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METHODOLOGY

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Blocking Abundant RNA Transcripts by High-Affinity Oligonucleotides during Transcriptome Library Preparation

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Abstract

Background RNA sequencing has become the gold standard for transcriptome analysis but has an inherent limitation of challenging quantification of low-abundant transcripts. In contrast to microarray technology, RNA sequencing reads are proportionally divided in function of transcript abundance. Therefore, low-abundant RNAs compete against highly abundant - and sometimes non-informative - RNA species.

Results We developed an easy-to-use strategy based on high-affinity RNA-binding oligonucleotides to block reverse transcription and PCR amplification of specific RNA transcripts, thereby substantially reducing their abundance in the final sequencing library. To demonstrate the broad application potential of our method, we applied it to different transcripts and library preparation strategies, including YRNAs in small RNA sequencing of human blood plasma, mitochondrial rRNAs in both 3′ end sequencing and long-read sequencing, and MALAT1 in single-cell 3′ end sequencing. We demonstrate that the blocking strategy is highly efficient, reproducible, specific, and generally results in better transcriptome coverage and complexity.

Conclusion Our method does not require modifications of the library preparation procedure apart from simply adding blocking oligonucleotides to the RT reaction and can thus be easily integrated into virtually any RNA sequencing library preparation protocol.

Keywords RNA sequencing, Oxford nanopore technologies, Single-cell RNA sequencing, Depletion

Introduction

RNA sequencing has become the gold standard for transcriptome characterization. Numerous RNA sequencing library preparation procedures have been developed to quantify various RNA biotypes, including amongst others polyA+ RNA sequencing, total RNA sequencing, 3′ end RNA sequencing and small RNA sequencing. Regardless of the library preparation method, RNA sequencing reads are distributed across RNA transcripts proportionally to their abundance. Consequently, highly abundant RNA species, often deemed non-informative, can dominate the RNA sequencing library and hamper the detection of lower abundant transcripts. A well-known example is ribosomal RNA (rRNA), which typically accounts for

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the pre-PCR blocked libraries) (Fig. 6A). For some cell types, e.g., erythrocytes and regulatory T-cells, the initial MALAT1 proportions were higher, resulting in a more drastic reduction (Supplemental Fig. 7). We observed a higher mitochondrial-derived RNA fraction for the pre-RT sample, which may indicate cell death (Fig. 6A). The LNAs are, in this case, combined with living cells for 18 min. We, therefore, focused our analysis on the pre-PCR protocol. UMAP representation of cells based on single-cell RNA-seq data from both the pre-PCR blocking and standard protocol revealed tight clustering of cell types independent of protocol (Fig. 6B), implying that the MALAT1 LNA oligonucleotide in the pre-PCR protocol has minimal impact on gene expression. This was further demonstrated by a perfect correlation (Spearman and Pearson correlation = 1.00) of gene expression values between the pre-PCR blocking and standard protocol (Supplemental Fig. 8). We observed a significant increase, albeit with a small effect size, in the mean number of detected genes per cell in the pre-PCR protocol; 1173 genes with at least two counts in the control sample and 1192 in the pre-PCR blocking sample ($p_{t\text{-test}} = 5.751e\text{-}05$) (Fig. 6A). The higher gene detection sensitivity might be related to the initial MALAT read fraction, the number of genes detected in the cells and the fraction of other highly abundant genes. The highest impact was seen in B memory cells where the mean number of detected genes increased from 1131 to 1235 (9.2% increase, $p_{adj,\ t\text{-test}} = 0.026$).

