

# Nucleic Acid Research Group 2008-2009 Study: A comparison of Different Priming Strategies for cDNA synthesis by Reverse Transcriptase, as evaluated by Real-Time RT-qPCR

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## INTRODUCTION

Real-time reverse transcriptase quantitative PCR (RT-qPCR) is widely used for measuring transcription levels. Assay-specific primers, although ideal for cDNA synthesis, are not always practical. Priming strategy and reverse transcriptase enzyme affect the sensitivity and variability of RT-qPCR and microarray results. The Nucleic Acid Research Group (NARG) designed a study to determine the optimal priming strategy for RT-qPCR. The NARG 2008-09 study was an extension of the 2007-08 study in which we evaluated the effect of reverse transcriptase priming strategies on RT-qPCR results. The previous study suggested a relationship between the assay sensitivity using cDNA generated with oligo-dT primers and qPCR assay placement relative to the 3-prime end of the transcript. This year's study was designed specifically to compare oligo-dT and random priming strategies as the assay target site varied. Because the previous study identified random hexamers or nonamers as most efficient of those tested, this year's study was designed specifically to compare oligo-dT, random 6-mers and 9-mers or gene specific primers and combinations. Four reverse transcriptases; Superscript II, Superscript III, Transcriptor and MultiScribe, were employed to determine the effect of enzyme. In addition, the qPCR assays looked at three genes of varying abundance,  $\beta$ -actin (high copy),  $\beta$ -glucuronidase (medium copy) and TATA binding protein (low copy) as well as varying distance from the 3' end for each transcript.

## RESEARCH PLAN AND STUDY TRANSCRIPTS

- To compare 13 different RNA priming strategies using three genes expressed at different levels: Human  $\beta$ -Actin (high),  $\beta$ -Glucuronidase (medium) and TATA Binding Protein (low)
- To compare the efficiency of 4 different RT enzymes that differ based on their RNase H activity and temperature sensitivity.
- 2008 Study Participants: The laboratories of all 8 NARG members
- To provide information useful in expanding study to real-time qPCR community

## REVERSE TRANSCRIPTION PRIMERS

**Oligo (dT)<sub>30</sub>**  
 5'-TTTTT-11TTTTTTTTTTTTTTT-3'

**Randomers: 6-9**  
 5'-NNNNNN-3'  
 5'-NNNNNNNN-3'

**Gene Specific Primers:**  
 $\beta$ -Actin (human)  
 5'-GCCGATCCACACGGAGTAC-3' (used in 2007-08 study)  
 5'-AATTACAGAAAGCAATGCATC-3'

$\beta$ -Glucuronidase (human)  
 5'-AAGATCCCTTTTATTTCC-3' (used in 2007-08 study)  
 5'-CGTTCGTGCATCAGGATC-3'  
 5'-GATACCAAGAGTAGTAGCTTCC-3'

**TATA Binding Protein (human)**  
 5'-AGGAATAACTTGGCTATAAC-3' (used in 2007-8 study)  
 5'-ATAGAGGTGGCTTTAAAC-3'

*All primers were synthesized by Integrated DNA Technologies*

## TAQMAN PROBE AND PRIMER SETS

$\beta$ -Actin I (Accession# NM\_001011, amplicon length=71 bases):  
**FWD Primer:** 5'-CCCTGGCCACCCAGAC-3'  
**REV Primer:** 5'-GCCGATCCACACGGAGTAC-3'  
**Probe:** 5'-FAM-ATCAAGATCATCTGCTCTCTGAGCCG-3'BHQ1

$\beta$ -Actin 2 (Accession# NM\_001101, amplicon length=89 bases):  
**FWD Primer:** 5'-CCACCCCACTTCTCTAAG-3'  
**REV Primer:** 5'-AATTACAGAAAGCAATGCATC-3'  
**Probe:** 5'-FAM-CCAGGCTCTCCCAAGTCCACACA-3'BHQ1

$\beta$ -Glucuronidase 1 (Accession# NM\_000181, amplicon length=66 bases):  
**FWD Primer:** 5'-GAATTTGGCAGTTTCAGT-3'  
**REV Primer:** 5'-AAGATCCCTTTATTTCC-3'  
**Probe:** 5'-FAM-CTGAGATACCCGACGAGAGTGC-3'BHQ1

$\beta$ -Glucuronidase 2 (Accession# NM\_000181, amplicon length=72 bases):  
**FWD Primer:** 5'-CTAACTGACGACCAAGG-3'  
**REV Primer:** 5'-GATACCAAGAGTAGTAGCTTCC-3'  
**Probe:** 5'-FAM-AACAGATCAATCCACATCGAGCC-3'BHQ1

