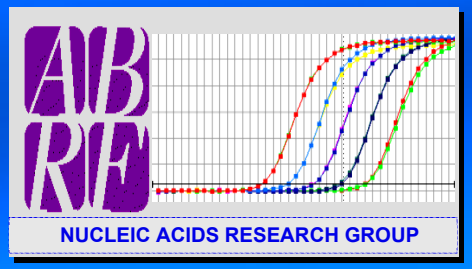

"Nucleic Acid Research Group (NARG) 2009-2010 Study : Optimal Priming Strategies for cDNA Synthesis in Real-Time RT-qPCR "

Presented by

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Nucleic Acid Research Group

Current Members

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Scott Tighe	University of Vermont
Anthony Yeung (EB liaison)	Fox Chase Cancer Center

http://www.abrf.org/NARG

Nucleic Acids Research Group

Past members involved in the Study Part I

Deborah S. Grove, Penn State University

Deborah J. Hollingshead, University of Pittsburgh

Gregory L. Shipley, UTHSC- Houston

Kathryn S. Lilley (EB), University of Cambridge

Part I: NARG "In-house" Study

Determine effects of priming strategies and enzyme on generating cDNA for qPCR

Determine contribution of assay type on qPCR

Part II: NARG "Benchmarking" Study

Opportunity for participants to use their in-house RT protocol on define sets of RNAs and primers

Identify optimal cDNA synthesis for use in the broadest range of qPCR assays

Determine general impact of RNA integrity on qPCR.

Part I: NARG "In-house" Study

A Two Years Investigation

YEAR 1: **Preliminary study to evaluate priming strategies used in the reverse transcriptase (RT) reaction to make cDNA for use in real-time qPCR**

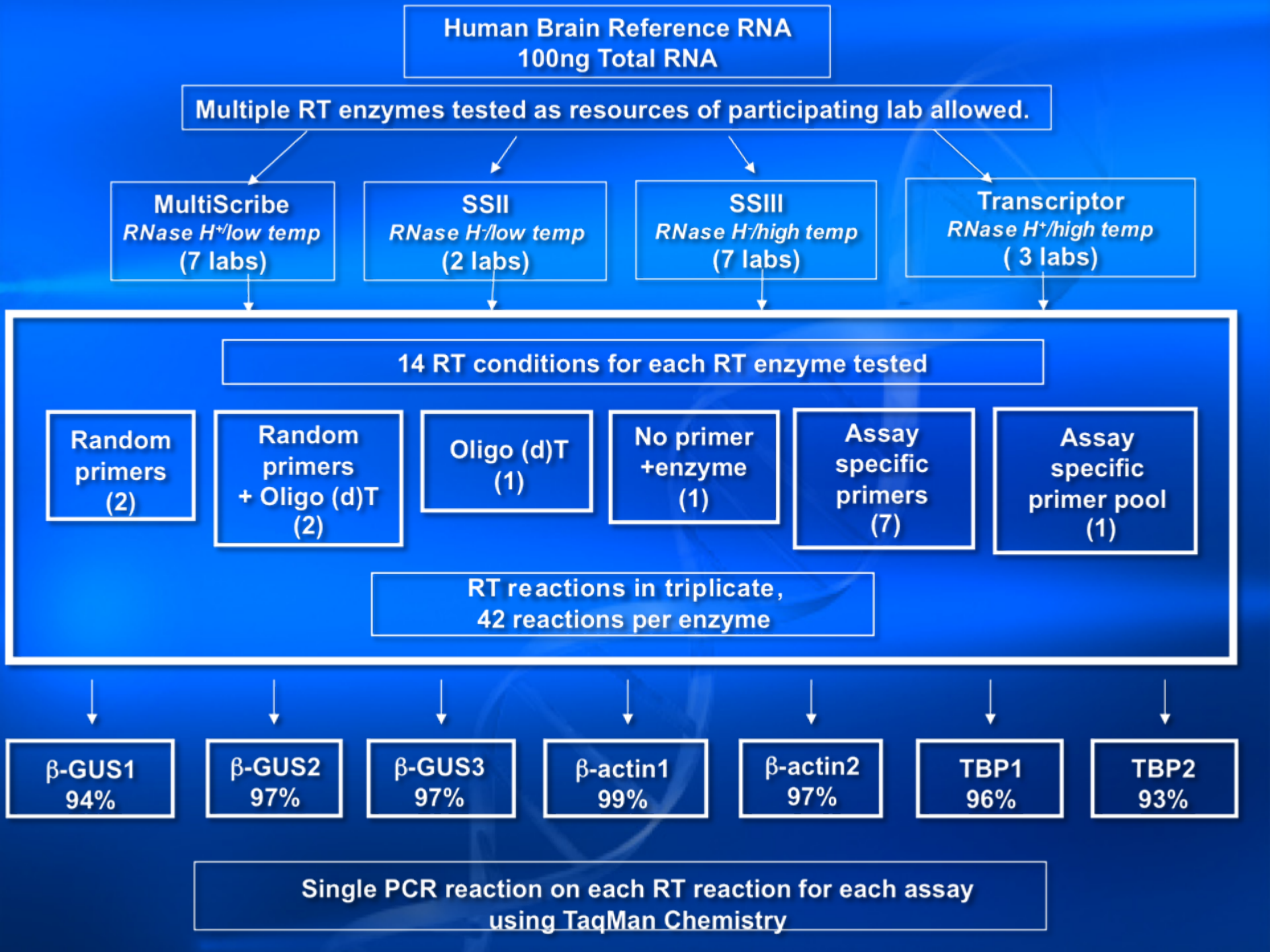
- **Compare randomers of different lengths (6 to 21 mers)**
- **Compare oligo(dT), anchored oligo(dT), gene specific primers, to no primer control (ΔC_q)**
- **Compare combinations strategies**

