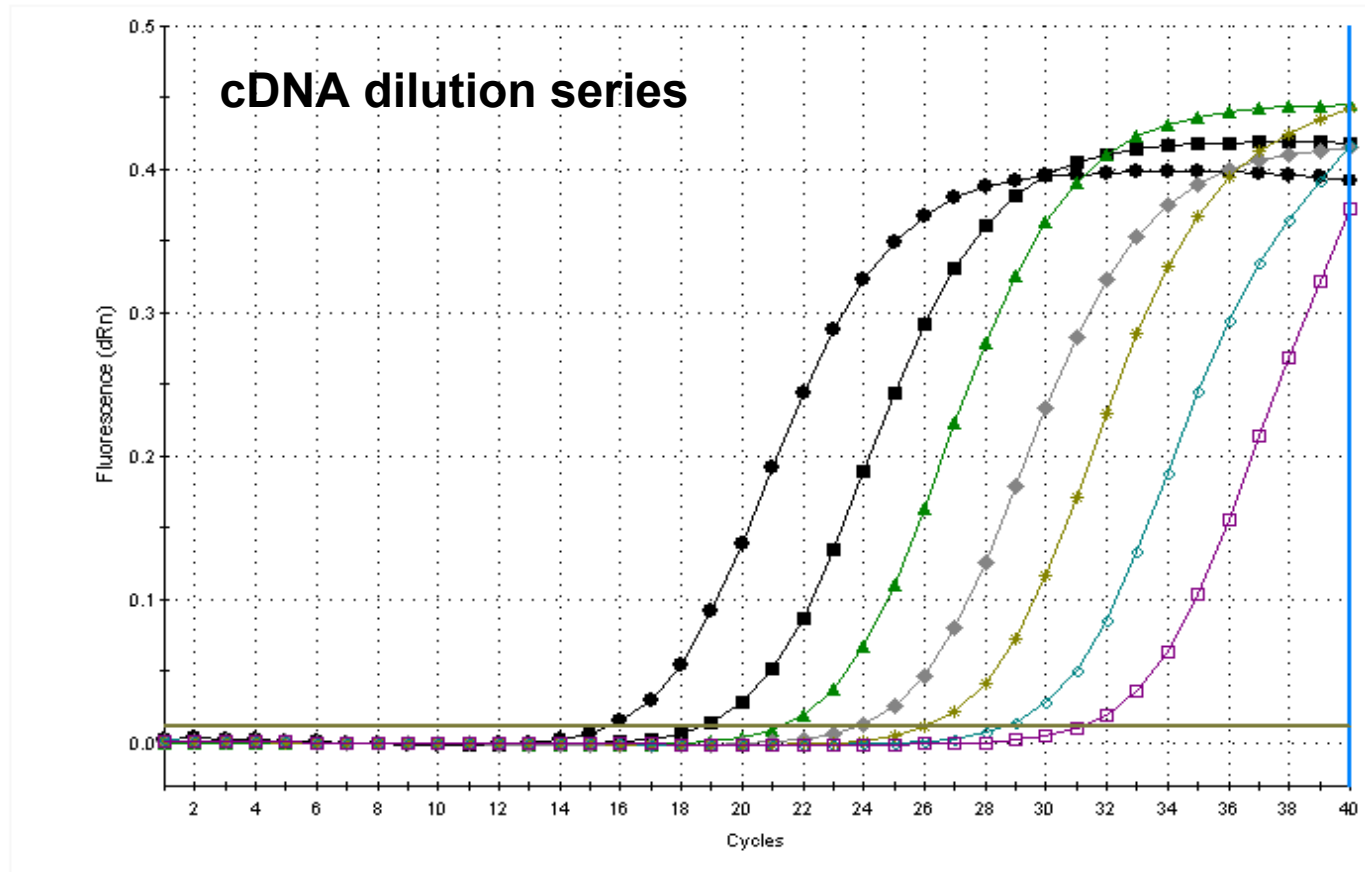
1. What is the dynamic range of RT reaction linearity?
  - **TEMPLATE:** 2 fold serial dilution of QPCR grade Human Universal Reference RNA
  - **RT PROCEDURE:** Global cDNA synthesis using random primers
  - **RT CONDITIONS:** Stratagene ProStar kit
  - **TEST:** QPCR of 2 different gene targets

# First questions first.....

1. Safety check.....
  - Check we can pipette reliably!
  - Check that the QPCR assay is reproducible and reliable on a cDNA template

