

Nucleic Acids Research Group 2009-2010 Study: Optimal Priming Strategies for cDNA Synthesis in Real-Time RT-qPCR

TC Hunter¹, SV Chittur², KL Knudtson³, V Nadella⁴, K Sol-Church⁵, WL Taylor⁶, S Tighe¹, AT Yueng⁷
¹University of Vermont, ²University at Albany, ³University of Iowa, ⁴Ohio University, ⁵Nemours Biomedical Research, A.I. duPont Hospital for Children, Wilmington DE, ⁶UTHSC-Memphis, ⁷Fox Chase Cancer Center.

INTRODUCTION

Real-time reverse transcriptase quantitative PCR (RT-qPCR) is a widely used technique for measuring transcription levels. Priming strategy and reverse transcriptase enzyme are key elements that affect sensitivity and variability of RT-qPCR and microarray results. The Nucleic Acid Research Group (NARG) had conducted preliminary studies within the group to examine the effects of priming strategy on generating cDNA for use with qPCR. This year's study is an open study in which the qPCR community has been invited to participate. Participants received the RT primers and RNA template and were asked to perform the RT reaction using their preferred reaction conditions. The RT products were returned to the NARG and all RT reactions were used in a qPCR reaction. Results from participating laboratories will be evaluated to determine the impact of priming strategy, assay chemistry and experimental setup on the RT step. Additionally, we are investigating the role of RNA integrity on cDNA synthesis.

RESEARCH PLAN

- To provide members of the real-time PCR community with an opportunity to test their laboratory technique for the handling of RNA and the synthesis of cDNA.
- To compare different primers for the synthesis of cDNA.
- To compare different chemistries for the synthesis of cDNA.
- To compare different experimental conditions for cDNA synthesis.
- To determine effect of RNA integrity on generating cDNA for use with real time RT-qPCR

REVERSE TRANSCRIPTION PRIMERS

Oligo (dT)₂₀:

5'-TTTTTTTTTTTTTTTTTTTTT-3'

Randomers: 6-mer

5'-NNNNNN-3'

Gene Specific Primers:

β-Actin (human)
5'-GCCGATCCACAGGAGTAC-3' (used in 2007-08 study)
5'-AATTACACGAAAGCAATGCTATC-3'

β-Glucuronidase (human)
5'-AAGATCCCCTTTTATTCCC-3' (used in 2007-08 study)
5'-CGTTCGTGCATCAGGTAC-3'
5'-GATACCAAGAGTAGTAGCTGTC-3'

TATA Binding Protein (human)
5'-AGGAAATAACTCTGGCTATAAC-3' (used in 2007-8 study)
5'-ATAGAGGTGGCTTTAACAC-3'

All primers were synthesized by Integrated DNA Technologies®

PROBE AND PRIMER SETS

β-Actin 1 (Accession# NM_001101, amplicon length=71 bases):
FWD Primer: 5'-CCCTGGCACCAGCAGC-3'
REV Primer: 5'-GCCGATCCACAGGAGTAC-3'
Probe: 5'-FAM-ATCAAGATCATGCTCTCTCTGAGCGC-3'BHQ1

β-Actin 2 (Accession# NM_001101, amplicon length=89 bases):
FWD Primer: 5'-CCACCCCACTCTCTAAGG-3'
REV Primer: 5'-AATTACACGAAAGCAATGCTATC-3'
Probe: 5'-FAM-CCAGTCTCTCCCAAGTCCACACA-3'BHQ1

β-Glucuronidase 1 (Accession# NM_000181, amplicon length=66 bases):
FWD Primer: 5'-GAATTTGCGGATTTATG-3'
REV Primer: 5'-AAGATCCCCTTTTATTCCC-3'
Probe: 5'-FAM-CTGAACATGACACGAGAGTGC-3'BHQ1

β-Glucuronidase 2 (Accession# NM_000181, amplicon length=72 bases):
FWD Primer: 5'-CTAACTATGACAGCAAGG-3'
REV Primer: 5'-GATACCAAGAGTAGTAGCTGTC-3'
Probe: 5'-FAM-AACAGATCACATCCACATACGAGCC-3'BHQ1

β-Glucuronidase 3 (Accession# NM_000181, amplicon length=87 bases):
FWD Primer: 5'-CAAAGTCGTCGCAAGTGG-3'
REV Primer: 5'-CGTTCGTGCATCAGGTAC-3'
Probe: 5'-FAM-CAGAGGCTGACACCTGGCACCT-3'BHQ1