Part I: NARG "In-house" Study

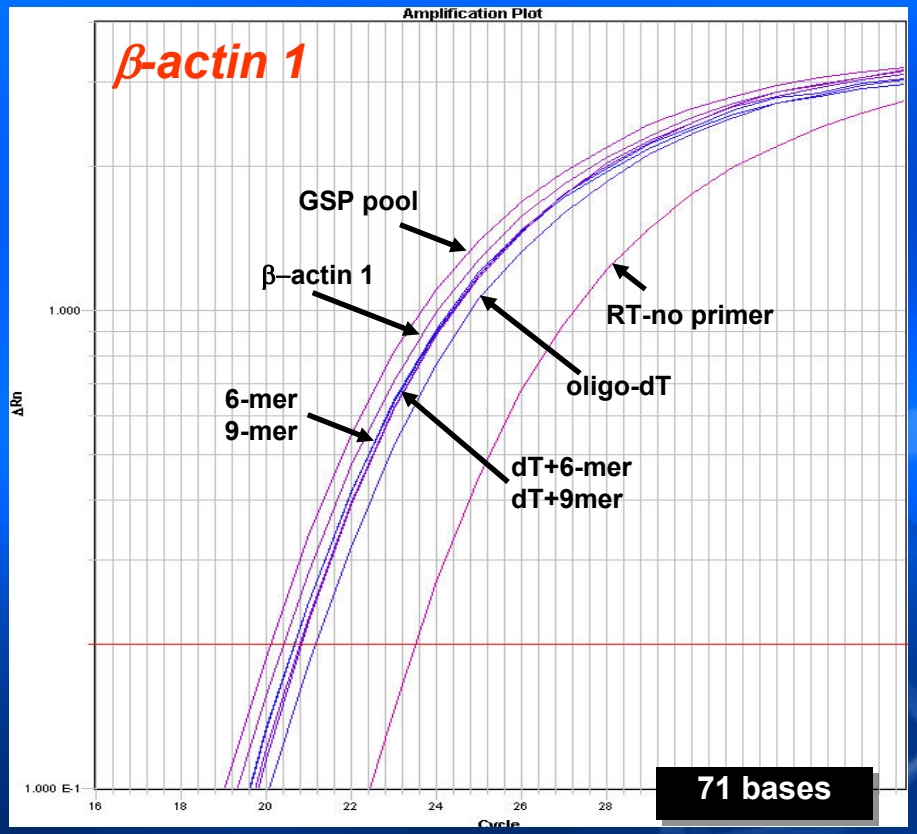
A Two Years Investigation

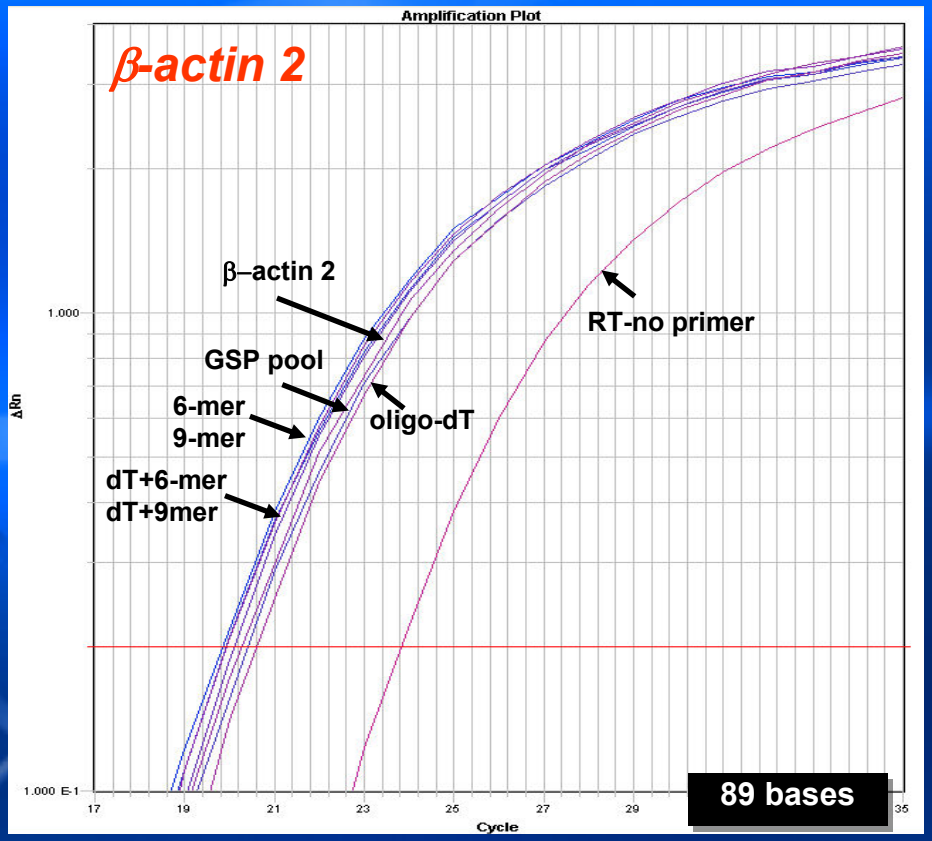
YEAR 2: Evaluate priming, enzyme strategies for RT AND assay location for qPCR

- Compare effect RT primers: 6 and 9mers, oligo(dT), gene specific primers (GSP), no primer control (ΔC_q)
- Compare effect of RT enzyme: AB's MultiScribe, Invitrogen SSII and III, Roche's Transcriptor
- Compare location qPCR assays (Actin1, Actin 2, GUS1,GUS2, GUS3, TBP1 and TBP2)