# Dilution series (5 fold) of cDNA from RT of 2.5ng RNA ( $\beta$ actin)

Amplification Plots

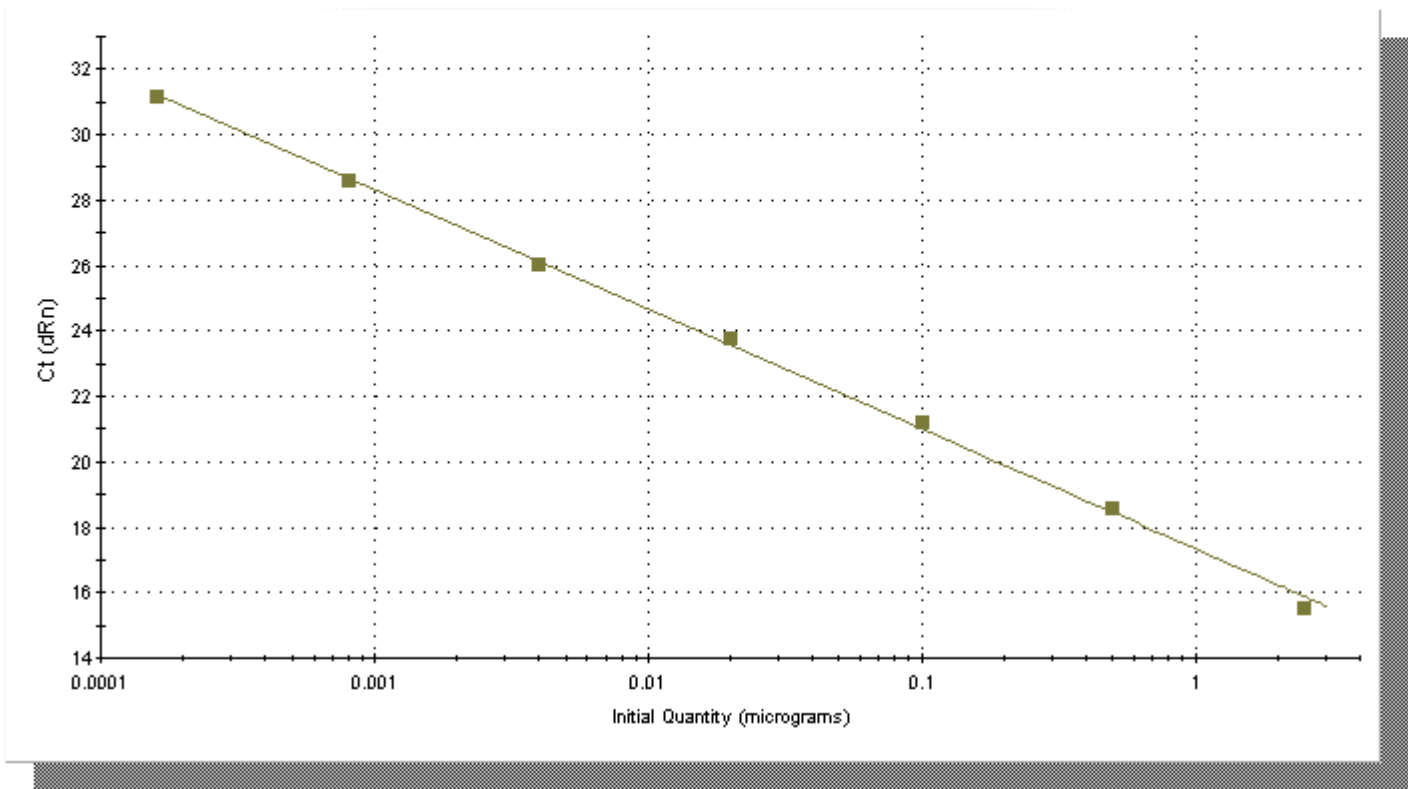


# cDNA Sample 2 dilution series (QPCR gene 1)

**cDNA dilution series**  
**Slope -3.66**

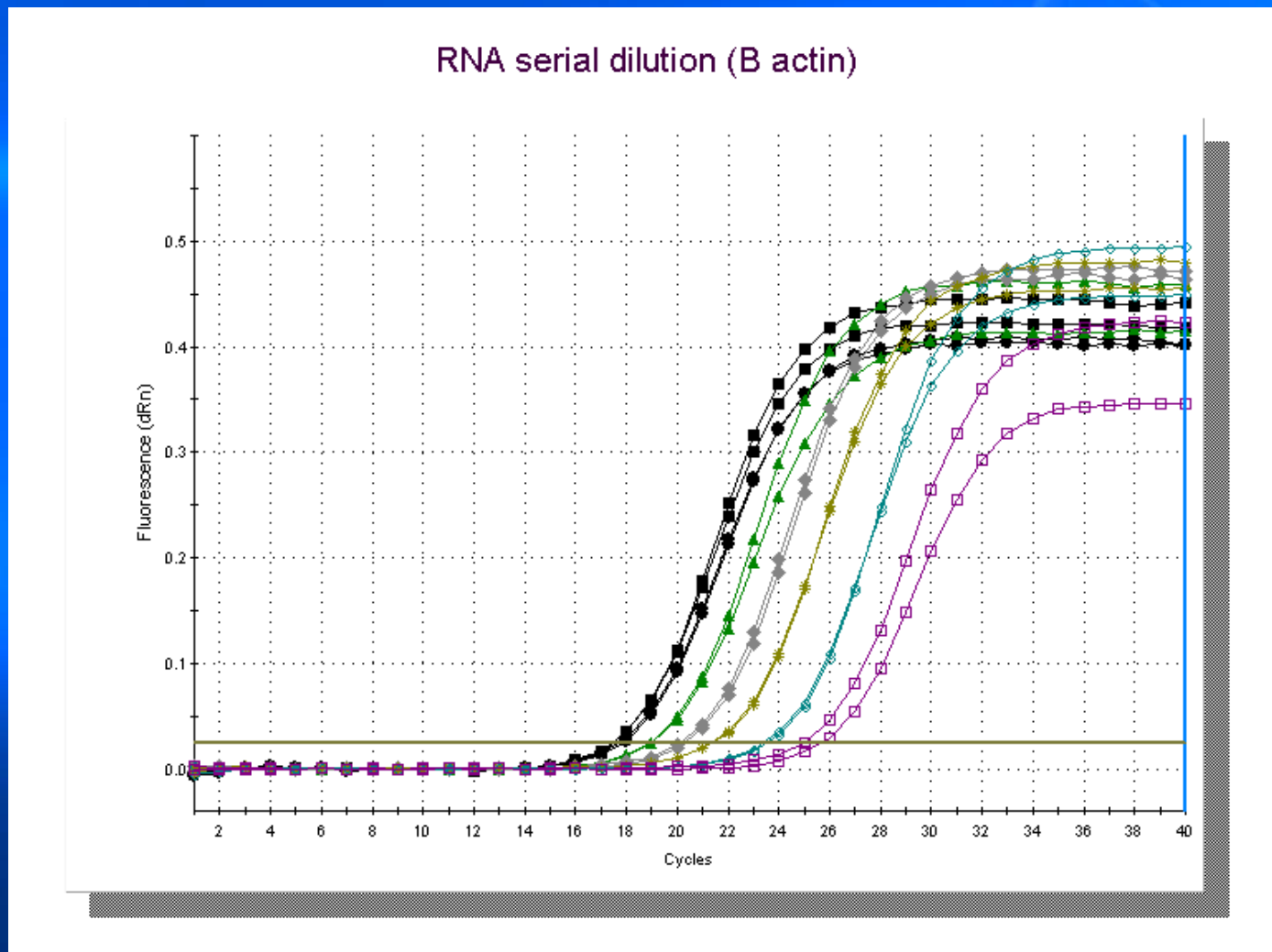
**Standard Curve**

Log fit values  
■ SYBR Standards, RSq:0.999  
— SYBR, Y = -3.665\*LOG(X) + 17.34, Eff. = 87.4%