LNA Blocking Simulation in Whole Blood 3′-End Sequencing

Although our wet-lab experiments indicate that high-affinity binding oligonucleotide blocking can efficiently deplete transcripts of interest, it remains to be determined what the relationship is between initial abundance and level of depletion in order to offer substantial benefit (such as increased library complexity). Some of our applications had a more significant impact on the number of additionally detected genes and on coverage increase than others. We assume that this impact is highly dependent on the initial fraction of targeted reads, the depletion efficiency, and the sequencing saturation. In order to investigate in detail, we simulated different abundances and depletion efficiencies of beta-globin (HBB) using publicly available whole blood 3′-end sequencing data, in which HBB accounted for 20.8% of all reads [68]). Figure 7 shows how the number of detected genes (with at least 10 counts) increases linearly with increasing depletion efficiency at shallow sequencing depth but increases exponentially at higher sequencing depths. The linear relation for low sequencing depths probably results from unsaturated sequencing. The relation becomes more linear as the initial unwanted fraction lowers in higher sequencing depths. We conclude that even inefficient depletion of high-abundant transcripts provides a substantial gain in the number of detected genes.

Discussion

We demonstrate that high-affinity binding oligonucleotides can be applied to block reverse transcription and PCR amplification of various RNA transcripts in different RNA-seq library preparation protocols. We present a flexible and robust method that can drastically increase the detection and coverage of (low abundant) genes in the library. While LNA oligonucleotides have been used before to block PCR amplification [45], we provided evidence that such oligonucleotides can block both reverse transcription and PCR amplification, indicated by evident fragment depletion in the PCR-independent Oxford Nanopore Technologies protocol and the post-RT single-cell sample, respectively. Moreover, we demonstrate that the impact of blocking specific RNA fragments on gene detection and coverage strongly depends on the abundance of the blocked fragments, the transcriptome complexity and the sequencing depth. For example, the impact of RNY4 blocking on gene (i.e., miRNA) detection and coverage was much more significant for PRP than PFP, most likely because of higher RNY4 abundance in PRP. We expect a more pronounced impact in PFP samples at lower sequencing depths. We provide support for this hypothesis using simulated depletion experiments.

Our method has several advantages compared to existing protocols. First, the method only requires a single additional step that can be implemented in any RNA-seq library preparation workflow. Second, no nucleic acid sample or library material is lost because of enrichment or washing steps, which we believe has a positive impact on detection sensitivity, especially for low-input samples.

While we generally observe potent blocking of targeted transcripts, we also observe a few minor unwanted effects. First, in single-cell RNA sequencing, adding LNA oligonucleotides to the GEMs during 3′ end sequencing resulted in a higher fraction of mtRNA reads. As living cells are incubated with LNA oligonucleotides for 18 min, the oligonucleotides may enter the cells and induce cell death. A large fraction of mtRNA co-occurred with few detected genes. Since adding the LNA oligonucleotides post-PCR also results in potent target blocking, we propose to use this approach instead. Second, the optimal concentration of LNA oligonucleotide may be application and target-dependent. A dedicated optimization step is warranted for optimal performance. This necessity is reflected in the single-cell RNA sequencing experiment, where the benefit (in terms of the number of detected genes) depends on the cell type. Factors to consider are

