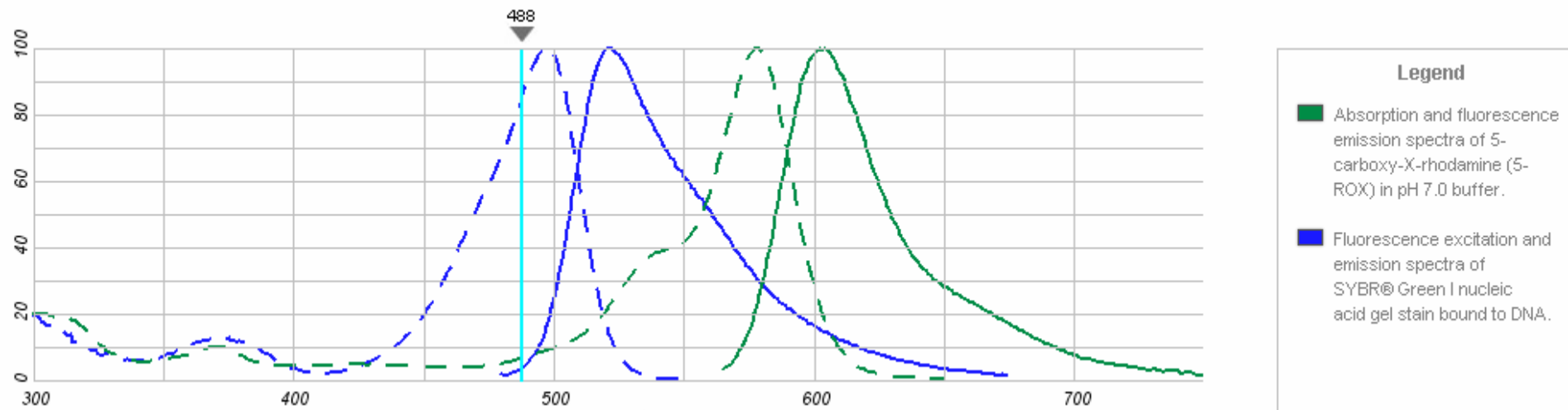


When does ROX rock?

Fabrice Magnino, PhD

Definition

- ROX is a fluorescent dye
- ROX is an acronym for 6-Carboxyl-X-Rhodamine



ROX and QPCR

- ROX was introduced by ABI in the 1990's
- It is mainly used as passive reference dye
- But it can also be used for probe labelling

Passive Reference Dye Definition

- ROX passive reference dye is used to normalize for non-PCR related fluorescence signal variation on certain real-time thermocyclers (ie., Applied Biosystems Prism™ 7000, 7700 and 7900 instruments). The traditional ROX passive reference dye does not take part in the PCR reaction and its fluorescence should remain constant during the PCR reaction. **BioCat Website (general supplier in Germany)**

Non-PCR related fluorescence signal variation

- The ROX passive reference consists of an inert dye whose fluorescence is stable along the qPCR reaction, allowing the normalization of well-to-well variations that may result from pipetting errors or instrument limitations. **Eurogentec Website (primer synthesis)**

Normalisation for pipetting errors or instrument limitations

- The passive reference dye does not participate in the 5' nuclease PCR, but instead provides an internal reference to which the reporter-dye signal can be normalized during data analysis. This is necessary to correct for fluorescent fluctuations due to changes in concentration or volume in the wells. Normalization is accomplished through ABI's Sequence Detection System (SDS) software, which divides the emission intensity of the reporter dye by the emission intensity of the passive reference to obtain a ratio defined as the R_n (normalized reporter) for a given reaction well.

University of Virginia website

Change of concentration or volume in the wells

$$\frac{\text{Reporter Dye intensity}}{\text{Passive reference intensity}} = R_n$$

www.qpcr.com.au

ABI's definition

- Passive reference:

A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations caused by changes in concentration or volume. A passive reference dye is included in all real-time PCR reagent kits.

Part Number 4348358 Rev.E 5/2005

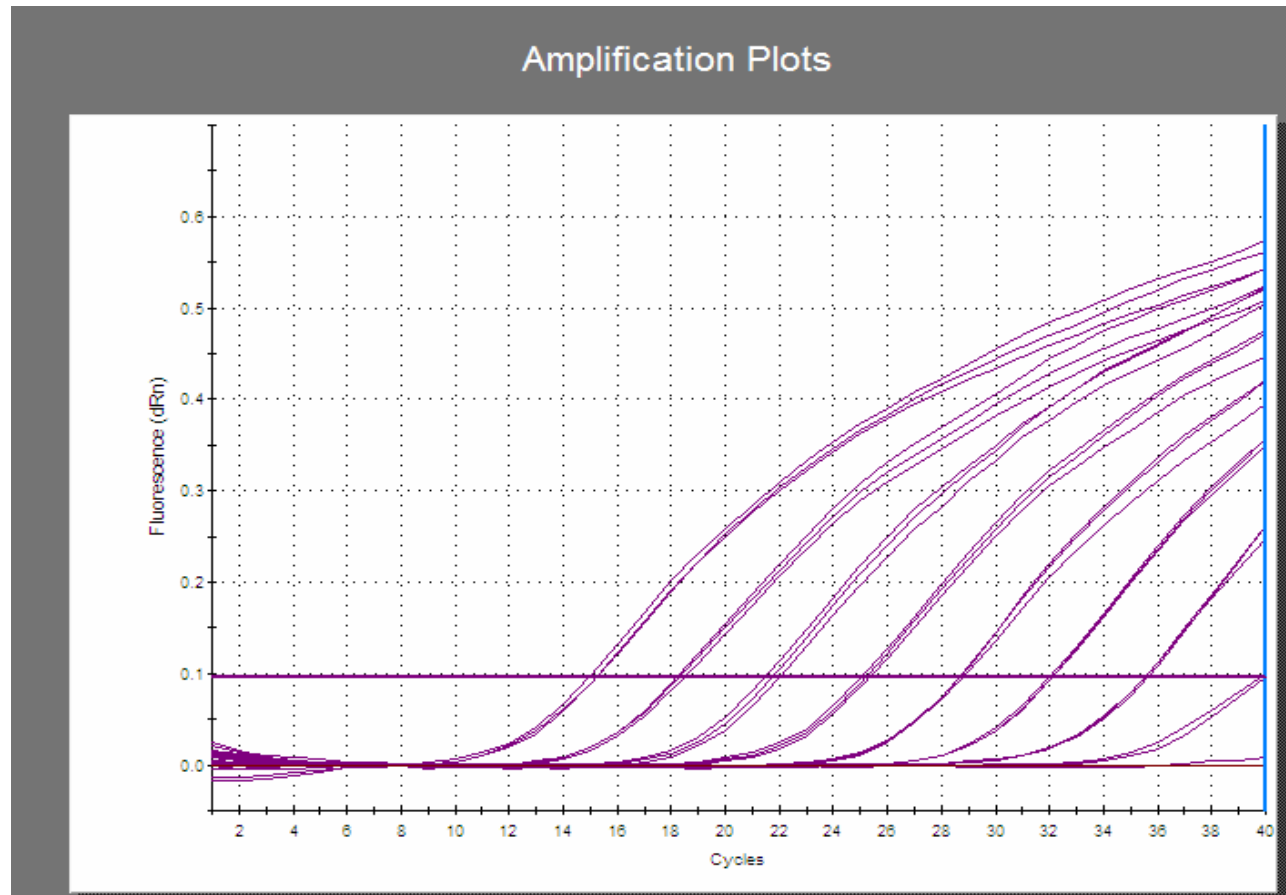
Summary

- ROX is a passive reference dye
- Normalisation for pipetting and instruments
- Normalisation is done by division $\frac{\text{Reporter Dye intensity}}{\text{Passive reference intensity}} = R_n$
- ROX is included in the Master Mix

Normalisation principle

$$\frac{\text{Reporter Dye intensity}}{\text{Passive reference intensity}} = R_n$$

$$\frac{26000}{16000} = 1.62$$

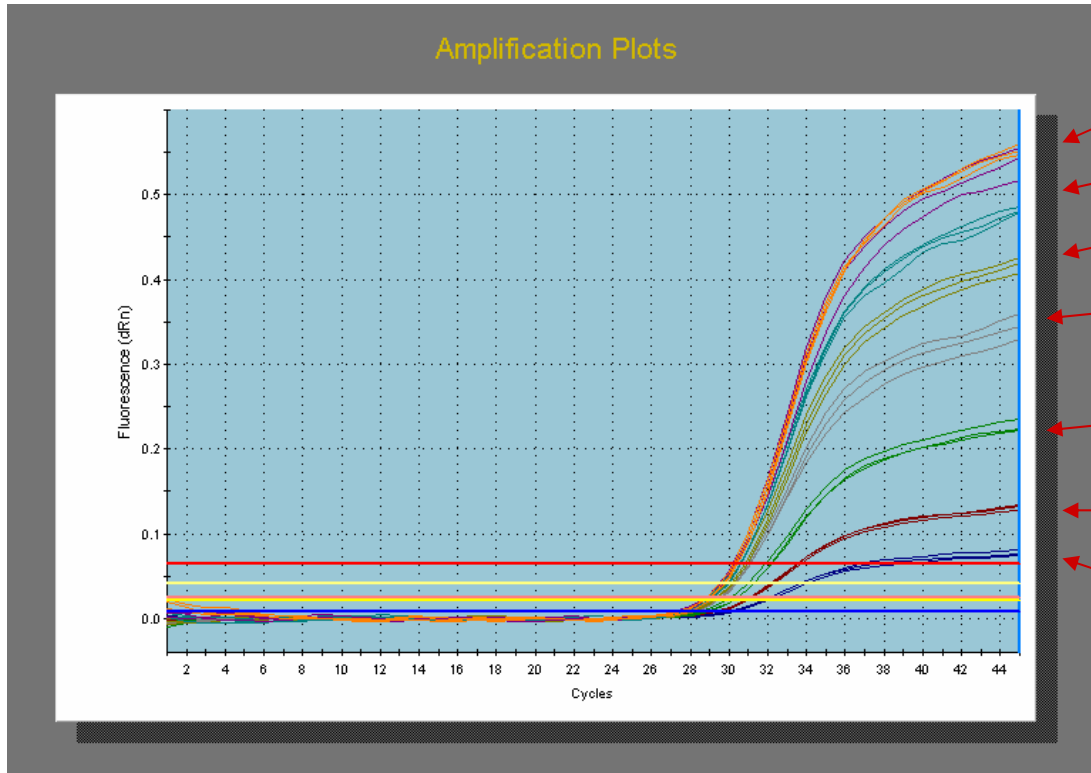


Is the [ROX] important?

$$\frac{\text{Reporter Dye intensity}}{\text{Passive reference intensity}} = R_n$$

[ROX]

No
With
ROX
Norm.



<0.015625 μM

0.015625 μM

0.03125 μM

0.0625 μM

0.125 μM

0.25 μM

0.5 μM

Why is ROX important?