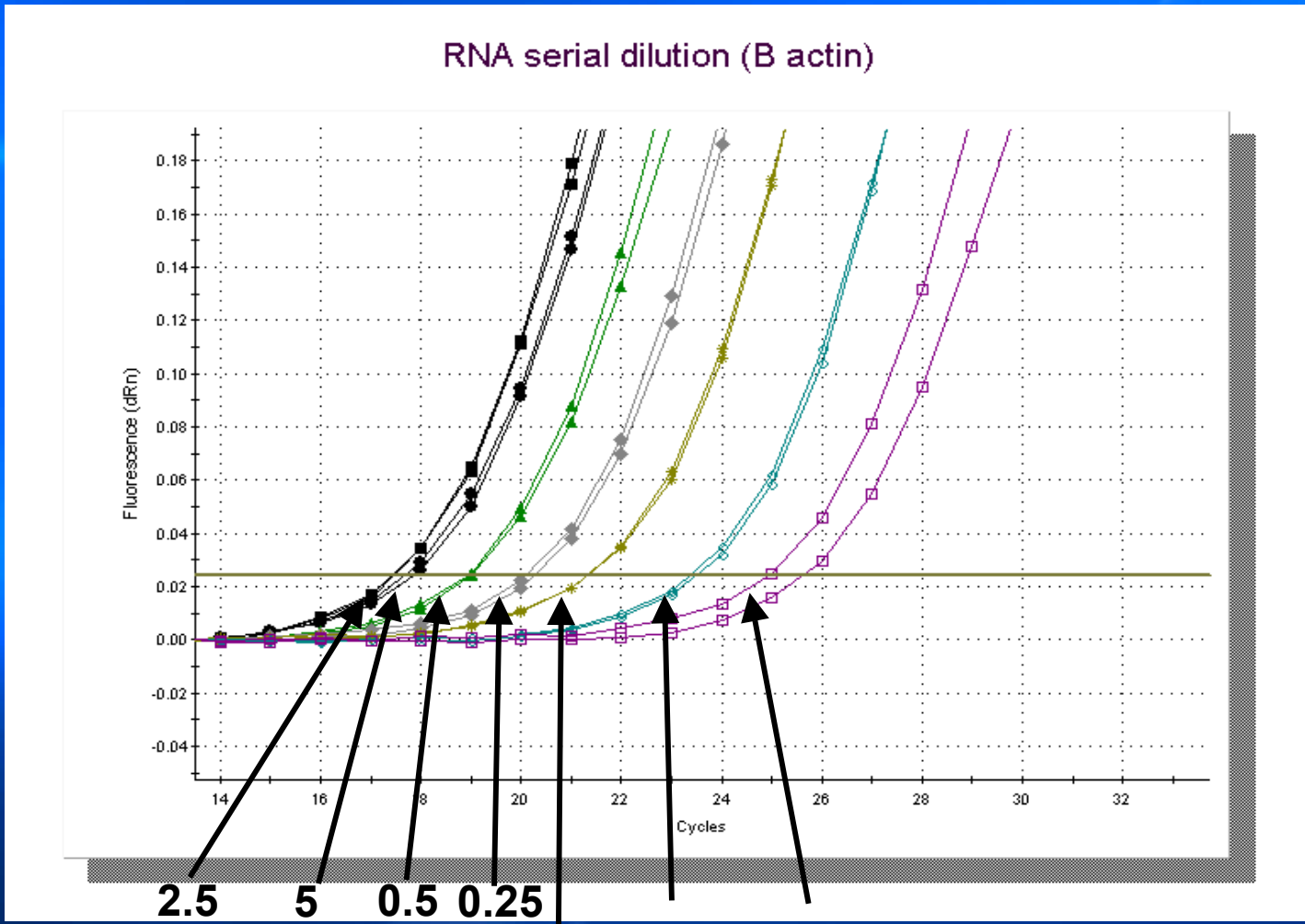


# Reverse transcribed RNA (2x) dilution series ( $\beta$ actin)

(5ng to 0.005ng)

