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Research paper

Evaluation of bias associated with high-multiplex, target-specific pre-amplification

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ABSTRACT

We developed a novel PCR-based pre-amplification (PreAmp) technology that can increase the abundance of over 350 target genes one million-fold. To assess potential bias introduced by PreAmp we utilized ERCC RNA reference standards, a model system that quantifies measurement error in RNA analysis. We assessed three types of bias: amplification bias, dynamic range bias and fold-change bias. We show that our PreAmp workflow introduces only minimal amplification and fold-change bias under stringent conditions. We do detect dynamic range bias if a target gene is highly abundant and PreAmp occurred for 16 or more PCR cycles; however, this type of bias is easily correctable. To assess PreAmp bias in a gene expression profiling experiment, we analyzed a panel of genes that are regulated during differentiation using the NTera2 stem cell model system. We find that results generated using PreAmp are similar to results obtained using standard qPCR (without the pre-amplification step). Importantly, PreAmp maintains patterns of gene expression changes across samples; the same biological insights would be derived from a PreAmp experiment as with a standard gene expression profiling experiment. We conclude that our PreAmp technology can facilitate analysis of extremely limited samples in gene expression quantification experiments.

1. Introduction

Pre-amplification (PreAmp) of nucleic acid is a powerful technique that allows for the analysis of large numbers of target genes from limiting samples. PreAmp can be achieved through whole transcriptome amplification [1] or at the level of targeted gene panels using PCR-based methodology [2,3]. However, there is a legitimate concern that PreAmp might change a sample to an extent that results generated from it are misleading or inaccurate. Better understanding of the limitations of a PreAmp-based workflow is necessary to ensure the reliability of research results.

To address such concerns, the National Institute of Standards and Technology (NIST), in conjunction with the External RNA controls consortium (ERCC) developed a set of reference standards to evaluate the performance of RNA quantification systems and workflows [4,5]. The ERCC standards are mixtures of up to 96 synthetic RNAs that are spiked into an RNA sample and processed and quantified along with the natural RNAs. The amount of each ERCC RNA in a mixture is precisely defined; the performance of an RNA quantification workflow/platform is determined by comparing the measured amount with the actual, defined amount of each ERCC control RNA. In addition, by spiking two sets of ERCC standards (with defined ratios of each ERCC target) into two different biological samples, the accuracy in quantifying gene expression differences between samples can be determined. ERCC standards have been used to assess qPCR, digital PCR, microfluidic qPCR, microarray and RNA-seq platforms for their precision, accuracy and detection limits in RNA quantification [6–10].

There are two fundamental challenges in PreAmp reactions because multiple targets are amplified simultaneously. The first challenge is increasing the capacity of the amplification reaction to allow targets of vastly different starting quantity to be efficiently amplified through every PCR cycle. The second challenge is maintaining target amplification specificity in the presence of large numbers of primers. Unless both challenges are addressed in a PreAmp reagent, the probability of having biased pre-amplification will be high. We developed a PreAmp reagent that utilizes an engineered DNA polymerase with improved binding affinity to DNA

Abbreviations: PreAmp, pre-amplification; ERCC, external RNA controls consortium; NIST, National Institute of Standards and Technology; NT2, NTera2; RA, retinoic acid.