$\beta$ -Glucuronidase 3 (Accession# NM\_000181, amplicon length=87 bases):  
**FWD Primer:** 5'-CAAGTCGTGGCGAATGG-3'  
**REV Primer:** 5'-CGTTCGTGCATCAGGATC-3'  
**Probe:** 5'-FAM-CAGAGGCTGCACCTGGCACCT-3'BHQ1

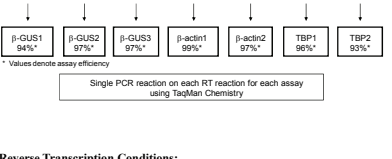
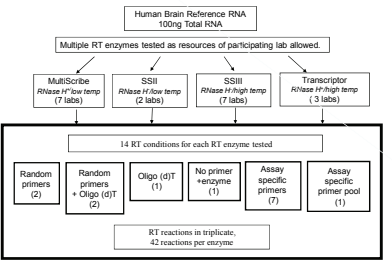
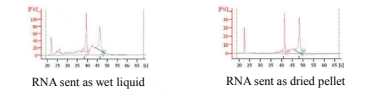
**TATA Binding Protein 1** (Accession# NM\_003194, amplicon length=80 bases):  
**FWD Primer:** 5'-ATGTGAAGTTTCTTAAGTTAG-3'  
**REV Primer:** 5'-AGGAATAACTTGGCTATAAC-3'  
**Probe:** 5'-FAM-CCTTGCTCTCCCAACCAACAT-3'BHQ1

**TATA Binding Protein 2** (Accession# NM\_003194, amplicon length=77 bases):  
**FWD Primer:** 5'-TATATGTAGATTTTAAACAATCGC-3'  
**REV Primer:** 5'-ATAGAGGTGGCTTTAAAC-3'  
**Probe:** 5'-FAM-TTTCCTCCTCAACCA-3'BHQ1 (INA bases underlined)

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

## METHODS

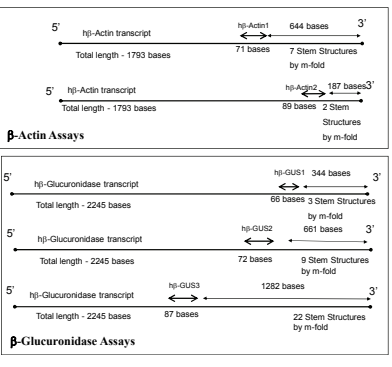
**RNA:** FirstChoice® Human Brain Reference RNAs was selected for this study and purchased from Ambion (Foster City, CA). This is one of the same reference RNA used as an External RNA Control (ERC) in the MAQC study (*Nature Biotechnology*; 24, 1123–1129, 2006). RNA quantity was confirmed on a NanoDrop spectrophotometer and integrity established by an Agilent 2100 Bioanalyzer. RNA was either dried down via a Speed Vac in an RNase-free environment or sent in liquid form spiked with 40 U/ug of SUPERase-In RNase Inhibitor (Invitrogen, Carlsbad, CA) for shipment to participating labs. RNA integrity was assessed by participating labs upon receiving.



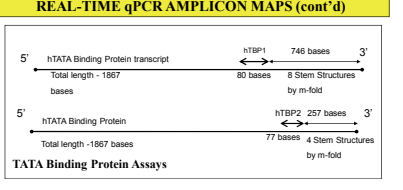
**Reverse Transcription Conditions:**  
 100 ng Total RNA (sent as dry pellet)/cDNA reaction  
**Primer:** Final Concentration (20 µl vol.)  
 Randomer 4.0 µM  
 Oligo (dT) 2.5 µM  
 Gene specific 400 nM  
 Combs 2 µM randomer + 2 µM oligo(dT) or 400 nM pool of 7 gene specific primers  
 RT Enzyme Individual Lab specifics

**qPCR Conditions:**  
**Primer:** Final Concentration (20 µl vol.)  
 cDNA 1/10 cDNA volume (10 ng RNA Equivalent)  
 Primer(s) 500 nM  
 Probe 250 nM  
 Master Mix ABI master mix