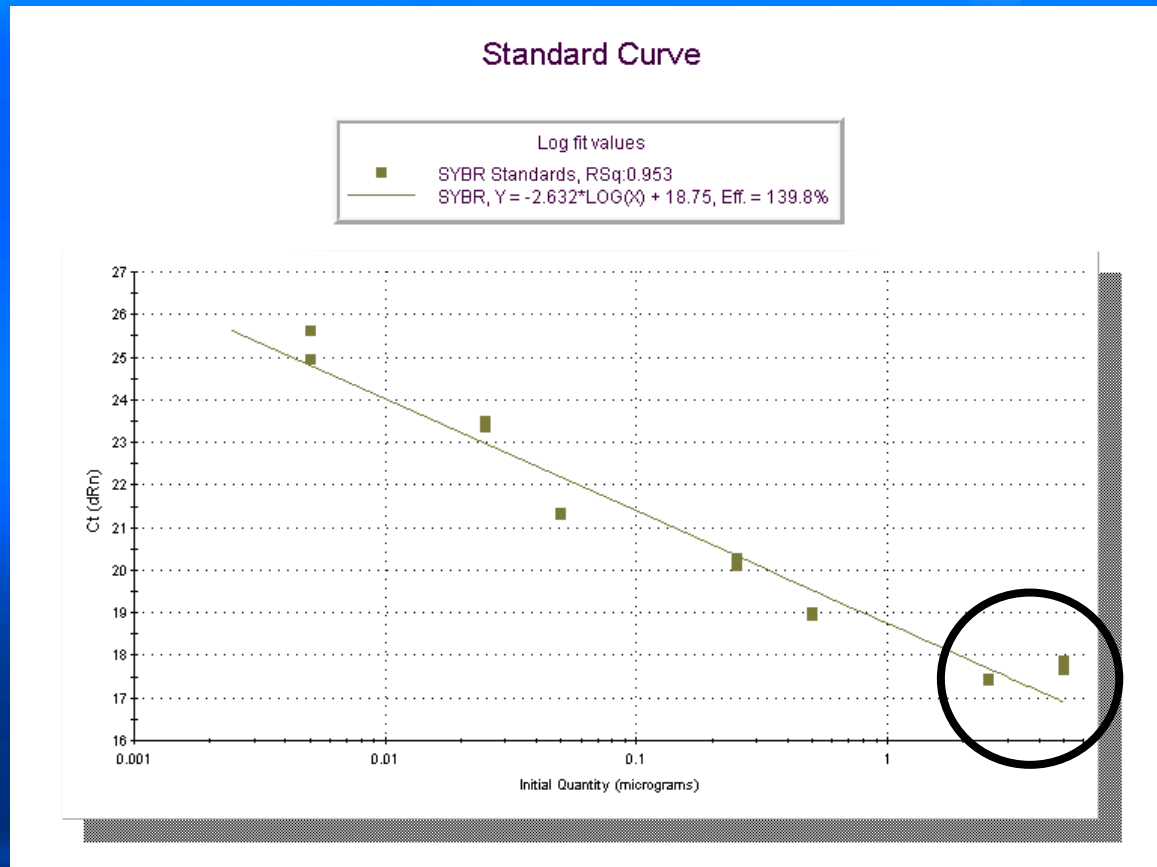


# Reverse transcribed RNA dilution series ( $\beta$ actin)

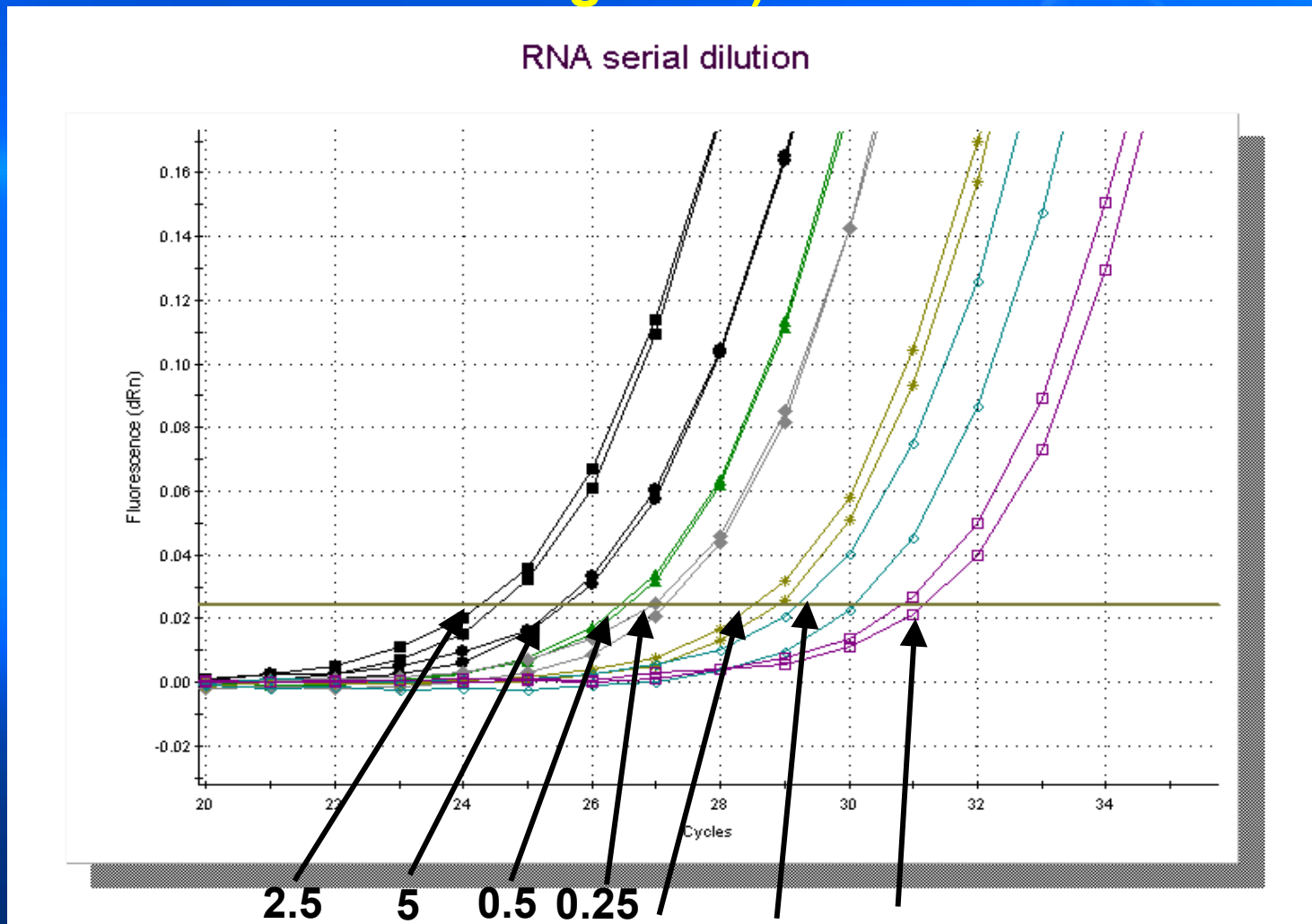


0.05 0.025 0.005

# Reverse transcribed RNA dilution series ( $\beta$ actin)

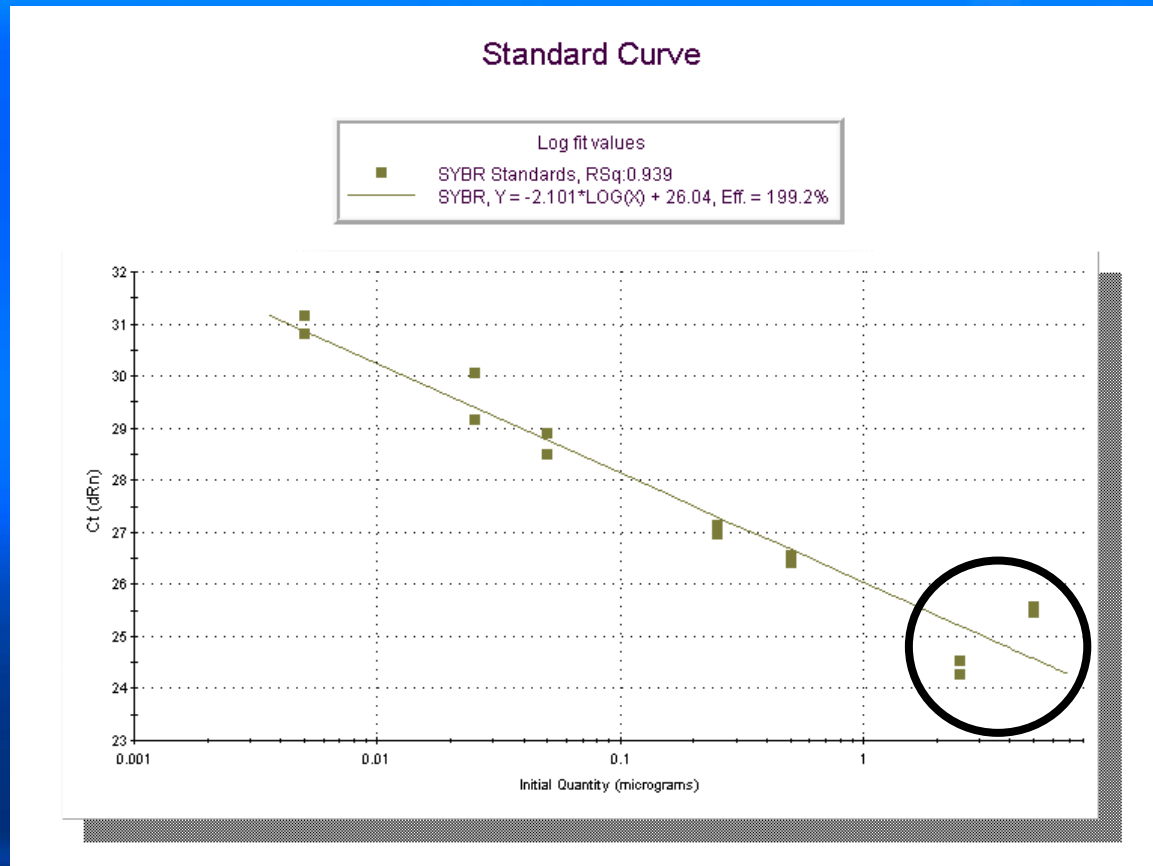


