Normalization of full-length cDNA libraries using double-strand specific DNase

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INTRODUCTION

Next generation sequencing technologies have become an essential tool for whole transcriptome analysis, enabling identification of new genes, expression patterns, transcripts, splicing isoforms and gene fusions. However, a major limitation associated with eukaryote transcriptome analysis is the significant variations in transcript concentrations, as copy numbers can vary by several orders of magnitude (1).

Normalization is a method developed to avoid the recurrent sequencing of abundant cDNAs (2). While the prevalence of highly abundant transcripts is decreased, rare transcripts are preserved. Thus, the discovery rate of low-copy transcripts is significantly enhanced. ArcticZymes has recently developed an easy and effective protocol for normalization of full-length cDNA transcripts using the double-strand specific dsDNase. The method is based upon the second-order reaction kinetics of renaturation of denatured DNA (3). Following denaturation of ds cDNA, abundant transcripts renature more efficiently compared to rare transcripts and become substrate for the dsDNase. Rare transcripts remain single-stranded, and are thus not recognized as substrate, resulting in a cDNA library enriched with low-copy transcripts. As the cDNA is flanked by known adaptor sequences, the normalized library can be amplified by PCR prior to directional/non directional cloning or immediate high-throughput sequencing.

Protocol and normalization efficiency have been evaluated using qPCR with several primers targeting abundant, intermediate and rare transcripts. Results have shown a strong decrease in the prevalence of highly abundant transcripts, while the effects on low copy transcripts have been negligible. Hence, the method yields a low redundancy, high quality full-length cDNA library, thereby dramatically increasing the rate of rare gene discovery and the efficiency of sequencing.

METHOD

Total RNA was isolated from human breast carcinoma cells by means of Trizol isolation. Using the Eukaryote Total RNA StaDry Sens Assay, Experion system (Bio-Rad), RO was measured to 9.8. Following first-strand synthesis of full-length cDNA transcripts using the SMARTer PCR cDNA Synthesis Kit (Clontech), cDNA amplification by long-distance PCR were obtained using the Advantage 2 PCR Kit (Clontech). All reactions were performed according to manufacturer’s instructions.

Normalization of the full-length cDNA library were initiated by combining 1μl Hybridization Buffer (4x) with 1μl dH2O and 2μl full-length cDNA(≈100ng/μl) for each sample to be normalized. Samples were incubated at 98°C for 2 minutes, followed by 68°C for 5 hours. Following incubation, hybridized cDNA was combined with 5μl preheated (38°C) dsDNase Reaction Buffer (2X) and 1μl Normalization dsDNase. Samples were incubated for 10 minutes at 38°C, before 1μl Stop Buffer (2X) was added.

Normalization efficiency was estimated using 9 different TaqMan® Gene Expression Assays (Applied Biosystems) representing 3 high, 3 intermediate and 3 low-copy transcripts. qPCR was performed using Brilliant III Ultra Fast qPCR MasterMix (Agilent Technologies) on the Mx3005 qPCR platform (Agilent Technologies).

RESULTS

High Copy Transcript

Intermediate Copy Transcript

Low Copy Transcript

REFERENCES


CONCLUSION

• cDNA normalization using dsDNase decreases the prevalence of high abundance transcripts and equalizes transcript concentrations
• Enables rare transcripts discovery and higher efficiency in next-generation sequencing
• Very simple protocol, no physical separation step

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Figure 1. Normalization of full-length cDNA by dsDNase resulted in a significant reduction of high copy transcripts. Primers targeting three high copy transcripts (GAPDH, UBC, B2M) were analyzed.

Left: Diagram illustrating the relative reduction of GAPDH in a normalized cDNA library compared to a non-normalized control. Right: qPCR results demonstrating the relative reduction of GAPDH as a significant shift in ΔCq

Figure 2. Results from qPCR demonstrated that normalization of full-length cDNA transcripts yielded a moderate reduction of intermediate copy transcripts. Primers targeting three intermediate copy transcripts (RPL13a, YWHAZ, PP1A) were analyzed.

Left: Diagram illustrating the relative reduction of RPL13a in a normalized cDNA library compared to a non-normalized control. Right qPCR results demonstrating the relative reduction of RPL13a as a moderate shift in ΔCq

Figure 3. The effect on low-copy transcripts after normalization of the full-length cDNA library were minimal. Primers targeting three low copy transcripts (JUN, IGF2R, NFKB1) were analyzed.

Left: Diagram illustrating the relative reduction of IGF2R in a normalized cDNA library compared to a non-normalized control. Right: qPCR results demonstrating the relative reduction of IGF2R as a negligible shift in ΔCq