## REAL-TIME qPCR AMPLICON MAPS



## METHODS



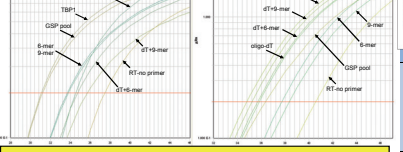
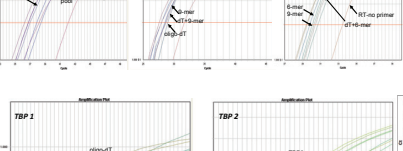
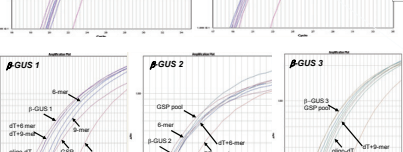
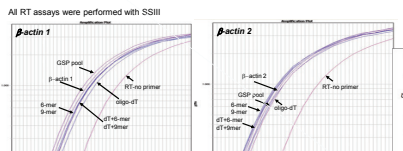
**Statistical Analysis Methods:**  
 The effect of each variable on  $C_t$  or  $\Delta C_t$  levels were assessed using a one-way analysis of variance (ANOVA) with the JMP v 5.01 Statistical Discovery Software (SAS Institute, Cary, NC).

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

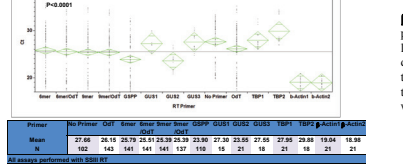
$\Delta C_t$  values were determined by subtracting the  $C_t$  value of priming strategy from the no primer  $C_t$  value for the same assay (i.e., no primer- $C_t$  value)

## RESULTS & DISCUSSION

### EFFECT OF RT STRATEGY AND ASSAY LOCATION



### EFFECT OF RT PRIMING STRATEGY ON $C_t$



## RESULTS & DISCUSSION

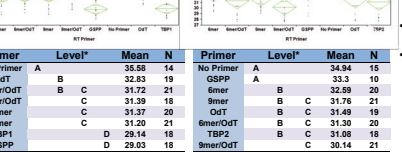
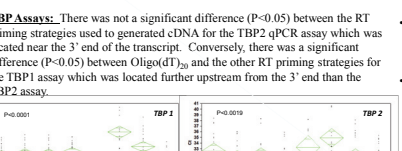
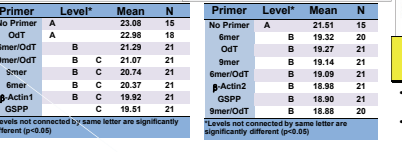
The mean  $C_t$  values for each of the gene-specific primers for the 2 assays against the  $\beta$ -actin and TBP gene transcripts were similar suggesting these assays were equally as effective in measuring transcript levels for their respective gene target. The mean  $C_t$  values for  $\beta$ -GUS1 and  $\beta$ -GUS3, however, were significantly different ( $P < 0.05$ ) than the mean values obtained using the  $\beta$ -GUS2 assay. The differences in transcript assessment between the 3 GUS assays may be the result of template non-specificity since primers used in the  $\beta$ -GUS2 assay also gave hits for non-coding RNA sequences in the genome.

The ANOVA using the combined data from all 7 assays showed that the non-specific priming strategies (i.e., randomers and oligo(dT)<sub>30</sub>) gave similar  $C_t$  values suggesting there was not an effect of priming strategy on the ability to generate cDNA for use with real time qPCR. As described below, however, there was a significant effect of priming strategy on the ability to generate cDNA when evaluated within each assay.

### EFFECT OF ASSAY LOCATION ON $C_t$

Last year's NARG study suggested that assay location relative to the 3' end of the gene transcript affected the relative ability of randomers and oligo(dT)<sub>30</sub> RT priming strategies to produce cDNA for use with qPCR. The study presented herein was designed to specifically explore the question of whether the assay location impacts the RT priming strategy. At least 2 qPCR assays that differed with respect to their location relative to the 3' end of the  $\beta$ -Actin, GUS, and TBP gene transcripts were designed. Relative levels of gene expression as denoted by  $C_t$  or  $\Delta C_t$  were acquired for the cDNAs generated using each priming strategy within each of the 7 qPCR assays. The results show that oligo(dT)<sub>30</sub> is more effective in generating cDNA when the qPCR assay location is closer to the 3' end of the transcript. For all assays, the gene specific primer appeared to be the most effective in generating cDNA for use with qPCR.

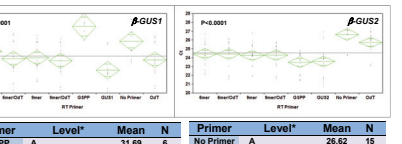
**$\beta$ -Actin Assays:** There was not a significant difference ( $P < 0.05$ ) between the RT priming strategies used to generate cDNA for the  $\beta$ -actin 2 qPCR assay which was located near the 3' end of the transcript. Conversely, there was a significant difference ( $P < 0.05$ ) between Oligo(dT)<sub>30</sub> and the other RT priming strategies for the  $\beta$ -actin 1 assay which was located further upstream from the 3' end than the  $\beta$ -actin 2 assay.