# Reverse transcribed RNA (2x) dilution series (QPCR gene 2)



0.05 0.025 0.005

# Reverse transcribed RNA (2x) dilution series (QPCR gene 2)



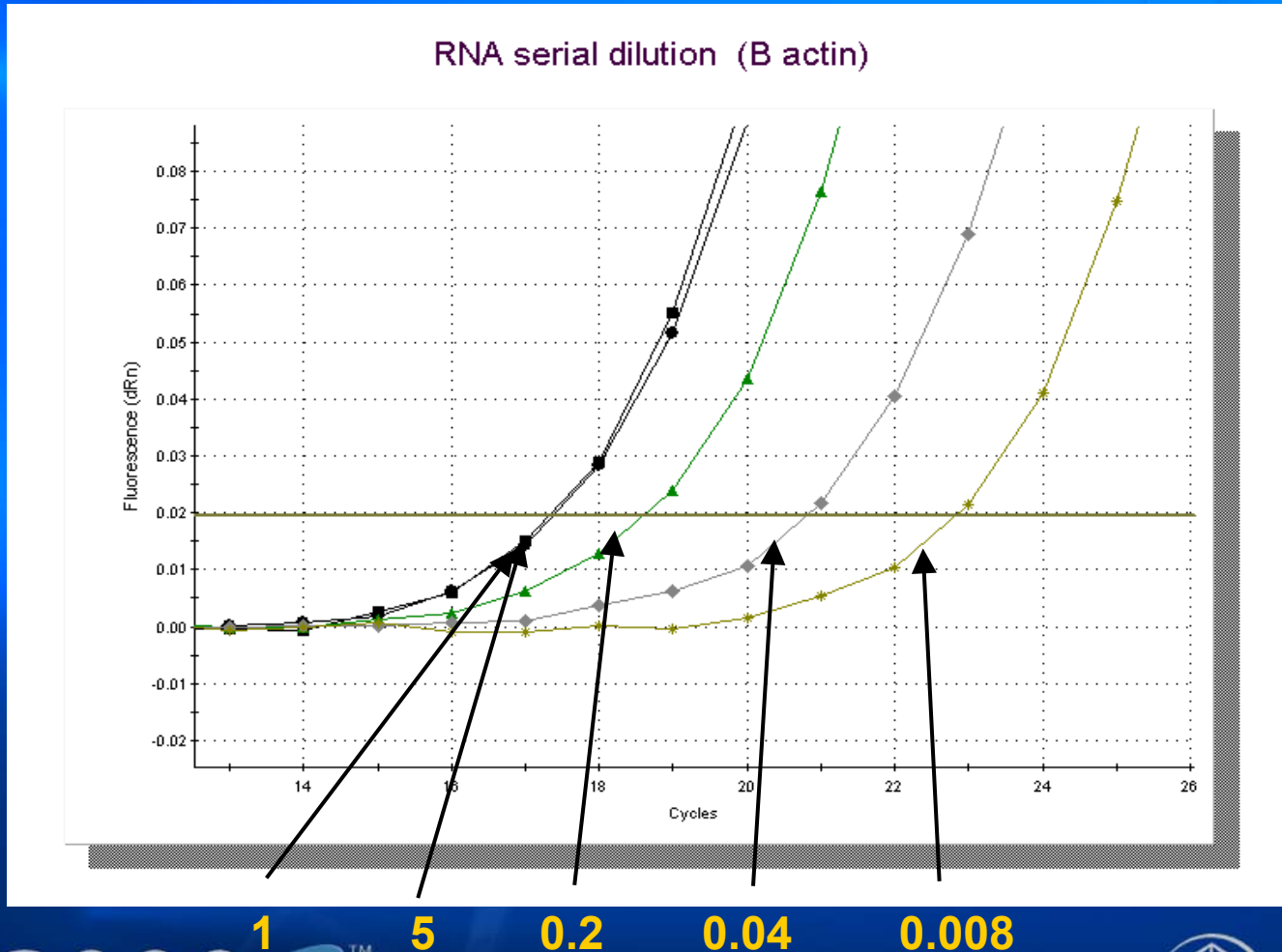
# What is causing the lack of linearity?