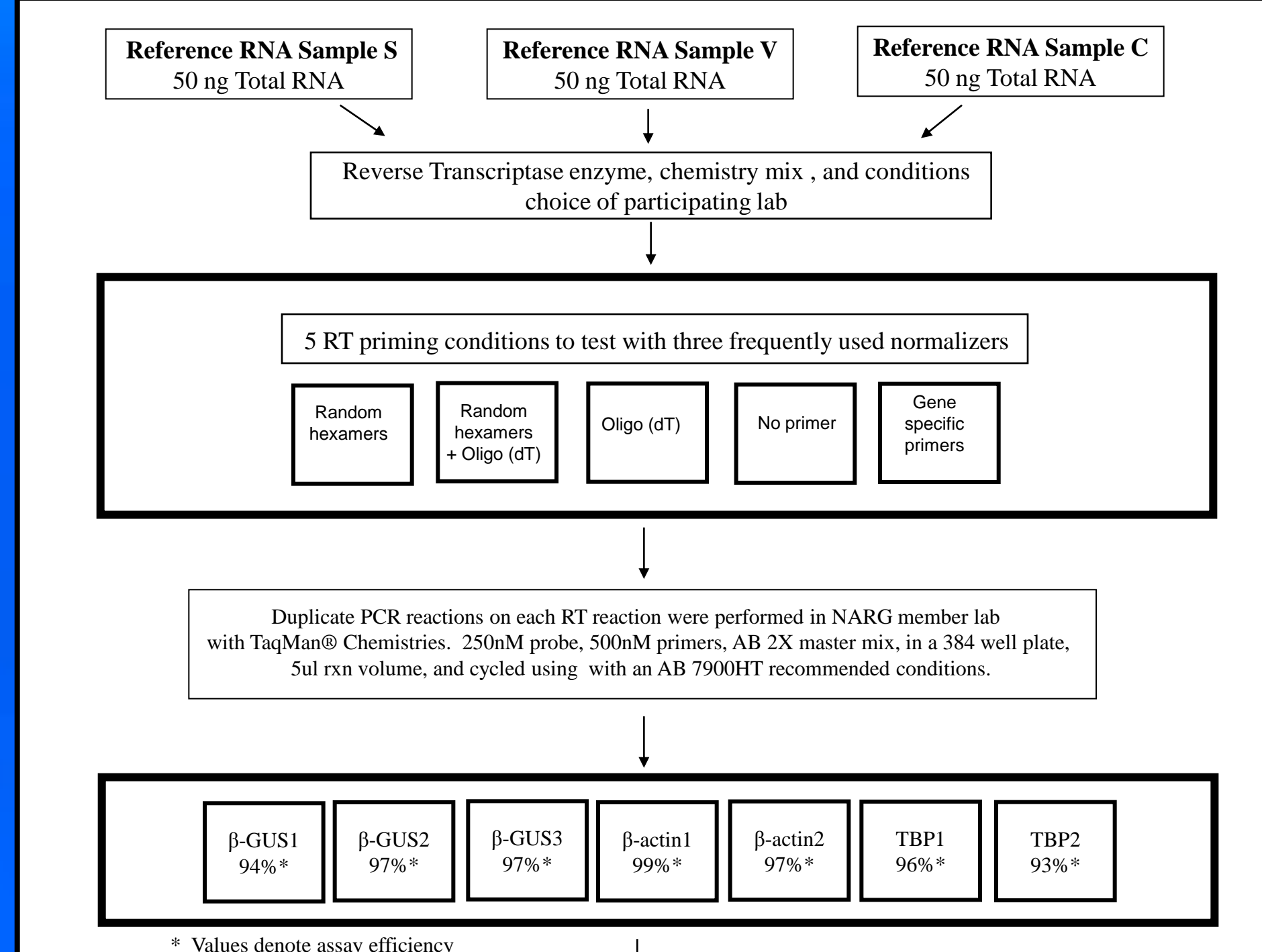
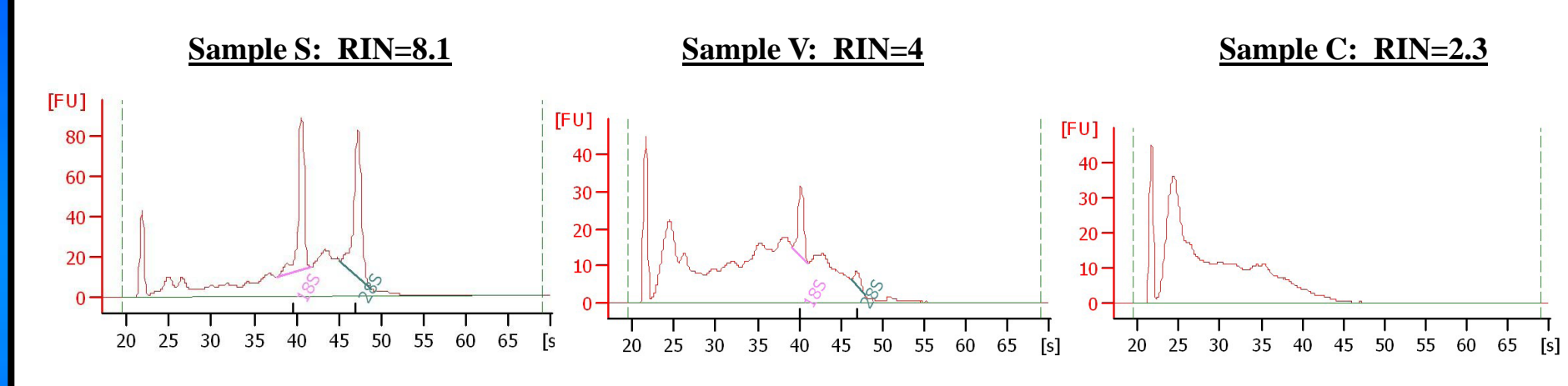
TATA Binding Protein 1 (Accession# NM_003194, amplicon length=80 bases):
FWD Primer: 5'-ATGTGAAGTTCTATAAGGTTAG-3'
REV Primer: 5'-AGGAAATAACTCTGGCTATAAC-3'
Probe: 5'-FAM-CCTTGTGCTCACCCACCAACAAAT-3'BHQ1