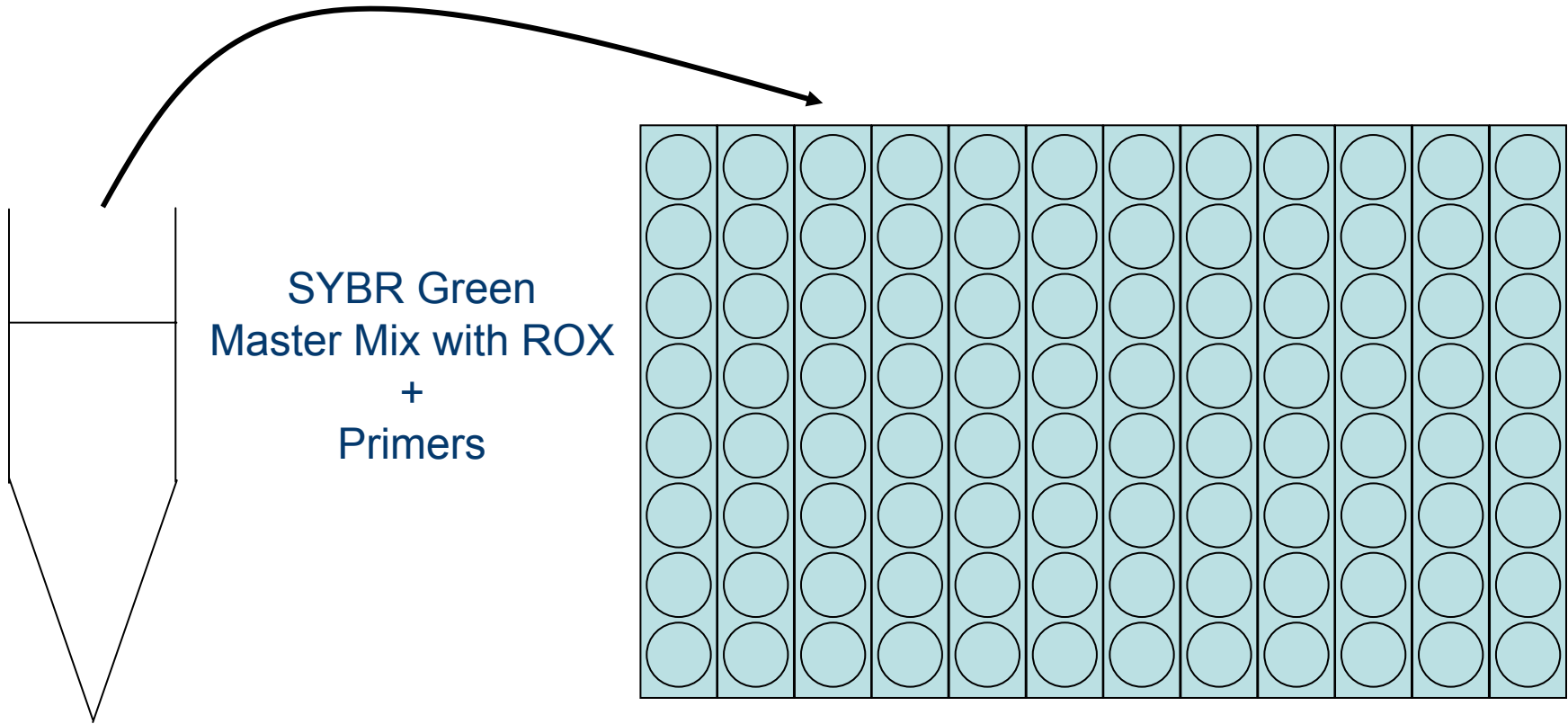
- ROX is a passive reference dye
- Normalisation for **pipetting** and **instruments**
- Normalisation is done by division $\frac{\text{Reporter Dye intensity}}{\text{Passive reference intensity}} = R_n$
- ROX is included in the Master Mix

How does it work?

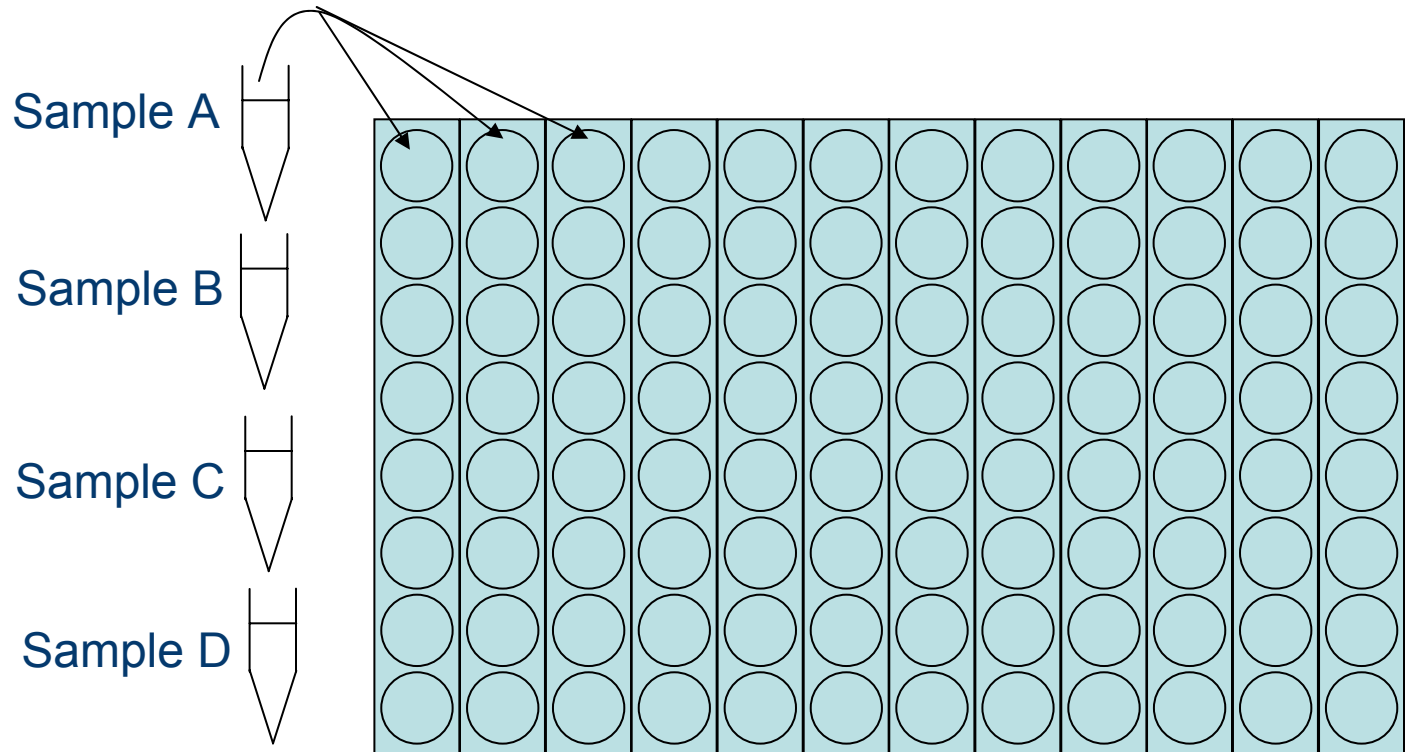
- Pipetting:
 - To correct the inherent variability
- Instrument:
 - To overcome instrument limitation

Pipetting correction: How does it work?

Distribute



Pipetting correction: How does it work?



Pipetting correction: Does ROX help?

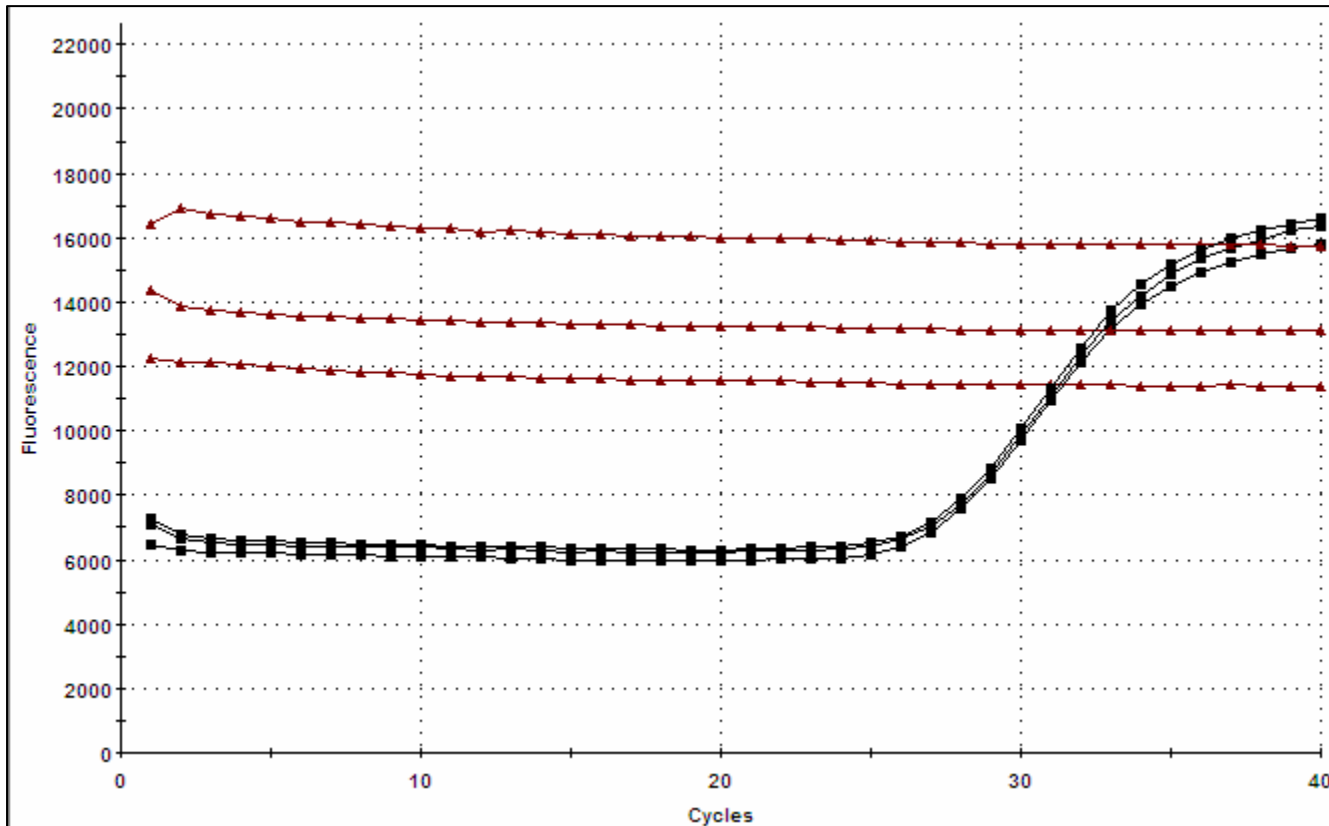
- ROX fluorescent levels will correlate with the quantity of Master Mix used.
- But ROX fluorescence will have no relation to the quantity of cDNA/DNA used in each reaction

~~Does this help?~~

Not really, because we are dividing the ROX fluorescence from the Master Mix by the reference dye!

$$\frac{\text{Reporter Dye intensity}}{\text{Passive reference intensity}} = R_n$$

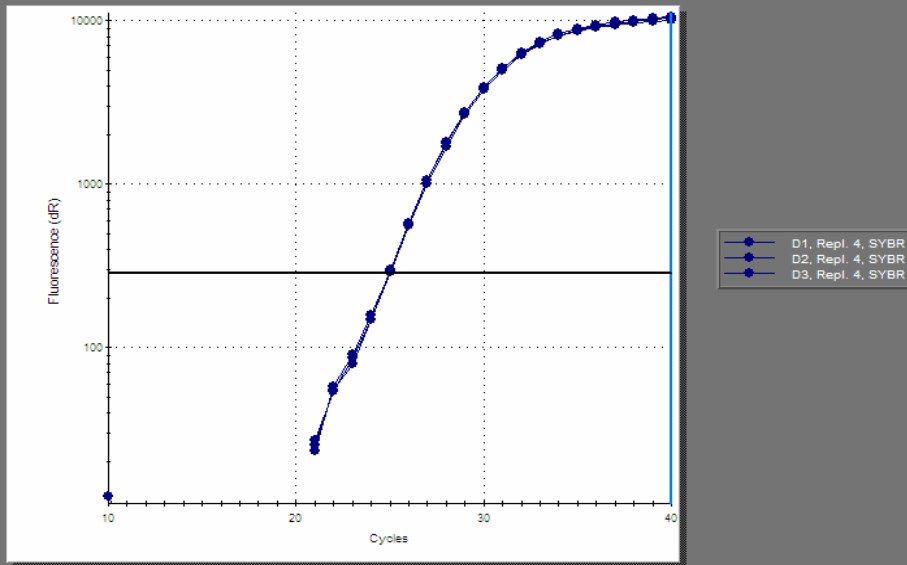
Pipetting correction: Does ROX help?