## 1. Is the RNA dilution series inaccurate?

- repeat the dilution series
- change the operator and template

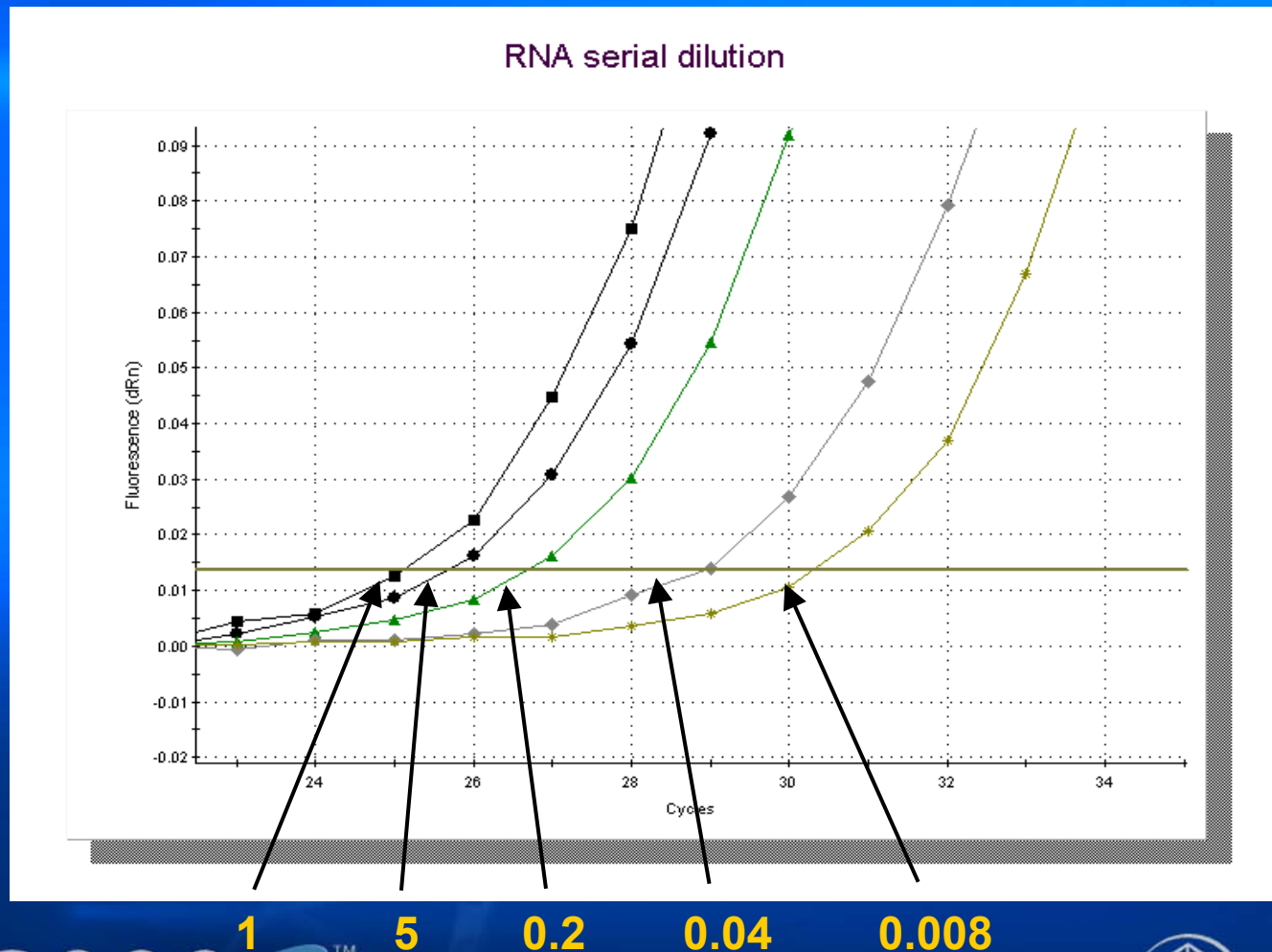
# Attempt 2

## Reverse transcribed RNA dilution series (5ng to 0.008ng) ( $\beta$ actin)



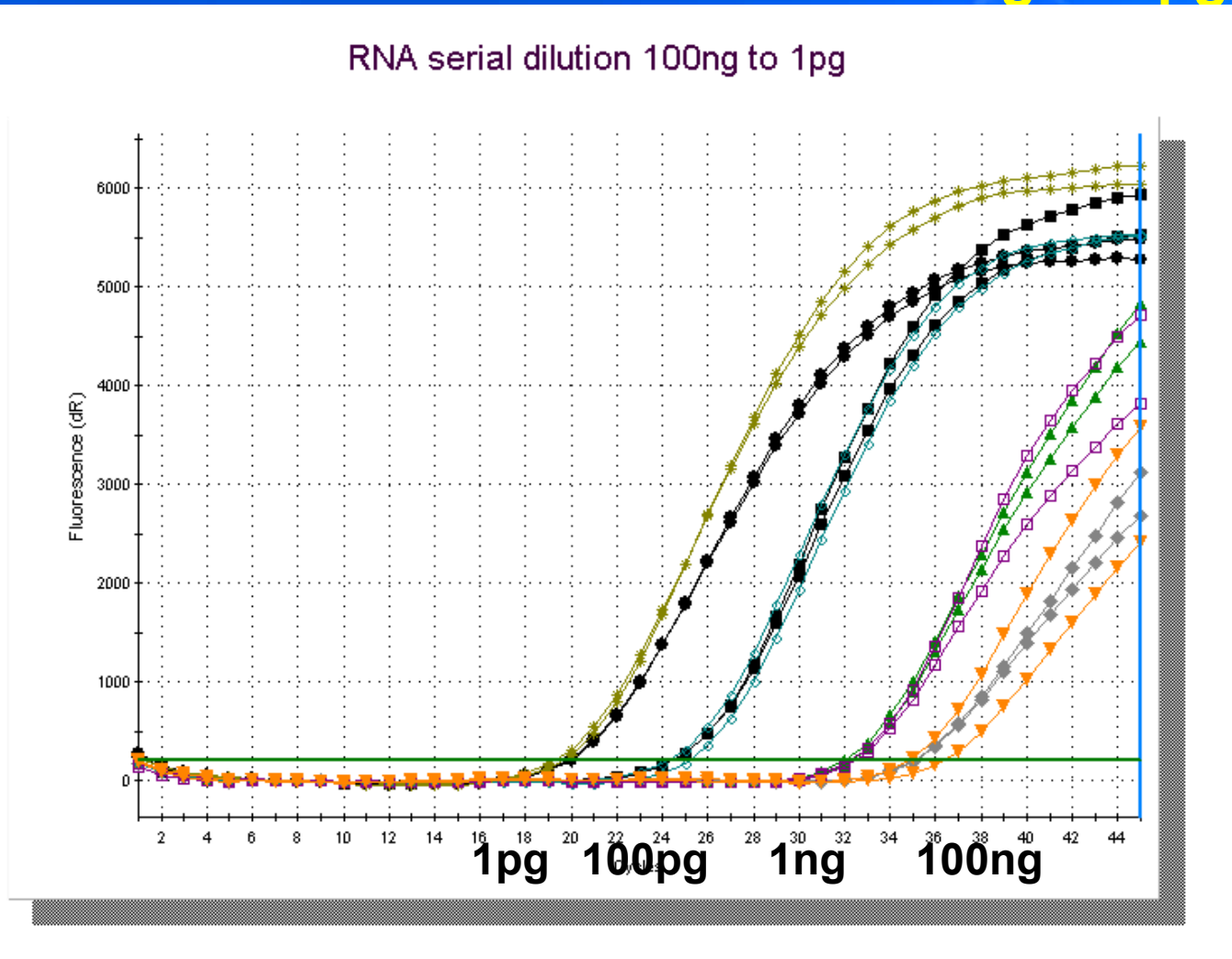
# Attempt 2

## Reverse transcribed RNA dilution series 5 fold (5ng to 0.008ng) (gene 2)



# New operator!

## RNA Serial Dilution 100x dilution 100ng to 1pg



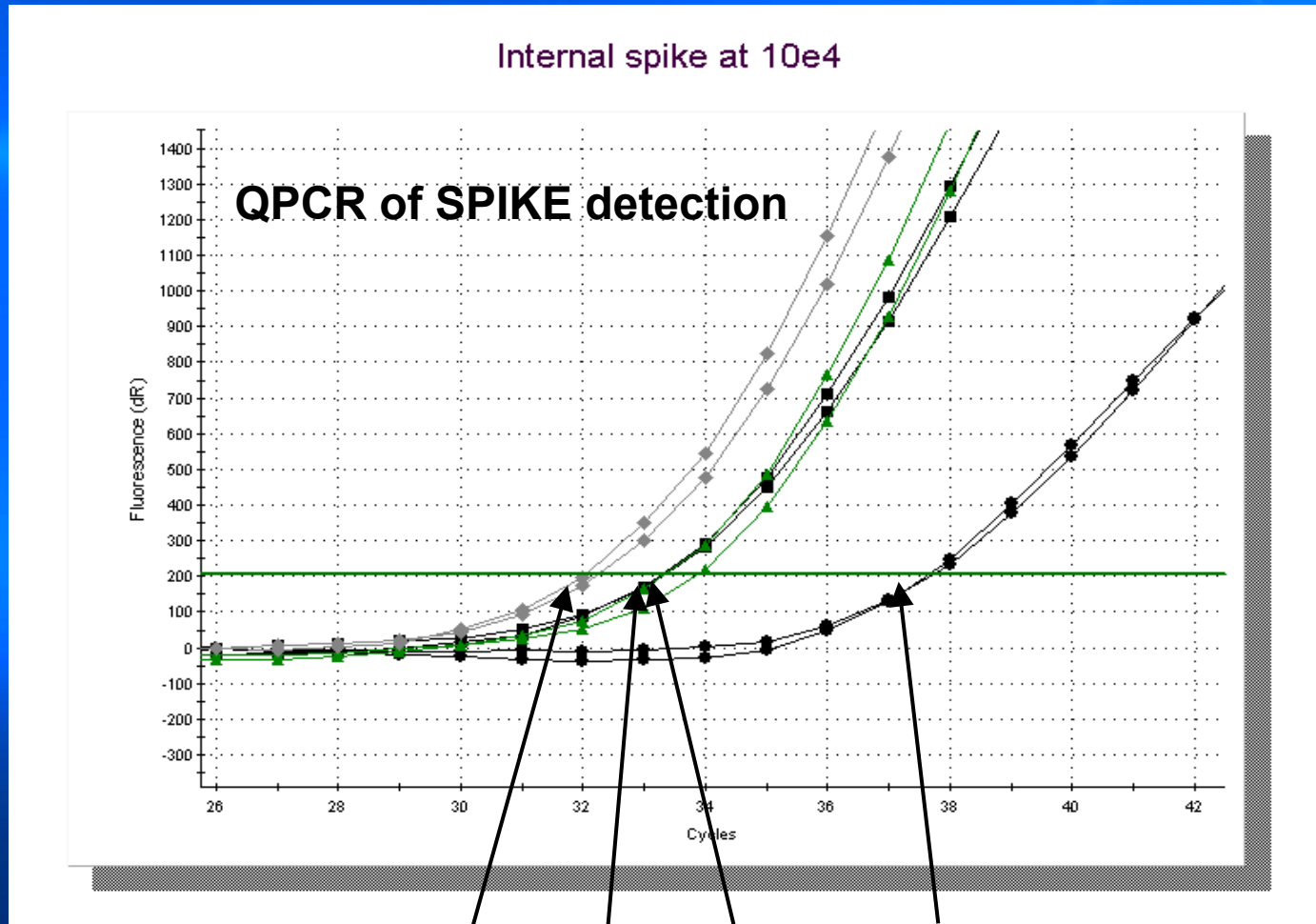
# Correct for RT efficiency using spiked RNA?

- Independent RNA serial dilution RT reactions show lack of linearity
- **Would adding a spike to the RT be helpful?**
- A constant concentration of a sequence should produce a constant ct for any gene
- Variation of that ct would indicate variation in RT efficiency

# Correcting for RT efficiency using spiked RNA

- A human total RNA serial dilutions was prepared (100 fold, 100ng to 1pg)
- $1 \times 10^4$  copies of a none human RNA transcript were added to each diluted RNA sample
- QPCR quantification was performed on each (in duplicate reactions)

# Constant input SPIKE quantification varies in presence of RNA serial dilution



1pg 100pg 1ng 100ng

Background  
RNA concentration

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# Reverse Transcription of total RNA to cDNA is concentration dependant

- Different concentrations of SPIKE cDNA are produced from the same input concentration
- SPIKE cDNA quantity is dependent on the background RNA quantity (inverse correlation)