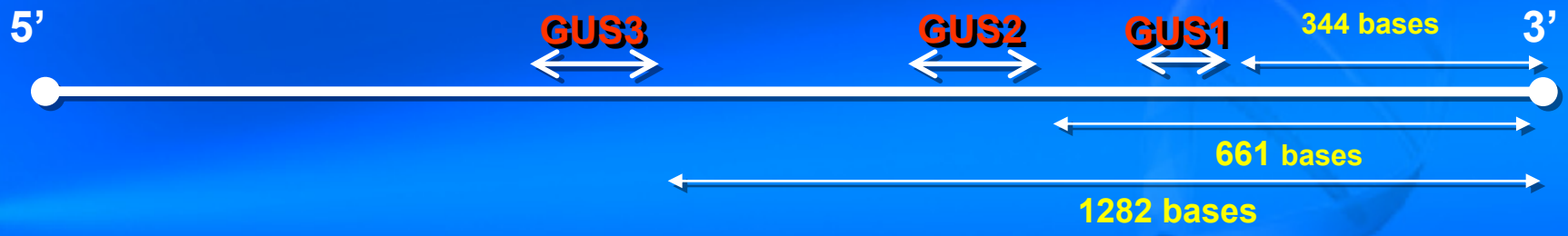

Human Brain Reference RNA
100ng Total RNA
Multiple RT enzymes tested as resources of participating lab allowed.
MultiScribe
RNase H+/low temp
(7 labs)
SSII
RNase H-/low temp
(2 labs)
SSIII
RNase H-/high temp
(7 labs)
Transcriptor
RNase H+/high temp
(3 labs)
14 RT conditions for each RT enzyme tested
Random primers (2)
Random primers + Oligo (d)T (2)
Oligo (d)T (1)
No primer +enzyme (1)
Assay specific primers (7)
Assay specific primer pool (1)
RT reactions in triplicate,
42 reactions per enzyme
β-GUS1 94%
β-GUS2 97%
β-GUS3 97%
β-actin1 99%
β-actin2 97%
TBP1 96%
TBP2 93%
Single PCR reaction on each RT reaction for each assay
using TaqMan Chemistry

