APPLICATION NOTE

Selective 2S rRNA blocking improves usable sequencing depth in *Drosophila melanogaster* small RNA-seq.

Introduction

The small RNA transcriptome is frequently characterized by a highly skewed abundance distribution, where a limited number of RNA species dominate sequencing output. When one small RNA species consumes a large share of reads, fewer reads remain available to detect lower-abundance small RNAs.

In human blood-derived libraries, for example, a small number of erythropoietic miRNAs account for a large fraction of reads, while in other tissues, degradation products or highly stable RNA fragments can dominate libraries. In *Drosophila melanogaster*, this issue is especially pronounced because 2S rRNA is an abundant ~30 nt mature ribosomal RNA component that falls within the size range targeted by small RNA library preparation workflows[1,2]. Because this fragment falls within the size range targeted by small RNA library preparation workflows, it is efficiently captured and enriched during library construction. As a result, 2S rRNA can account for a large fraction of reads in *Drosophila* small RNA libraries.

To overcome this limitation, sequence-specific blocking can be employed to suppress dominant RNA species during library preparation. The NEXTFLEX™ Custom small RNA blockers are short synthetic oligonucleotides designed to hybridize with high affinity to a target RNA. Upon binding, blockers interfere with adapter ligation by reducing ligase accessibility to the RNA termini, effectively excluding the target molecule from downstream amplification and sequencing without the need for enzymatic depletion or prior manipulation of the input RNA.

Blockers can be designed against a wide range of interfering molecules, including rRNA fragments, tRNAs, or highly abundant miRNAs, and have been previously applied to highly abundant miRNA species in human blood[3].

Here, we evaluate a custom small RNA blocker targeting 2S rRNA in *D. melanogaster* and show, consistent with previous reports, that suppressing this dominant RNA species improves library complexity.

Methods

Libraries were prepared according to the manufacturer's instructions. A total of 590 ng of total RNA extracted from wild-type *D. melanogaster* adults was used as input.The 2S rRNA blocker was resuspended to 5 µM and added during Step A of the NEXTFLEX Small RNA-seq Kit v4 workflow. Five replicate libraries were prepared for each condition. Final libraries were quantified by fluorometry (Tecan Infinite® M Nano), pooled equimolarly, and sequenced on an Illumina® NovaSeq 6000™ platform at 9 pM with 3 % PhiX spike-in using 2 × 50 bp paired-end reads, targeting 20 million reads per sample. For small RNA analysis, reads were aligned to the *D. melanogaster* reference assembly GCF_000001215.4 and annotated using FlyBase, with downstream analyses performed using custom scripts.