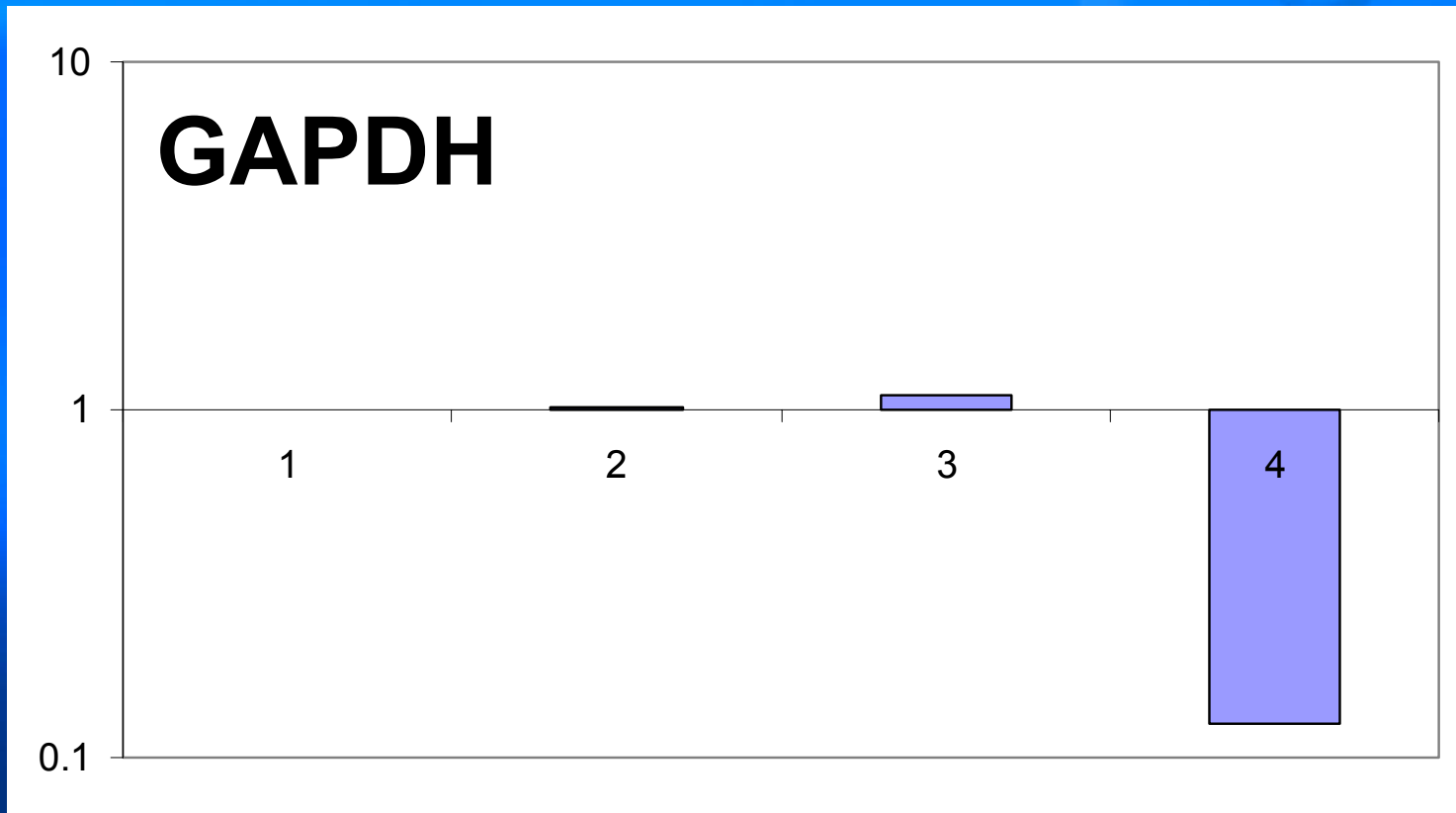
## CONCLUSION:

- An equal RNA concentration should be added to all RT random primer reactions

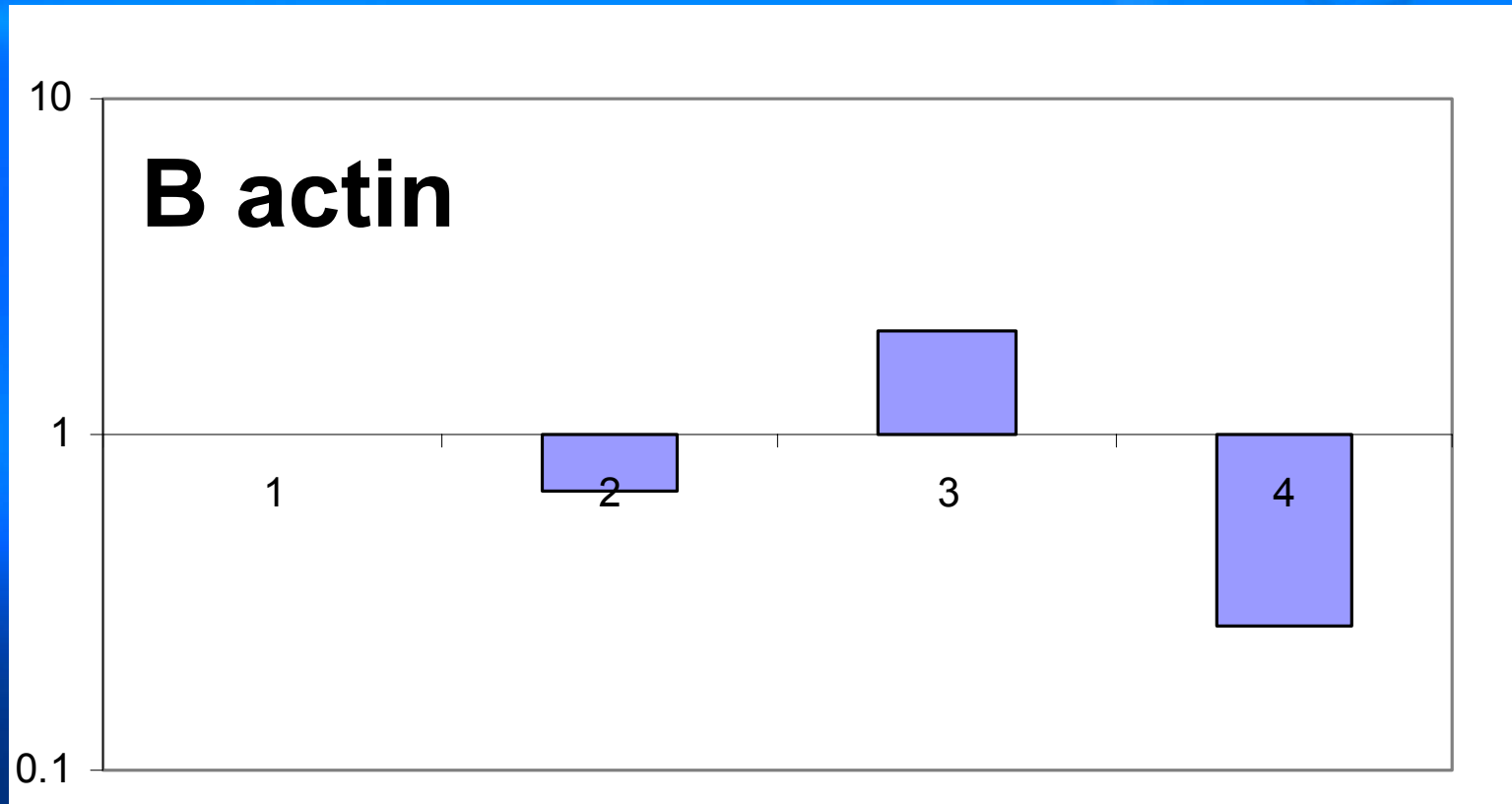
# How reproducible is the RT reaction?

- Four independent RT reactions were performed on a positive control RNA (calibrator)
- Constant RNA concentration (2.5ng) was included in every RT
- Genes were quantified relative to a cDNA standard curve