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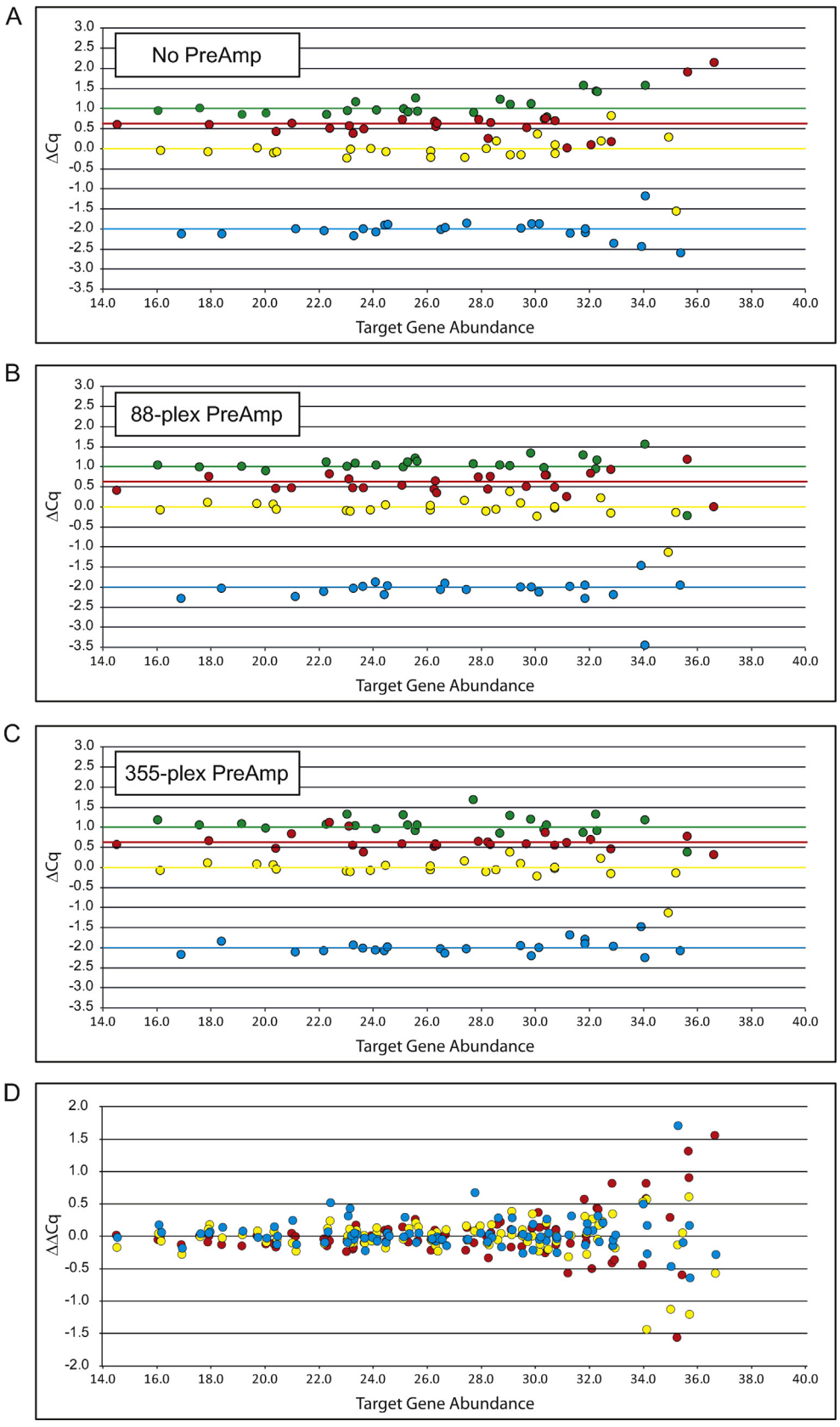

Fig. 1. Fold-change bias of ERCC standards. ExFold spike in-mixtures were added to 10 ng NT2 RNA, reverse transcribed and pre-amplified for 14 PCR cycles. ERCC targets were quantified by qPCR using three technical replicates, the experiment was performed one time. Fold-change measurements for each ERCC target are plotted against target abundance. Results show the measured difference, between samples, of the amount of each ERCC target (colored circles) and the actual difference that should be detected (Fig. 1A–C; colored lines at 1.0, 0.585, 0 and −2.0 ΔCq units representing RNA abundance ratios of 2–1, 1.5–1, 1–1 and 1–4 respectively). (A) Analysis of samples that were not pre-amplified. (B) Analysis of samples in which 88 ERCC targets were pre-amplified. (C) Analysis of samples in which 355 targets were pre-amplified. (D) Overall fold-change bias for 88 ERCC targets. No PreAmp samples (red circles), 88-plex PreAmp samples (yellow circles), 355-plex PreAmp samples (blue circles).