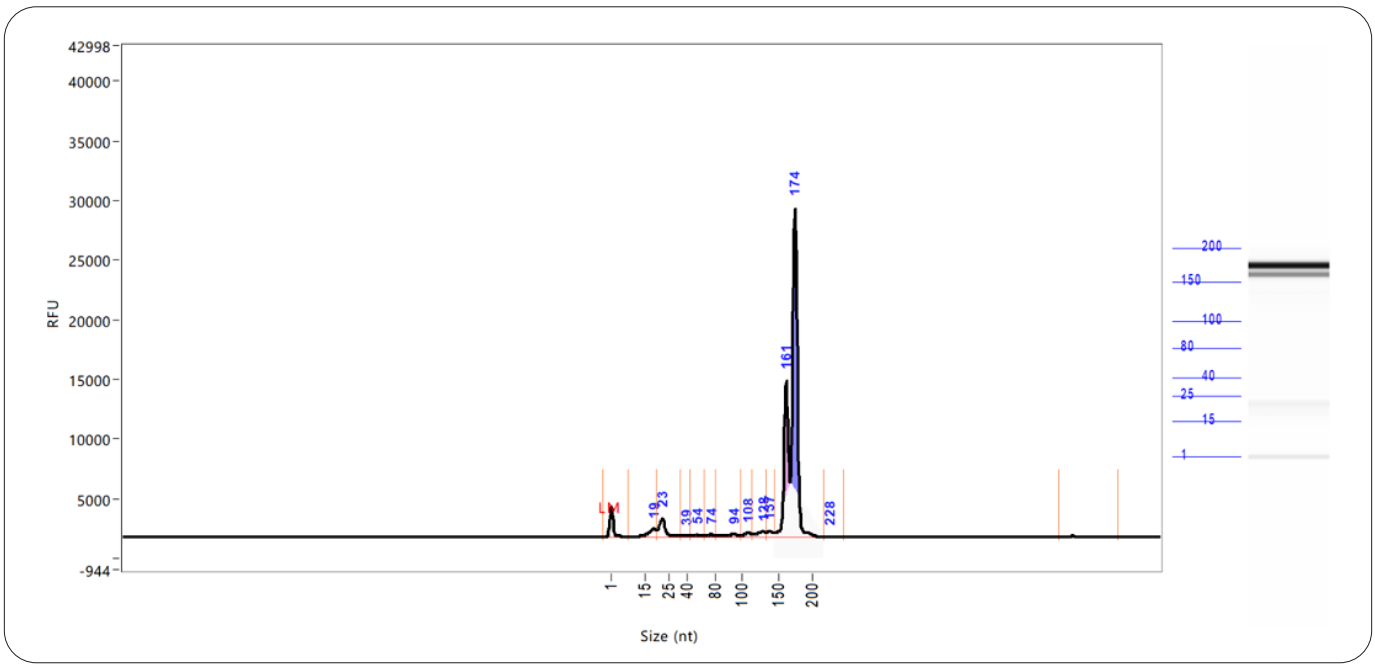

Figure 1: Example small RNA library prepared from whole-body RNA extracted from adult, wild-type *D. melanogaster*.

Results

2S rRNA blocking

In unblocked libraries, reads mapping to the 2S rRNA reference accounted for an average of 75 % of total sequencing output. Following application of the blocker, that fraction fell to 0.5 %, a reduction of ~150-fold (Figure 2A). At a target depth of 20 million reads per sample, this reduction corresponds to approximately 15 million fewer reads consumed by 2S rRNA and substantially more sequencing space available for other small RNA species. The impact of blocking is also reflected in FastQC per-base sequence content profiles. In unblocked libraries, the 2S rRNA sequence drives a strong positional nucleotide bias across read positions, consistent with a dominant overrepresented template. After blocking, base composition becomes more balanced across positions, consistent with a more diverse small RNA population. This shift is concordant with the reduction in 2S-mapping reads observed in the alignment data (Figure 2B).

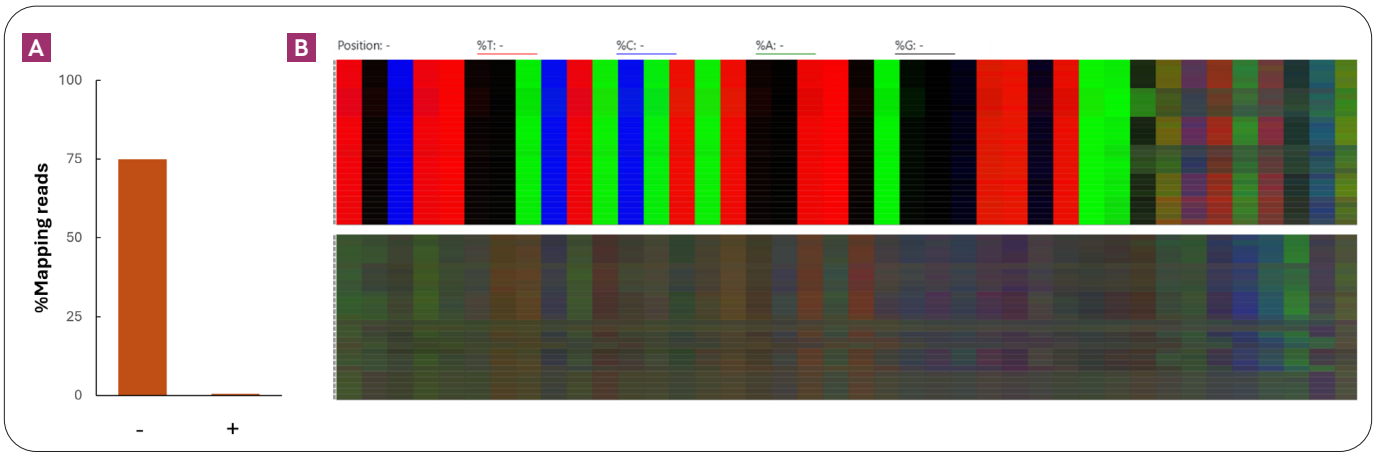

Figure 2: A. Percentage of reads mapping to 2S rRNA in unblocked (-) and blocked (+) libraries. B. Per-base sequence content (FastQC). In unblocked samples (top), base composition is dominated by the 2S rRNA sequence, causing strong positional bias. In blocked samples (bottom), this bias is markedly reduced, consistent with effective 2S rRNA removal.