Pipetting correction: Does ROX help?

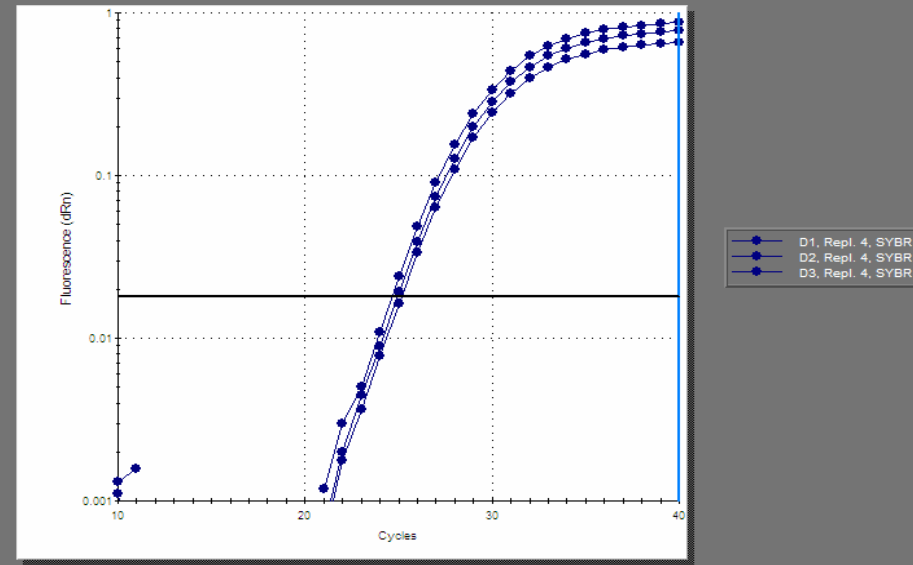
Without

Amplification Plots



With

Amplification Plots



Why use ROX then?

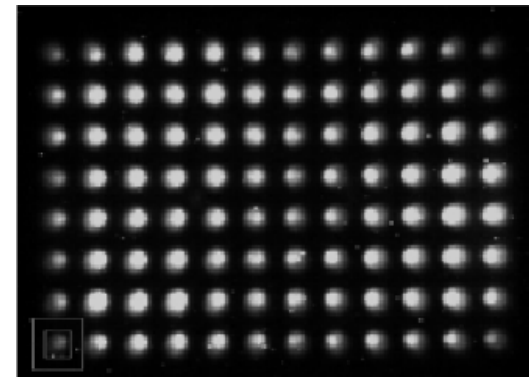
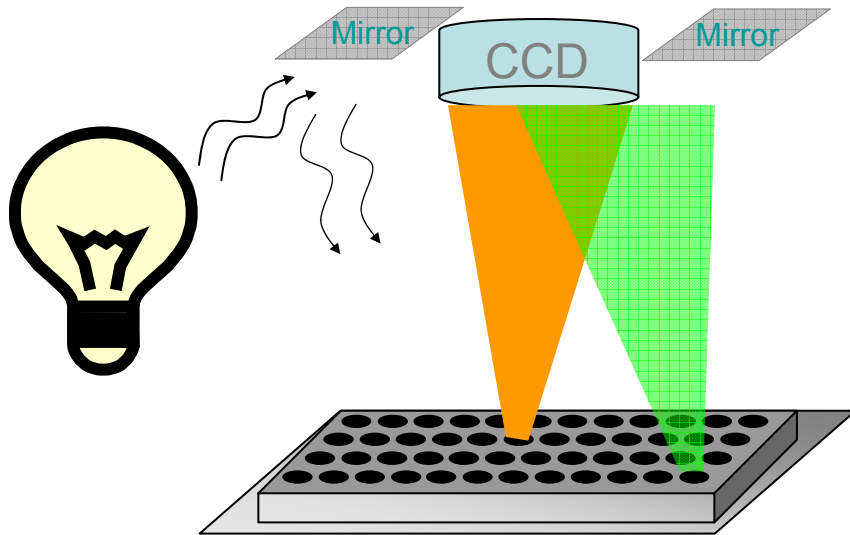
- Pipetting:
 - To correct the inherent variability

In fact it is the opposite,
Master Mix pipetting errors
will affect accurate pipetting
of cDNA/DNA

- Instrument:
 - To overcome instrument limitation

Why use ROX then?

- Instrument limitation:



96 well uniformity RUN.

Intensity depends on position.

Why use ROX?

- Pipetting:
 - To correct the inherent variability

- Instrument:
 - To overcome instrument limitation

Does ROX rock?

- Not really, correcting for instrument limitation is good, but correcting for inaccurate pipetting of cDNA/DNA would have been better
- Moreover when using ROX, inaccurate Master Mix pipetting will affect the data.

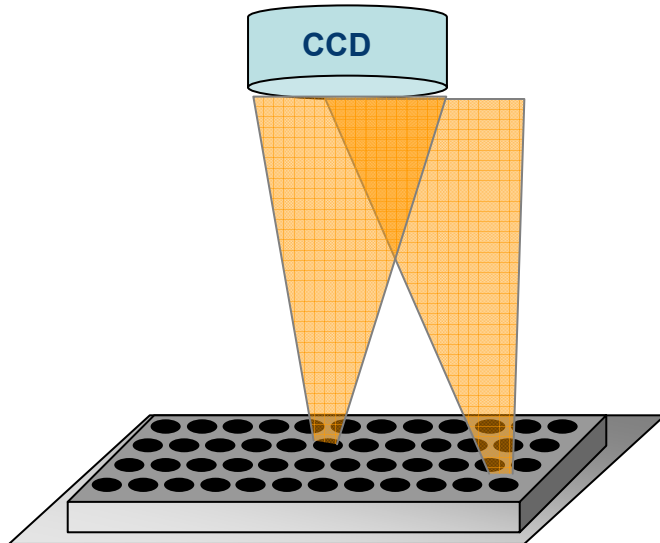
Can ROX rock then?

Maybe!

How can ROX rock?

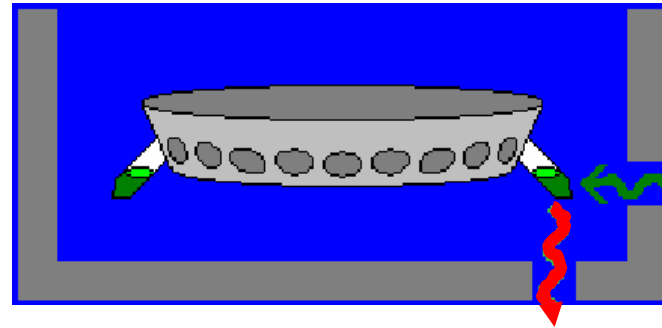
Simultaneous (CCD)

Intensity depends on well position



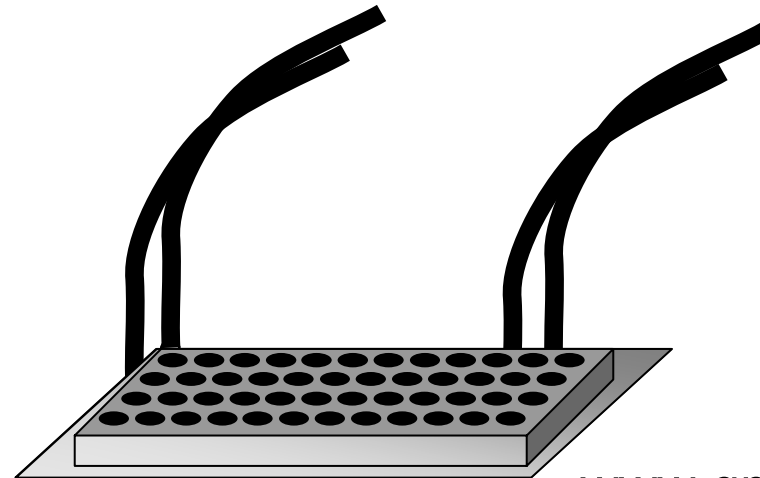
Asynchronous (scanning)

Constant intensity (no position effects)



Roche
LC1.0 & 2.0

Corbett
Rotorgene



Stratagene
Mx4000
Mx3000P
Mx3005P

Why use ROX then?

- Pipetting:
 - To correct the inherent variability
- Instrument:
 - To overcome instrument limitation

**Indeed those instruments do not require ROX
and therefore their users do not use it**

Can ROX rock on asynchronous reading instruments?

- Instrument:
 - To overcome instrument limitation
- Pipetting:
 - To correct the inherent variability

Was a great concept!

New Concept for asynchronous reading instruments

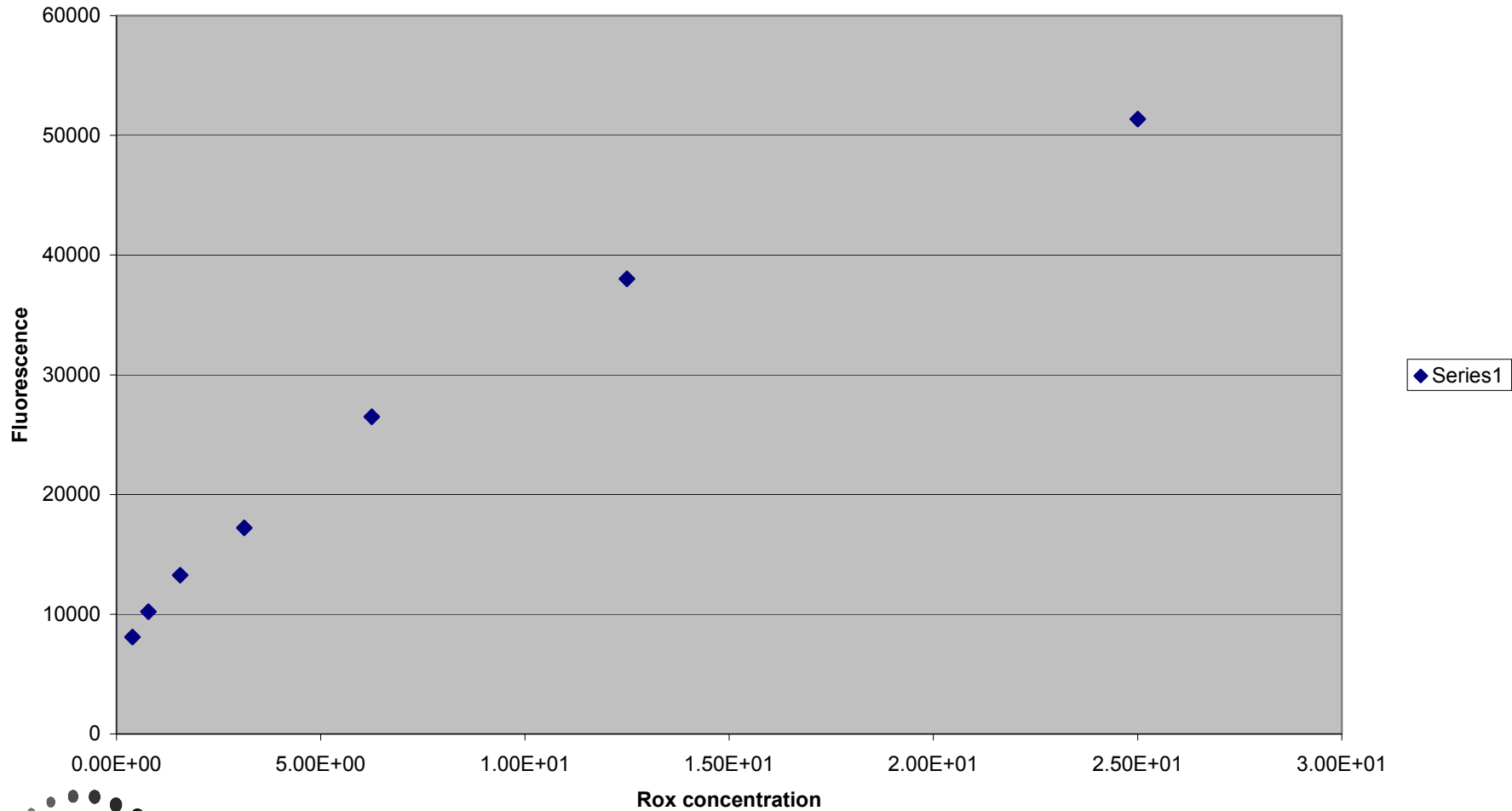
- What happens if we include ROX in the cDNA rather than the Master Mix?
- Would we be able to normalise against ROX?