TATA Binding Protein 2 (Accession# NM_003194, amplicon length=77 bases):
FWD Primer: 5'-TTATATGTAGATTTTAAACACTGC-3'
REV Primer: 5'-ATAGAGGTGGCTTTAACAC-3'
Probe: 5'-FAM-TTTCCTCAACCAA-3'BHQ1 (LNA bases underlined)

Assays were developed and optimized in the lab of Dr. Greg Shipley

METHODS

RNA: Ambion's First Choice Brain Reference RNA was non-chemically degraded to RIN values of 8.1, 4, and 2 and then stopped by adding an RNase inhibitor. RNA was dried down via a Speed-Vac in an RNase-free environment in the presence of Biomatrix's RNAsable for shipment to participating labs. RNA quantity was confirmed on a NanoDrop spectrophotometer and integrity established by an Agilent 2100 Bioanalyzer. RNA integrity was also assessed by participating labs upon receiving and after resuspension.



RESULTS: IN-HOUSE STUDY (2008/2009)

In-House Study: NARG Member Labs

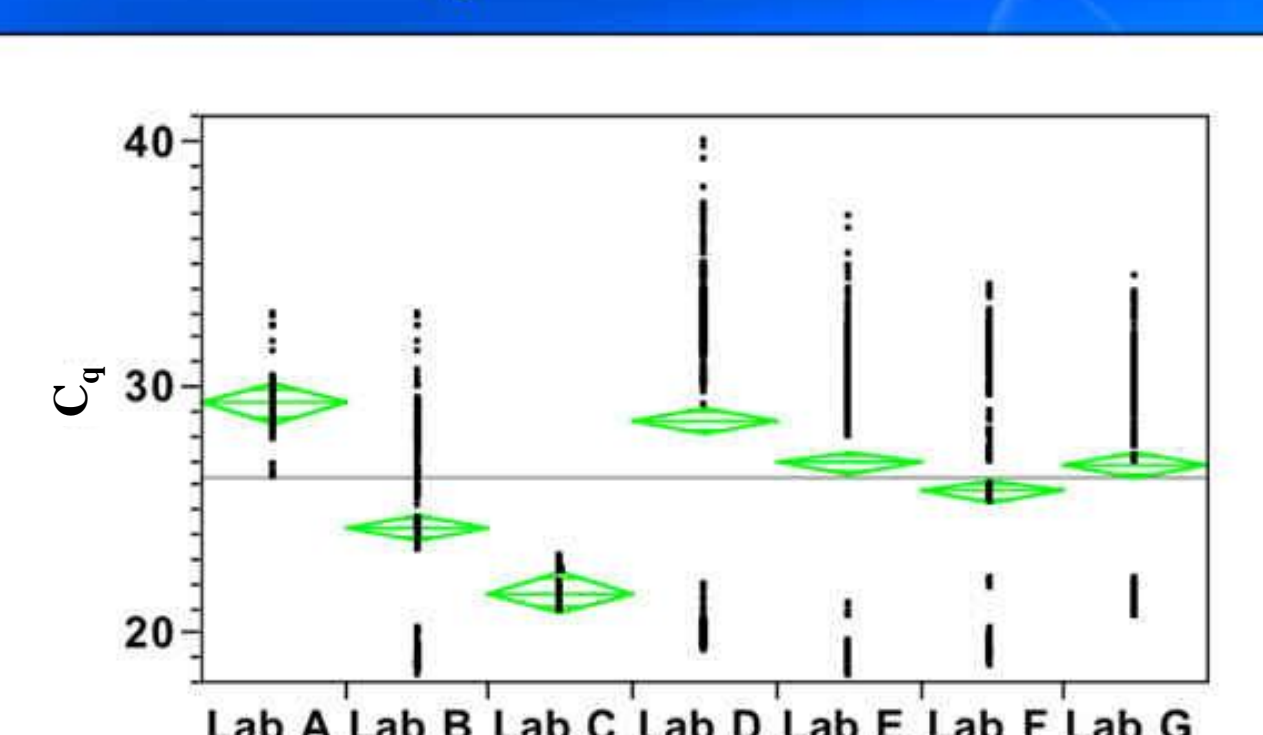


Table I: Mean C_q values derived from all seven assays by NARG member labs only (p<0.0001)

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)

ANALYSIS

Statistical Analysis Methods:

-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).
-A Student's *t*-test was used to assess for significant difference levels (P < 0.05) between the groups contained within each variable.
-ΔC_q values were determined by subtracting the C_q value of priming strategy from the no primer C_q value (i.e., no primer -C_q value)