Impact on miRNA

Although the overall fraction of miRNA-mapping reads remained near 8 %, blocking increased the number of miRNA annotations passing the detection threshold from 33 to 55 on average. This indicates that the primary benefit was not a bulk increase in the total miRNA fraction, but improved detection of lower-abundance miRNA species after removal of the dominant 2S rRNA species from the sequencing pool.

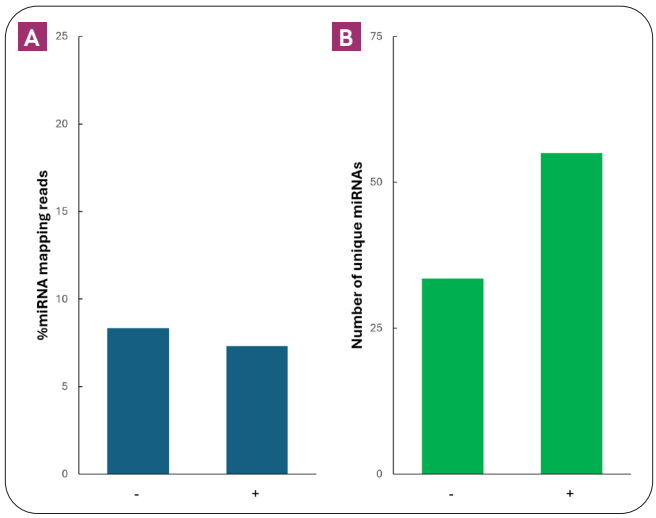

Figure 3: A. The percentage of miRNA-mapping reads is similar in unblocked (-) and blocked (+) libraries. B. Removal of 2S rRNA redistributes sequencing depth, leading to a 66 % increase in the number of unique miRNAs detected compared with unblocked libraries.

Conclusion

These results highlight a structural limitation of small RNA sequencing in *D. melanogaster*: 2S rRNA is an abundant endogenous ribosomal fragment that co-migrates with the small RNA fraction and cannot be effectively removed by size selection. When a large fraction of reads map to this species, the effective sequencing depth available for other small RNAs is substantially reduced.

These results demonstrate that blocking can effectively mitigate dominant small RNA species and improve library composition in systems where conventional workflow adjustments are insufficient. Because blocker design is sequence-specific, this approach can be adapted to other species when the sequence of the target RNA is known.

References

1. Pavlakis, G.N., et al. (1979). Sequence and secondary structure of Drosophila melanogaster 5.8S and 2S rRNAs and of the processing site between them. *Nucleic Acids* Res. 7(8):2213–2238. doi:10.1093/nar/7.8.2213.
2. Jordan, B.R., et al. (1976). Late steps in the maturation of Drosophila 26S ribosomal RNA: generation of 5.8S and 2S RNAs by cleavages occurring in the cytoplasm. *J Mol Biol.* 101(1):85–105. doi:10.1016/0022-2836(76)90067-x.
3. Juzenas, S., et al. (2020). Depletion of erythropoietic miR-486-5p and miR-451a improves detectability of rare microRNAs in peripheral blood-derived small RNA sequencing libraries. NAR Genom Bioinform. 2(1):lqaa008. doi: 10.1093/nargab/lqaa008.
4. Wickersheim, M.L., Blumenstiel, J.P. (2013). Terminator oligo blocking efficiently eliminates rRNA from Drosophila small RNA sequencing libraries. *BioTechniques*. 55(5):269–272. doi: 10.2144/000114102.
5. Fowler, E.K., et al. (2018). Small RNA populations revealed by blocking rRNA fragments in *Drosophila melanogaster* reproductive tissues. *PLoS One.* 13(2):e0191966. doi: 10.1371/journal.pone.0191966.