SIGMA-ALDRICH

CORPORATION

Accurate gene expression profiling – Facing the issues of normalisation and efficiency

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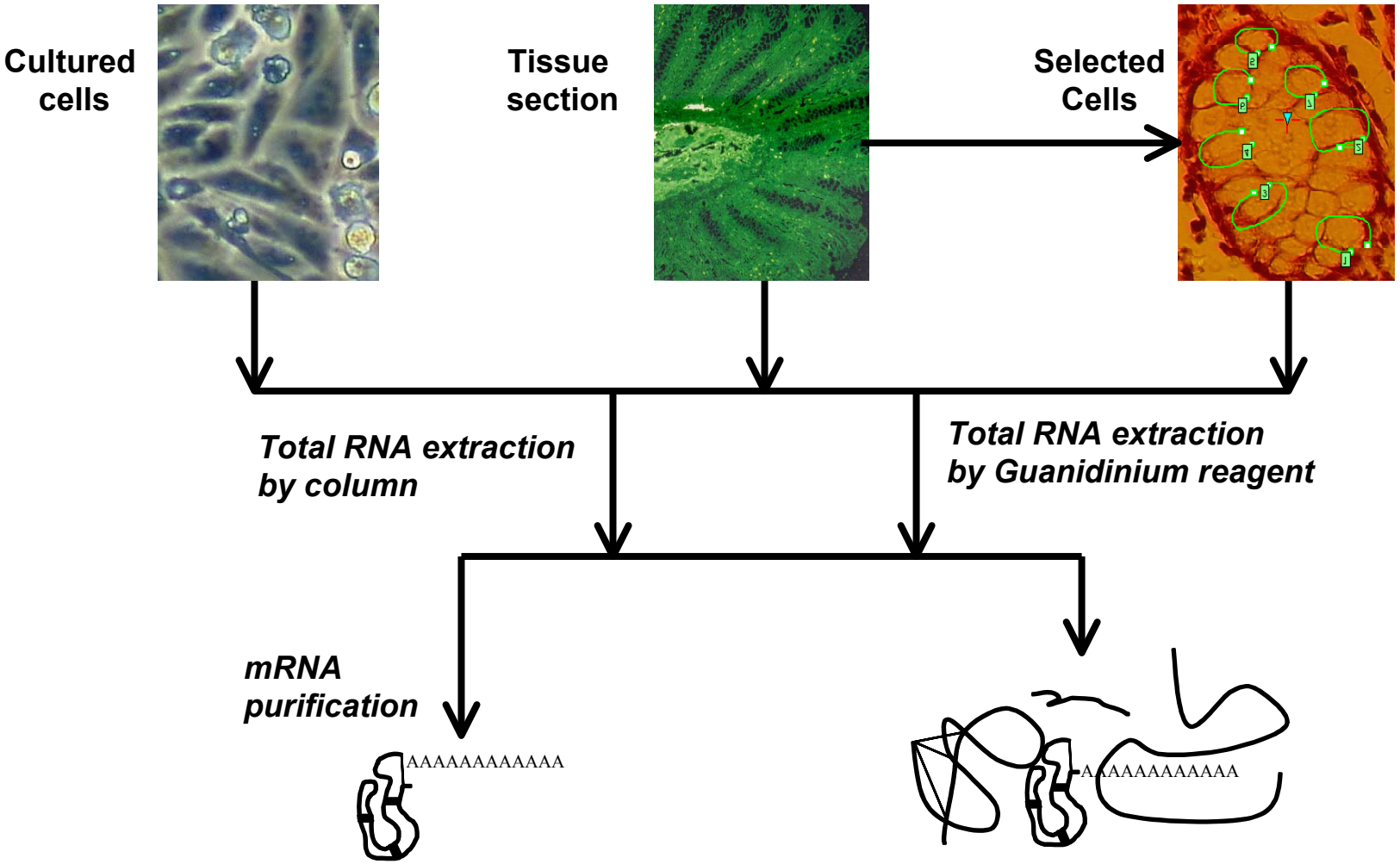
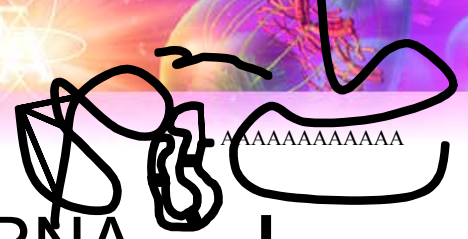
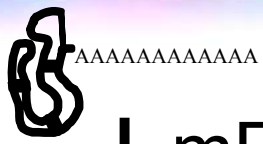
Variations in RNA extraction





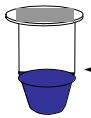
Total RNA



mRNA

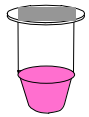
One tube assay

Two tube assay



target-specific primers

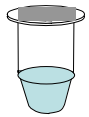
Tth **polymerase or RTase + *Taq* pol**



oligo-dT ***or***
random primers ***or***
random primers and oligo-dT ***or***
target-specific primers

RT priming

Reverse Transcriptase

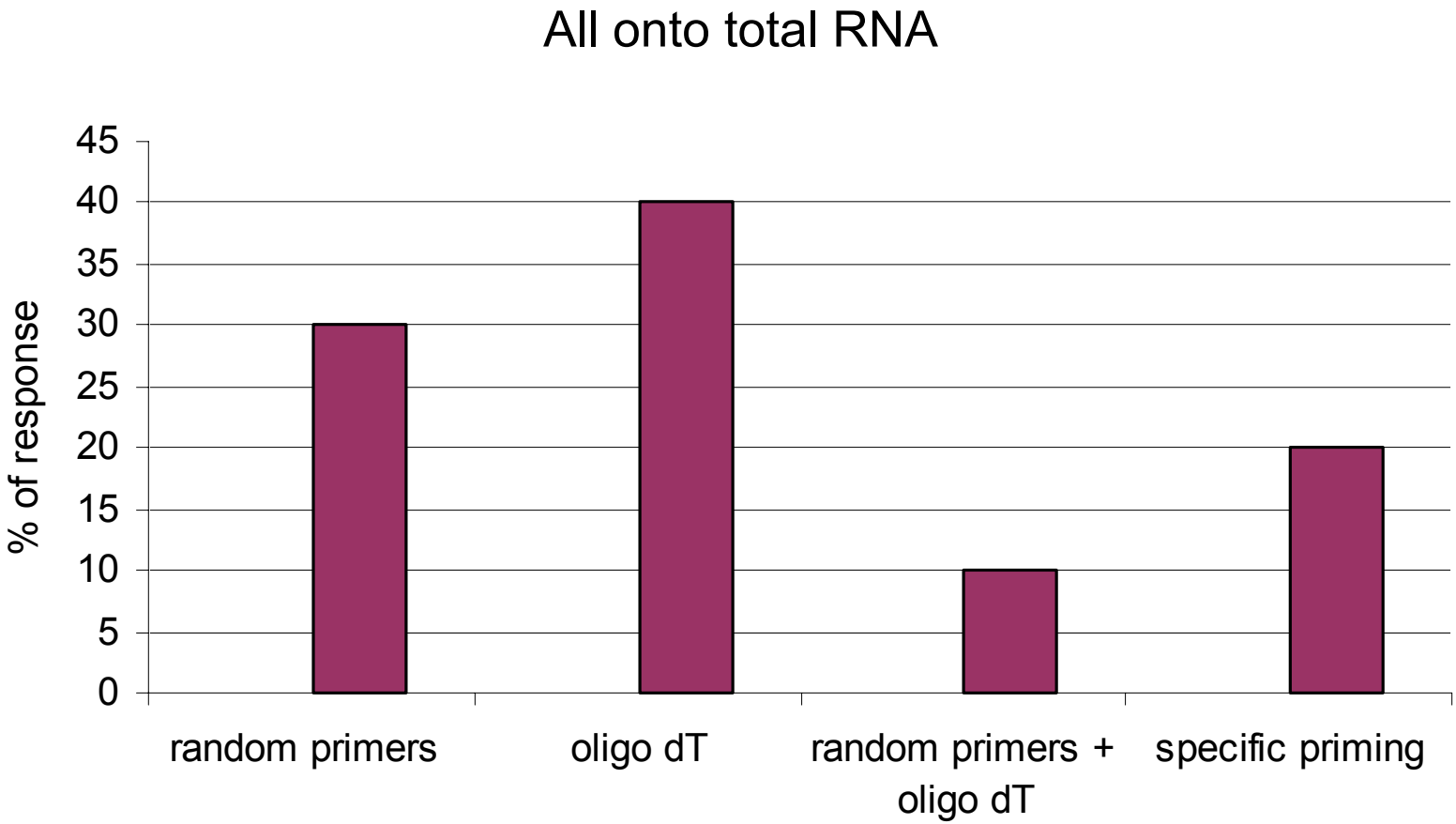


Target-specific PCR primers

Taq polymerase

QPCR

Favourite QRT-PCR Priming strategies



Data from participants of yahoo groups; qpcrlistserver
Bustin, Benes, Nolan, Pfaffl (2005) JME in press

