Nucleic Acids Research Group 2008 Study: A Comparison of Different Priming Strategies for cDNA Synthesis as Measured by Real-Time RT-qPCR

KL Knudtson[1], DS Grove[2], DJ Hollingshead[3], KS Lilley[4], GL Shipley[5], K Sol-Church[6], WL Taylor[7] and TC Hunter[8]

[1]University of Iowa, [2]Penn State University, [3]University of Pittsburgh, [4]University of Cambridge , [5]UTHSC-Houston, [6]A.I. duPont Hospital for Children, [7]UTHSC-Memphis, [8]University of Vermont

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INTRODUCTION

Real-Time Reverse Transcriptase Quantitative PCR (RT-qPCR) has become the method of choice to quantify transcript levels. Unfortunately, the ability to generate adequate quantities of cDNA for use with qPCR continues to challenge investigators. Recent publications (Stahlberg, *et. al.*,Clinical Chem., 50:509, 2004; Stangegaard, *et. al.*, BioTechniques 40:649, 2006) focused on the reverse transcription step and more specifically the impact on assay sensitivity of the random primer used in the generation of the cDNA. While many laboratories use assay-specific primers for priming cDNA synthesis, the need to also utilize non-specific priming strategies during the RT step has become paramount for some RT-qPCR assay designs (e.g., multiplexed assays). The Nucleic Acid Research Group (NARG) has conducted an internal study to evaluate priming strategies for generating cDNA for use with real-time RT-qPCR. The goal of this study is to determine which single or combinatorial priming strategies are best suited for the most comprehensive synthesis of cDNA from total RNA for real-time RT-qPCR. The study was designed to evaluate the ability of random primers of increasing length (6 to 21 bases in steps of 3 bases) in initiating cDNA synthesis. The set of random primers was compared to oligo-(dT), anchored oligo-(dT), combinations of random primer and oligo-(dT), assay-specific primer and no primer with and without reverse transcriptase. A high temperature RNaseH- RT enzyme commonly used in this community (NARG Survey 2007), Superscript III was selected as the reverse transcriptase enzyme.

RESEARCH PLAN AND STUDY TRANSCRIPTS

-To Compare 25 different RNA priming strategies using three genes expressed at different levels: Human **β** -Actin (high), **β**-Glucuronidase (medium) and TATA Binding Protein (low)

-First Year Study Participants: The laboratories of all 7 NARG members

-To Provide information useful in expanding study to real-time qPCR community

REVERSE TRANSCRIPTION PRIMERS

Randomers: 6, 9, 12, 15, 18, 21
5'-NNNNNN-3'
5'-NNNNNNNNN-3'
5'-NNNNNNNNNNNN-3'
5'-NNNNNNNNNNNNNNN-3'
5'-NNNNNNNNNNNNNNNNNN-3'
5'-NNNNNNNNNNNNNNNNNNNNN-3'

Oligo (dT)$_{20}$:
5'-TTTTTTTTTTTTTTTTTTTT-3'

Anchored Oligo (dT)$_{20}$:
5'-TTTTTTTTTTTTTTTTTTTTV-3'

Gene Specific Primers:
β -Actin
5'-GCCGATCCACACGGAGTAC-3'
β-Glucuronidase
5'-AAGATCCCCTTTTTATTCCC-3'
TATA Binding Protein
5'-TGTTCACTTGGGCAGGAC-3' *All primers were synthesized by Integrated DNA Technologies®*

PROBE AND PRIMER SETS

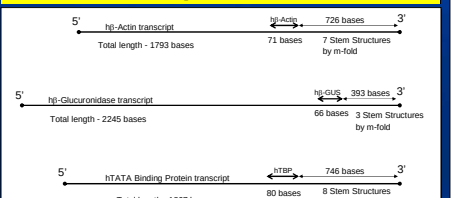
β-Actin (Accession# NM_001101, amplicon length=71 bases) **:**
FWD Primer: 5'-CCCTGGCACCCAGCAC-3'
REV Primer: 5'-GCCGATCCACACGGAGTAC-3'
Probe: 5'FAM-ATCAAGATCATTGCTCCTCCTGAGCGC-3'BHQ1

β-Glucuronidase (Accession# NM_000181, amplicon length=66 bases) **):**
FWD Primer: 5'-GAATTTTGCCGATTTCATG-3'
REV Primer: 5'-AAGATCCCCTTTTTATTCCC-3'
Probe: 5'FAM-CTGAACAGTCACCGACGAGAGTGC-3'BHQ1