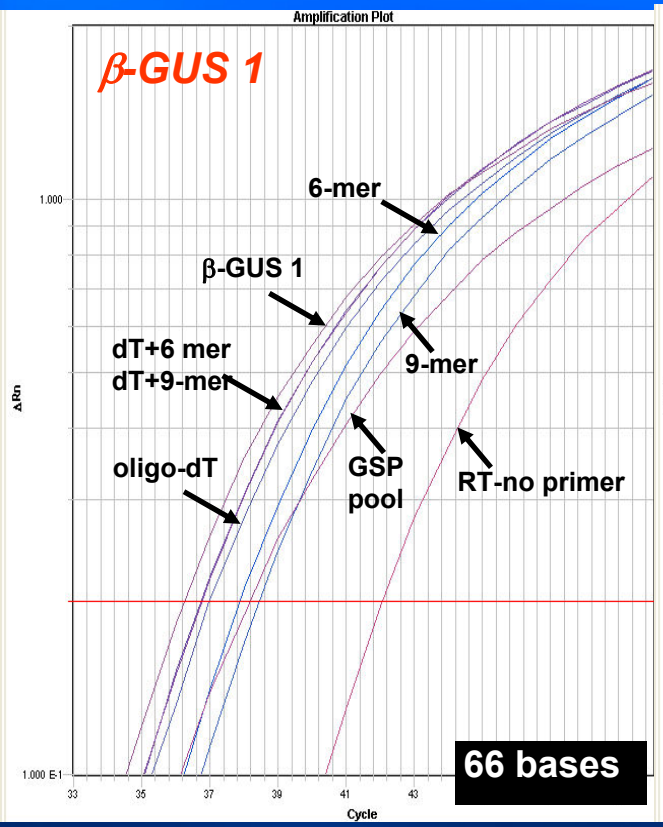
β-Actin Amplification Plots

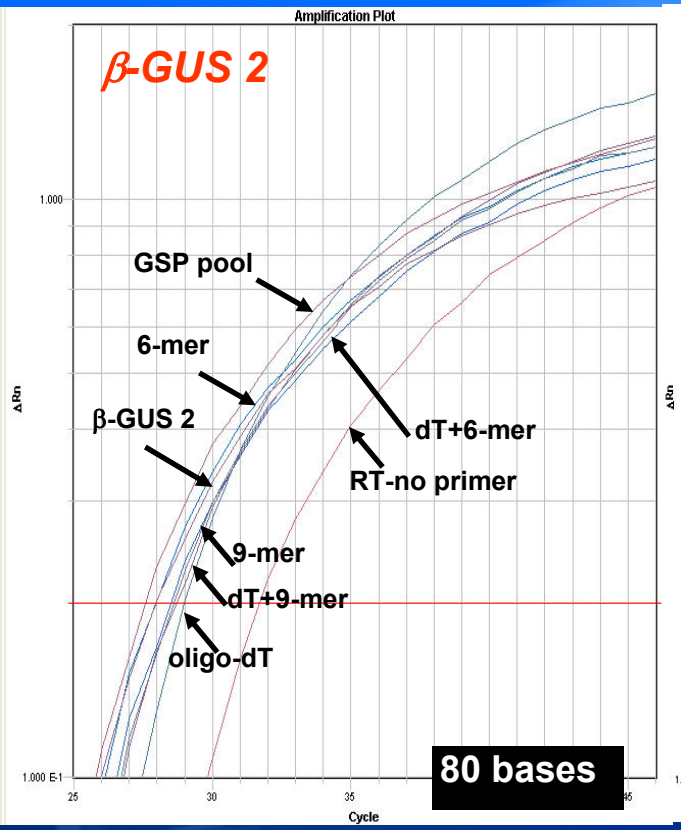


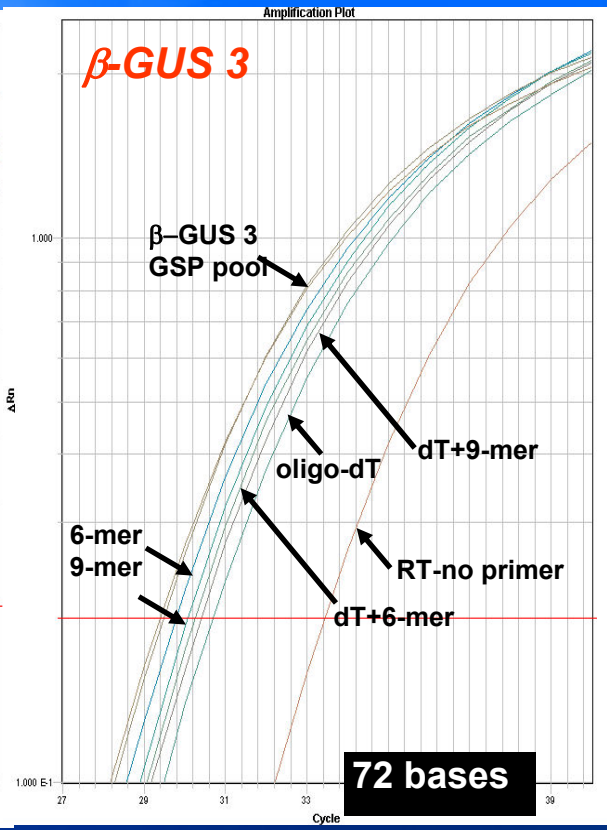


β-Glucuronidase Amp Plots

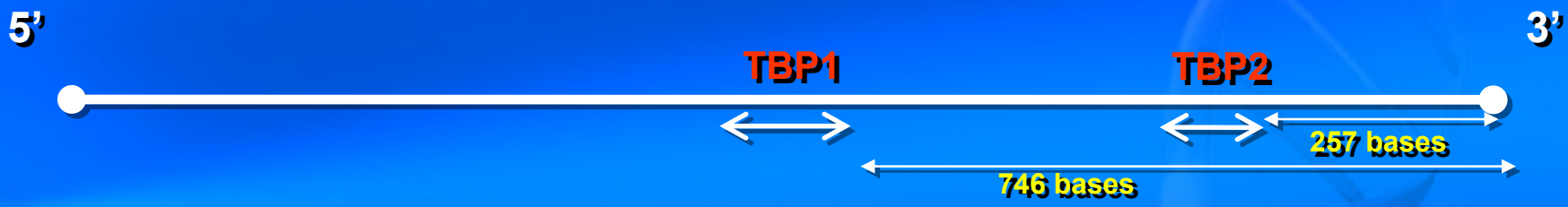


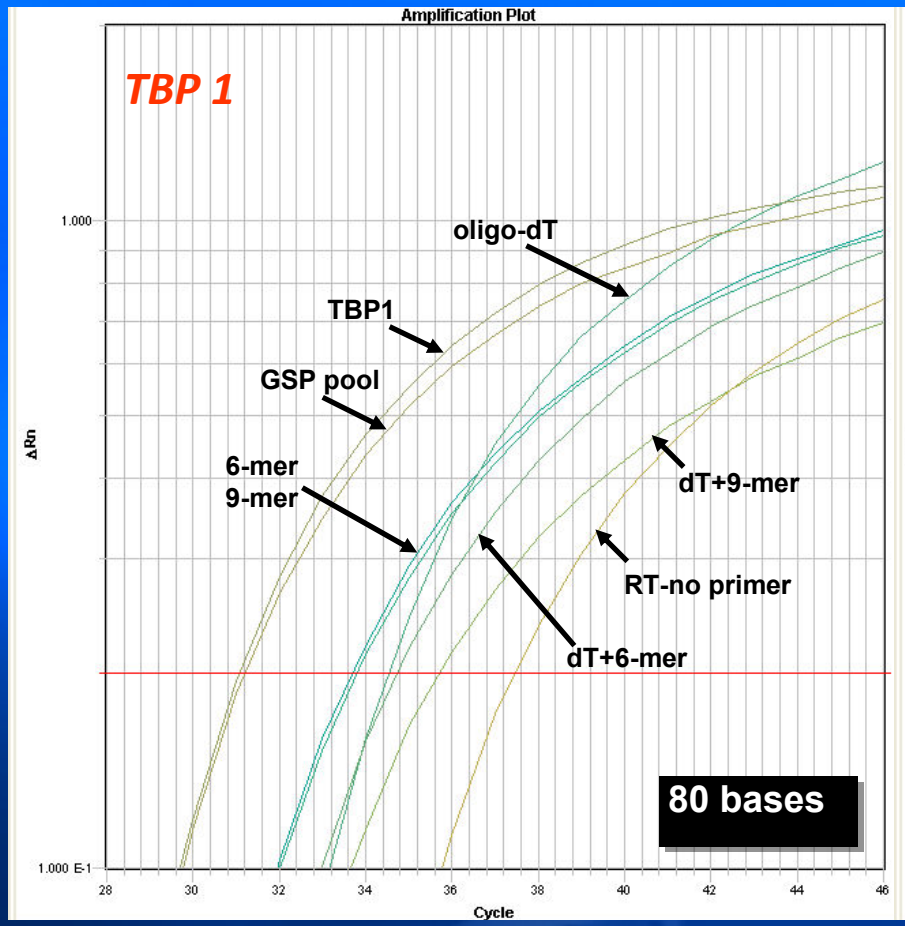


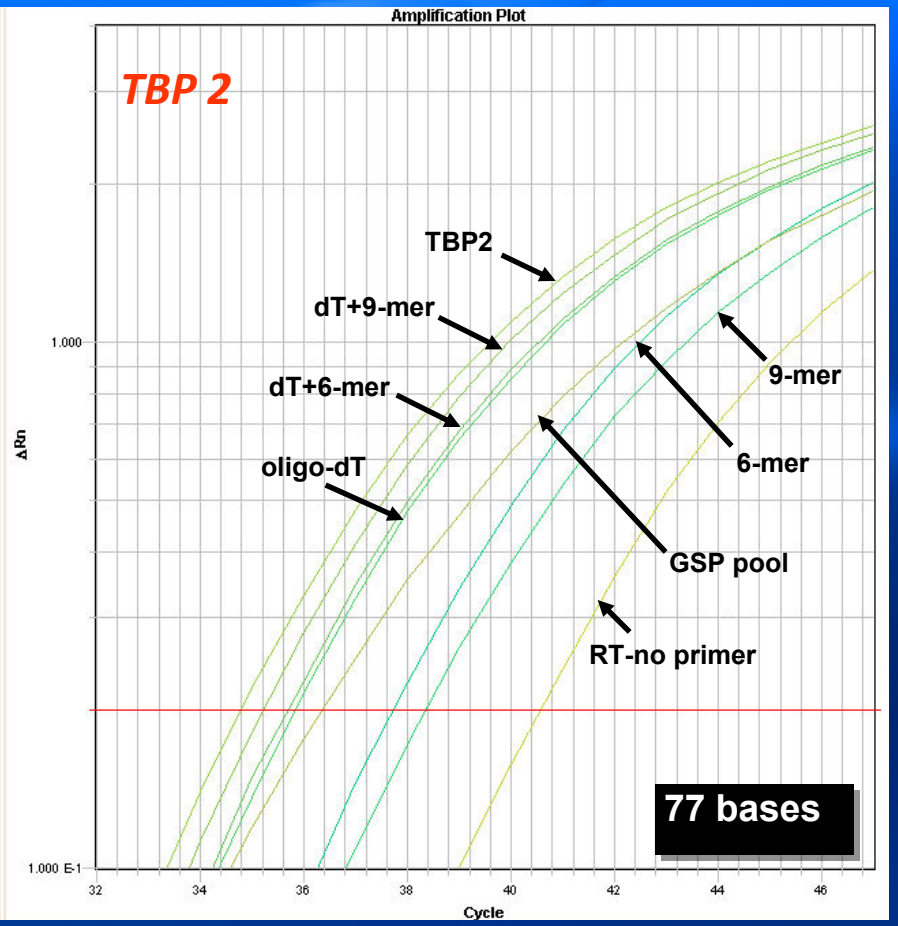




TATA Binding Protein Amp Plots







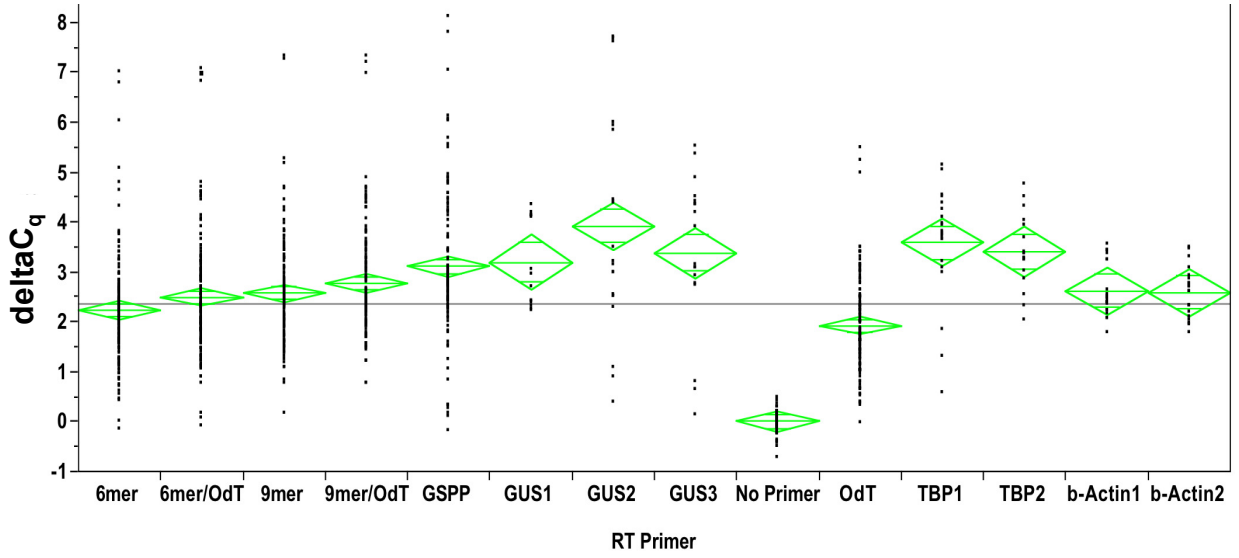
Statistical Analysis

The effect of each variable on C_q or ΔC_q levels were assessed using a one-way analysis of variance (ANOVA) with the JMP v 5.01 Statistical Discovery Software (SAS Institute, Cary, NC).

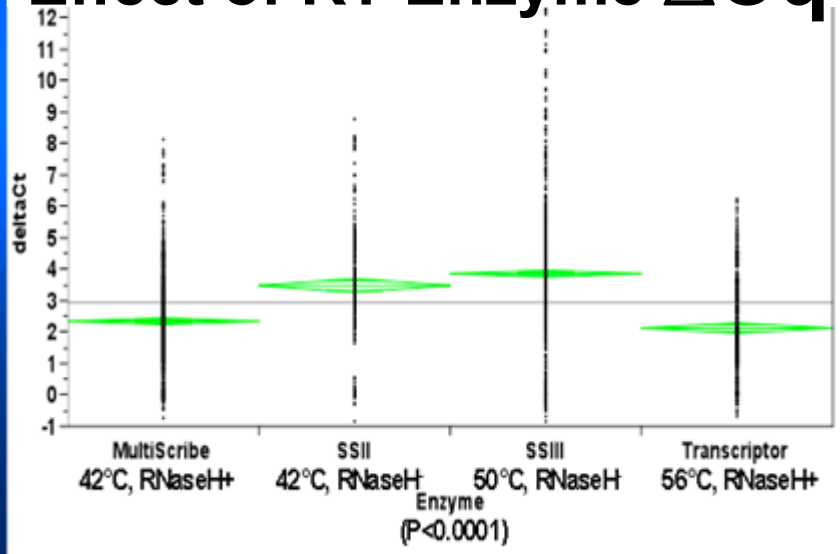
The green diamonds represent the mean and the standard error which is a pooled estimate of the variance.

A Student's *t*-test was used to assess for significant difference levels ($P < 0.05$) between the groups contained within each variable.