STUDY PARTICIPANTS RESULTS

Figure I: Effect of Lab on C_q values across all assays and Samples V (RIN=8.1) and S (RIN=4), No primer data included:

Average C_q values by Lab for all 7 assays and both templates

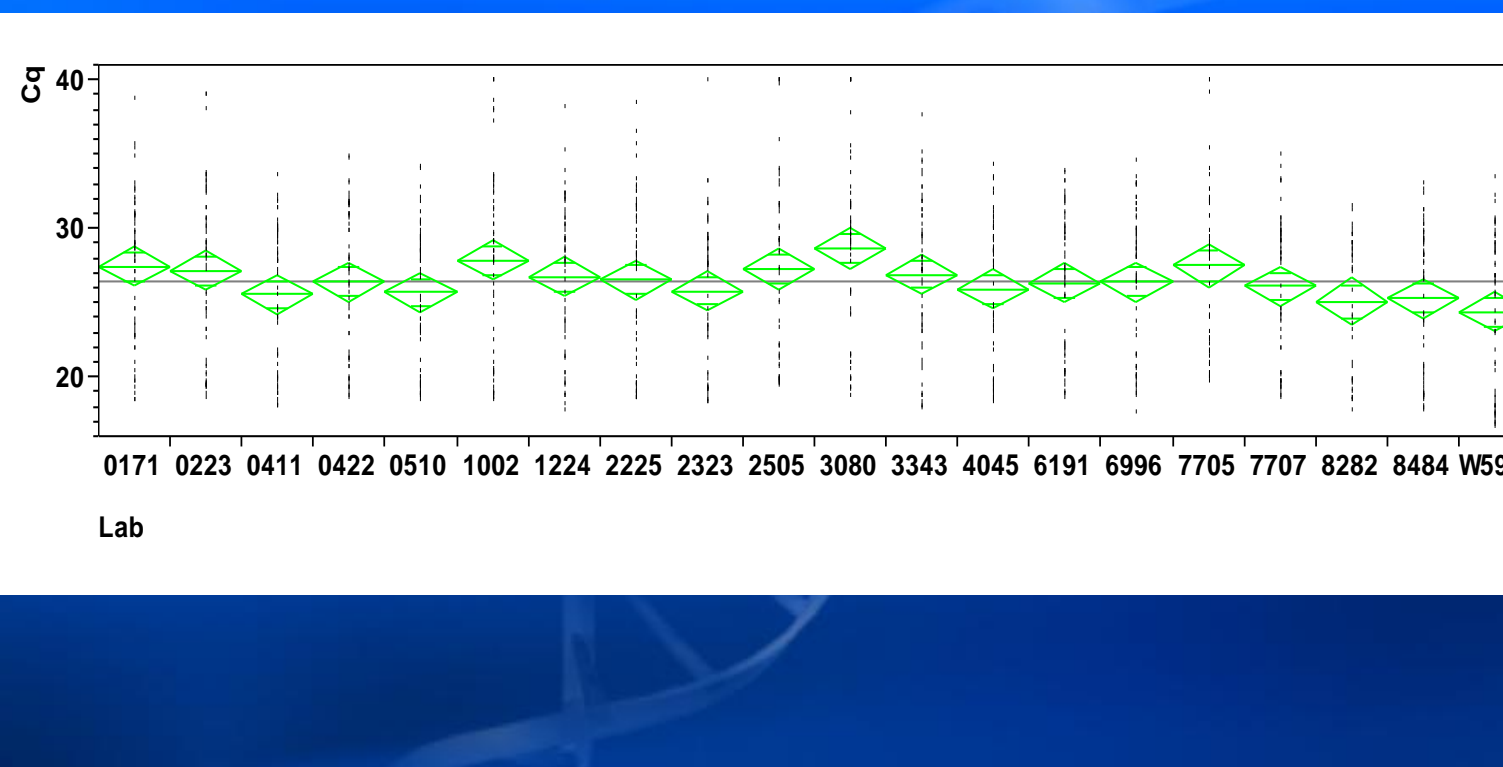


Table II: Participant benchmarking: C_q mean values from samples V and S for all assays