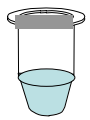
Total RNA

Two tube assay

oligo-dT *or*
random primers

RT priming

Reverse Transcriptase

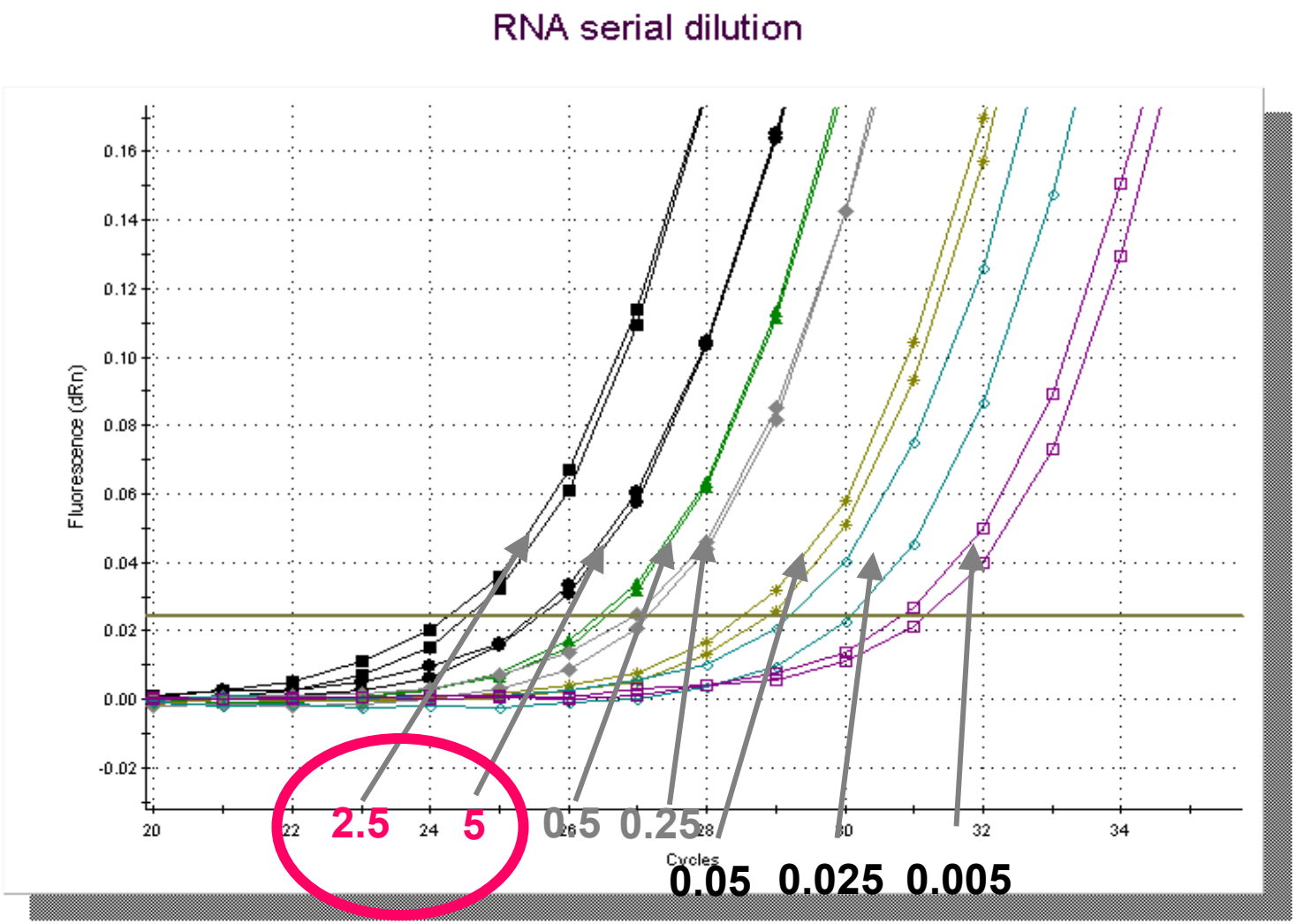


Target-specific PCR primers and fluorescent probe

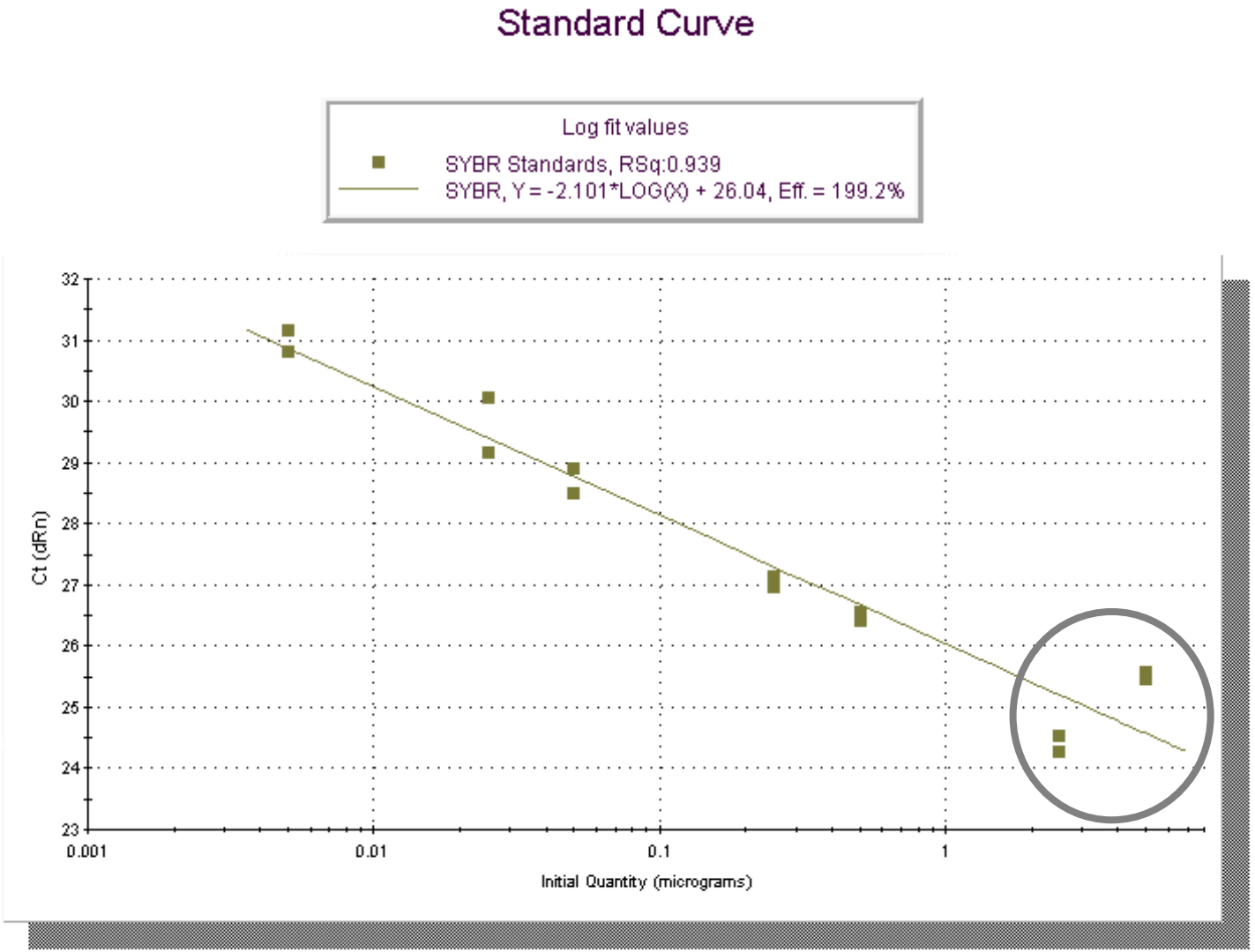
Taq polymerase

QPCR

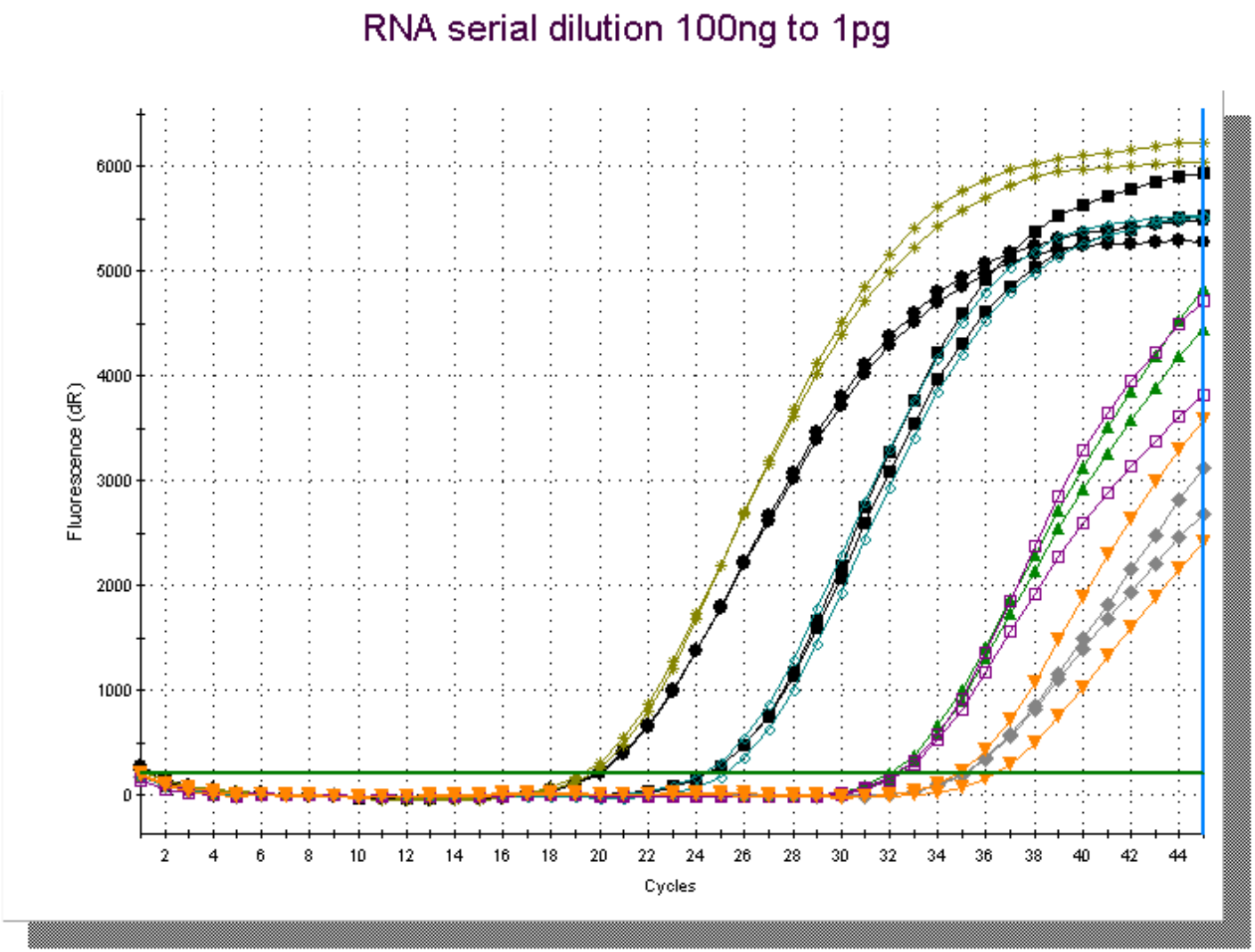
Random primed RNA (2x) dilution series (QPCR NHE1)



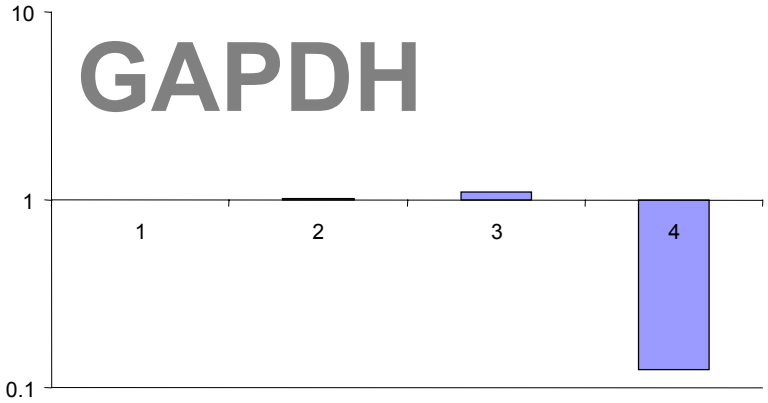
Random primed RNA (2x) dilution series (QPCR NHE1)

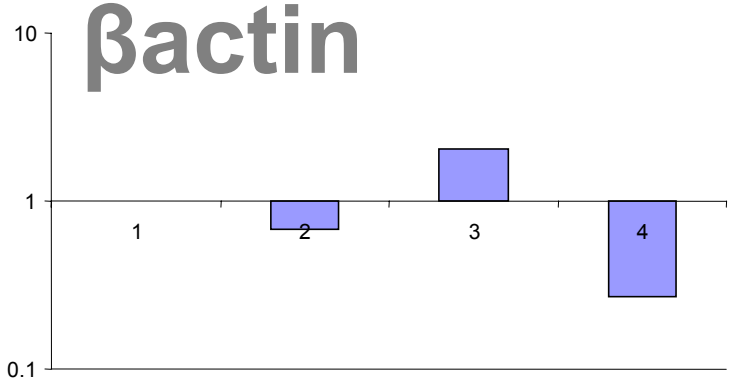


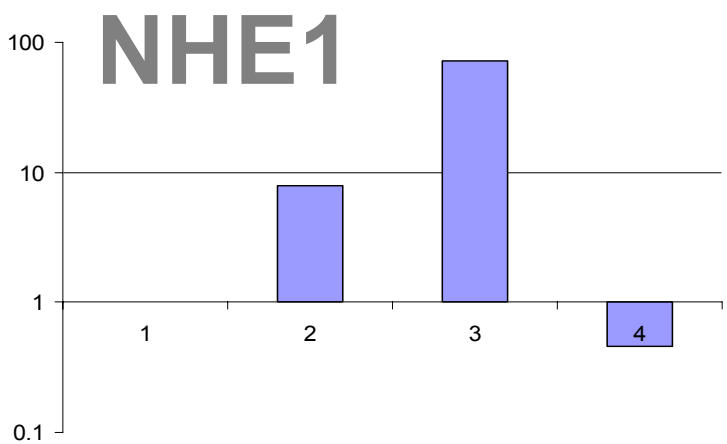
Random Priming RT and QPCR (100 fold dilutions, ß-actin) is reproducibly none linear



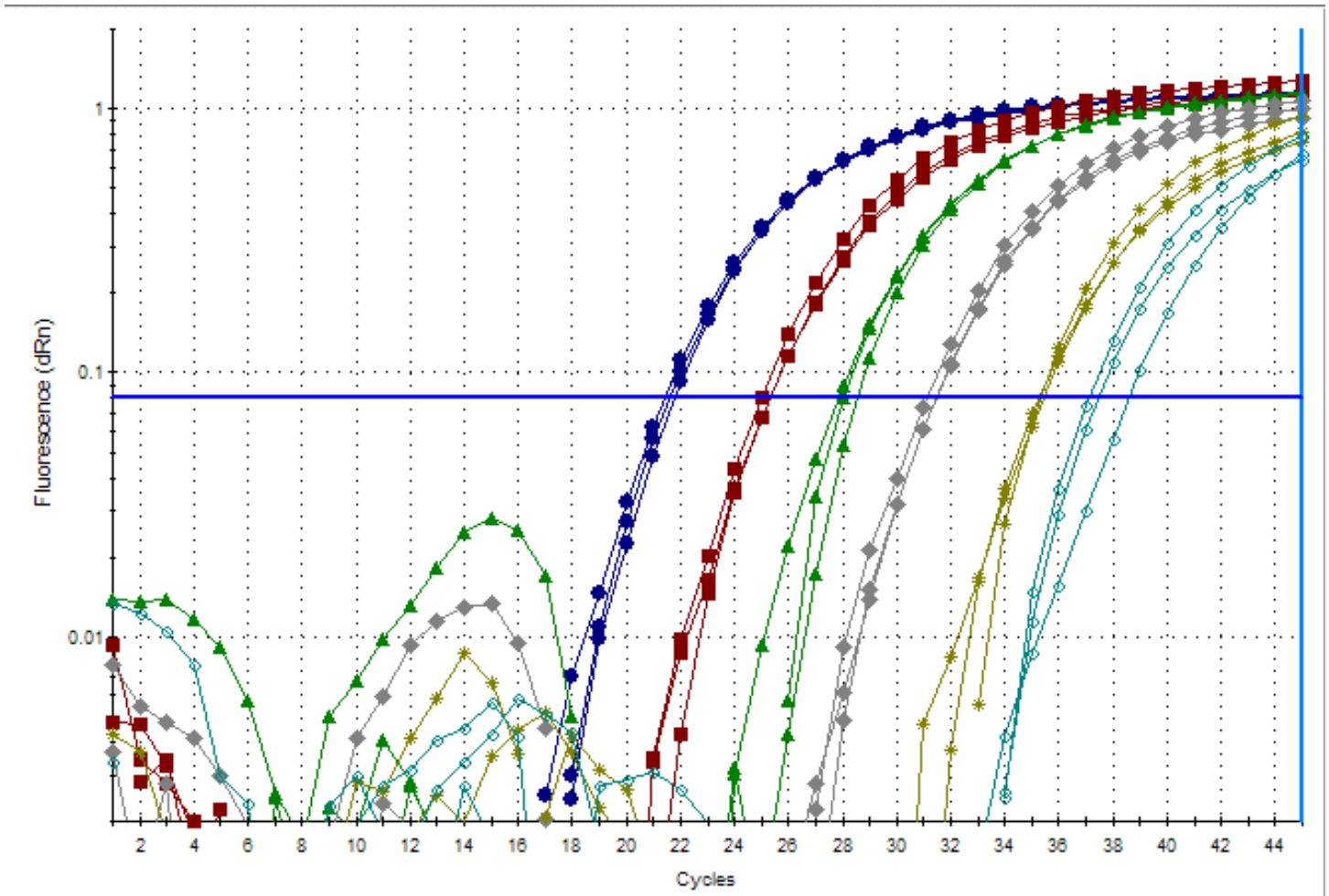
Gene quantification is not reproducible between different RT reactions