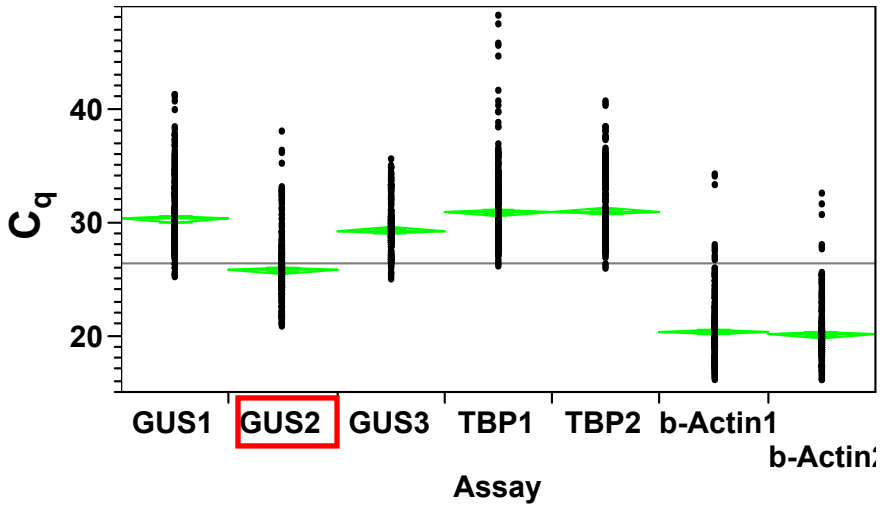
Effect of RT Priming Strategy on ΔCq



Effect of RT Enzyme ΔCq



Effect of qPCR assay on Cq



Conclusions of In-House Study

- **RNA has a high capability to self priming**
- ***Gene specific RT priming strategy generates the lowest C_q values regardless of assay location. Multiple genes can be assayed using pools of GSP in the RT***
- **If GSP not an option, a combination of Oligo(dT) and randomer will insure best result regardless of assay location**
- **If use oligo-dT to generate the cDNAs, the closer to the 3'end the qPCR assay is, the better**

Part II:
NARG "Benchmarking" Study

Opportunity for participants to use their in-house RT protocol on define sets of RNAs and primers

Identify optimal cDNA synthesis for use in the broadest range of RNA quality and qPCR assay locations

Study Design

A benchmarking opportunity, with a twist!