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

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

VARIABLES EXAMINED THAT MAY IMPACT RESULTS

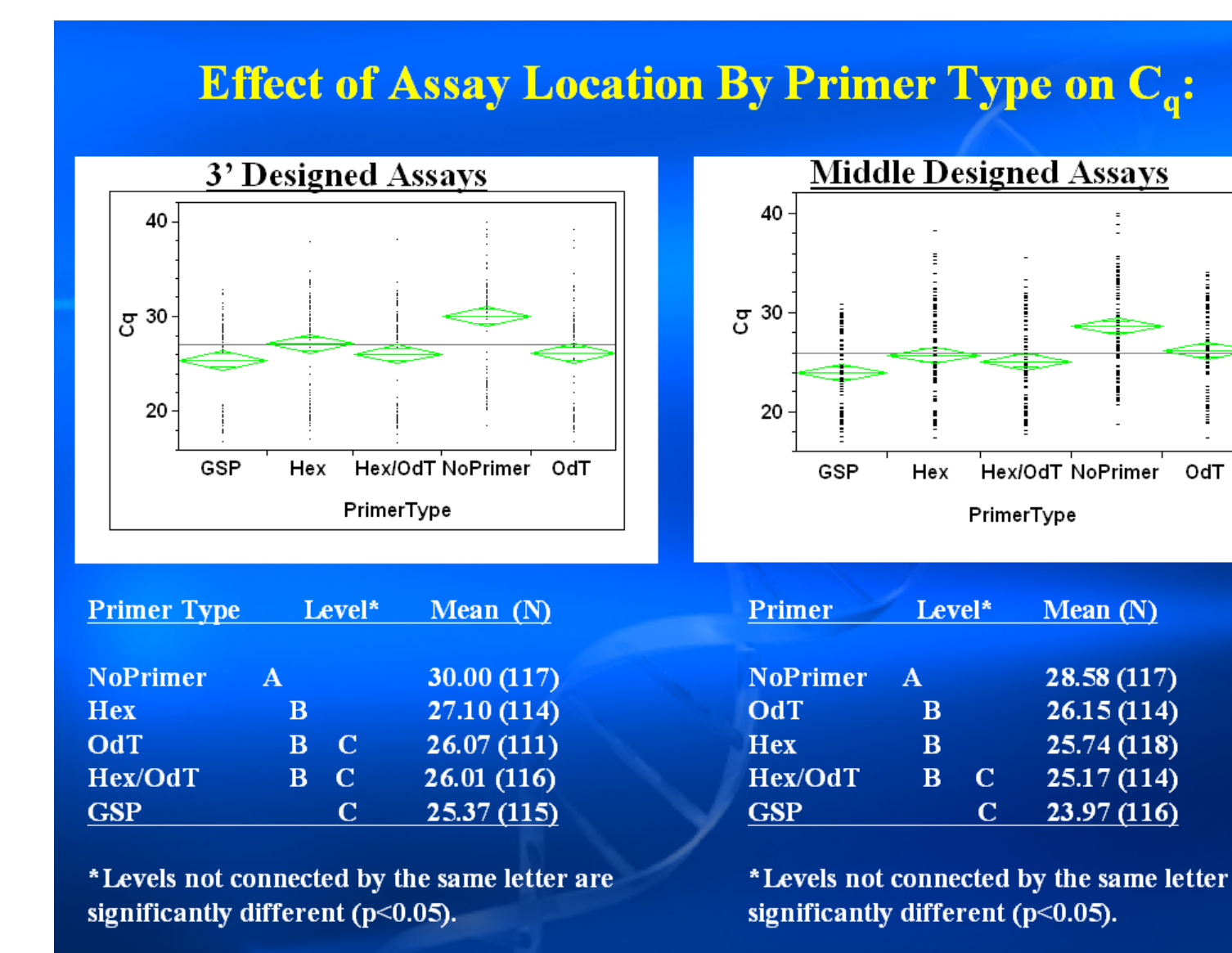
The effect of several variables were examined to assess the impact on results observed:

- Effect of assay location by primer
- Effect of Lab on C_q
- Effect of enzyme on C_q
- Effect of RT temperature on C_q
- Effect of RNA quality on C_q