Gene specific priming RT and QPCR (10 fold dilutions, GAPDH)



From RNA to cDNA to gene expression data

- Random priming of total RNA dilutions did not give a linear response
- ✓Random primed RT reactions appear to be reproducibly none linear from reaction to reaction when run together
- Random primed RT reactions do not appear to be reproducible from reaction to reaction when run on different occasions
- ✓Specific priming of RNA dilutions appeared to give a linear response

From RNA to cDNA to gene expression data

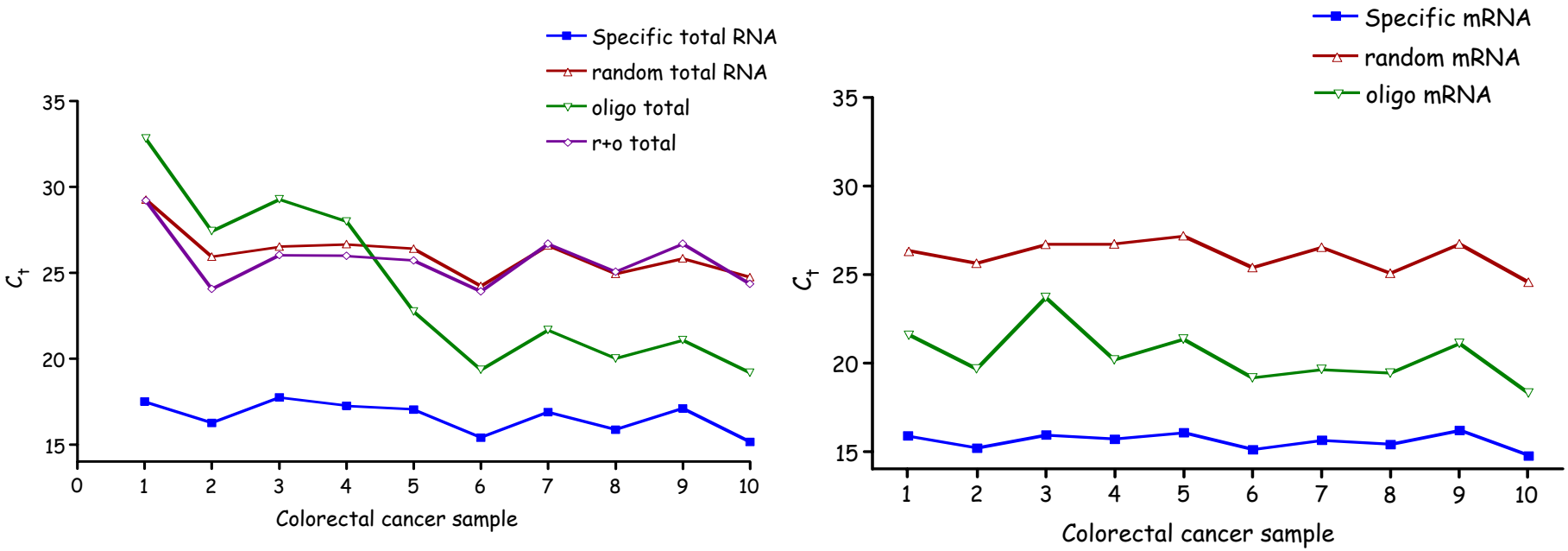
Further Study:

- **Investigate different RT priming strategies**
- **Investigate priming total RNA and mRNA**
- **Investigate the effects of these variables on data interpretation**
- **Investigate effect of template structure on RT- QPCR sensitivity and efficiency**

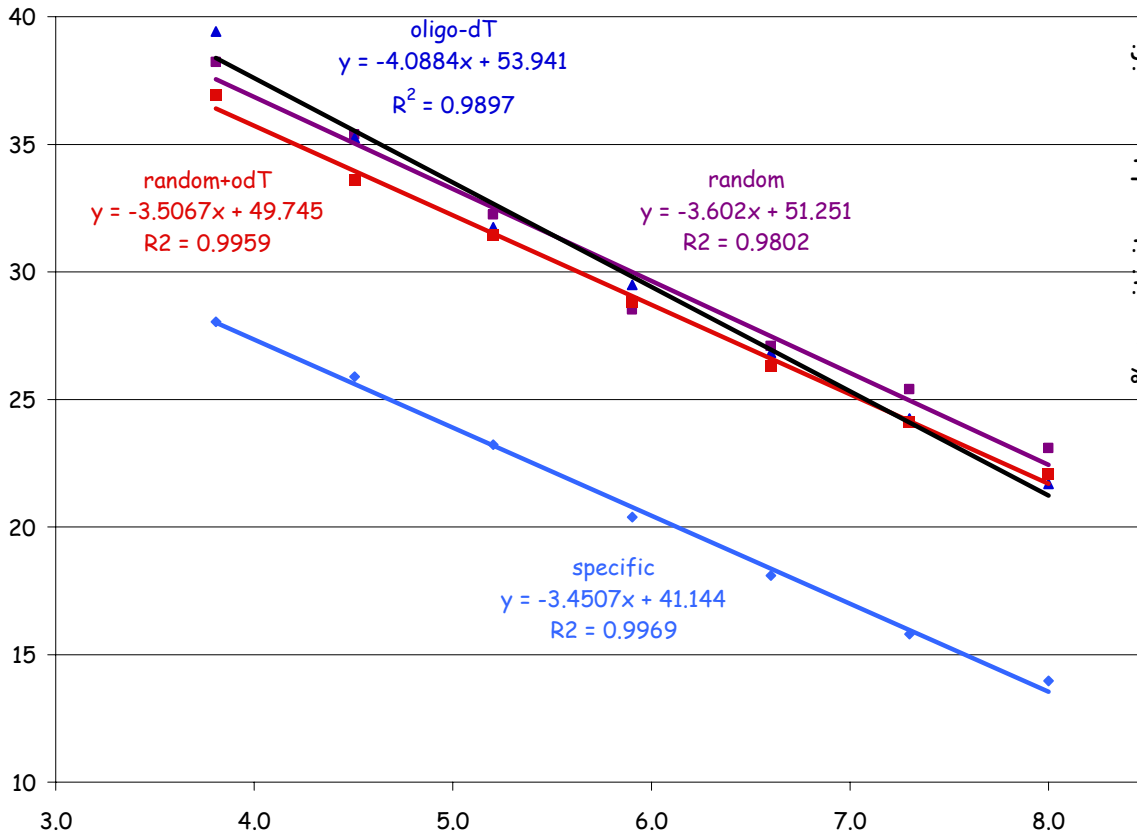
Comparing RT Priming Strategies

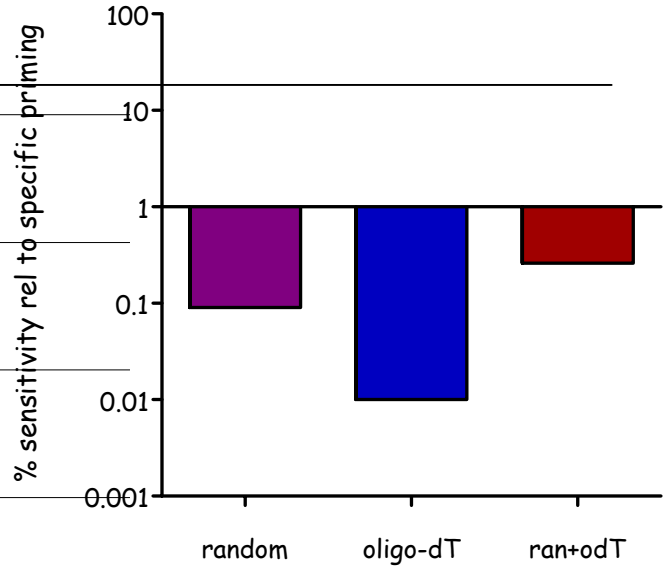
GAPDH

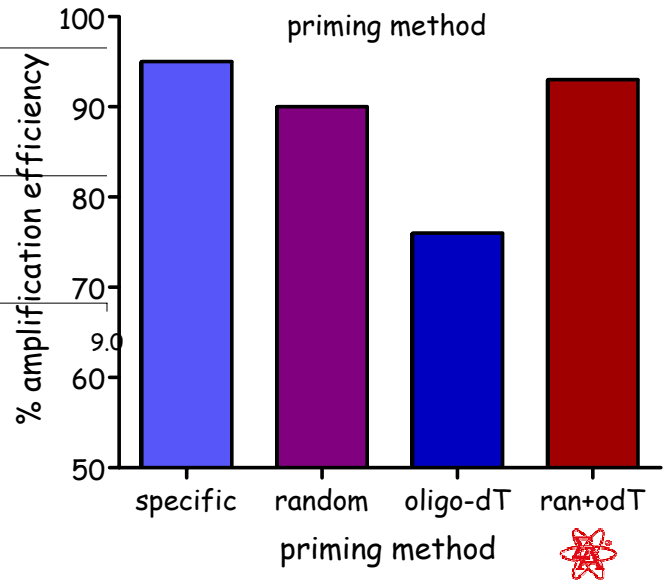
Constant RNA input concentration



Sensitivity and efficiency using mRNA - GAPDH







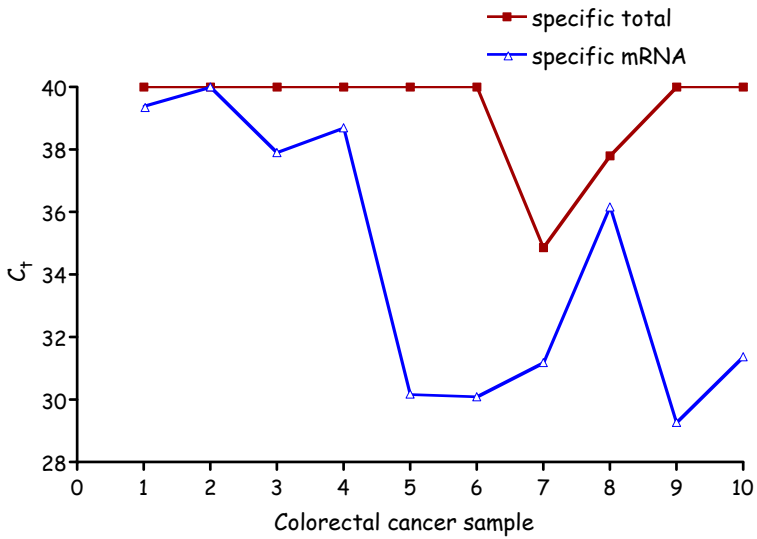
GAPDH - mRNA serial dilution

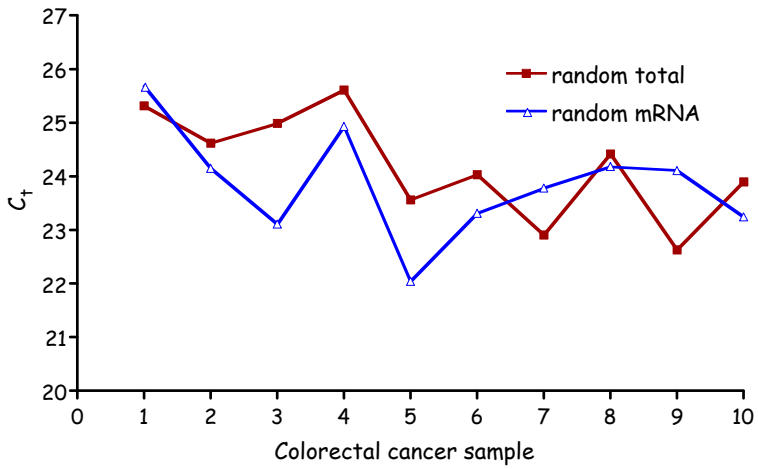
From RNA to cDNA to gene expression data - GAPDH