Reference RNA Sample S
50 ng Total RNA, RIN 7.9

Reference RNA Sample V
50 ng Total RNA, RIN 4.3

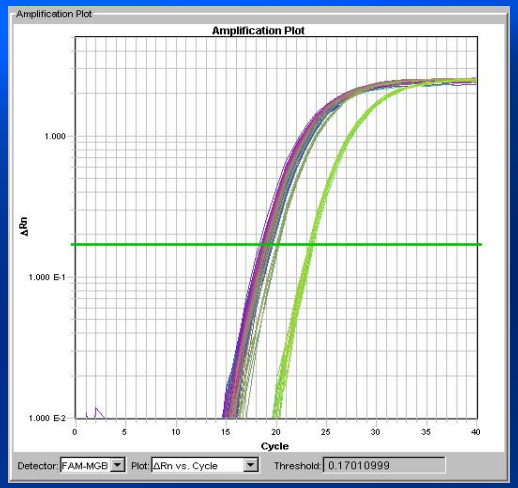
Reference RNA Sample C
50 ng Total RNA 2.2

Participant Labs for Reverse Transcription using in-house RT conditions, preferred RT-enzyme and chemistry mix

No primer control	Random hexamers	Oligo (dT)	Hex + Oligo(dT)	Gene specific primers

All cDNAs were shipped back to NARG for qPCR processing.

- 5 ng RNA equivalents cDNA were amplified using the ABI 2x master mix using 500 nM primers and 250 nM probe in a 5ul reaction volume.
- Cycling was performed on 384-well plates using the standard conditions recommended for the AB7900HT platform.



Benchmarking Study

Participants from 20 labs

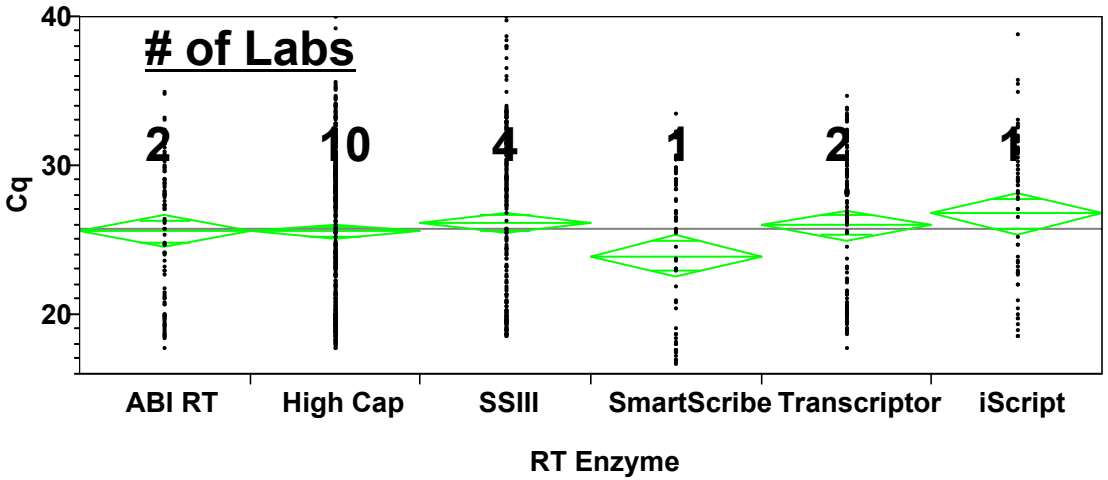
6 different types of RT enzymes

- **10** labs used AB's High Capacity RT at 37C (7) or 42C (3)
- **4** labs used Invitrogen's SuperScriptIII at 50C
- **2** labs used AB's reverse Transcriptase at 37C
- **2** labs used Roche's Transcriptor RT at 50 or 55C
- **1** lab used Clontech's SmartScribe at 42C
- **1** lab used BioRad's iScript at 42C

One-way analysis of variance (ANOVA) to determine:

- Effect of enzyme type and RT temperature on Cq
- Effect of RT priming strategy and qPCR assay location
- Effect of Lab on Cq
- Effect of overall RNA quality on RT-qPCR