**TBP Assays:** There was not a significant difference ( $P < 0.05$ ) between the RT priming strategies used to generate cDNA for the TBP2 qPCR assay which was located near the 3' end of the transcript. Conversely, there was a significant difference ( $P < 0.05$ ) between Oligo(dT)<sub>30</sub> and the other RT priming strategies for the TBP1 assay which was located further upstream from the 3' end than the TBP2 assay.

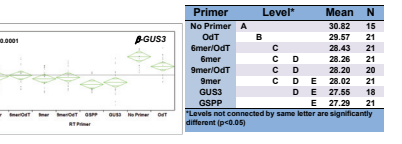
**$\beta$ -GUS Assay:** There was not a significant difference ( $P < 0.05$ ) between the RT priming strategies used to generate cDNA for the  $\beta$ -GUS1 qPCR assay which was located near the 3' end of the transcript. Conversely, there was a significant difference ( $P < 0.05$ ) between Oligo(dT)<sub>30</sub> and the other RT priming strategies for the GUS2 and GUS3 assays which were located further upstream from the 3' end than the GUS1 assay. The gene specific primer pool priming strategy did not work with the GUS1 assay.

## RESULTS & DISCUSSION



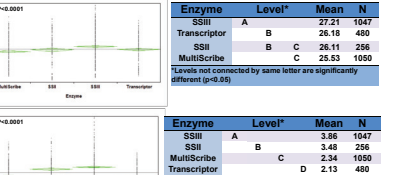
Primer	Level*	Mean	N
No Primer	A	31.69	6
ODT	A	30.21	12
6mer	B	29.18	18
9mer	C	28.55	18
6mer/ODT	B	28.44	20
9mer/ODT	B	24.26	21
GUS2	C	23.24	18
GUS2	C	23.24	21
GSSP	C	23.55	20
GUS1	D	27.3	15

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



### EFFECT OF RT ENZYME

ANOVA and *t*-test means comparison by  $C_t$  for each of the RT enzymes. Levels not connected by same letter are significantly different. While MultiScribe fared better on the basis of  $C_t$  values, SSIII scores better when comparing  $\Delta C_t$ .



Enzyme	Level*	Mean	N
SSIII	A	30.82	15
Transcriptor	B	28.18	480
MultiScribe	B	26.11	256
MultiScribe	C	25.53	1060

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

RNA has a high capability to self prime even when a high temperature RT enzyme like SuperScript™ III is used.

- Each assay generates its own unique profile for optimal cDNA priming strategy that appears to be impacted by:
  - Location of assay design
  - Lab performing assay (data not shown)
- Gene specific priming generates the lowest  $C_t$  values for  $\beta$ -Actin, TBP and  $\beta$ -GUS. However this may not be a simple task when performing qPCR for multiple transcripts and in fact the GSP pool fared inconsistently between the assays.
- While no discernible differences were observed between the ODT and random priming for all three transcripts measured in the 3' most assays, ODT by itself fared poorly in the assays designed further away from the 3'-end. This suggests that location of the assay is a very important component but using a mixture of ODT/randomer would improve the overall results.
- The behavior of the GUS2 primer is attributed to similarity between the transcript sequence to sequence of non-coding RNA.
- No significant differences were found between manual and robotic setups of the reactions (data not shown)
- No significant differences could be discerned from the enzymes used in the study.

**Future Directions:**  
**NARG Study Part II:**  
**Rationale:** Allow users to benchmark their in-house RT protocol using provided RNA template and primers.  
**Goal:** To find effective primer(s) that will provide optimal cDNA synthesis for use in the broadest range of qPCR assays.  
 Real-time qRT-PCR community performs the Reverse Transcriptase (RT) Reaction  
**Provided:** RNA, Primers (6-mer, 9-mer, oligo (dT)<sub>30</sub>, mixes, GSP)  
**Protocols:** Questionnaire/Survey  
**Return:** cDNA to the NARG for qPCR, Questionnaire/Survey

### ACKNOWLEDGEMENTS

We gratefully acknowledge all the participants of this study. We would like to acknowledge the hard work of David Frank (University at Albany), Meghan Kohlmeier and Scott Tighe (University of Vermont), Eilat Snir and Mary Boes (University of Iowa), Xiaoyang Wang and Scott Hutto (UTHSC-Houston), and Jennifer Holbrook and Deborah Stables (Nemours COBRE Center for Pediatric Research) with whom this study would not have been possible.