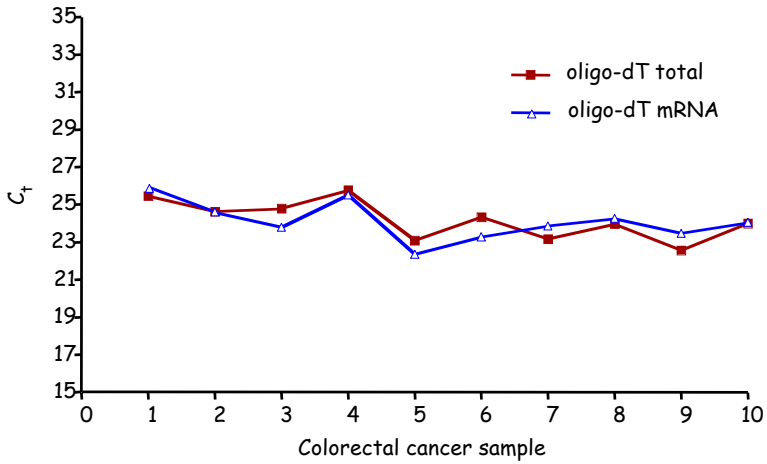
GAPDH Comparisons:

- **Using total or mRNA specific priming appears most sensitive**
- **When priming strategies are compared using mRNA serial dilutions specific priming appears most sensitive and results in highest amplification efficiency**

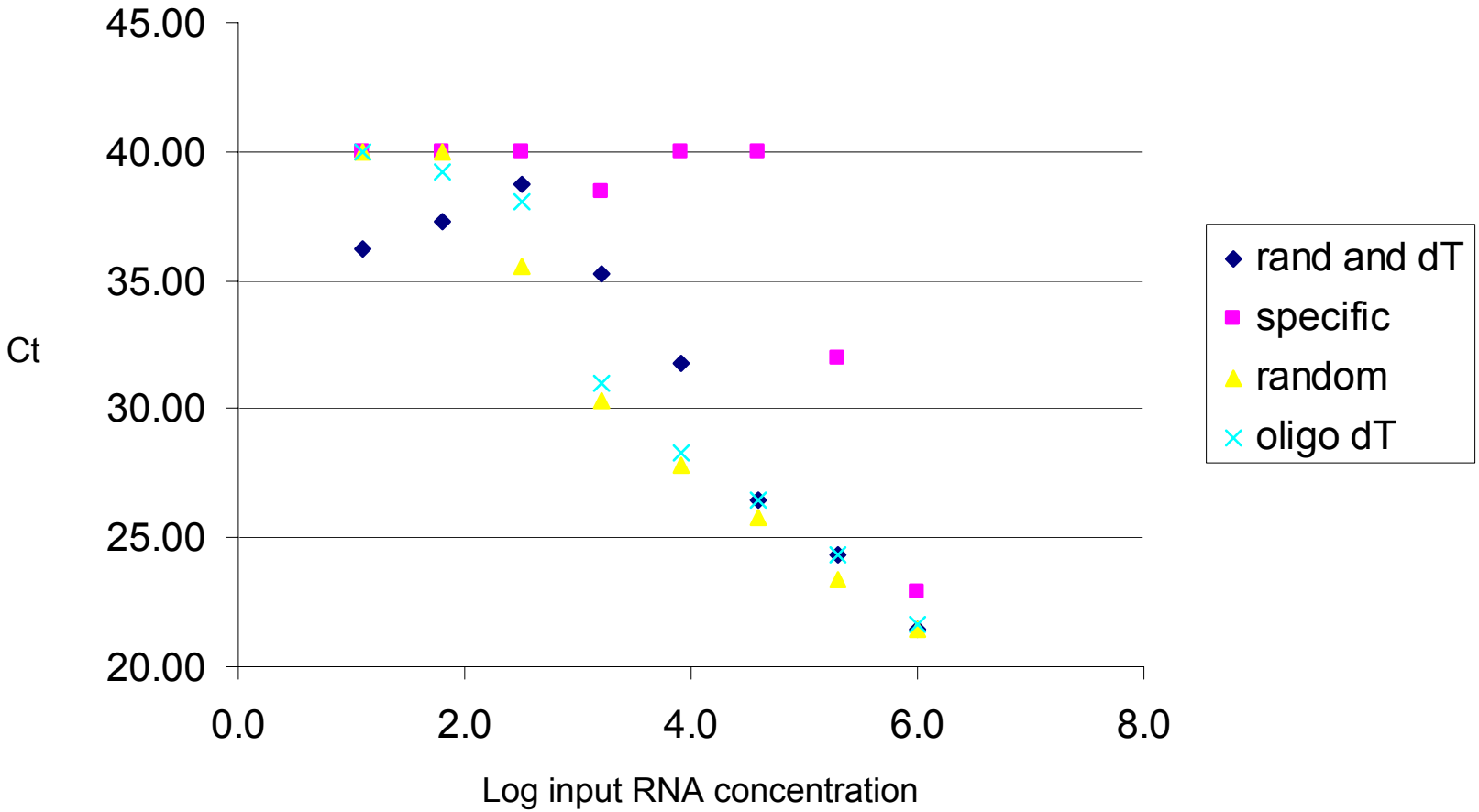
Total RNA vs mRNA - IGF-I



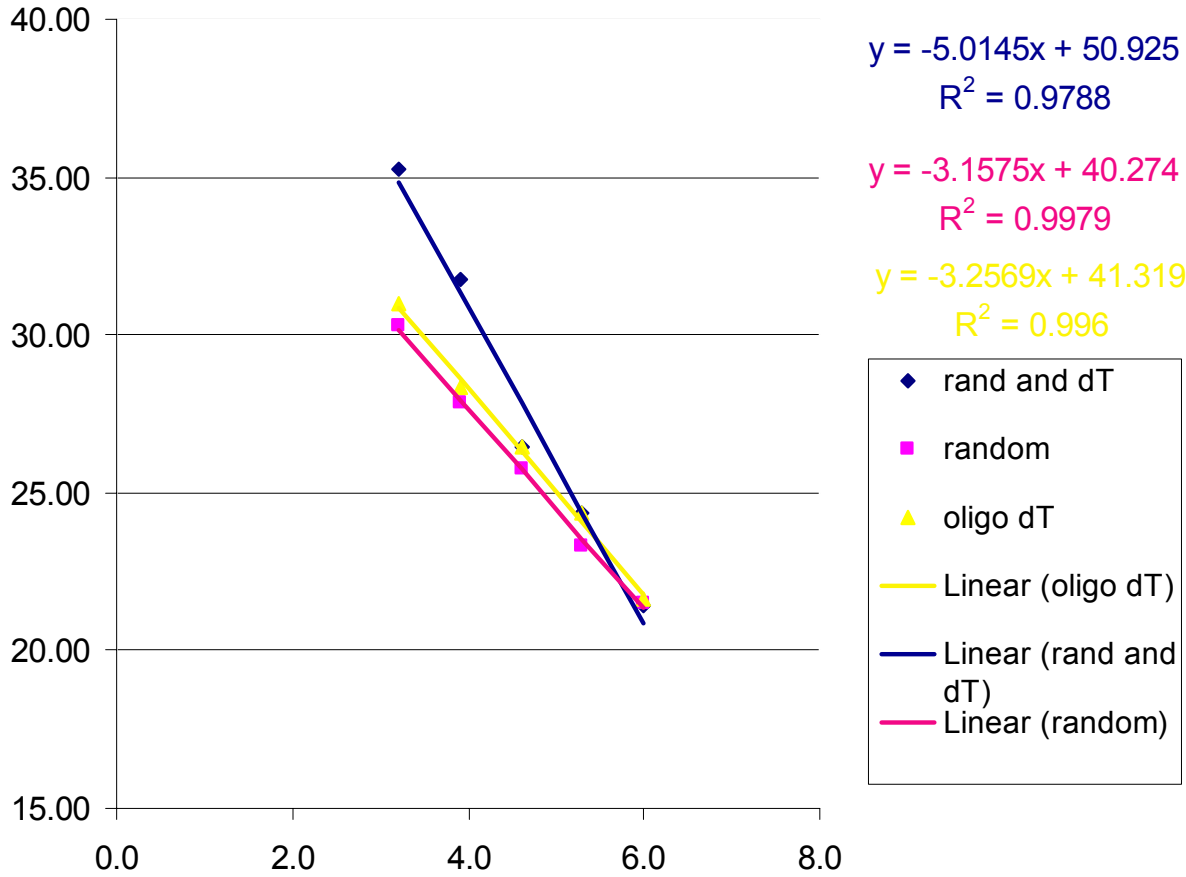




Sensitivity and efficiency using mRNA – IGF1



Sensitivity and efficiency using mRNA – IGF1

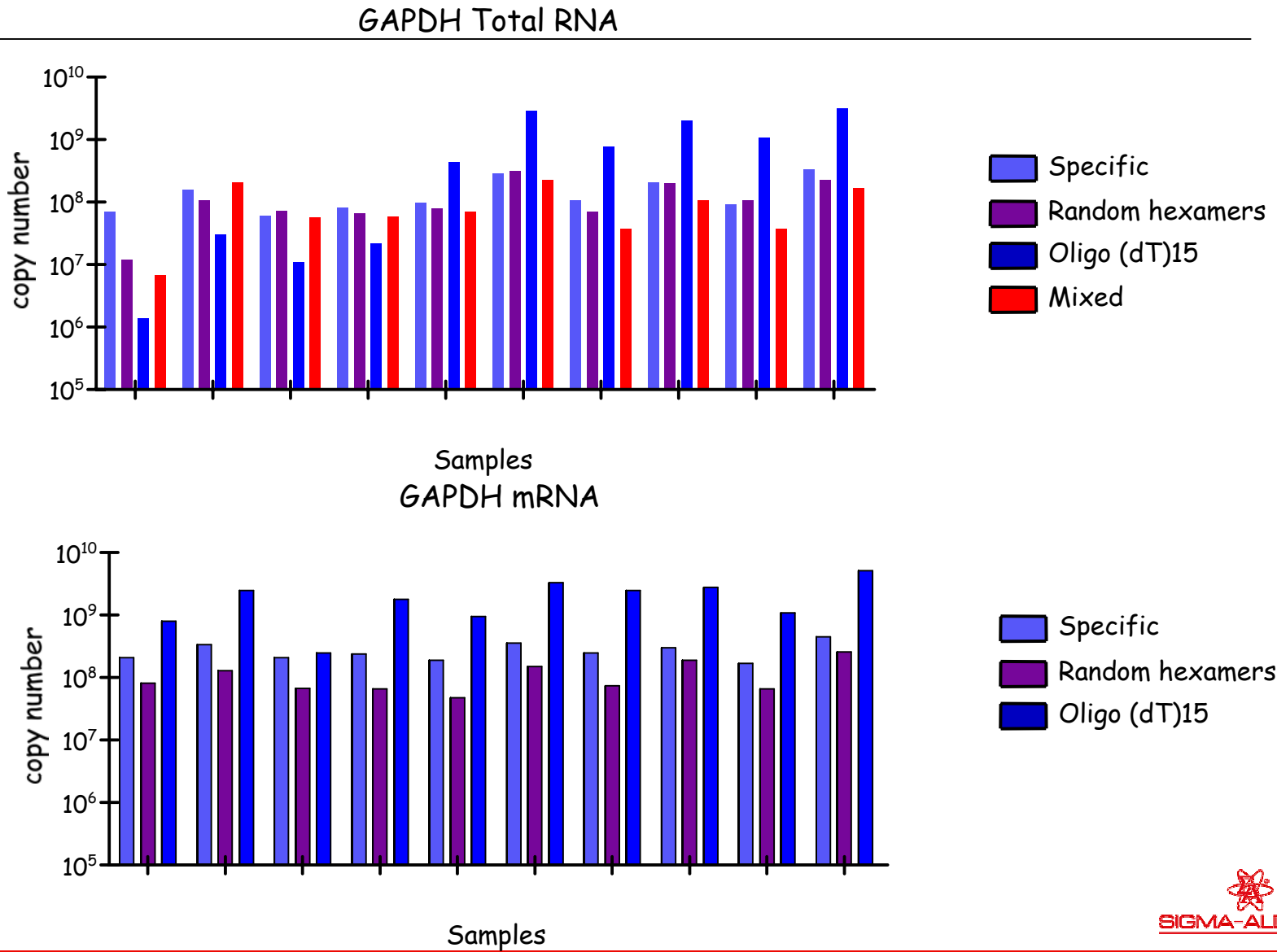


From RNA to cDNA to gene expression data – IGF1

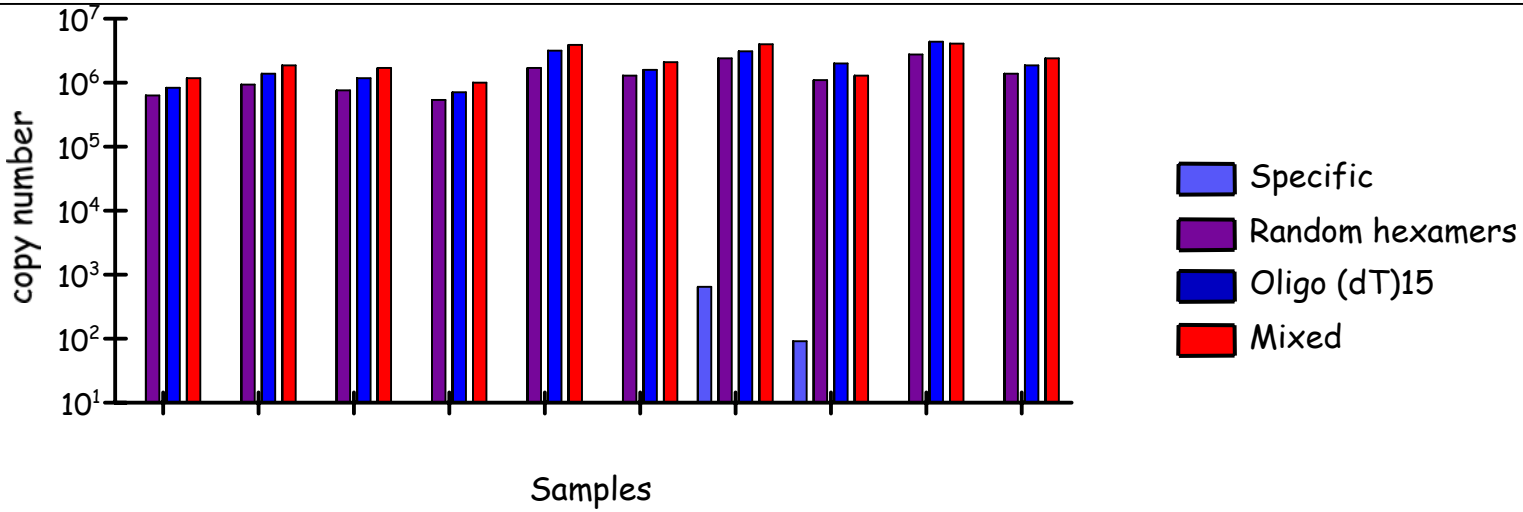
IGF1 Comparisons:

- **All priming strategies relatively insensitive**
- **Using total or mRNA Oligo dT priming appears most sensitive**
- **When priming strategies are compared using mRNA serial dilutions Oligo-dT or random priming appear most sensitive and results in highest amplification efficiency**
- **Specific priming is very poor (slight improvement on mRNA)**

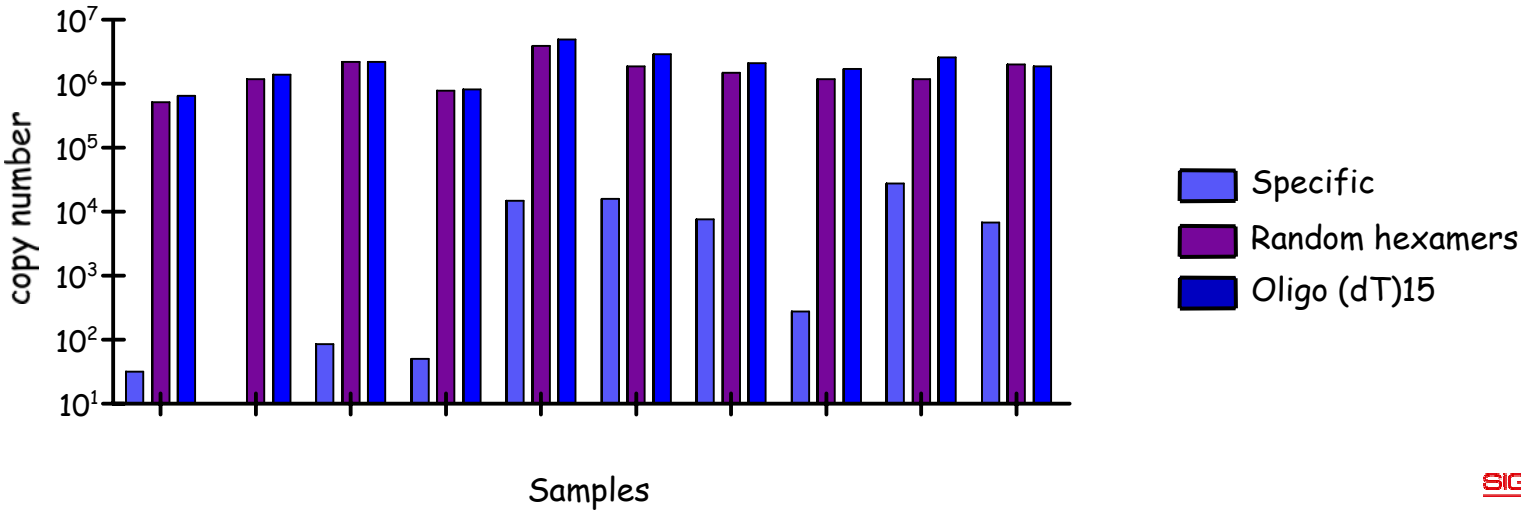
Data interpretation



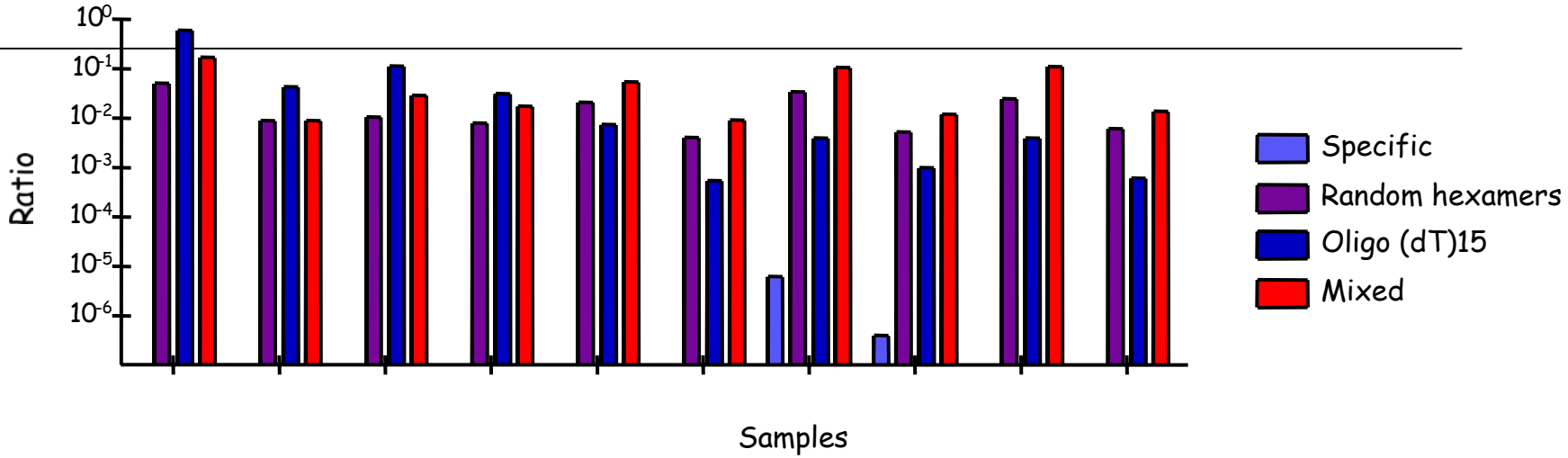
IGF-I Total RNA



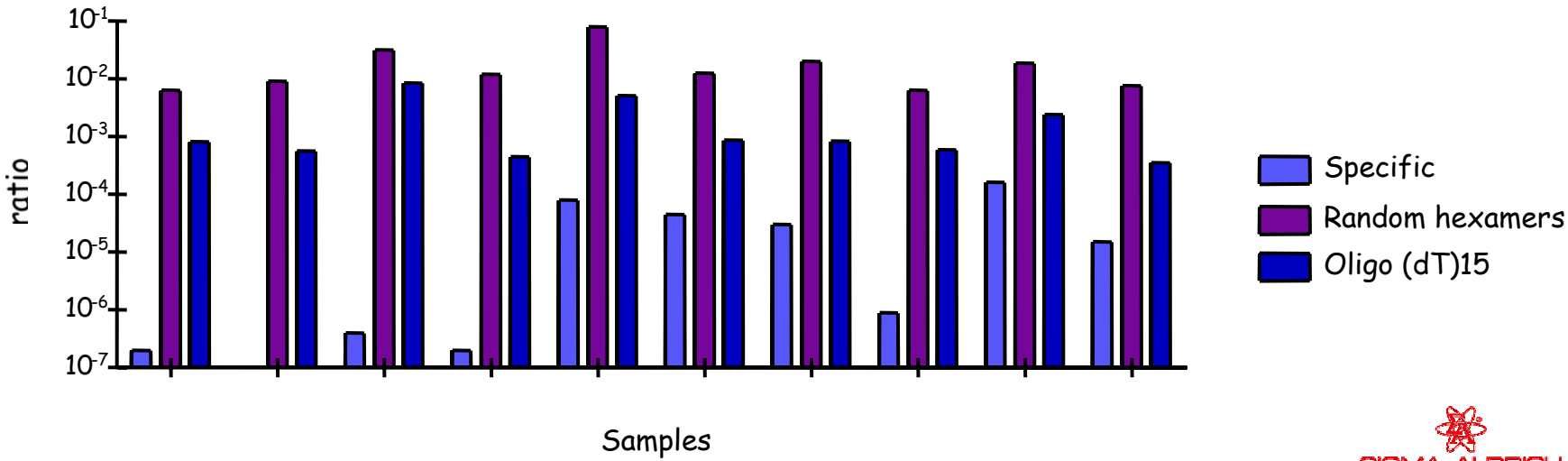
IGF-I mRNA



IGF-I/GAPDH total RNA



IGF-I/GAPDH mRNA



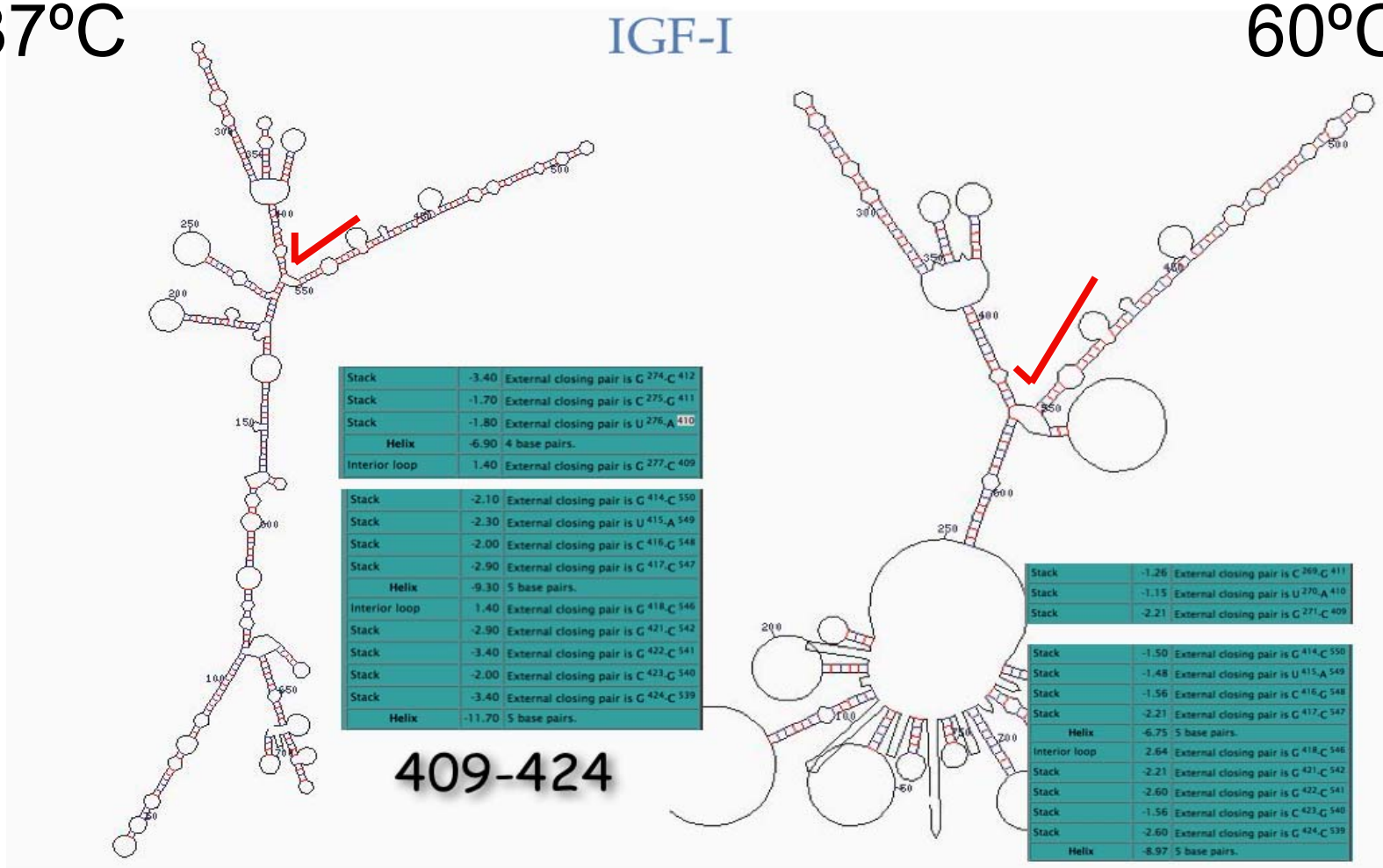
Influence of priming strategy on data interpretation