Project in Collaboration with Nanhua Cheng, Army Malaria Institute, QLD

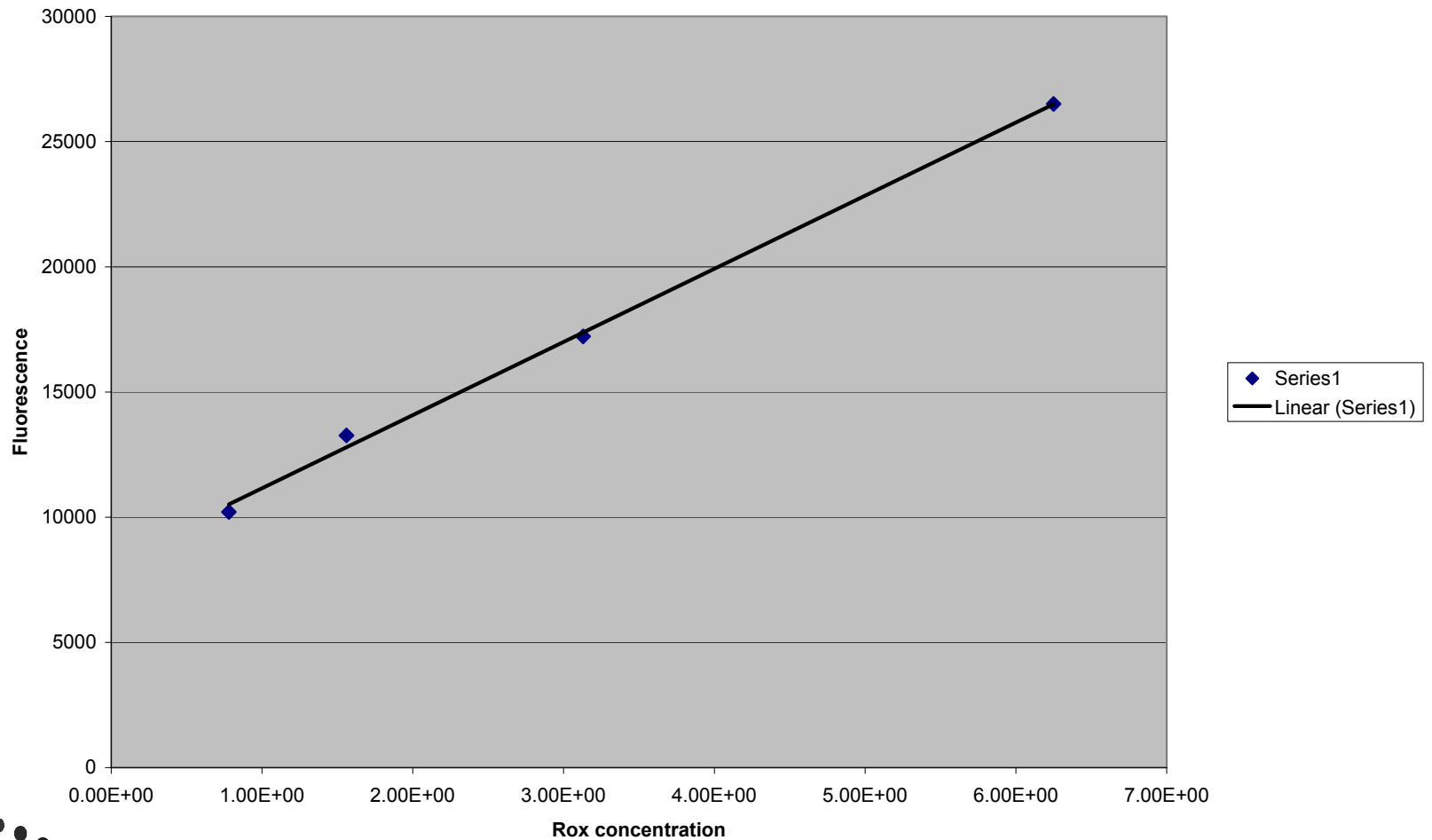
- Evaluation on the dynamic range of ROX
- Setup an experiment with ROX in DNA sample
- Test on 2 different genes
- Analysis with or without ROX normalisation

Dynamic range

Rox linearity

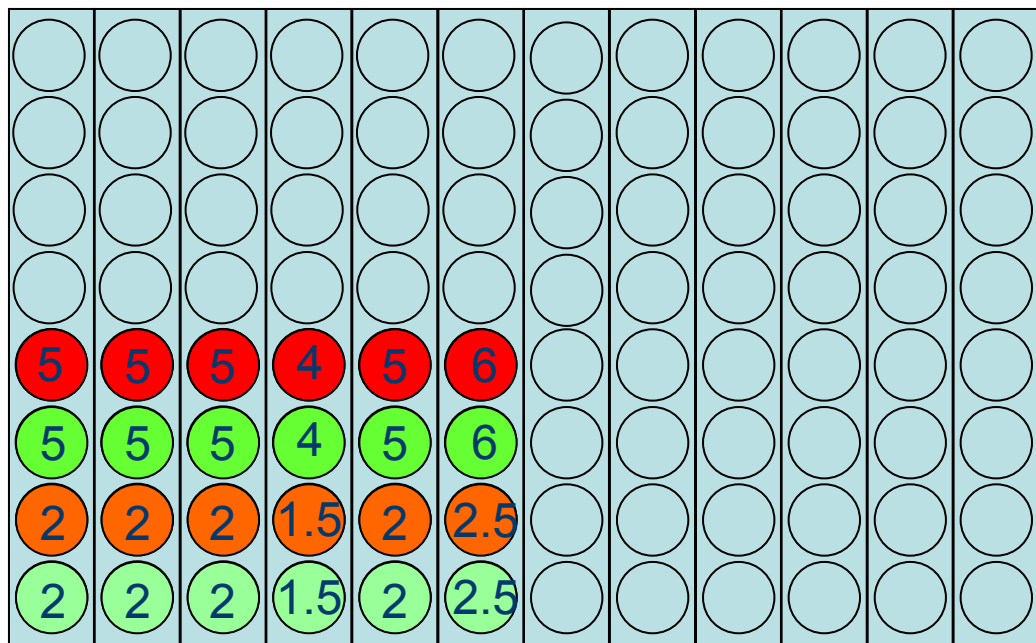


Dynamic range



Protocol

- Stratagene Brilliant II Master Mix
- Stratagene Mx4000 and Mx3005P
- Triplicates of 5 μ l DNA in 20 μ l Mmix
- Then 4, 5 and 6 μ l DNA in 20 μ l Mmix
- Triplicates of 2 μ l DNA in 23 μ l Mmix
- Then 1.5, 2, 2.5 μ l DNA in 23 μ l Mmix