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

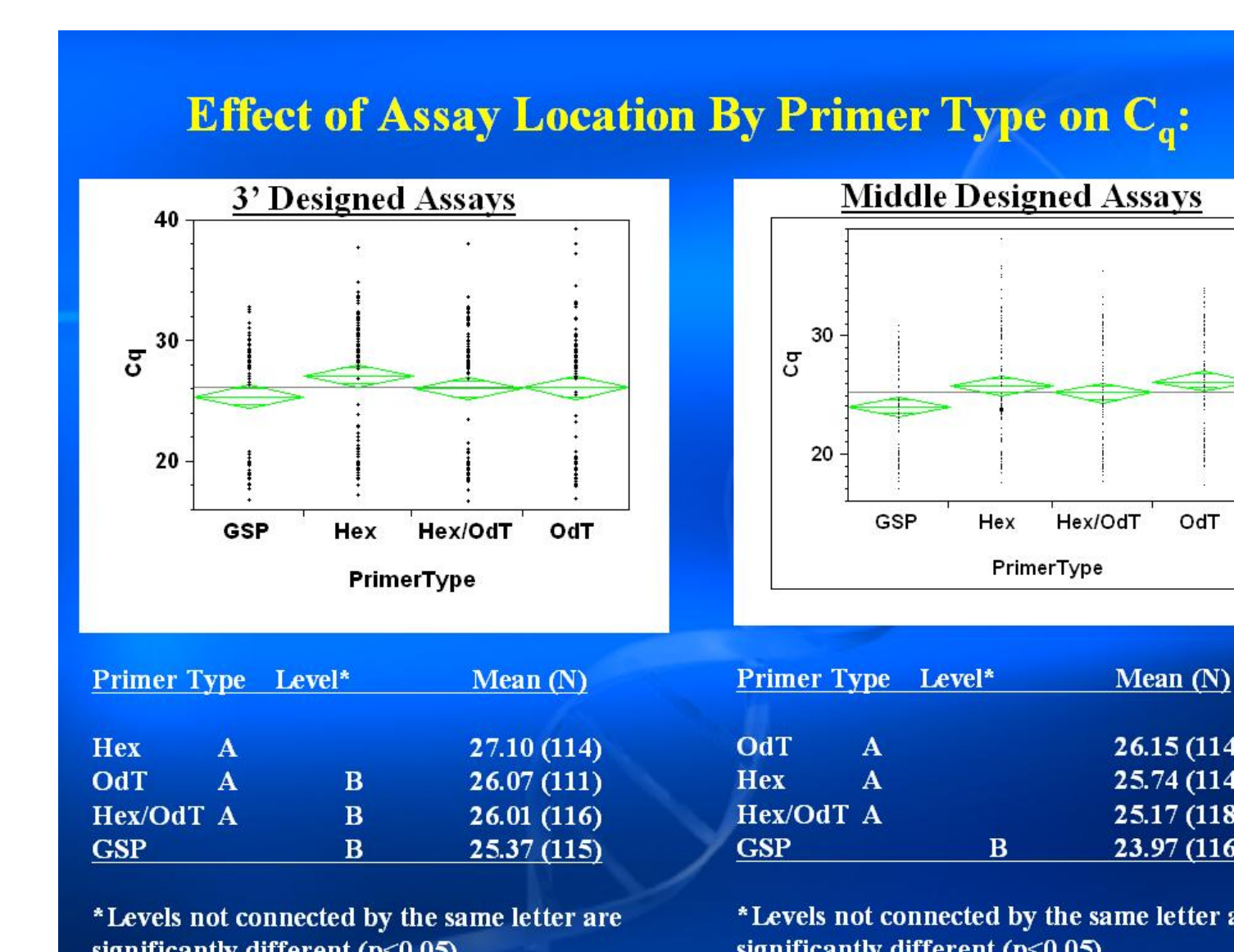
ANALYSIS

Figure II: Effect of Assay Location on C_q with no primer strategy included:



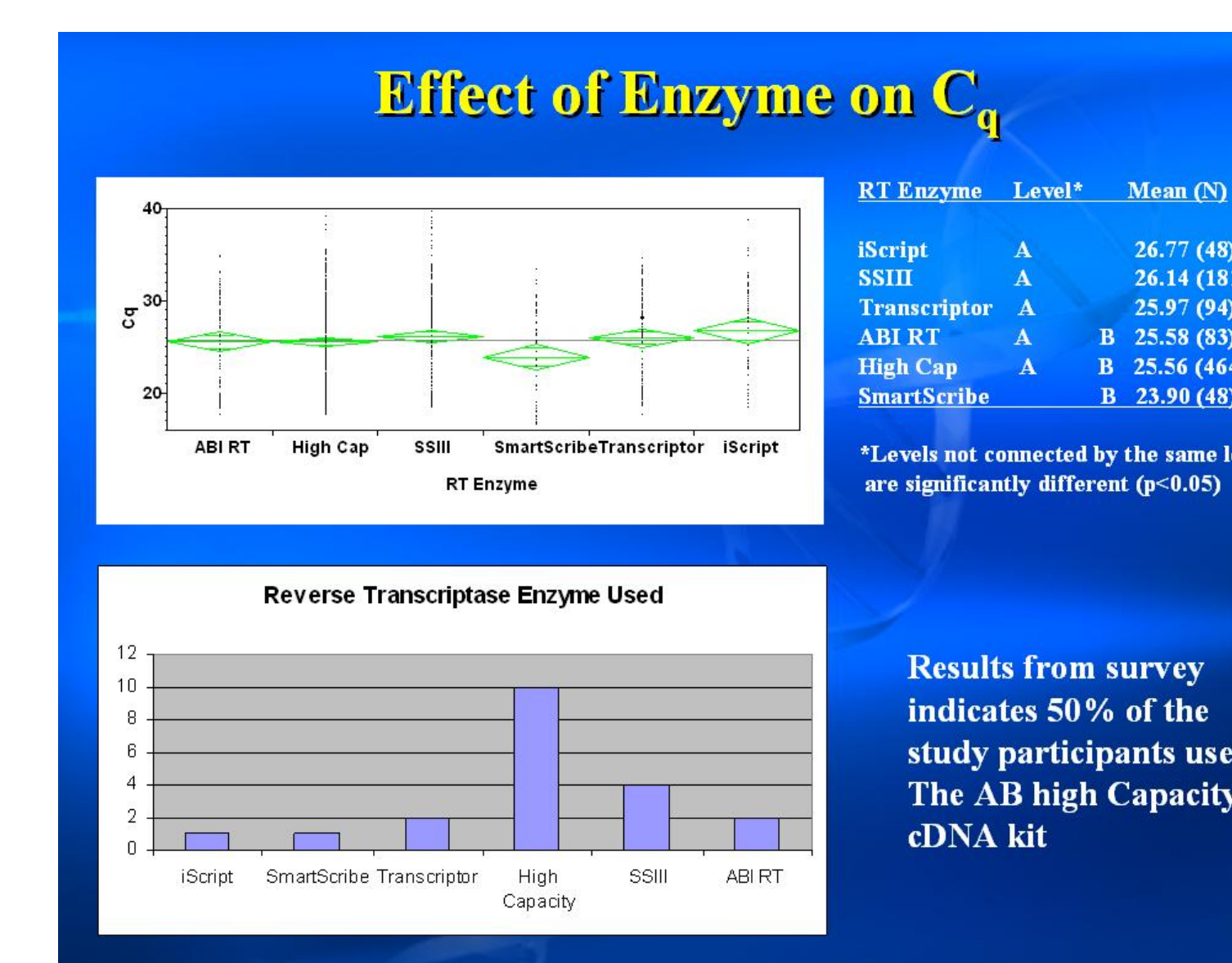
-There was no significant difference between RT priming strategies used to generate cDNA for qPCR assays with regards to the location of the assay design.