Effect of Enzyme & Temperature on C_q



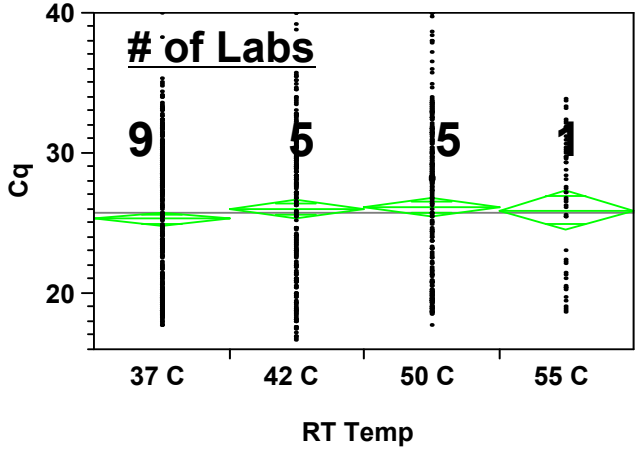
RT master mix?
- 16 kits
- 4 home-brew

Enz inactivation?
- 17 Yes
- 3 No

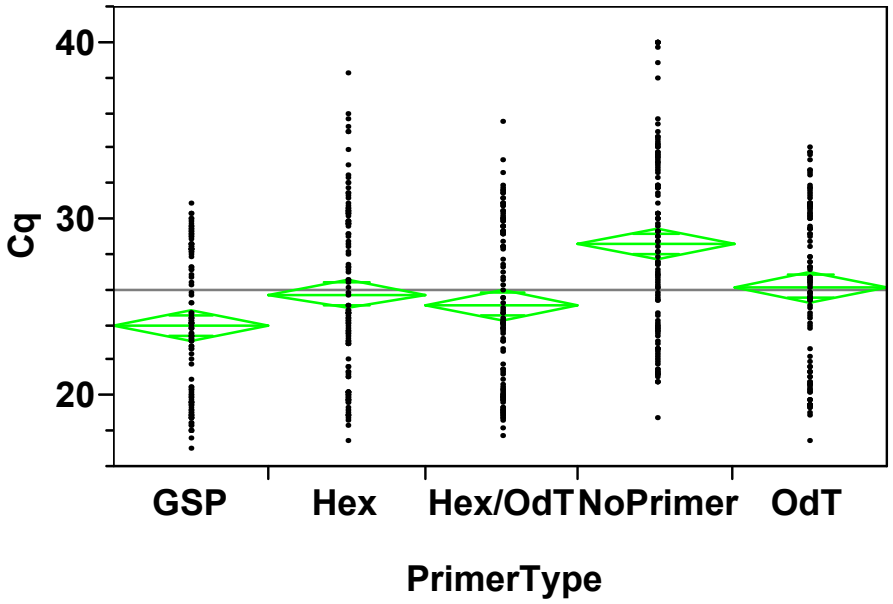
Set-up?
- 13 One well at time
- 6 Multi channel

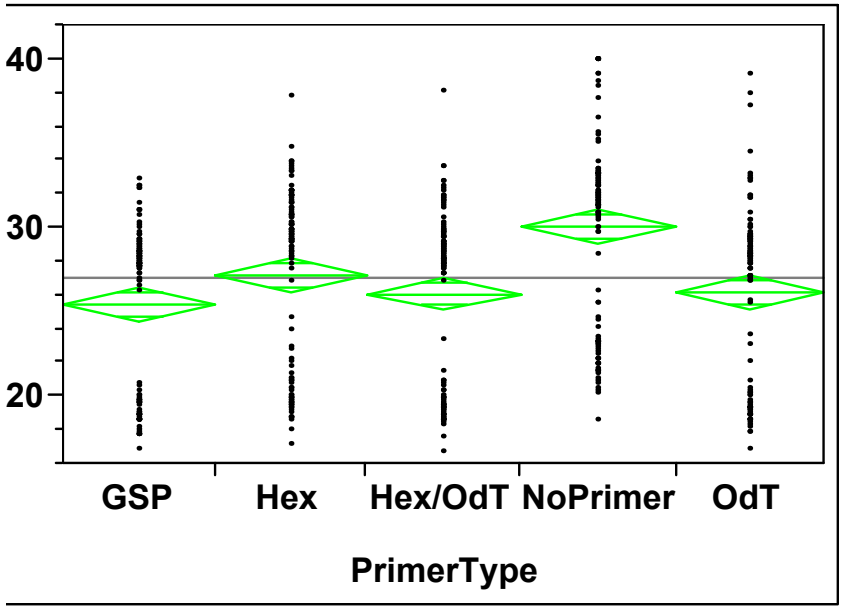
RT Enzyme	Level*		Mean (N)
iScript	A		26.77 (48)
SSIII	A		26.14 (181)
Transcriptor	A		25.97 (94)
ABI RT	A	B	25.58 (83)
High Cap	A		25.56 (464)
SmartScribe		B	23.90 (48)

*Levels not connected by the same letter are significantly different ($p<0.05$)