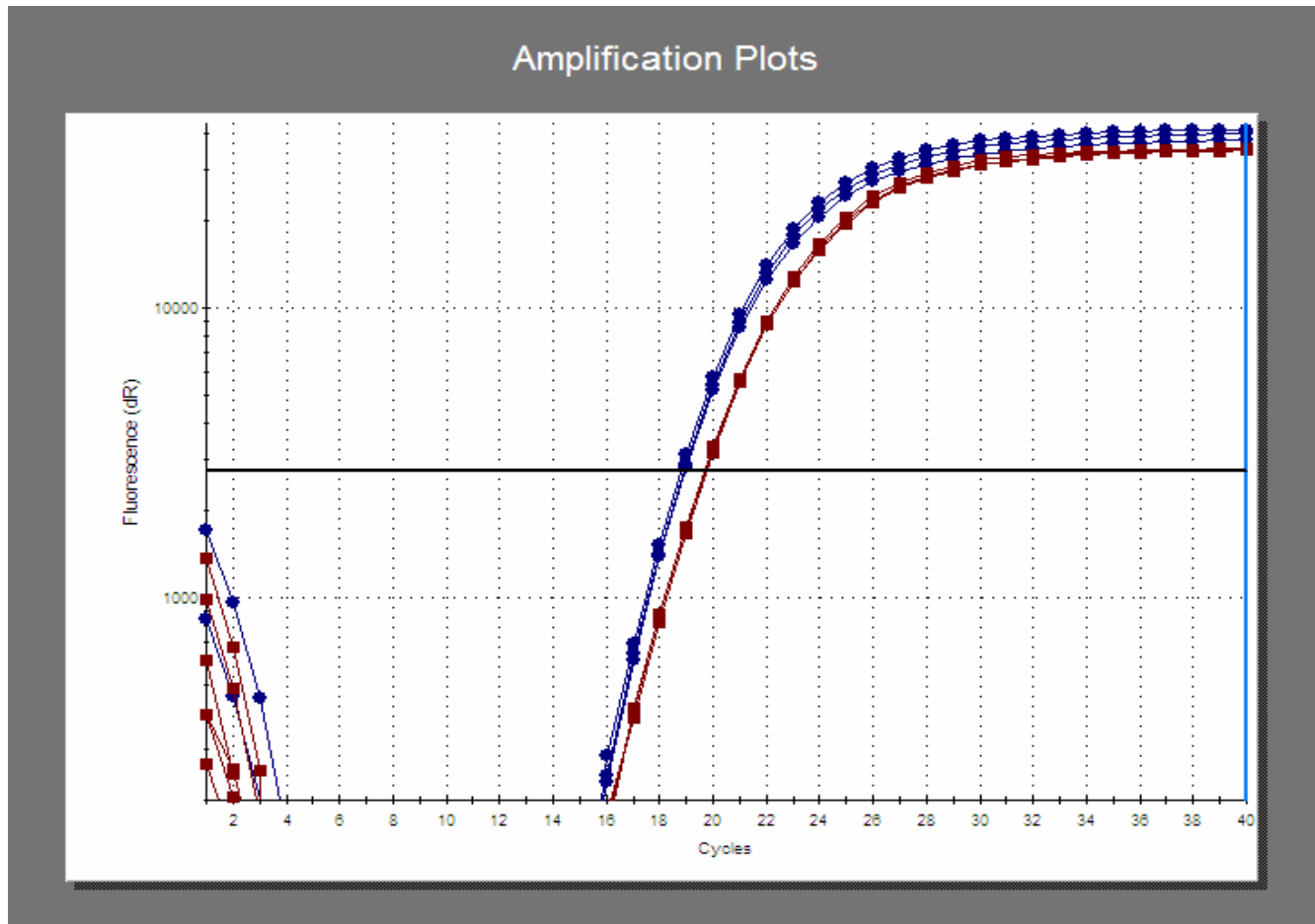
  Target A

  Target B

Results

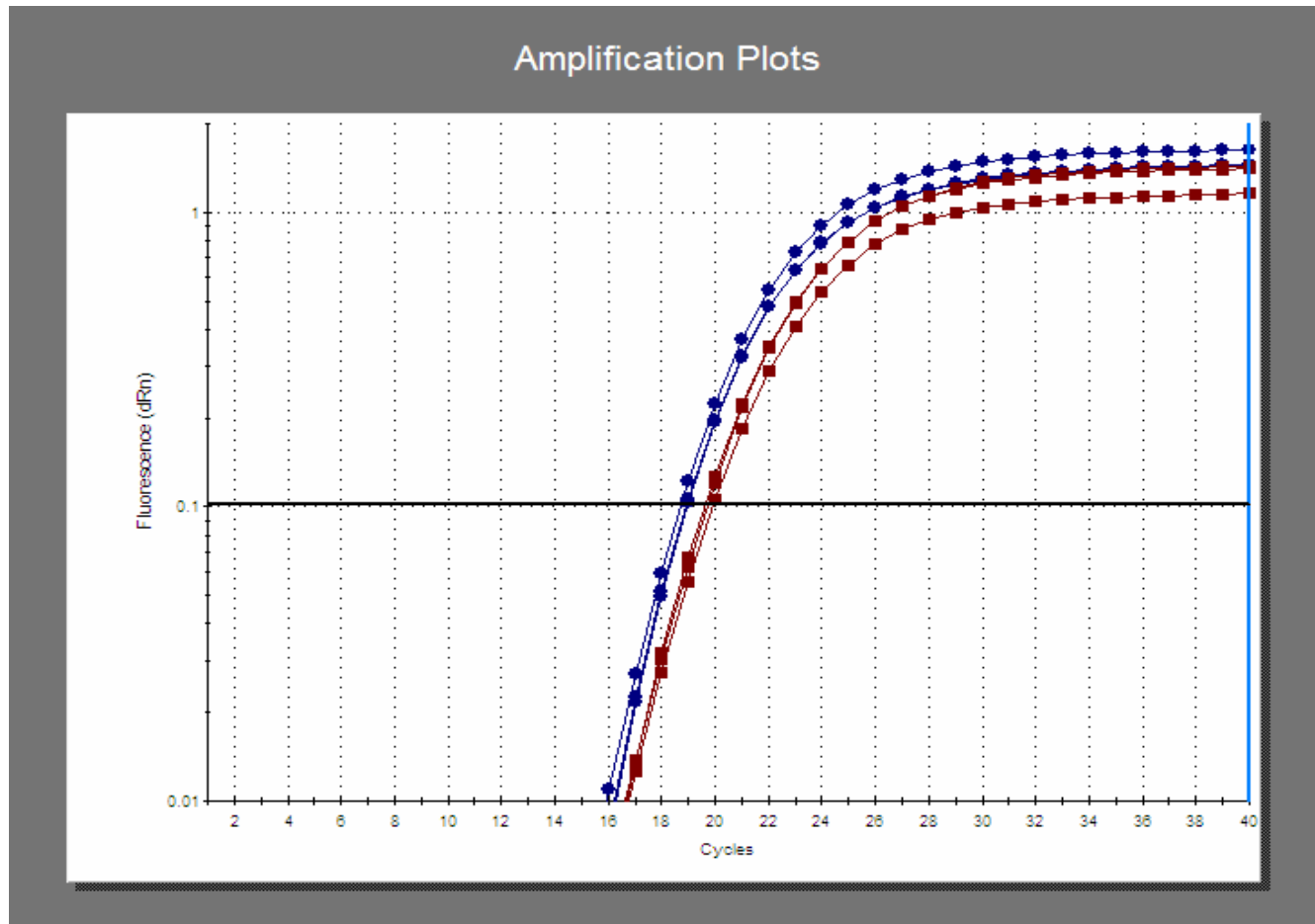
Amplification Plots

Without ROX, 5 μ l triplicates



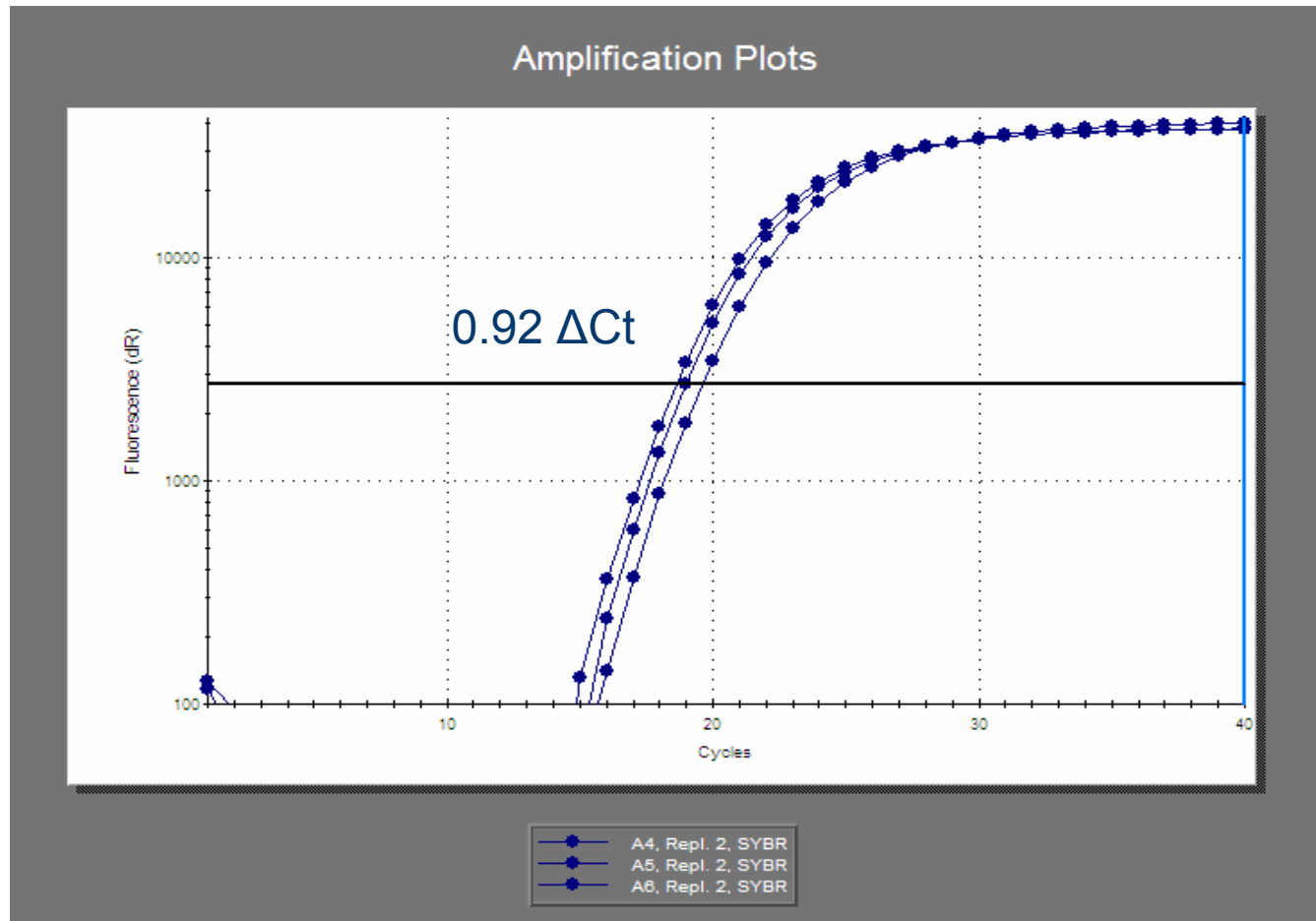
Amplification Plots

With ROX, 5 μ l triplicates



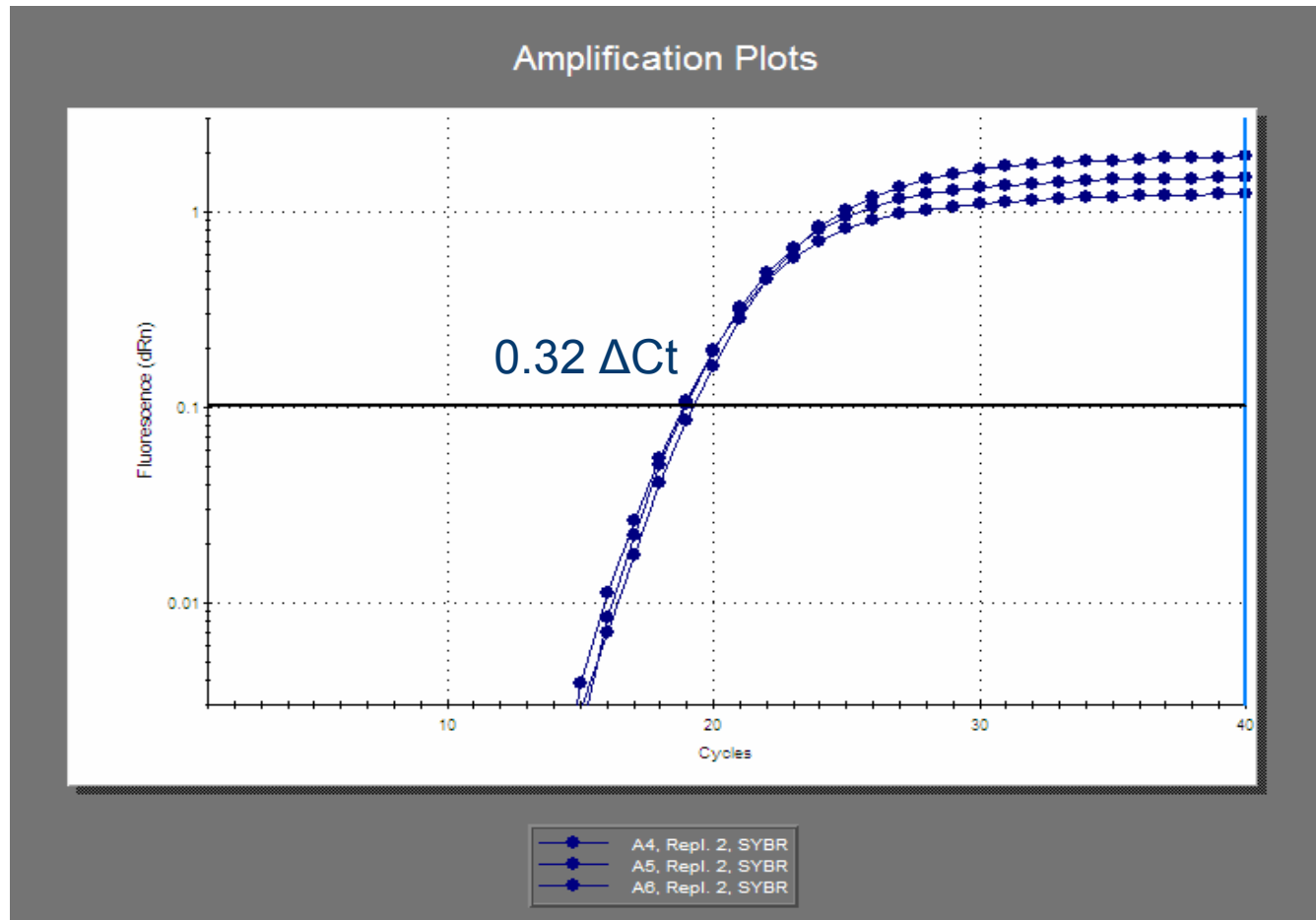
Amplification Plots Target A

Without ROX, 4, 5, 6 μ l



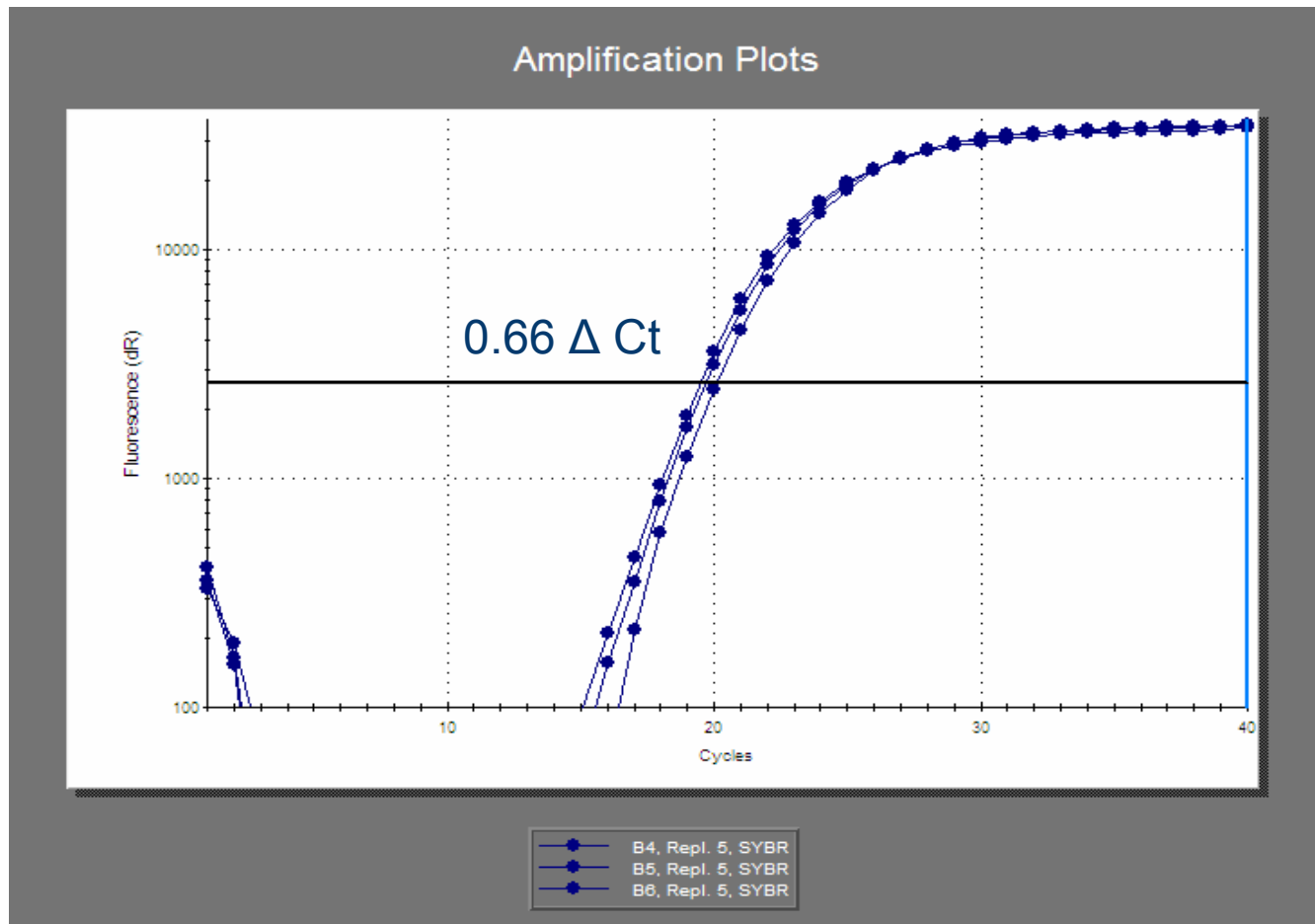
Amplification Plots Target A

With ROX, 4, 5, 6 μ



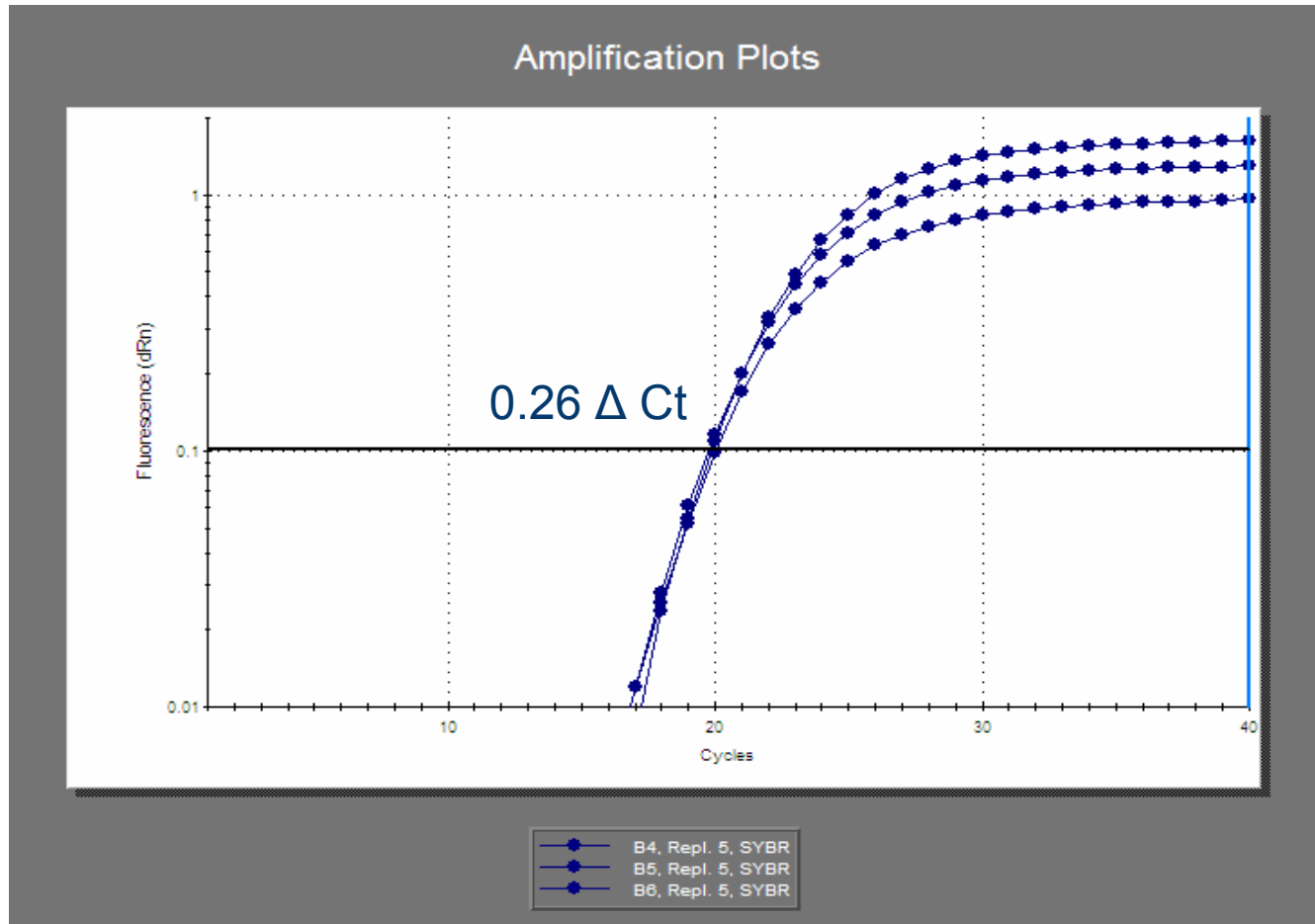
Amplification Plots Target B