Figure III: Effect of Assay Location on C_q with no primer excluded



-With no primer data removed, the C_q values decrease, but no significant difference was observed for RT priming strategies between those assays designed on the 3' end of transcript as compared to a middle assay design.

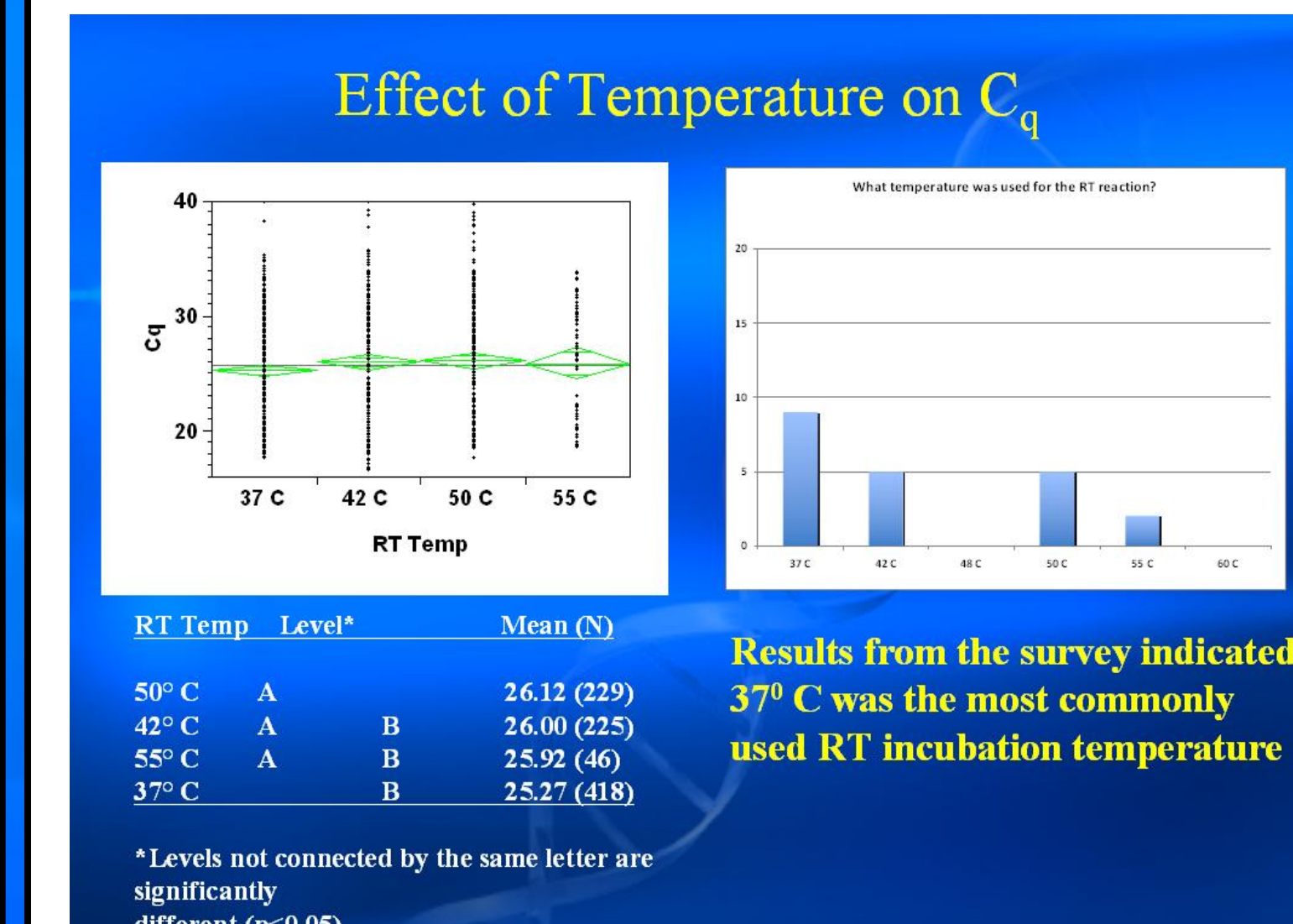
Figure IV: Effect of RT enzyme on C_q



-SmartScribe showed a significant RT enzyme effect on generation of C_q values, but only one sample set was submitted.