Effect of Assay Location on C_q

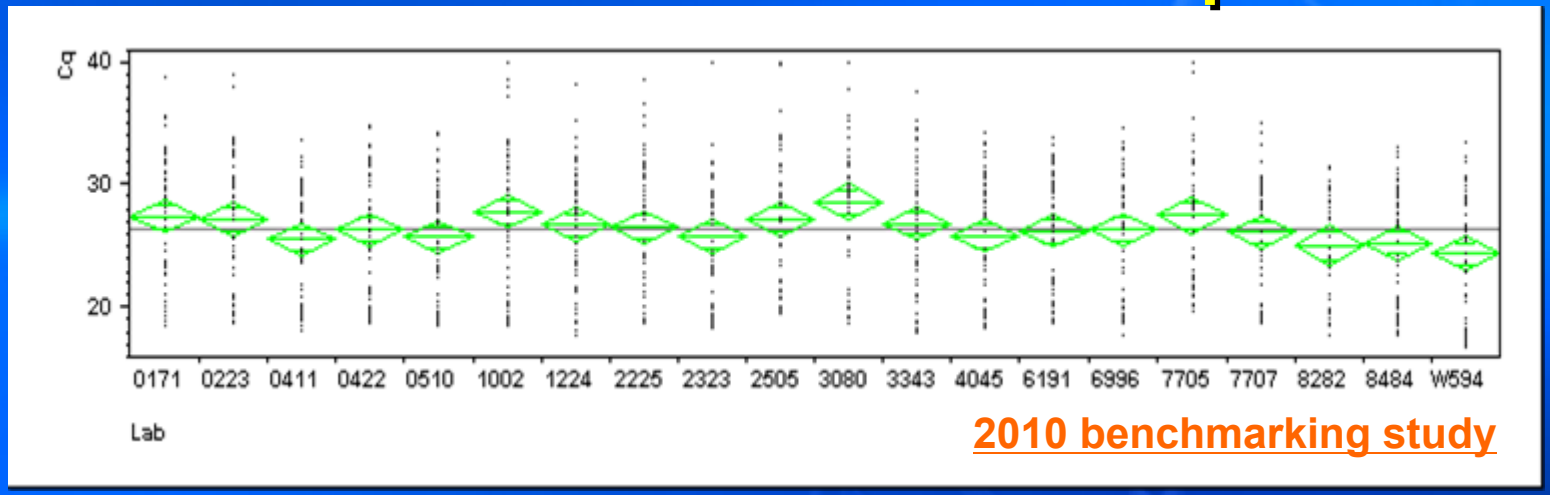




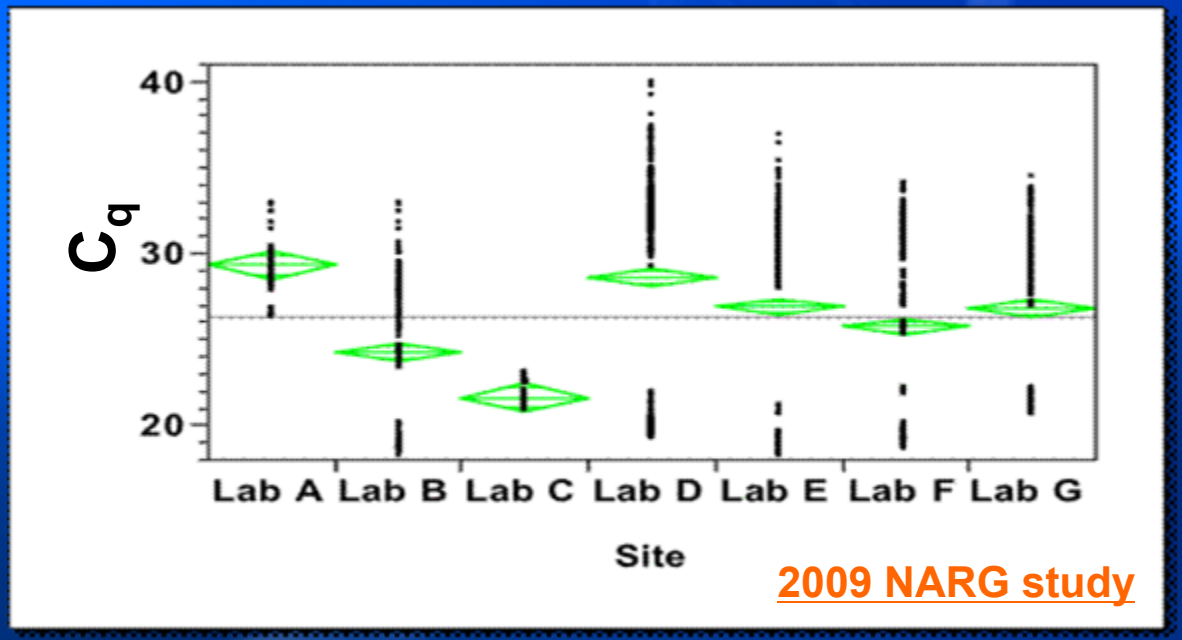
Assays located away from 3'end

Assays located near 3'end

Effect of Lab on C_q



2010 benchmarking study



2009 NARG study

Effect of Lab on C_q

2009 NARG study

Lab	Levels*	Mean (N)
Lab A	A	29.33 (132)
Lab D	A	28.59 (388)
Lab E	B	26.88 (391)
Lab G	B	26.81 (396)
Lab F	C	25.77 (396)
Lab B	D	24.27 (396)
Lab C	E	21.61 (124)

*Levels not connected by the same letter are significantly different ($p<0.05$).

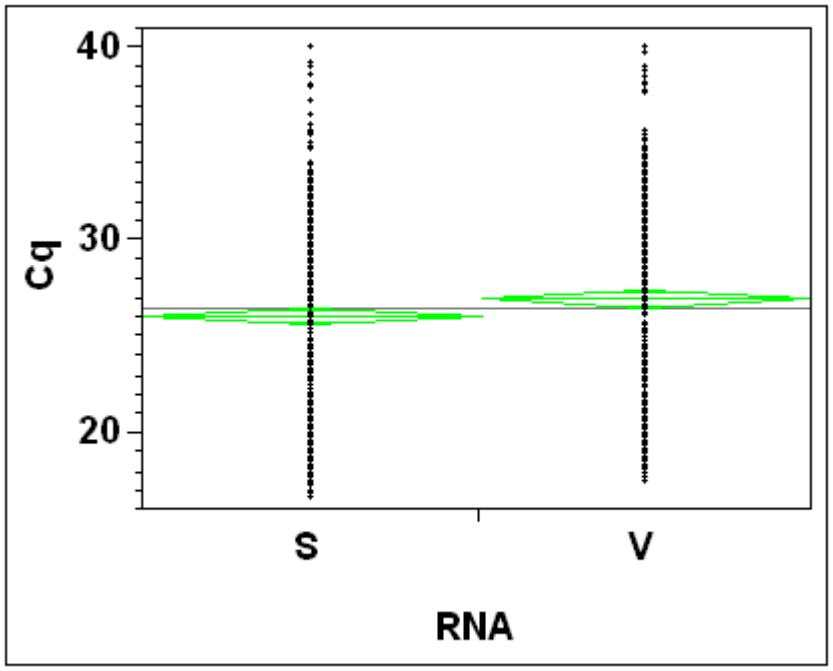
2010 benchmarking study

Lab	Level*			Mean (N)
0171	A			26.77 (48)
3080	A	B		26.71 (43)
1002	A	B		26.65 (47)
7705	A	B		26.59 (38)
0223	A	B		26.30 (48)
1224	A	B		26.28 (48)
3343	A	B		26.23 (48)
0422	A	B		26.15 (48)
6996	A	B		26.02 (48)
6191	A	B		25.92 (46)
2505	A	B	C	25.87 (39)
2225	A	B	C	25.70 (47)
7707	A	B	C	25.30 (48)
4045	A	B	C	25.17 (48)
0411	A	B	C	25.00 (48)
0510	A	B	C	24.97 (48)
2323	A	B	C	24.90 (48)
8282	A	B	C	24.78 (35)
8484		B	C	24.76 (47)
W594			C	23.90 (48)

Effect of RNA Template Quality on C_q

RNA	Level*	Mean (N)
V	A	26.91 (567)
S	B	25.96 (585)

*Significance level p<0.05.



What is the Best Priming Strategy to Use to Generate cDNA for Use in qPCR?

If the cDNA will be used for only 1 gene assay, the appropriate gene-specific primer may be a better choice

The use of randomer-oligo(dT) combinations in the RT reaction appear to give universally lower C_q values and higher ΔC_q differences regardless of the assay location

RNA quality will significantly impact your qPCR results, however trends may be teased out even using a sub-optimal template in the RT (as sample V with RIN 4.3)

From Part I and II results: Main data variation may be the result of the qPCR not the RT step