Without ROX, 4, 5, 6 μ l



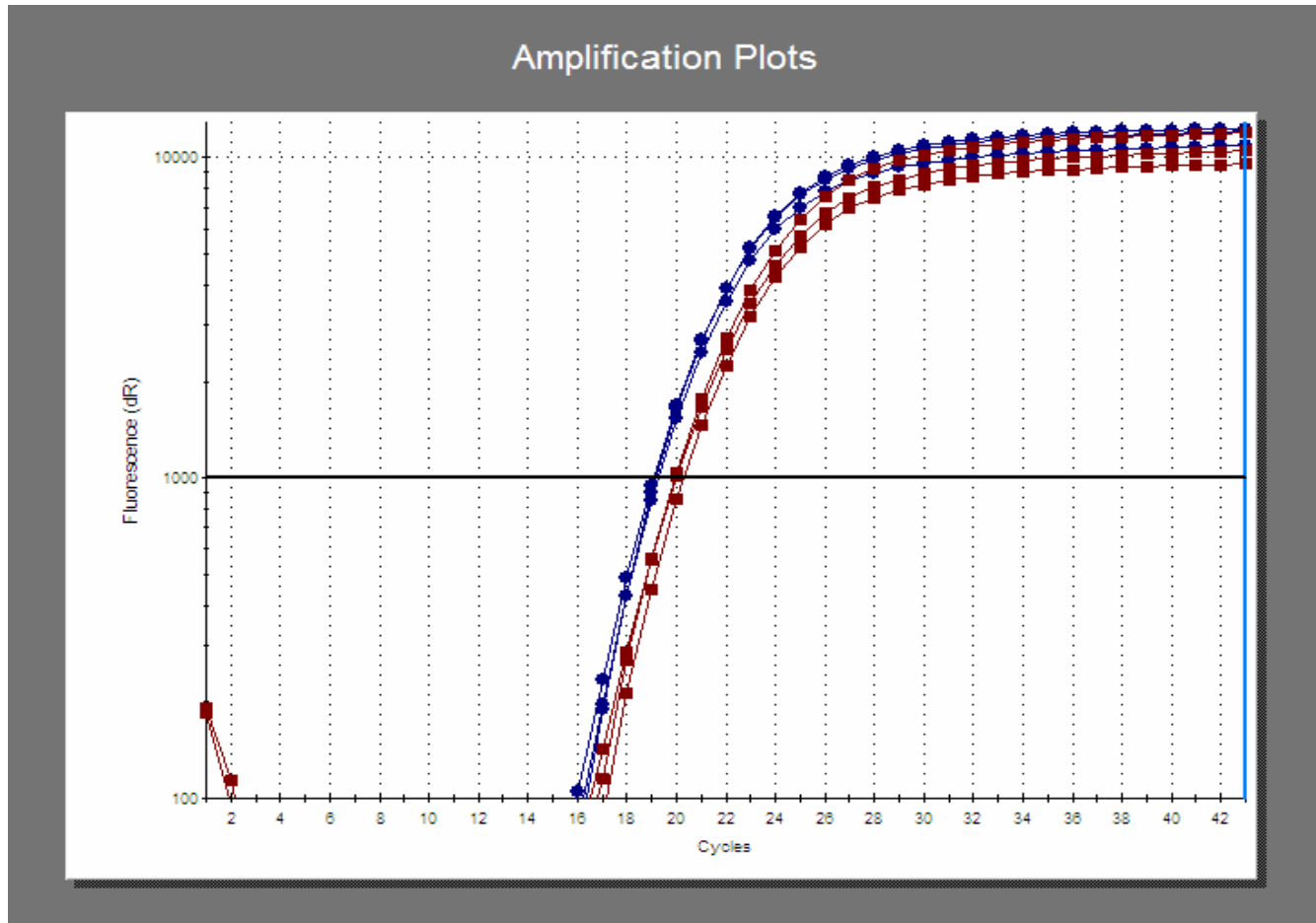
Amplification Plots Target B

With ROX, 4, 5, 6 μ l



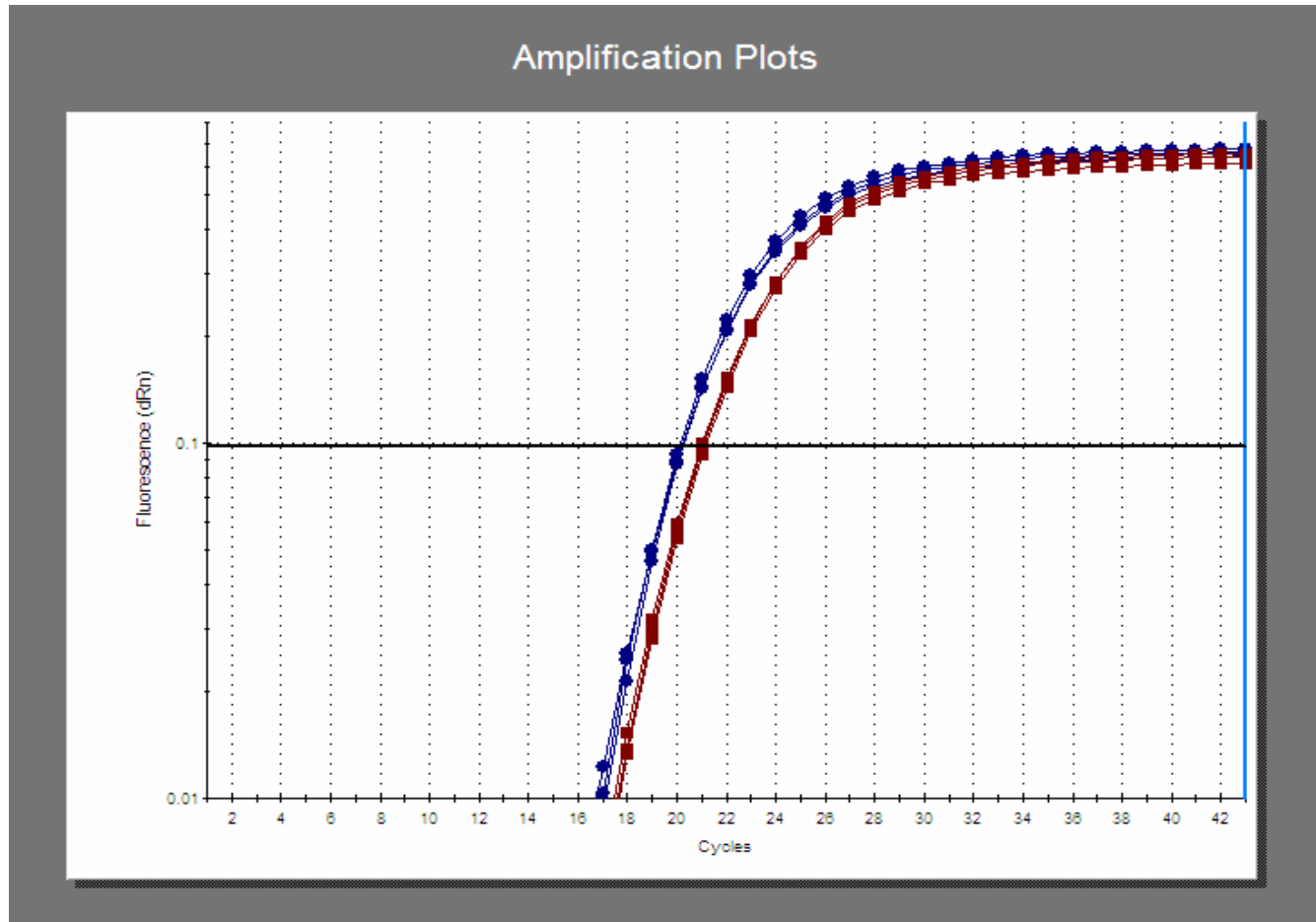
Amplification Plots

Without ROX 2 μ l triplicate



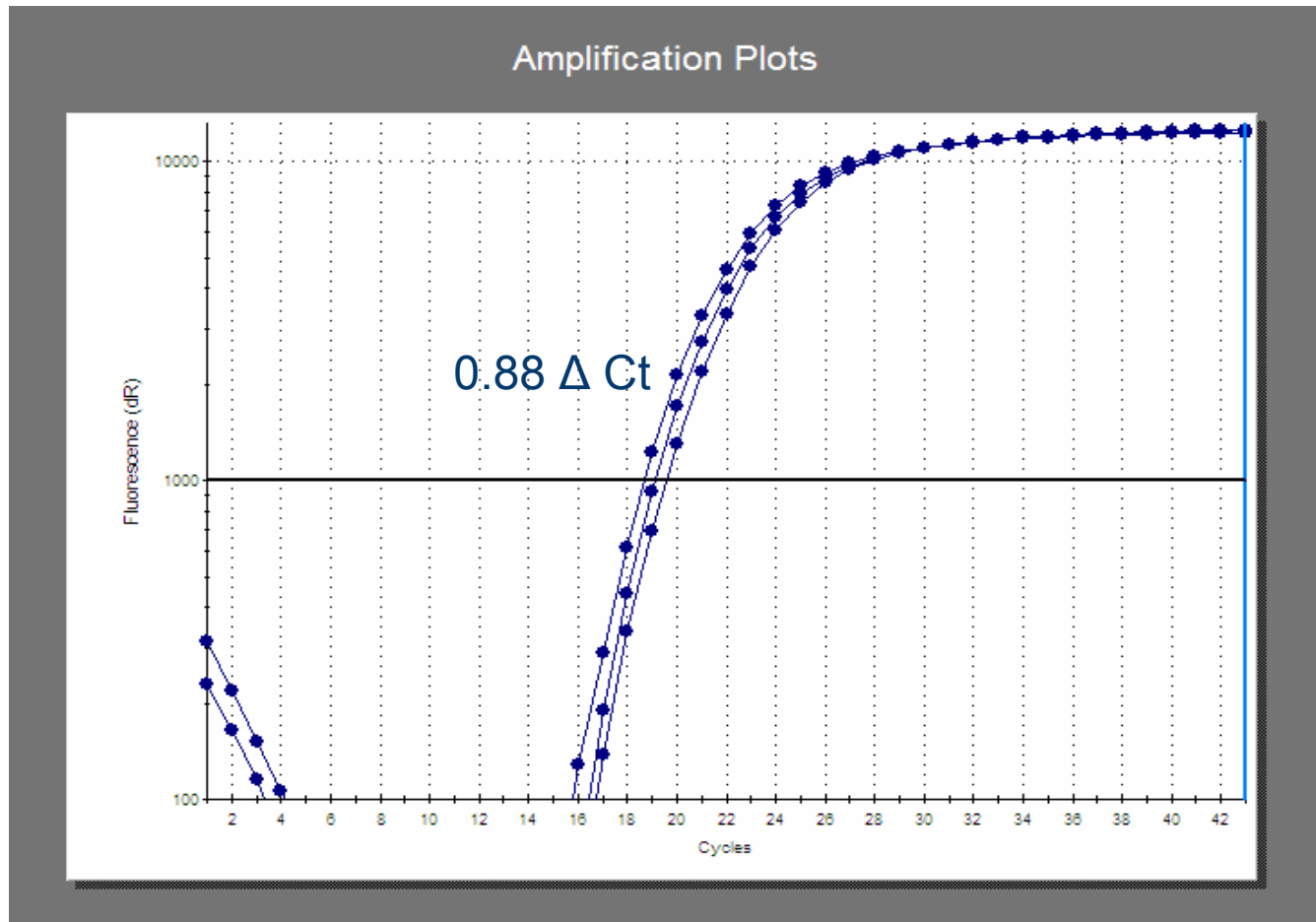
Amplification Plots

With ROX 2 μ l triplicate



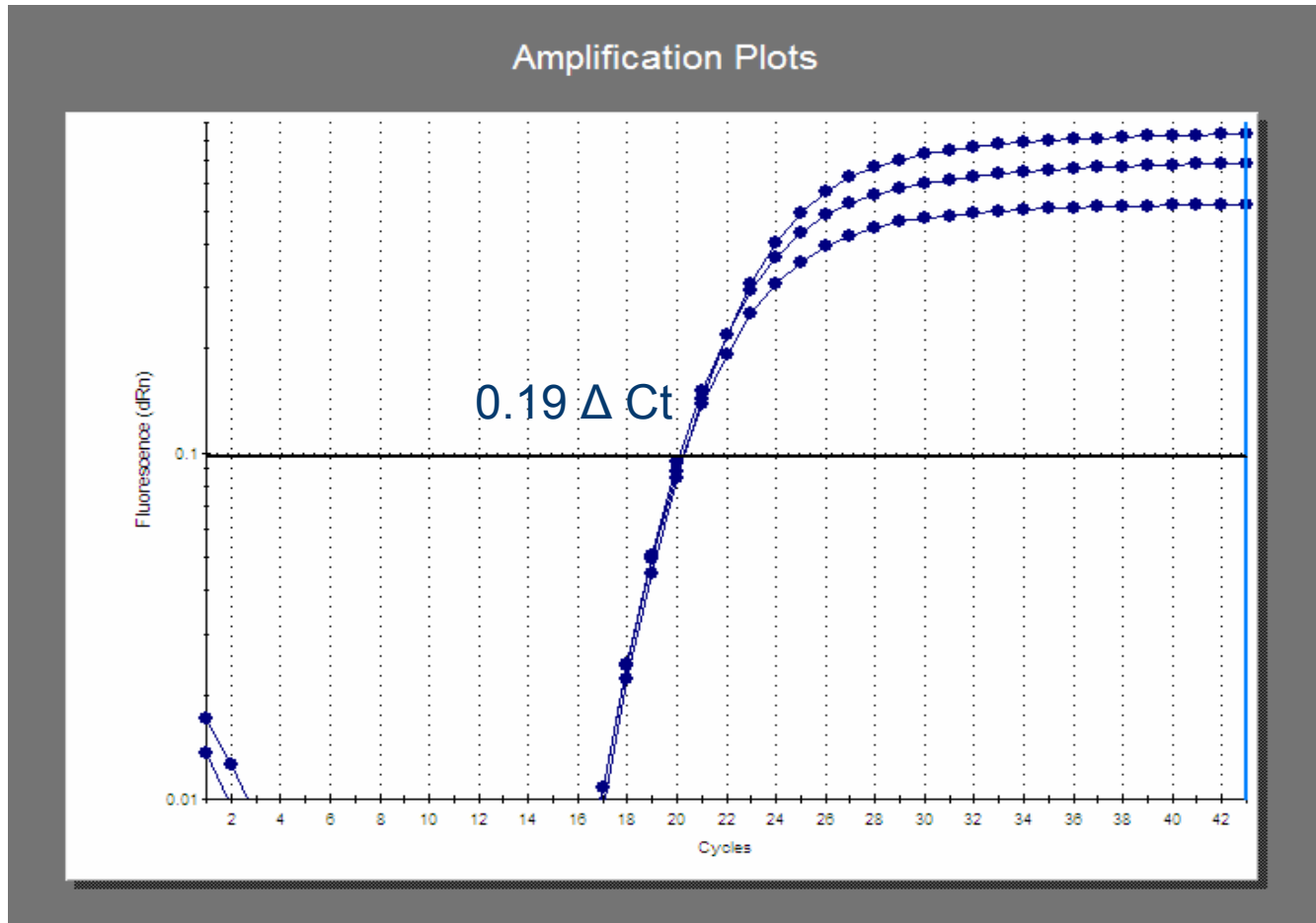
Amplification Plots Target A

Without ROX 1.5, 2, 2.5 μ l



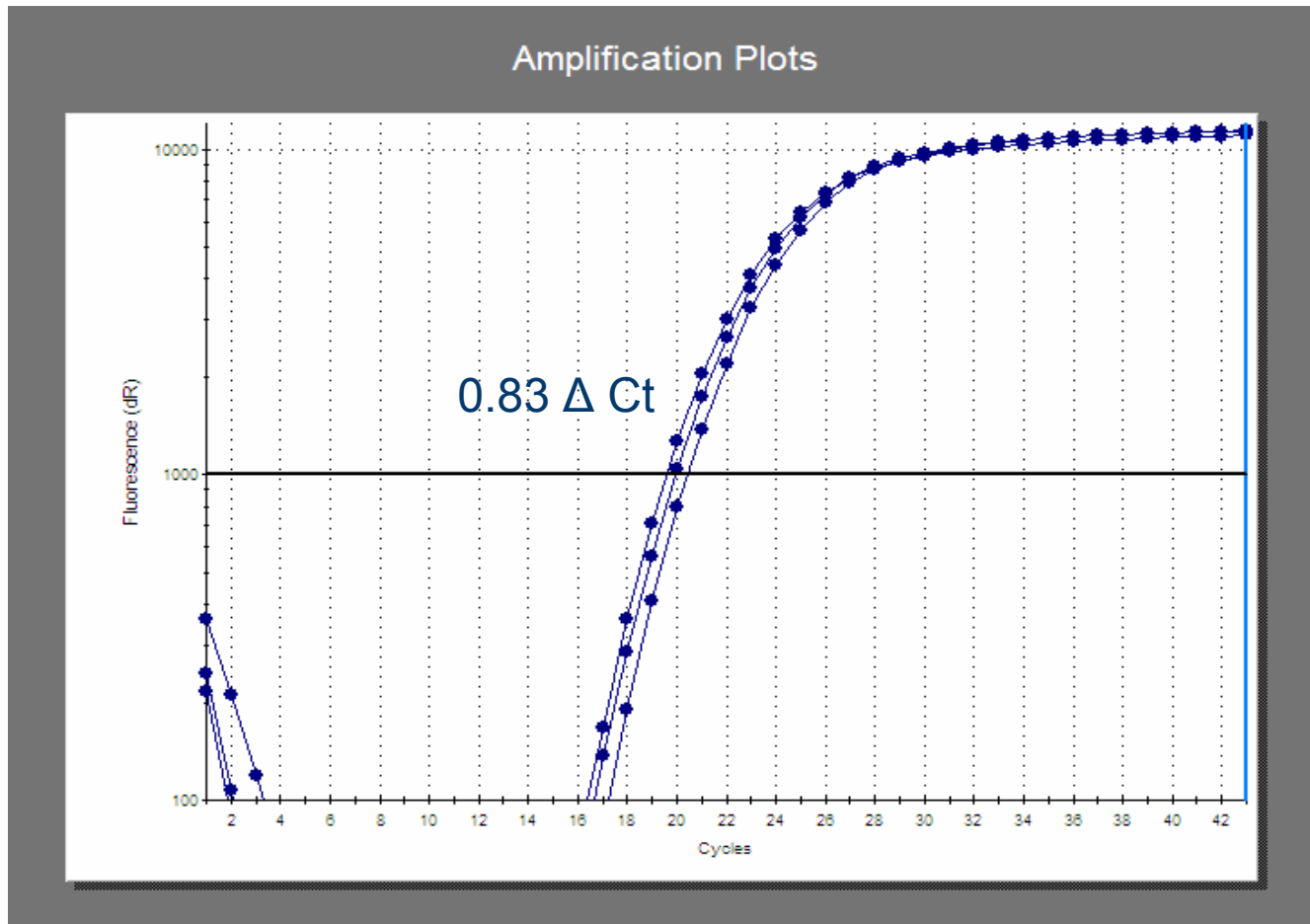
Amplification Plots Target A

With ROX 1.5, 2, 2.5 μ l