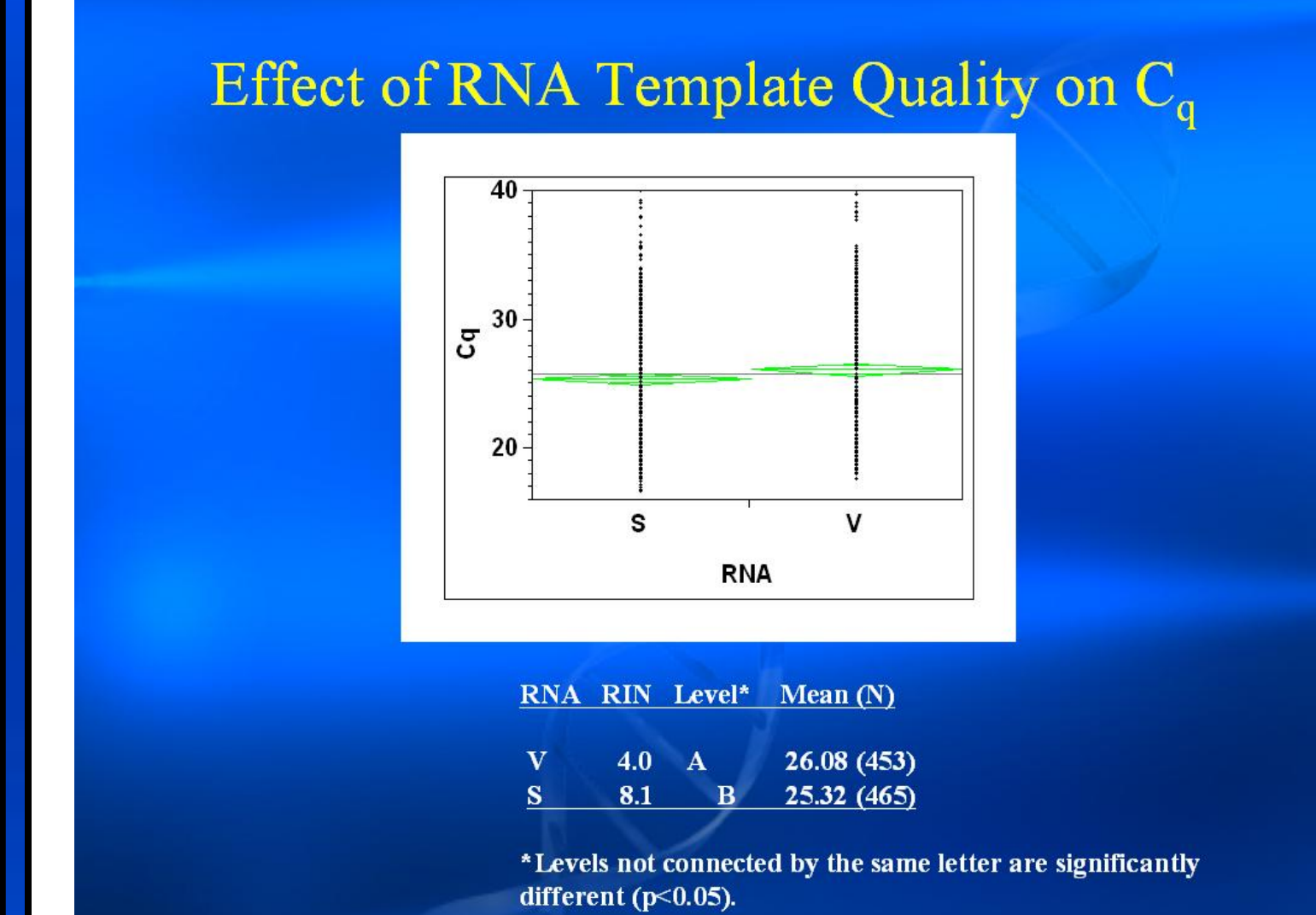
ANALYSIS

Figure IV: Effect of RT rxn temperature on C_q



-The 37° C RT incubation temperature was used most often and generated lower C_q values, but was not significantly different from 42° C and 50° C.

Figure V: Effect of RNA integrity on C_q



-The RNA integrity does impact significantly the C_q values. When comparing a sample with a RIN 8.1 vs. 4.0

CONCLUSIONS

-The C_q values generated from the 2008/2009 "in-house" study showed greater variation between labs as compared to the 2009/2010 open benchmarking study. This variation from the in-house study may have been introduced in the qPCR amplification step as this was not controlled.

-RNA has a high capability to self prime.

-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.

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

-A gene specific primer pool might be considered as well, but the cDNA product should be tested in all assays.

-RNA integrity does impact C_q values, although the ΔC_q between sample S (RIN 8.1) and sample V (RIN 4.0) was only one (2-fold difference) at 100% PCR efficiency.

ACKNOWLEDGEMENTS

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