TATA Binding Protein (Accession# NM_003194, amplicon length=80 bases)**:**
FWD Primer: 5'-CCGTGGTTGTGAAGAAGATC-3'
REV Primer: 5'-TGTTCACTTGGGCAGGAC-3'
Probe: 5'FAM-CCGTGGTTGTGAAGAAGATC-3'BHQ1

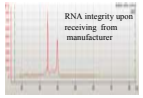
Assays were developed and optimized in the lab of Dr. Greg Shipley, UTHSC, Houston, TX

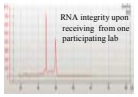
REAL-TIME qPCR AMPLICON MAPS

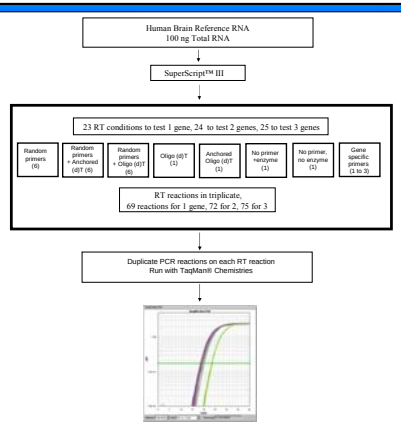


METHODS

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, 1132 – 1139, 2006). RNA quantity was confirmed on a NanoDrop spectrophotometer and integrity established by an Agilent 2100 Bioanalyzer. RNA was dried down via a Speed Vac in an RNase-free environment for shipment to participating labs. RNA integrity was assessed by participating labs after resuspension.







Reverse Transcription and qPCR reaction Conditions:

100 ng Total RNA (sent as dry pellet)/cDNA reaction

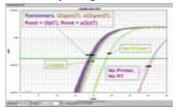
Primer:	Final Concentration (20 µl vol.)
Randomer	4.0 µM
Oligo (d)T	2.5 µM
Anchored oligo(d)T	2.5 µM
Gene specific	2.5 µM
Combos	2 µM randomer +2 µM oligo(dT) or anchored oligo(dT)

PCR RXN:

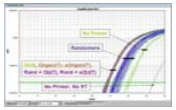
cDNA	1/10 cDNA volume (10 ng RNA Equivalent)
Primer(s)	Individual Lab specifies
Probe	Individual Lab specifies

RESULTS

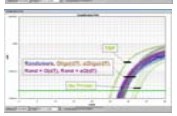
Amplification plots by assay: Typical amplification plots generated for each assay illustrates the ability of RNA (no primer) to self-prime the cDNA reaction more efficiently than commonly thought



β-Actin



β-Glucuronidase



TATA Binding Protein

ANALYSIS

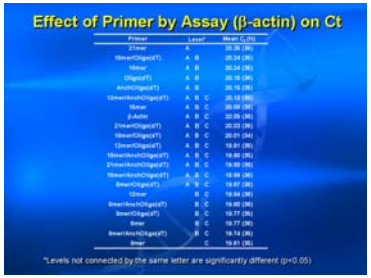
Statistical Analysis Methods:

-The effect of each variable on C_t or ΔC_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).

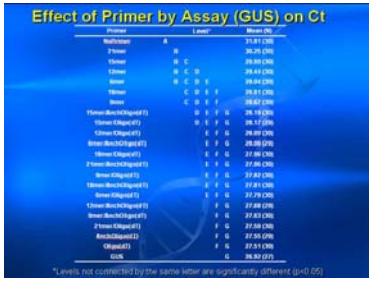
-A Student's *t*-test was used to assess for significant difference levels ($P < 0.05$) between the groups contained within each variable.

-ΔC_t values were determined by subtracting the $C_{t\ value}$ of priming strategy from the no primer C_t value (i.e., no primer $-C_t$ value)

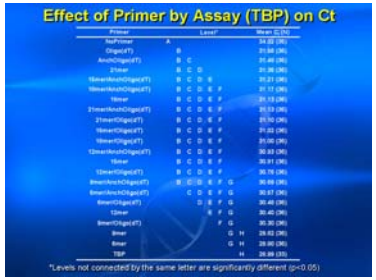
Effect of Primer on C_t by Assay: t-test means comparison by C_t for each priming strategies. Levels not connected by same letter are significantly different. Each assay appears to generate its own unique profile for best cDNA priming strategy



-The shorter randomers and mixture of randomers with oligo(dT) and anchored oligo(dT)were similarly effective priming strategy as the gene specific.



