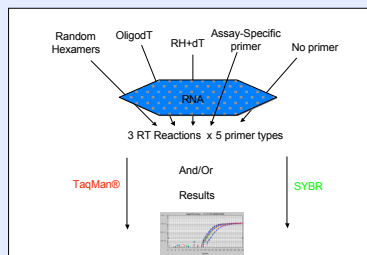


## What was the effect of different priming strategies on quantification?

### Experimental Design



**Abstract**

The Nucleic Acids Research Group (NARG) study for 2005-2006 invited scientists to participate in a study designed to gain crucial information about the variability of the reverse transcription (RT) step of the quantitative (q)PCR assay and about the comparability of qPCR results obtained using different cDNA priming strategies. The study was designed with two primary goals: 1) to provide members of the real-time community with an opportunity to appraise their technique and 2) to contrast different cDNA priming strategies utilizing the participants' assay reagents and instruments. Two differently-expressed genes were chosen, human  $\beta$ -glucuronidase ( $\beta$ GUS) and human TATA-Binding Protein (TBP). A RNA template, primers and probes were provided by the NARG and participants were asked to test five reverse transcription priming strategies for preparing cDNA: no primer, random hexamers, oligo-dT, assay-specific primer and random hexamers:oligo-dT (1:1). The participants then performed real-time PCR using the cDNA templates generated by the different priming strategies, using either TaqMan<sup>®</sup> or SYBR Green I reagents and real-time instrumentation commonly used in their laboratory. Results were submitted to the NARG for analysis and will provide feedback as to individual's technique as well as information to the general community on how priming strategies affect the final PCR data.

### Study Goals

To compare 5 different RNA priming strategies using two genes expressed at differing levels  
 To provide evaluation/education for study participants

### Research Plan

Each Laboratory-  
 Perform reverse transcription (RT) on the provided reference RNA template using 5 priming strategies  
 Amplify cDNA prepared with each priming strategy using the NARG experimental protocol and supplied assay materials  
 Complete a web-based survey on assay reagents, instruments and laboratory methodology  
 Send the raw data and jpg files of amplification plots to the NARG committee

### Selected Genes

Human GUS ( $\beta$ -Glucuronidase) and TBP (TATAA Binding Protein) were selected as genes with different transcript levels

GUS: Medium-abundance transcript  
 TBP: Low-abundance transcript

### Methods

**Project Kit:**  
 Reverse Transcription Reagents:  
 4000 ng Stratagene hReference RNA Template  
 125 pmole Random hexamers  
 125 pmole Oligo dT  
 125 pmole hGUS R primer for RT: 5'CGAGTGAAGATCCCCTT3'  
 125 pmole hTBP R primer for RT: 5'TGGACTGTCTTCTCACTCTGGC3'

**PCR Reagents:**  
 1000 pmole hGUS F primer: 5'CTCATTGGAAATTTGCC3'  
 1000 pmole hGUS R primer: 5'CGAGTGAAGATCCCCTT3'  
 200 pmole hGUS Probe: 5'FAM-TGAACAGTCACCGACGAGAGTGCTGG-3'BHQ1  
 1000 pmole hTBP F primer: 5'TTCGGAGAGTCTGGGATGTA3'  
 1000 pmole hTBP R primer: 5'TGGACTGTCTTCACTCTGGC3'  
 200 pmole hTBP Probe: 5'FAM-CCGTGGTTCGTGGCTCTTATCTCCTAT-3'BHQ1

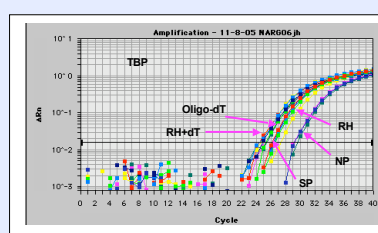
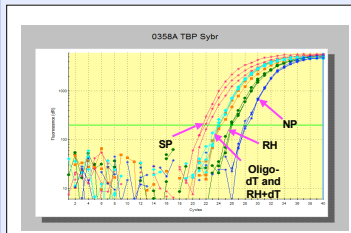
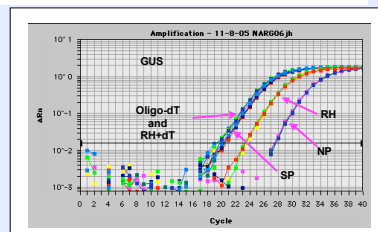
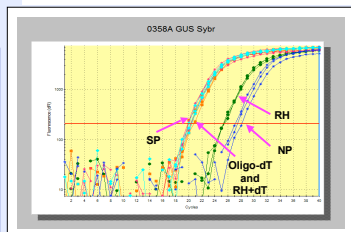
Clin. Chem. 2002 Aug;48(8):1329-37; J Thorac Cardiovasc Surg. 2002 Mar;123(3):484-91; BBRC. 313 (2004) 856-862.

Participants were given directions on how to perform the RT and they were asked to run PCR reactions using the chemistry and machine(s) in their laboratory.

Information concerning the chemistry, platform, assay conditions, etc., was submitted using a web-based survey form; jpg files of amplification plots were sent with the final exported numerical data via e-mail.