Using constant input total or mRNA

- **Interpretation of normalised data using random or oligo dT priming results in similar interpretation**
- **Specific priming results in great variation (due to IGF1 priming)**
- **mRNA appears to predict lower relative quantities than total RNA (by around 100 fold)**

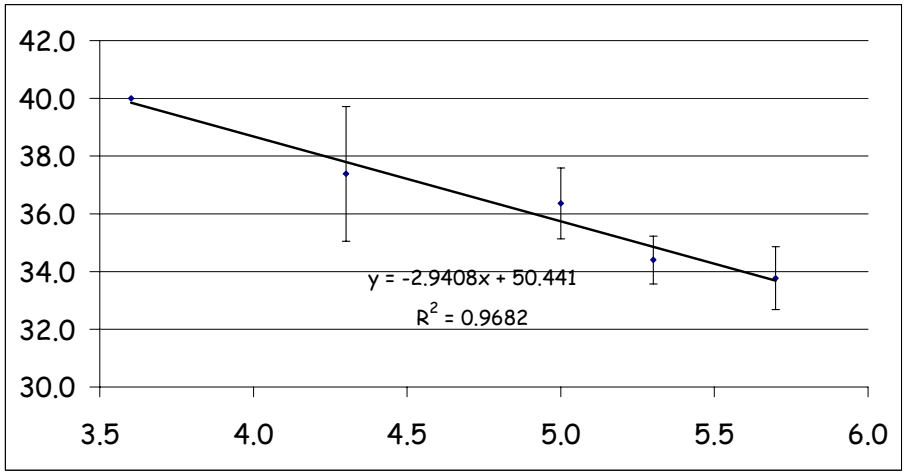
IGF1 structure

37ºC



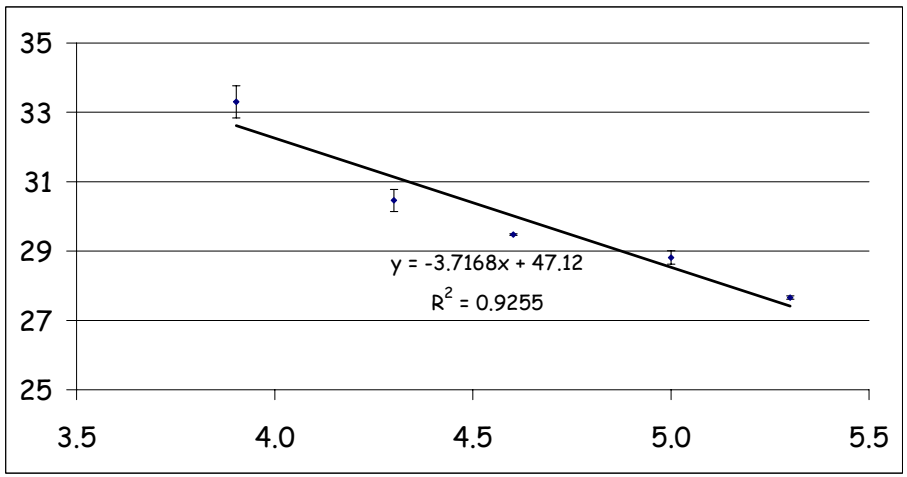
60ºC

Comparing priming: IL-15



One tube assay (RNA dilutions) - Specific primers

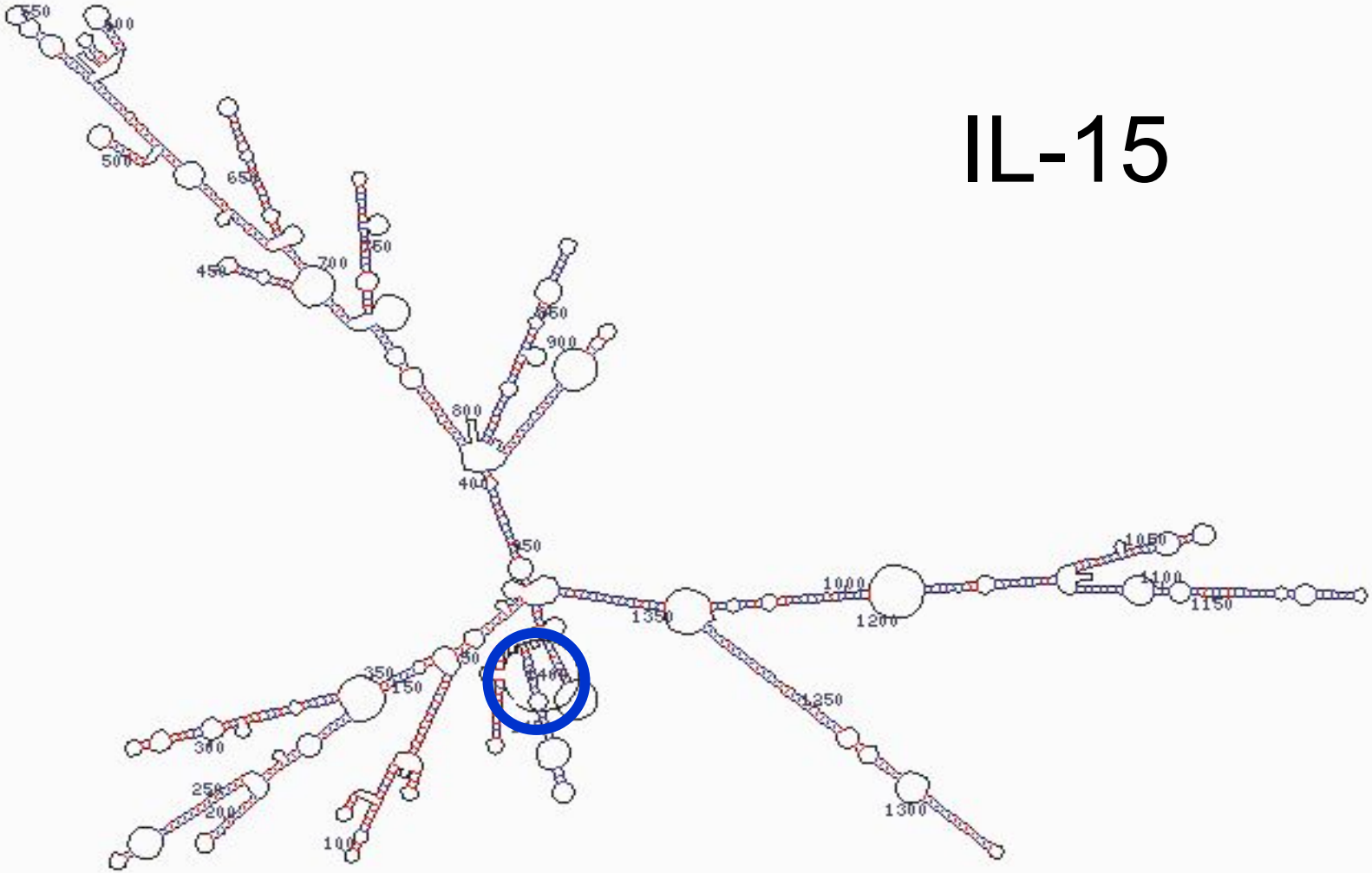
Slope = -2.9



Two tube assay (cDNA dilutions) - Random primers

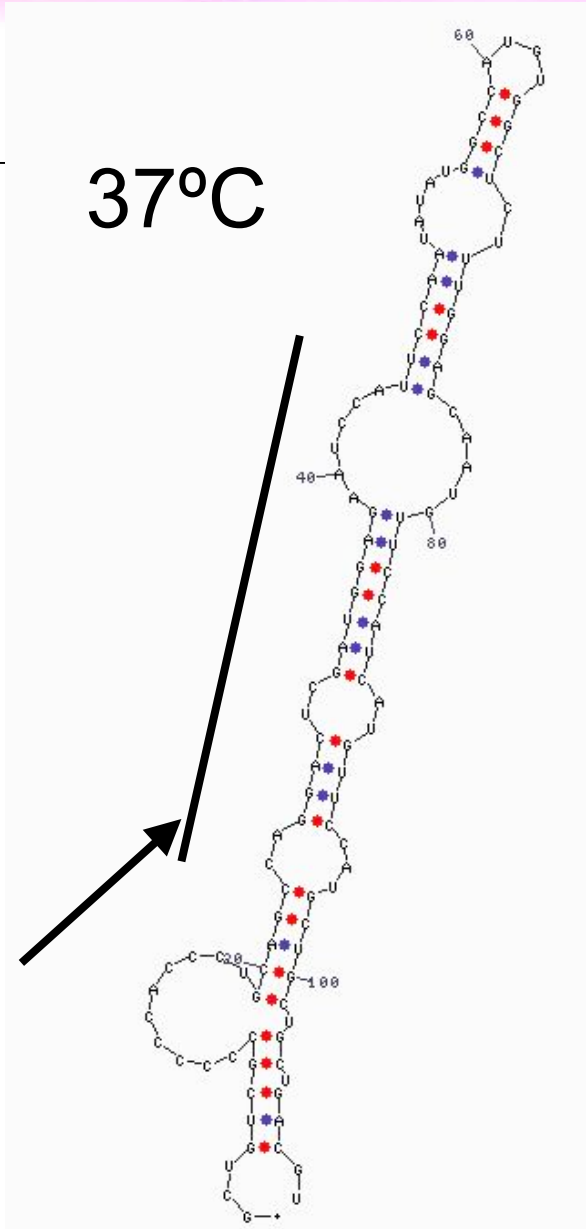
Slope = -3.7

IL-15



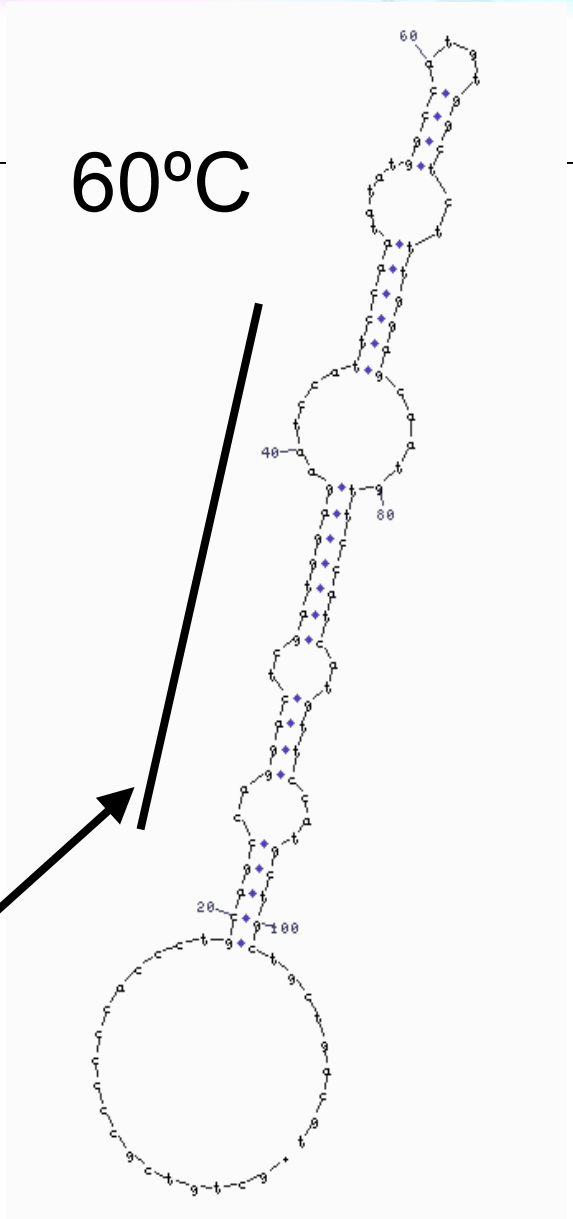
IL-15

37ºC



Reverse primer

60ºC



Reverse primer

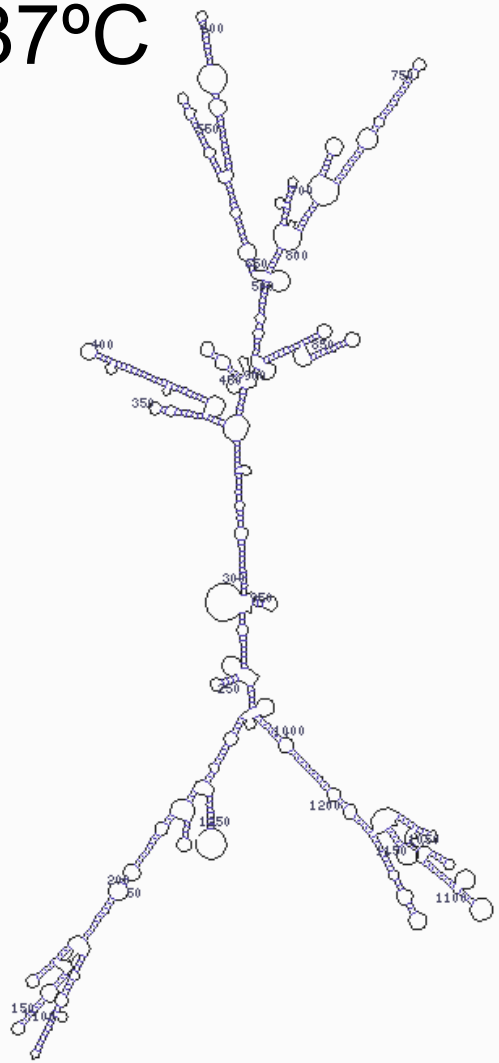
Influence of target structure on priming efficiency

