The Nucleic Acids Research Group (NARG) study for 2005-2006 involved 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 DNA 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 DNA priming strategies utilizing the participants' assay reagents and instrumentation. Two differently-expressed genes were chosen, human β-glucuronidase (βGUS) 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 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 to each participant'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 DNA priming strategies using two genes expressed at different 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 jpeg files of amplification plots to the NARG committee

Selected Genes

Human GUS (β-Glucuronidase) and TBP (TATA Binding Protein) were selected as genes with different transcript levels
GUS: Medium-abundance transcript
TBP: Low-abundance transcript

Methods

Project Kit:
Reverse Transcription Reagents
400 ng Reference cDNA Template
125 pmol Random hexamers
125 pmol Oligo-dT
125 pmol hGUS R primer for RT: 5’CCAGTGAAGATCCCTTCT
125 pmol hTBP R primer for RT: 5’TGGACTTTCTTTCTTGGC3’
PCR Reagents:
1000 pmole hGUS F primer: 5’TCTCATTGTTACTTTGGC3’
1000 pmole hGUS R primer: 5’TGGACTTTCTTTCTTGGC3’
200 pmole hGUS F probe: 5’FAM-TGAGACTCTACGAGAAGATTGGC-3’
200 pmole hTBP F primer: 5’TGGACTTTCTTTCTTGGC3’
200 pmole hTBP R primer: 5’TGGACTTTCTTTCTTGGC3’
200 pmole hTBP F probe: 5’FAM-CCTGCTTGGTCTCTGACTTTCTC-3’

Participants were given guidelines on how to perform the RT and they were asked to run PCR reactions using the chemistry and machines in their laboratory.

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

Results

The data were analyzed using the ABI 7900 SDS software of the Applied Biosystems iCycler. All RT reactions were run in triplicate in the presence of SYBR Green I dye. The PCR reaction mixture contained 2.5 μl reaction mix (2x Power SYBR Green PCR Master Mix; Applied Biosystems), 500 nM each of forward and reverse primers, and 100 ng cDNA template in a total volume of 25 μl. The thermal cycling conditions were 95° C for 10 min followed by 40 cycles of 95° C for 15 sec, 58° C for 1 min, and 72° C for 1 min. After the final cycle, melting curve analysis was performed to detect the presence of nonspecific products.

Conclusion

Overall, primer with an assay-specific primer resulted in the lowest Ct.

The assay-specific primer was overwhelmingly the most effective primer strategy for TBP (8%) 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 was the second favored for TBP but third for GUS.

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