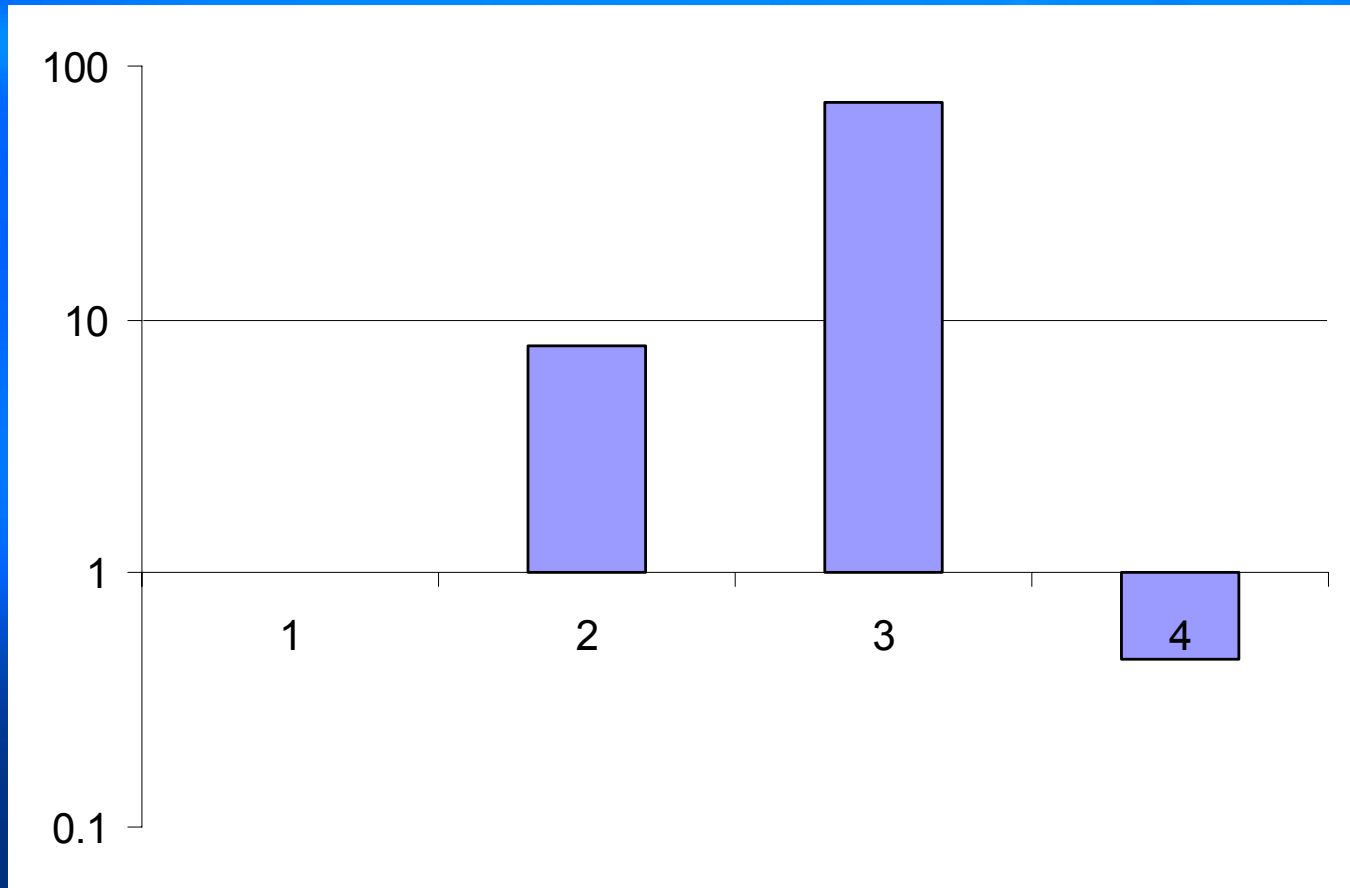
# GAPDH quantification in four independent RT batches



# B actin quantification in four independent RT batches



# Quantification of a low expression gene in four independent RT batches



# How reproducible is the RT reaction?

- There appears to be batch to batch variation in gene specific reverse transcription
- This is more apparent if the gene is expressed at a low level
- This suggests practical evidence against the procedure of normalisation to a housekeeping (highly expressed)

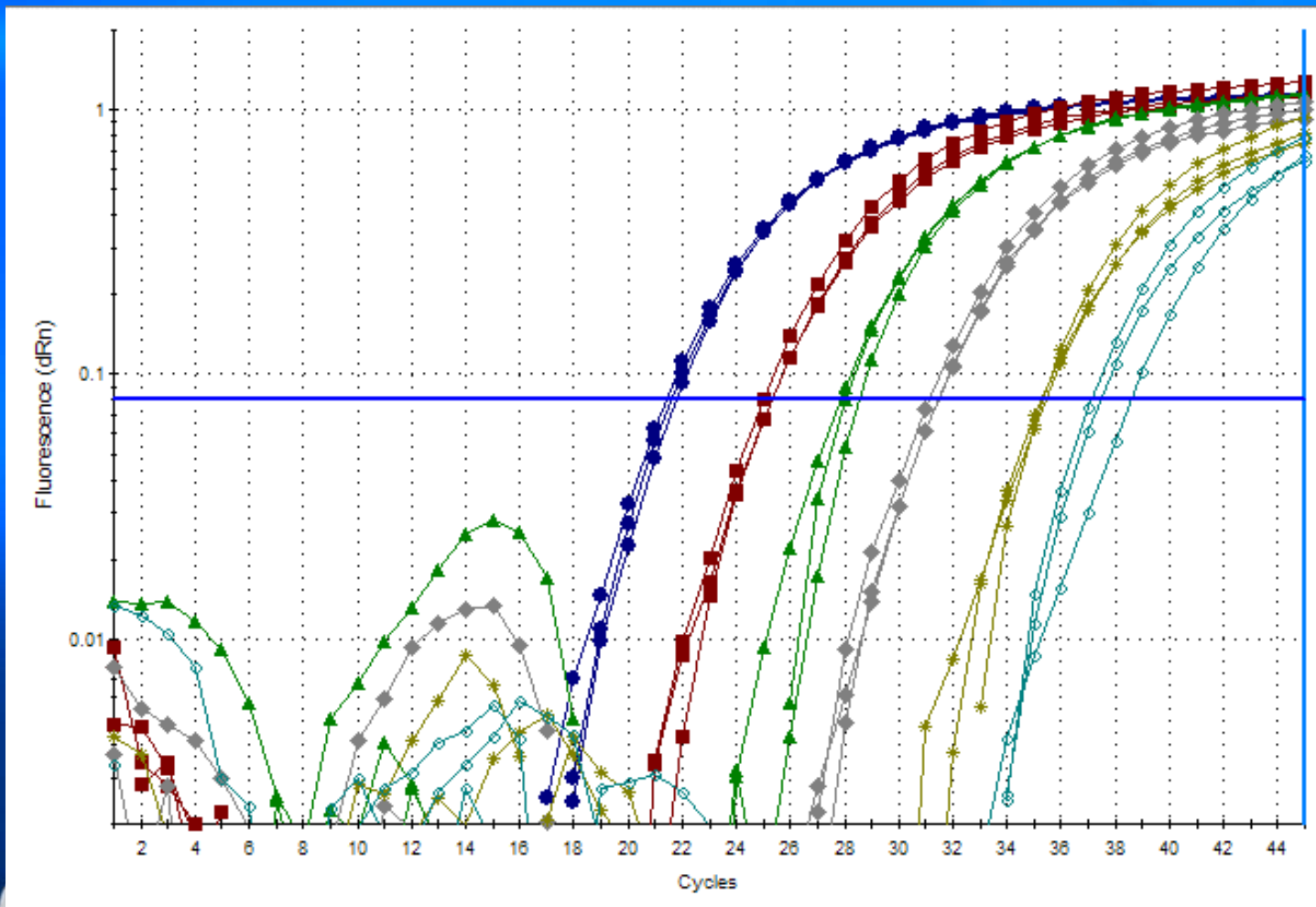
gene

# A Revised Protocol for Gene Expression Profiling

- Collect tissues samples
- Extract total RNA (usually “small amount”) and QUANTIFY
- Include a constant RNA amount into each RT reaction (2.5ng in Prostar)
- Include a calibrator (constant) sample with each RT batch
- Measure transcript quantity for samples and calibrator relative to a cDNA standard curve
- Normalise sample transcript quantity relative to calibrator quantity for each RT batch

# Alternatively.....

## Gene specific RT and QPCR (10 fold dilutions) IS linear



# Many Thanks to:

- Helen Lacey and Colin Sibley, St Mary's Hospital, Manchester, UK
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