Using total RNA or cDNA dilution series

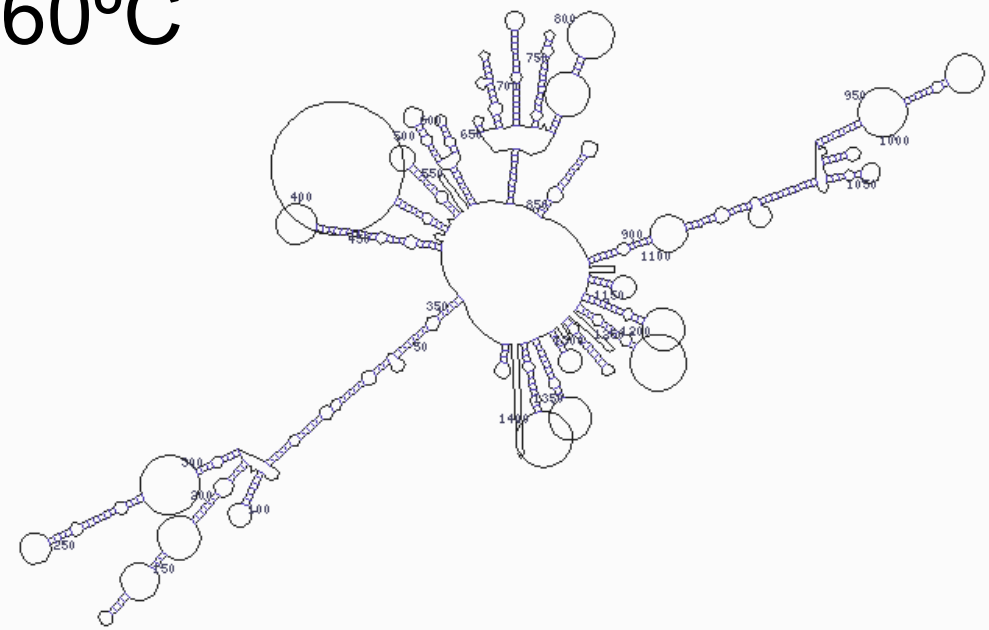
- **Random priming appears to be more forgiving of secondary structure**
- **Specific primers need to be located within open regions (at 60°C or RT temperature)**

GAPDH

37ºC

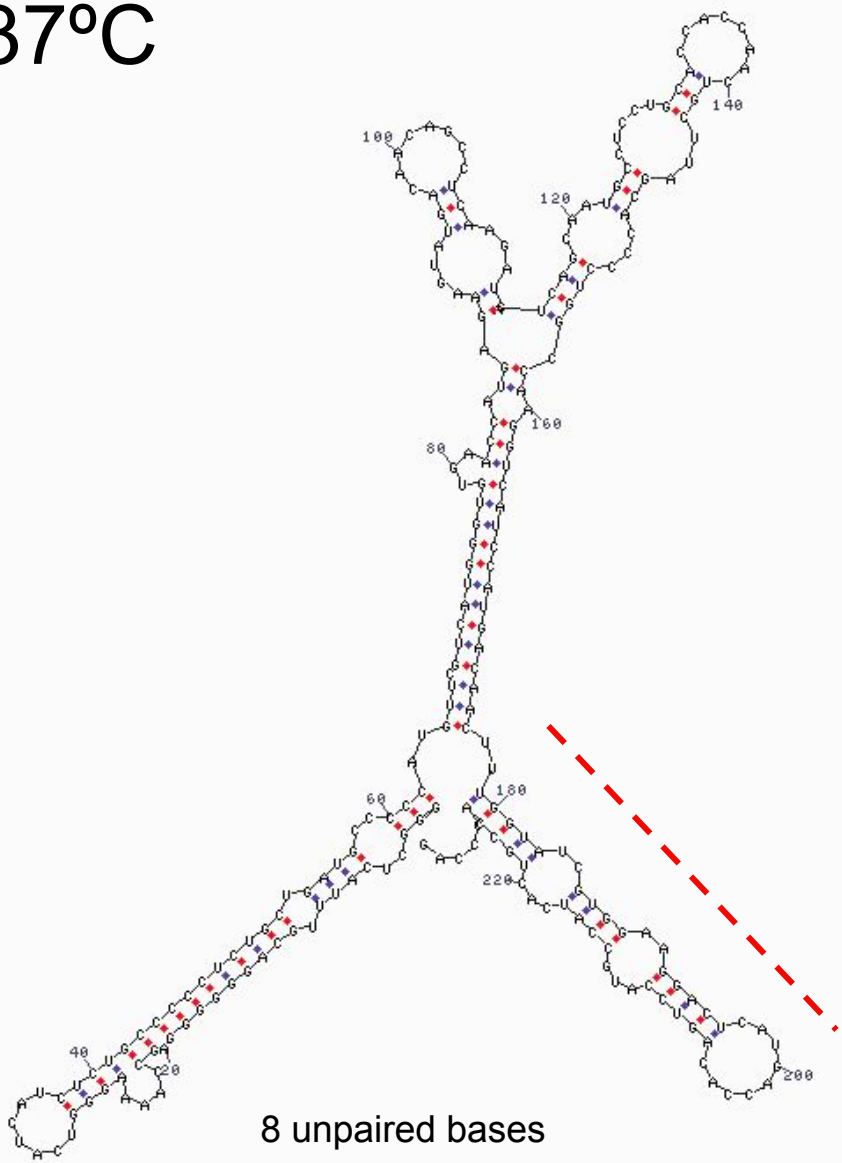


60ºC



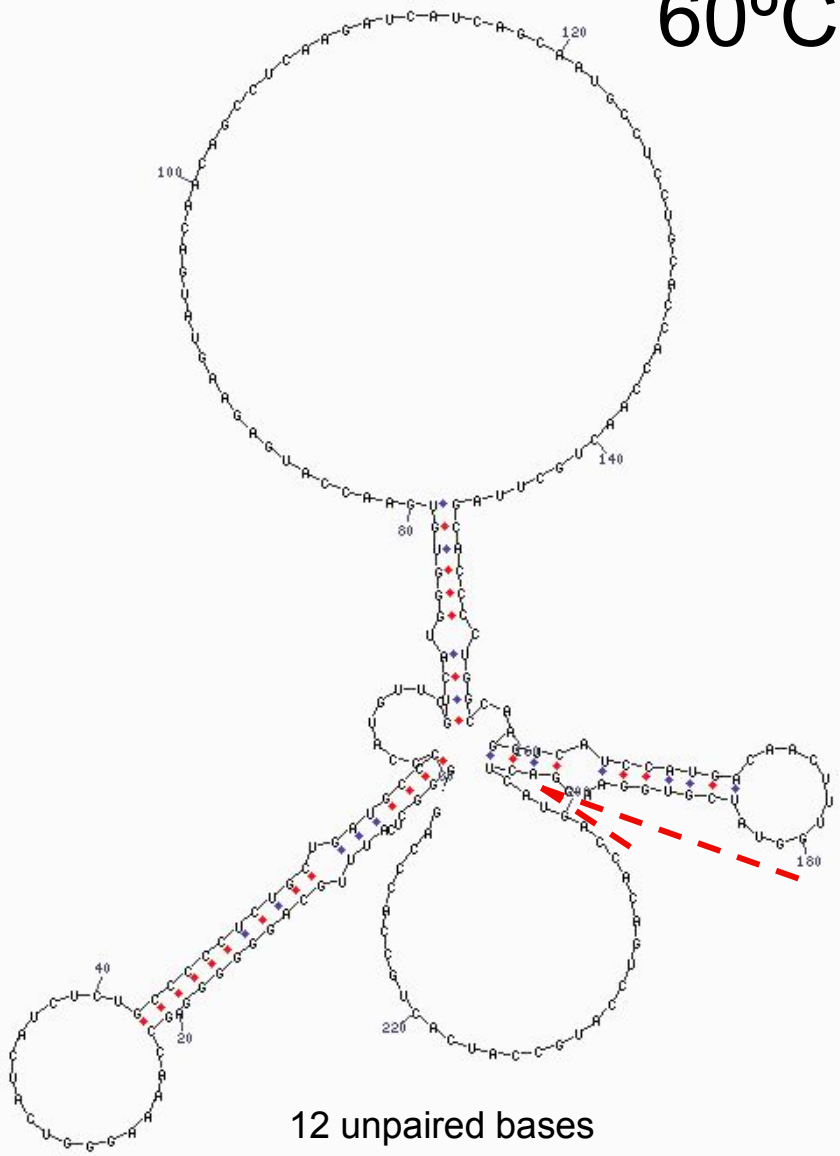
GAPDH 5’