**Primer/Probe Synthesis:** The probes were synthesized in the DNA Chemistry Laboratory at the Centers for Disease Control. The probe was synthesized on an Applied Biosystems (Foster City, CA) 3400 DNA synthesizer using standard phosphoramidite chemistry, starting with a BHO-1 CPG. 0.2 mmole (Biosearch Technologies, Novato, CA) and labeled at the 5' end with 6-carboxy-fluorescein (FAM) (Glen Research, Sterling, Virginia). Primers were synthesized on a MerMade 12 DNA synthesizer (BioAutomation, Plano TX) using standard phosphoramidite chemistry and 0.05 umole columns from Biosearch Tech (Novato CA). The probe was purified to greater than 90% purity by reverse-phase HPLC.

## Examples of typical amplification plots for GUS and TBP from two participants showing data produced with the different priming strategies.



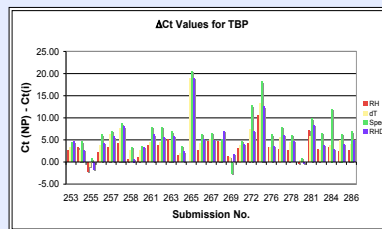
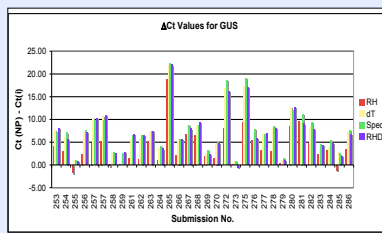
## How were the data analyzed?

Examine the differences among the priming strategies

Express the differences as the  $\Delta C_t$  between an individual strategy, I, and no primer (NP)

$$\Delta C_t(I) = C_t(NP) - C_t(I)$$

The graphs below show the differences, for each submission, between the Ct values for each priming strategy and the Ct value obtained with no priming.

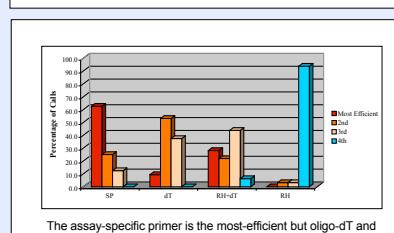


Use the calculated  $\Delta C_t$  values to rank each priming reagent from each laboratory's data set

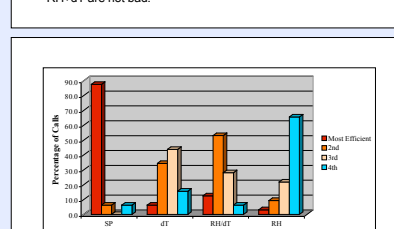
Assign a value of 1 to the strategy with the lowest  $\Delta C_t$   
 Assign a value of 4 to the strategy with the highest  $\Delta C_t$

Calculate a call percentage of all rankings for each priming strategy

$$\text{Call percentage} = 100 \times \frac{\text{\# of 1st place (2nd, etc) rankings}}{\text{total \# of submissions}}$$



The assay-specific primer is the most-efficient but oligo-dT and RH+dT are not bad.



The assay-specific primer is the most-efficient.

### Summary

Overall, priming with an assay-specific primer resulted in the lowest  $\Delta C_t$ .

The assay-specific primer was overwhelmingly the most effective priming strategy for TBP (88%) but it was only slightly better than Oligo-dT for GUS(63%).

Oligo-dT was the second best primer for GUS and third for TBP, RH + dT the second favored for TBP but third for GUS.

### Conclusions

Optimal priming strategy may be target-dependent.

In this study random hexamers appear to be a poor choice for priming.

### Questions for Future Studies

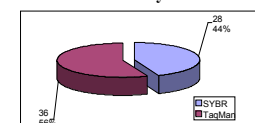
What specifications of random primers are required for better priming?  
 e.g., length of oligomers, experimental conditions.

What is the "pulse" of the real-time community in terms of the technologies, chemistries, protocols etc. being used?

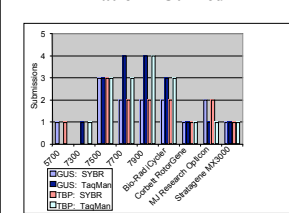
I.e., survey laboratories to see what they are doing.

### Submission Information

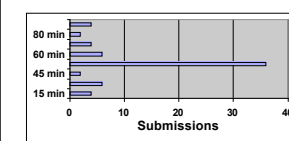
#### Chemistry Choice



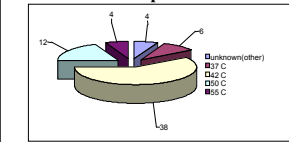
#### Platform Utilized



#### RT Incubation Time



#### RT Temperature



#### Participation by Country



#### Acknowledgements

We gratefully acknowledge all the participants of this study. We would like to acknowledge the hard work of Jessica Hoffman of Trudeau Institute, Karen McCausland of the CDC, Emmanuelle Nicolas of FCCC, Mary Sobieski of UTHSC-Houston, and Ashley Price of Penn State University, without whom this study would not have been possible. We would also like to thank our ABRF Executive Board *ad hoc*, Dr. Susan Hardin of the University of Houston for her guidance.

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