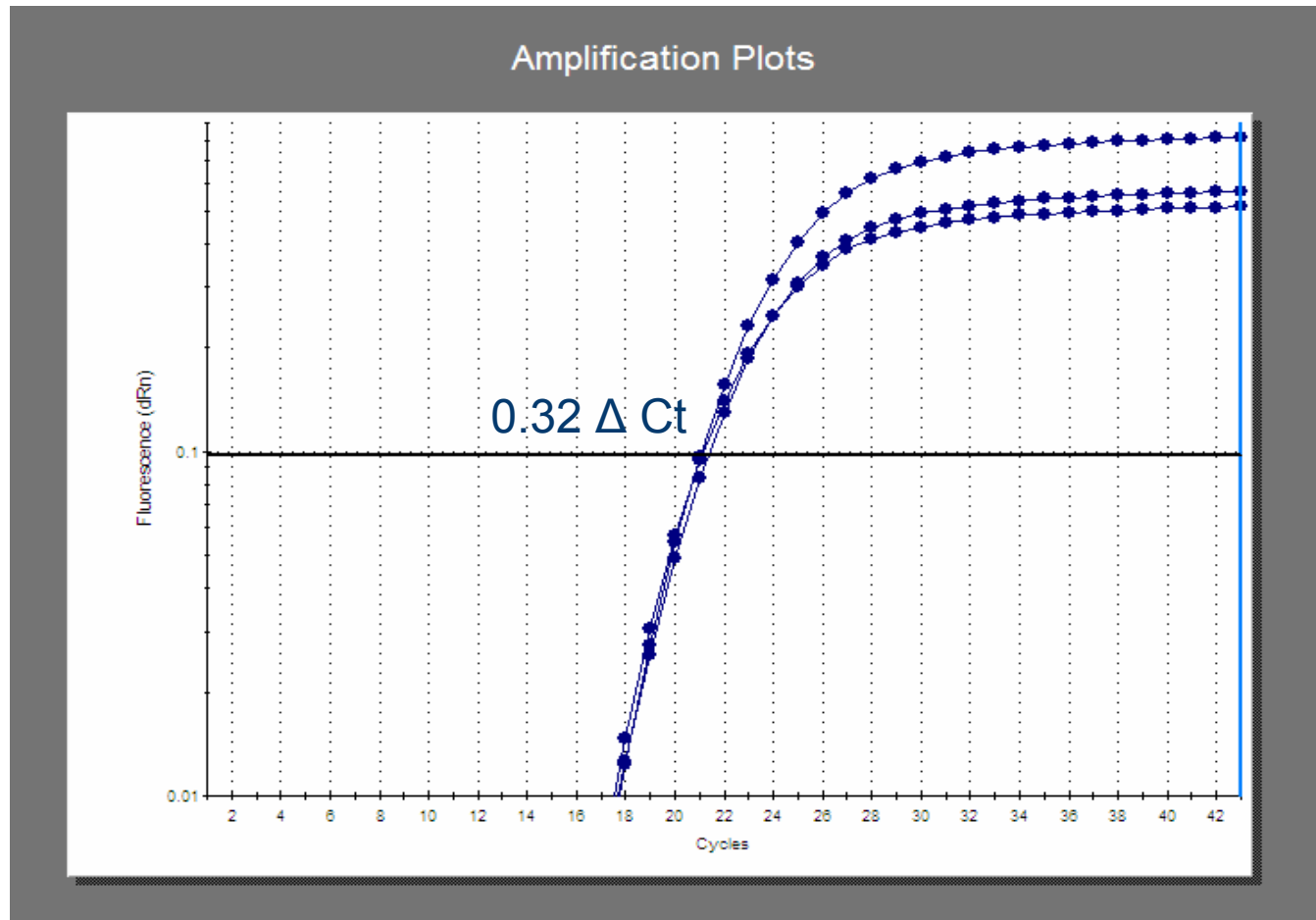
Amplification Plots Target B

Without ROX 1.5, 2, 2.5 μ l



Amplification Plots Target B

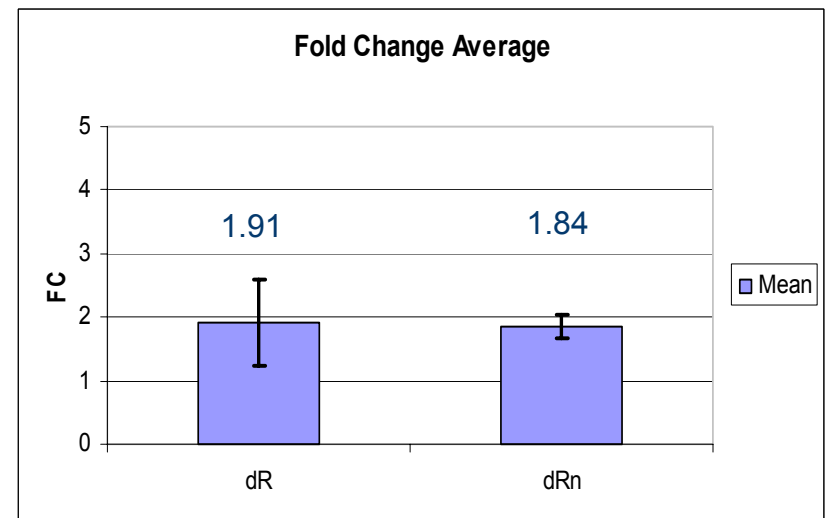
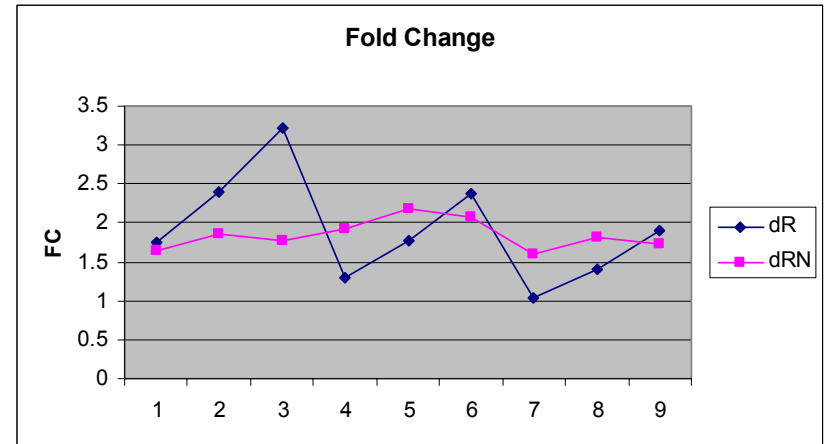
With ROX 1.5, 2, 2.5 μ l



Fold Change analysis between both targets (FC=1.83 based on triplicates dR)

$2^{-\Delta\Delta Ct}$ dR	1.5 μ l	2 μ l	2.5 μ l
1.5 μ l	1.75	2.39	3.23
2 μ l	1.29	1.77	2.38
2.5 μ l	1.04	1.41	1.91

$2^{-\Delta\Delta Ct}$ dRn	1.5 μ l	2 μ l	2.5 μ l
1.5 μ l	1.65	1.87	1.78
2 μ l	1.92	2.17	2.07
2.5 μ l	1.60	1.82	1.73



Conclusion

- ROX can be used for pipetting normalisation even for mistakes of up to 25%
- However it seems that mixing ROX directly with DNA adds some variability
- Pure ROX might not be the best solution
- Next step is to test with a labelled oligo

Perspectives

This new way to use the ROX normalisation could overcome the need for replicates and increase the overall precision of real time PCR experiments run on asynchronous reading instruments

Acknowledgment

- Nanhua Cheng
- Jenny Peters



Thank you!