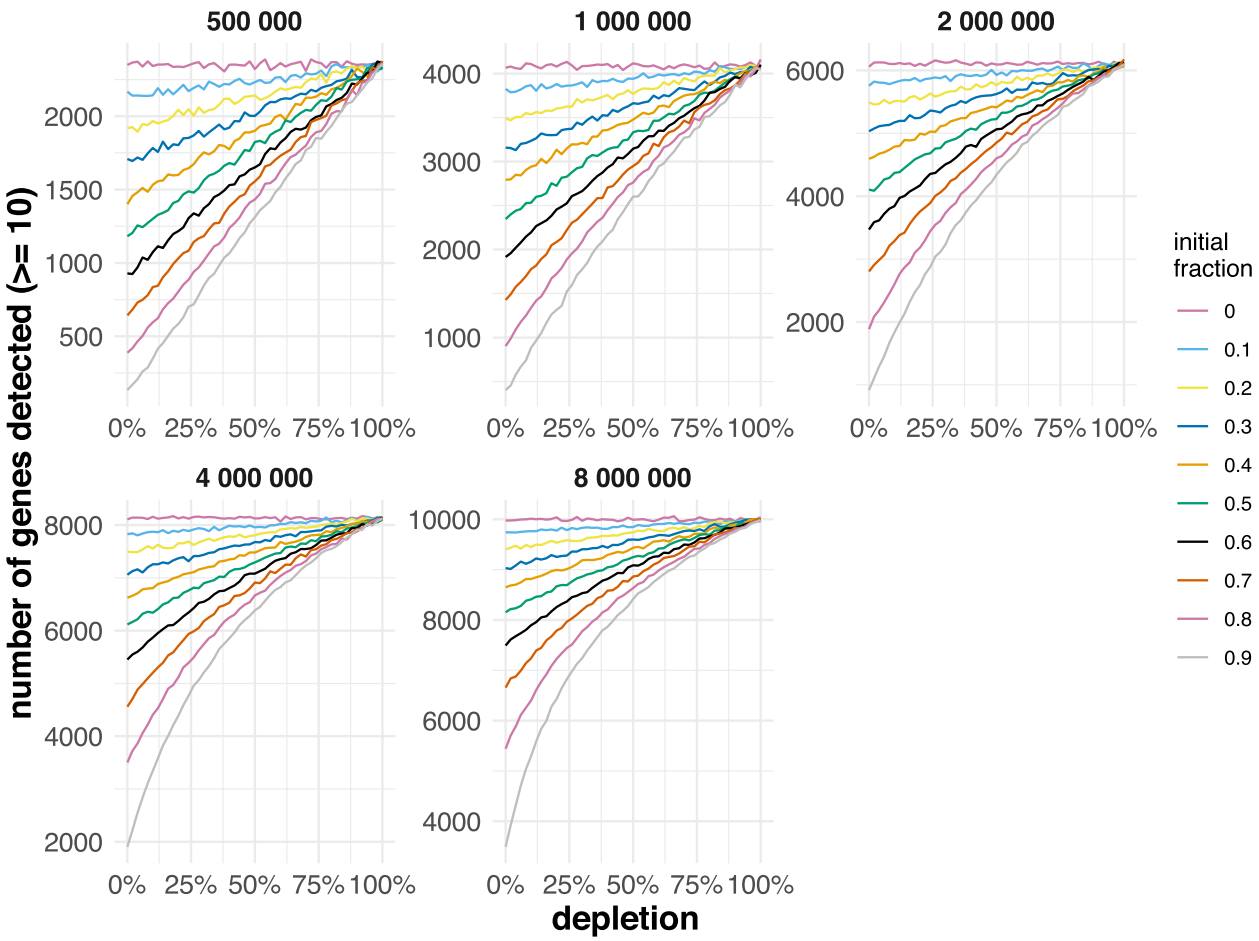

Fig. 7 Simulations of HBB LNA oligonucleotide blocking in 3-end RNA sequencing data from whole blood. Each separate figure shows the number of genes with a count higher than 10 for each simulation with varying HBB depletion efficiency (0% means no depletion, 100% mean total depletion). The lines are colored by the initial fraction of HBB counts (of the total amount of counts). The graphs are separated by the number of counts the data was subsampled to

the original fraction of the targeted RNA transcript and the input RNA concentration of the library preparation protocol. We advise to combine samples in one single library prep to exclude batch effects, as is generally advised for RNA-seq experiments. Third, we observed a limited number of off-target effects upon adding specific LNA oligonucleotides (for instance, MT-AT8 and H4C3 in the 3′ end sequencing experiment). We did not observe significant sequence complementarity between the LNA oligonucleotides and the presumed off-targets. Nevertheless, off-target effects are not entirely unexpected given the relatively short length of the LNAs, their high RNA-binding capacity, and the small design space. The latter lowers the number of possible oligonucleotides and thereby the chances of designing one without off-target effects. Increasing oligonucleotide length or reducing the number of LNA nucleotides to lower binding affinity may improve specificity. A fourth limitation of the method is that it may only be applicable to small RNA sequencing or RNA-sequencing library prep methods employing an oligo(T) or a gene-specific RT primer. When the priming is random, it is impossible to design a single LNA oligonucleotide to block reverse transcription of the whole fragment. One option would be to design multiple LNA oligonucleotides spanning the entire transcript, but this could become prohibitively expensive, depending on the length of the fragment. Fifth, LNA synthesis is costly. Nevertheless, the amount of oligo that is required for efficient blocking is limited. Even at low synthesis scale, several hundreds of reactions can be performed, resulting in a limited per-sample cost. As fully and partially modified LNA oligonucleotides are equally efficient for YRNA depletion, partially modified LNA oligonucleotides could be used to further reduce oligo synthesis cost (although additional validation would be required as we only demonstrated this for a single RNA target sequence). Notably,