37ºC



8 unpaired bases

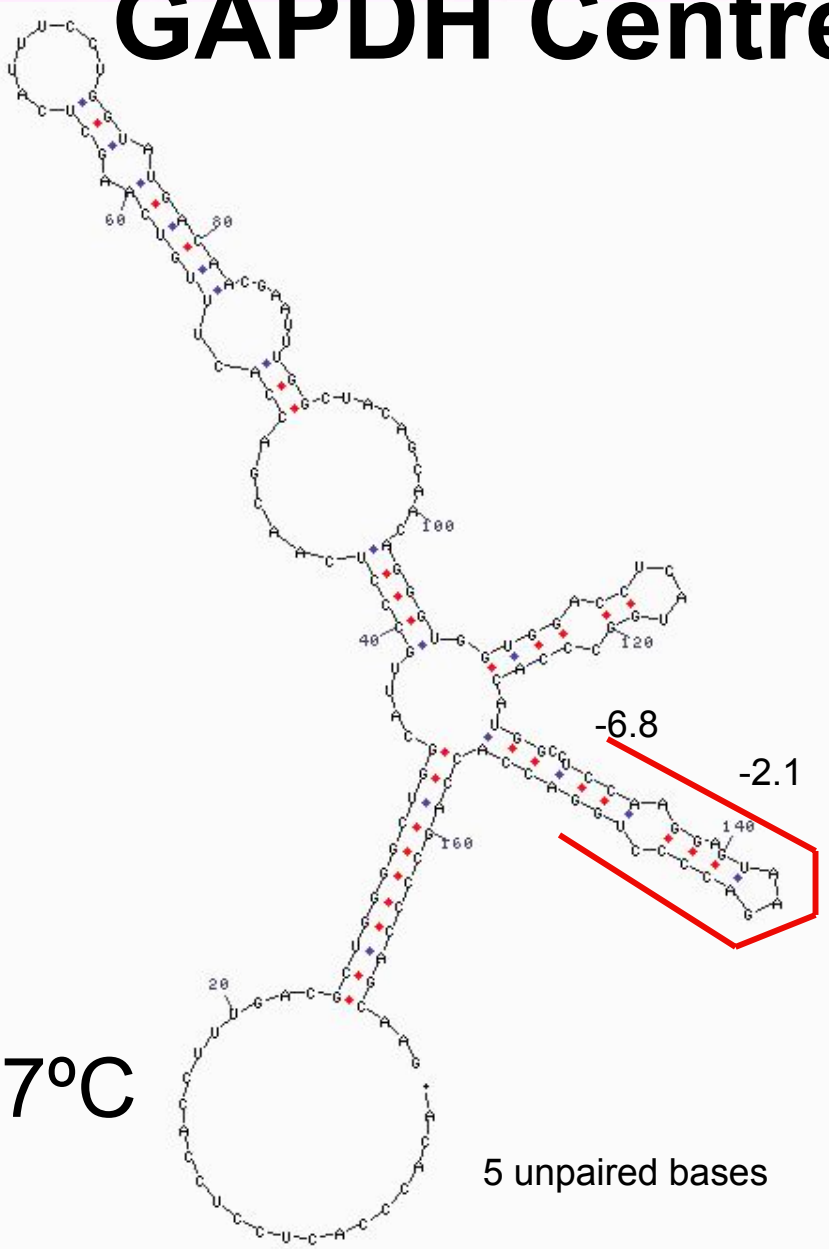
60ºC



12 unpaired bases

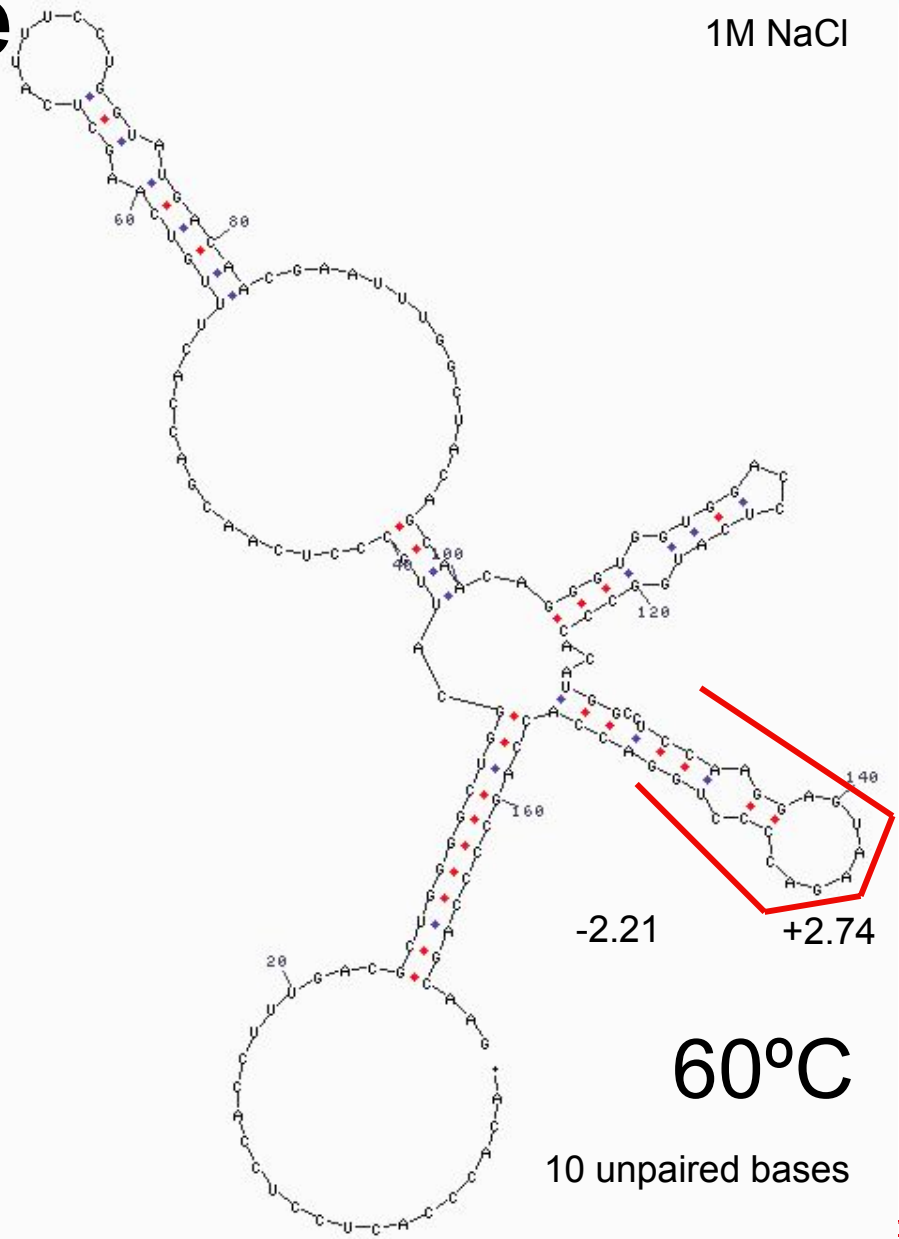
GAPDH Centre

1M NaCl



37ºC

5 unpaired bases

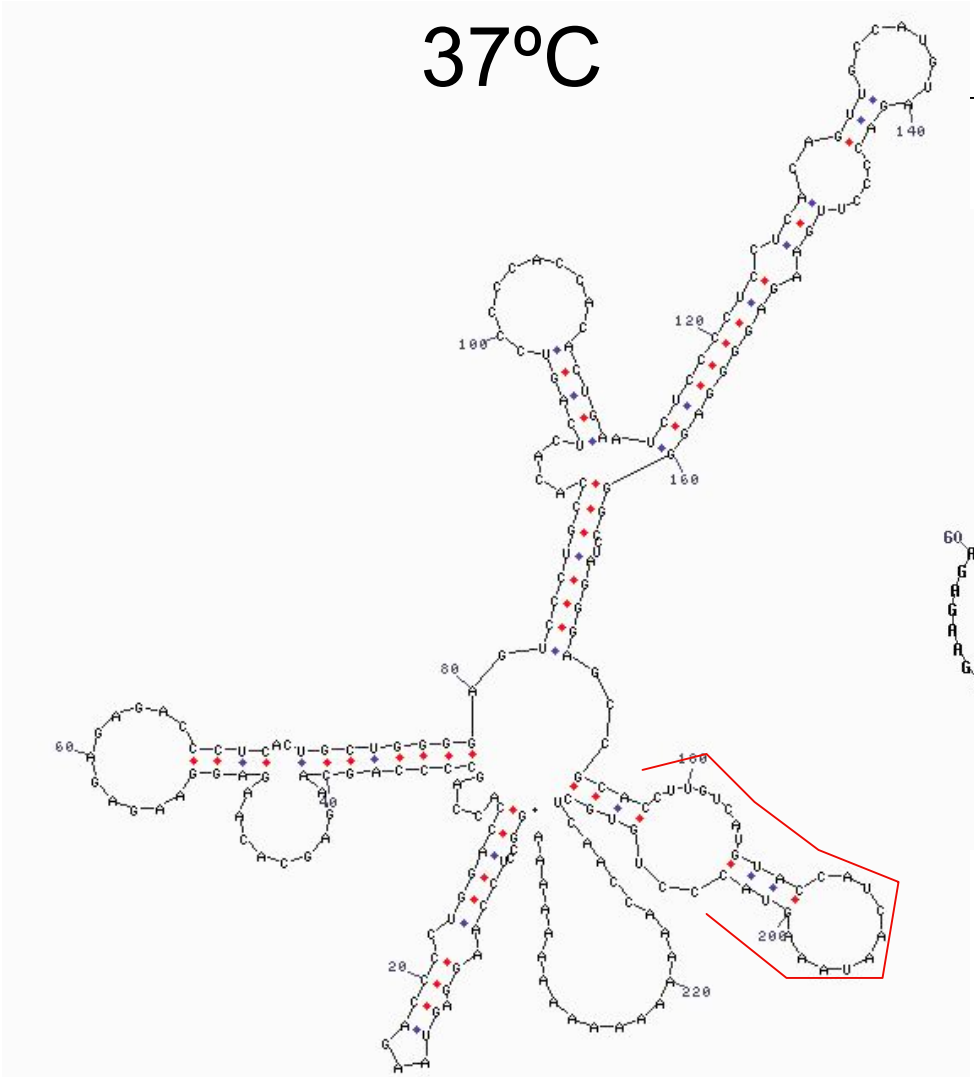


60ºC

10 unpaired bases

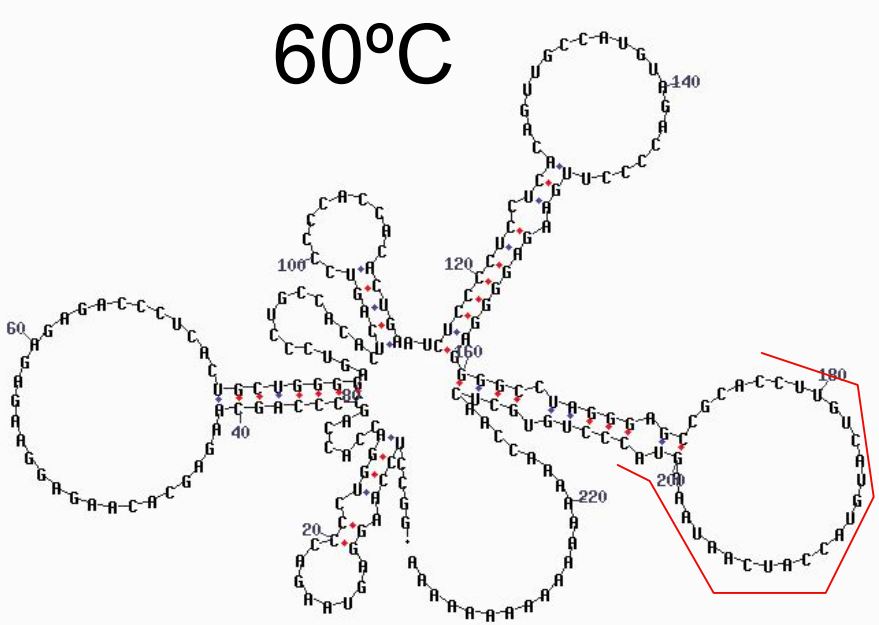
GAPDH 3’

37ºC



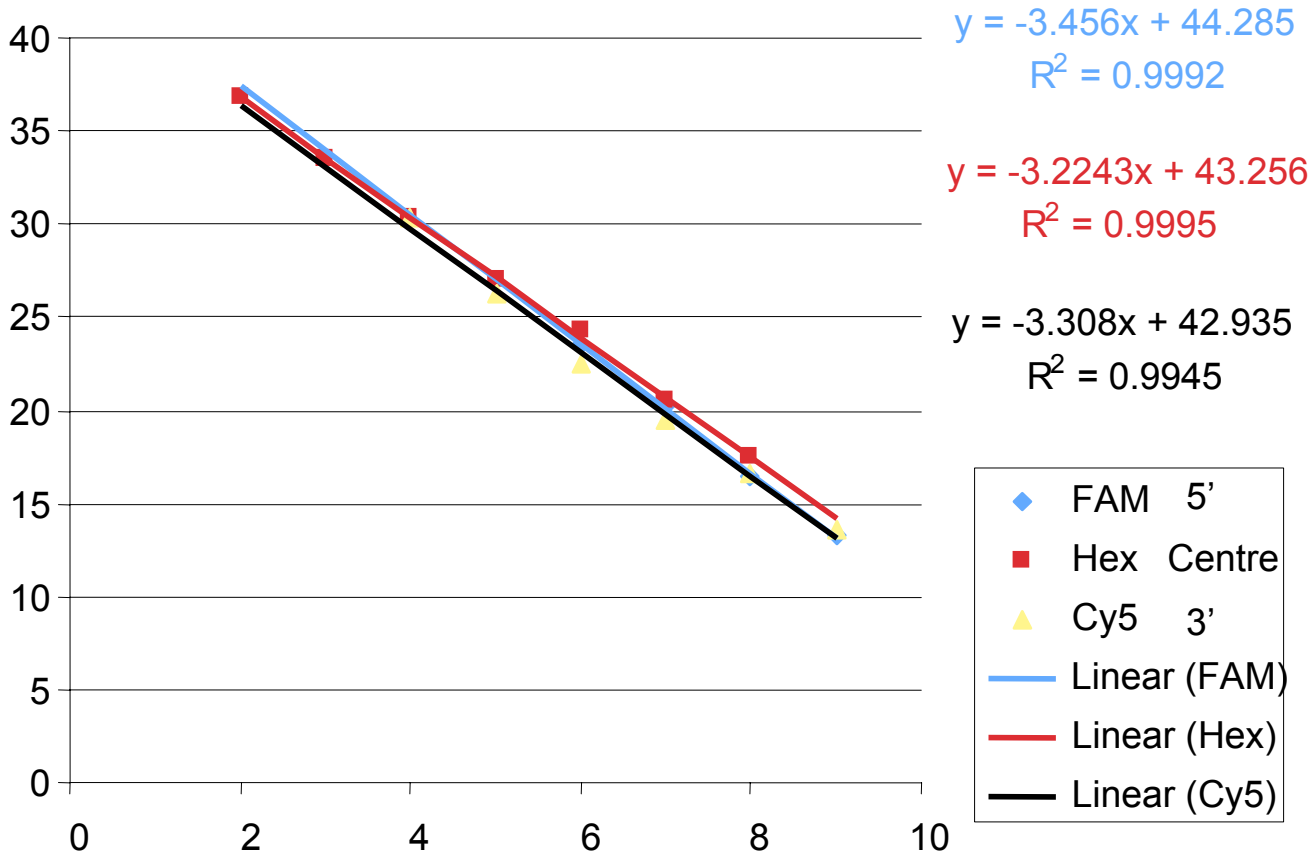
18 unpaired bases

60ºC



24 unpaired bases

Total RNA target
GAPDH specific primed dilution series



Assays designed by Natalie Simpson at www.designmyprobe.com

QRT-PCR protocol considerations

- When possible quantify input RNA
- Include a constant RNA amount into each RT
- Correct for RT batch to batch variations *(Placenta 26 (2005) 93-98)*

QRT-PCR protocol considerations

For unknown amounts of RNA:

- **Design specific reverse primers to open regions of transcript (at RT temperature) [www.designmyprobe.com]**
- **Use specific primers to increase sensitivity (especially when a large difference in transcript quantities is expected)**
- **Or use mRNA since all priming strategies appear to give limited linear response**
- **Whichever system is chosen check linearity and dynamic range of priming strategy for all genes to be measured.**

Many Thanks to:

- Prof Stephen Bustin (QMUL, London, UK)
- Dr Reinhold Mueller and Gothami Padmabandu (Stratagene, La Jolla, USA)
- Dr Helen Lacey and Prof Colin Sibley (Manchester University Medical School, UK)
- Natalie Simpson (Sigma-Genosys, Cambridge, UK)