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. This study was designed to determine the optimal priming strategy for RT-qPCR. The NARG 2008-09 study was an NARG multi-site study designed to evaluate the effect of reverse transcription priming strategies on RT-qPCR results. The previous study suggested a correlation between the assay sensitivity and variability 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 was done using random primers and more efficient RTs than those used in this study, this study was designed specifically to compare oligo(dT), random 6-mer and 9-mer specific primers across the entire human transcriptome. SuperScript III Transcriptase and MultiScribe, were employed to determine the effect of enzymes in addition to the qPCR assays which lacked the three genes of varying abundance, β-glucuronidase (medium copy) and TATA binding protein (low copy).

RESEARCH PLAN AND STUDY DESIGN

- To compare 13 different RNA priming strategies using these genes expressed at different levels.
- To test whether the assay location impacts the RT priming strategy. At least 2 qPCR assays were done for each primer concentration in each of the 7 qPCR assays.
- To determine the effect of enzyme. In addition, the qPCR assays looked at three genes of varying abundance, β-glucuronidase (medium copy) and TATA binding protein (low copy).
- To find effective primer(s) that will provide optimal cdNA synthesis for use with RT-qPCR.
- To determine the effect of primer length and layout.
- To compare the efficiency of 4 different RT enzymes that differ based on their high or low activity and temperature sensitivity.
- To test whether manual or robotic setups of the reactions give the same results.
- To provide information useful in expanding study to real-time qPCR community.

RESULTS & DISCUSSION

- The ANOVA using the combined data from all 7 assays showed that the non-specific priming strategies (i.e., random and oligo(dT)) gave similar Ct values suggesting that there was no significant advantage in terms of sensitivity or variability in generating cdNA for use with real time qPCR.
- As described above, however, there was a significant difference in the primers able to bring about cdNA when evaluated within each assay.

EFFECT OF ASSAY LOCATION ON Ct

- The mean Ct values for each of the gene-specific primers for the 2 assays against the β-actin and TBP gene templates were similar suggesting these assays were equally as effective in measuring transcription levels for their respective gene targets.
- The mean Ct values for β-glucuronidase and TBP gene templates were significantly different (P<0.05) from the mean values obtained using the β-actin or TBP assay. The differences in transcription assay between the β-glucuronidase and TBP gene templates may be due to the presence of non-specific primers used in the β-glucuronidase assay also give rise to non-coding RNA sequences in the cdNA.

To compare β-glucuronidase and TATA binding protein 1 gene transcripts were designed. Relative levels of β-glucuronidase (medium copy) and TATA binding protein (human) were determined using a one-way analysis of variance (ANOV A) with the JMP v.8.0 software. The ANOV A using the combined data from all 7 assays showed that the non-specific priming strategies (i.e., random and oligo(dT)) gave similar Ct values suggesting that there was no significant advantage in terms of sensitivity or variability in generating cdNA for use with real time qPCR.

EFFECT OF RT PRIMING STRATEGY ON Ct

- There was not a significant difference (P<0.05) between the RT priming strategies used to generate cdNA for the β-na2728 PCR assay which was located near the 3' end of the transcript. Conversely, there was a significant difference (P<0.05) between oligo(dT) and the other RT priming strategies for the β-na2728 PCR assay which was located further upstream from the 3' end than the βTBP assay.

- There was not a significant difference (P<0.05) between the RT priming strategies used to generate cdNA for the β-glucuronidase (medium copy) and TATA binding protein 1 gene transcripts were designed. Relative levels of β-glucuronidase (medium copy) and TATA binding protein (low copy) were determined using a one-way analysis of variance (ANOV A) with the JMP v.8.0 software. The ANOV A using the combined data from all 7 assays showed that the non-specific priming strategies (i.e., random and oligo(dT)) gave similar Ct values suggesting that there was no significant advantage in terms of sensitivity or variability in generating cdNA for use with real time qPCR.

CONCLUSIONS

- A study was not significant difference (P<0.05) between the RT priming strategies used to generate cdNA for the β-na2728 PCR assay which was located near the 3' end of the transcript. Conversely, there was a significant difference (P<0.05) between oligo(dT) and the other RT priming strategies for the β-na2728 PCR assay which was located further upstream from the 3' end than the βTBP assay.
- The behavior of the GUS2 primers is attributed to similarity  between the GUS2 primer concentration and the rna specific primers across the entire human transcriptome. SuperScript III Transcriptase and MultiScribe, were employed to determine the effect of enzymes in addition to the qPCR assays which lacked the three genes of varying abundance, β-glucuronidase (medium copy) and TATA binding protein (low copy).

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