blocking unwanted transcripts may also help reduce the sequencing cost. Finally, the observed shortening in read length with increasing LNA concentration in the Oxford Nanopore Technologies experiment is problematic, as it suggests off-target binding of the LNAs. Although the LNA oligonucleotides are expected to preferentially bind sequences with a lower number of mismatches, the steady decline in coverage towards the 5′-end of RNA transcripts points towards close to non-specific binding of the LNA oligonucleotides when supplied at high concentration. The possibility of the LNA oligonucleotides inhibiting the sequencing by binding to the final library can be dismissed by investigating the adaptor-to-adaptor reads (which signify complete sequencing of the read).

We believe the method presented here is versatile and can be used for other applications not investigated here, including hemoglobin mRNA blocking in whole blood samples (up to 70% of all mRNA in whole blood [69]) or trypsin mRNA in pancreatic RNA samples. As we have shown, samples dominated by a few fragments have a higher potential of benefitting from LNA oligonucleotide-transcript blocking. We suggest the users perform an initial computational analysis to define the expected benefit prior to implementing and optimizing our proposed method. While we only investigated mixtures of up to four different LNA oligonucleotides, it would be possible to combine more and block multiple fragments in one sample. Such mixtures can be designed specifically for unique and challenging sample types, containing several highly expressed, uninformative fragments [70].

Conclusion

In conclusion, we present a novel and broadly applicable method to specifically block unwanted RNA transcripts during RNA sequencing library preparations by simply adding a target-specific high-affinity oligonucleotide to the RT or PCR reaction.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12575-023-00193-3.

Additional file 1: Supplemental Figure 1. Count distributions of noLNA, LNA1x, and LNA10x in PFP and PRP. **Supplemental Figure 2.** Read distribution for each sample and treatment. **Supplemental Figure 3.** Correlation plots comparing all biological replicates. **Supplemental Figure 4.** Length distribution of full-length transcripts after Oxford Nanopore direct-RNA sequencing. **Supplemental Figure 5.** Quality score distribution of full-length transcripts after Oxford Nanopore direct-RNA sequencing. **Supplemental Figure 6.** Illustration of problematic MALAT1 fragments and design space. **Supplemental Figure 7.** MALAT1 transcription in each cell type. **Supplemental Figure 8.** Correlation plot between prePCR and noLNA samples. **Supplemental Table 1.** Sequences of designed synthetic oligonucleotides. For each modified oligonucleotide the identification, gene target and sequence are provided. The sequences contain modification information in the generally accepted standard of notification.

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Authors' Contributions
Celine Everaert: Conceptualization, Methodology, Software, Formal Analysis, Resources, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization, Funding Acquisition. Jasper Verwilt: Conceptualization, Methodology, Software, Formal Analysis, Investigation, Resources, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization. Kimberly Verniers: Investigation, Writing – Review & Editing. Niels Vandamme: Investigation, Resources, Writing – Review & Editing, Funding Acquisition. Alvaro Marcos Rubio: Investigation, Resources, Writing – Review & Editing. Jo Vandesompele: Conceptualization, Resources, Writing – Review & Editing, Supervision, Funding Acquisition. Pieter Mestdagh: Conceptualization, Resources, Writing – Review & Editing, Supervision, Funding Acquisition. The author(s) read and approved the final manuscript.

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Availability of Data and Materials
The datasets generated and/or analysed during the current study are available in EGA, EGAS00001006023.

Declarations

Ethics Approval and Consent to Participate
Our analyses have been approved under EC/2017/1207 by the Ghent University Hospital ethical committee.

Consent for Publication
Not applicable.

Competing Interests
The authors declare no competing interests.

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