-Oligo(dT), anchored oligo(dT), and the mixtures of randomers with oligo (dT) and anchored oligo (dT) generated lowest C_t values for the GUS assay. It should be noted that the GUS assay was designed 393 bases from the 3' end of the transcript and may contribute to the success of the oligo (dT) priming strategy

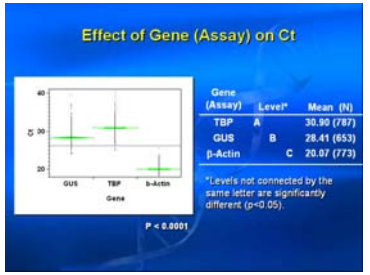


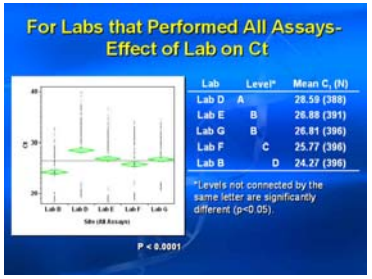
-Gene specific priming and the shorter randomers (6-mer and 9-mer) generated the lowest Ct values for the TBP assay.

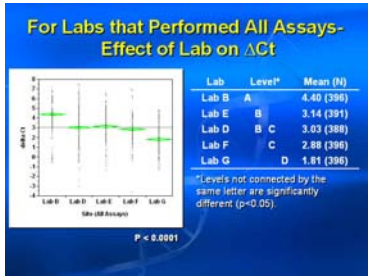
Variables examined that may impact results:

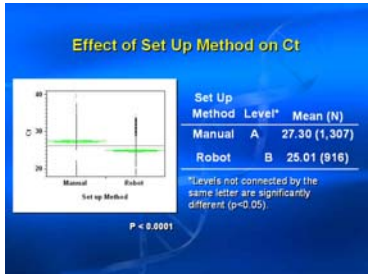
-The effect of several variables were examined to assess the impact on results observed:
- -Effect of Gene Assay on Ct
- -Effect of Lab on Ct
- -Effect of Lab on ΔCt
- -Effect of Set Up Method on Ct

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



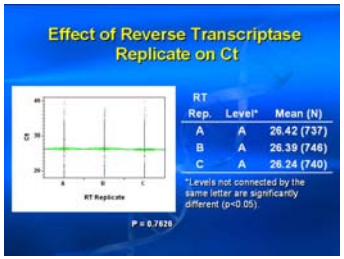




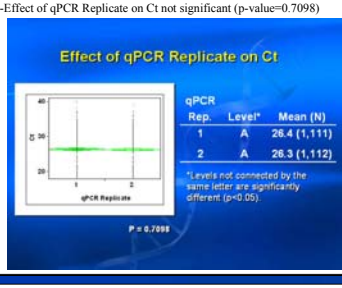


Variables examined that may impact results:

-Effect of RT replicate on Ct not significant (p-value=0.726)



-Effect of qPCR Replicate on Ct not significant (p-value=0.7098)



Results by Assay:

β-Actin
There were not any significant differences between any of the primers or combination in their ability to affect C_t or ΔC_t levels

GUS
The effects of oligo (dT) and anchored oligo (dT) alone or in Combination with a randomer on C_t or ΔC_t were significant compared to the randomers alone

TBP
The effects of the gene specific primer, followed by the randomers On C_t or ΔC_t were significant compared to the effects of oligo (dT) and anchored oligo (dT) alone or in combination

Conclusions:

-RNA has a high capability to self prime even when a high temperature RT enzyme like SuperScript™ III is used
- -Amount of self-priming was lab specific in this experiment

-Each assay generates its own unique profile for optimal cDNA priming strategy that appears to be impacted by:
- -Level of expression
- -Location of assay design
- -Manual vs. robotic plate set up
- -Lab performing assay

-Gene specific priming generates the lowest Ct values for TBP and GUS

-No discernable differences were observed between the anchored or non-anchored oligo (dT) priming for all three transcripts measured

-Different priming strategies for a high expressing transcripts such as β-Actin may not define one specific strategy as optimal

Future Directions:

-To examine the impact of assay location in relation to the 3'-end by using three new assays for the transcripts used in this study with varying distances from the 3'-end

NARG Study Part II:

-Rationale: Allow users to to benchmark their in-house RT protocol Using provided RNA template and primers.

-Goal: To find an 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)$_{20}$, mixes, GSP Protocols, Questionaire/Survey

Return: cDNA to the NARG for qPCR, Questionaire/Survey

ACKNOWLEDGEMENTS

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