APPLICATION NOTE

Small RNA sequencing of challenging samples using the Element Biosciences® AVITI™ platform.

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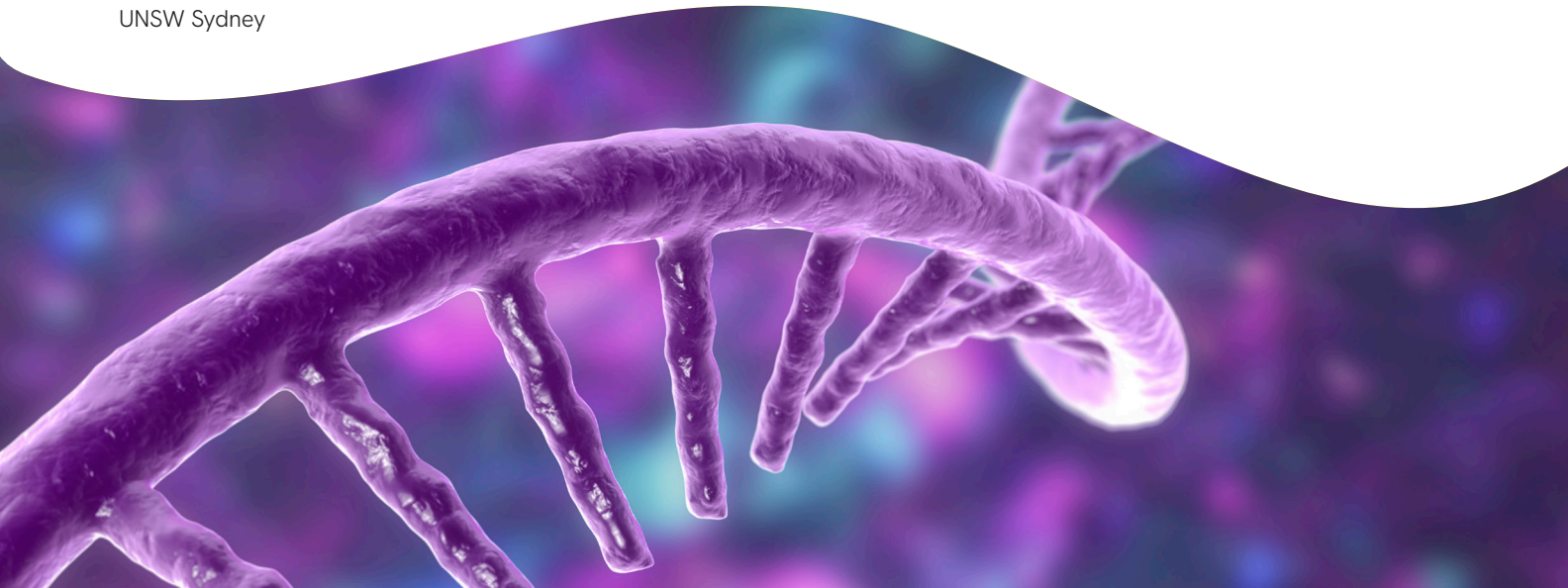
Introduction

Small RNA sequencing has become a fundamental tool for characterizing regulatory RNA species such as microRNAs (miRNAs), small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs). These molecules play critical roles in gene regulation, development, and disease. However, extracting high-quality small RNA data from degraded or preserved tissue samples remains a technical challenge due to RNA fragmentation, which can impact both library preparation efficiency and sequencing accuracy.

When extensive degradation takes place, fragments are enriched in 5′ phosphate and a 3′ hydroxyl group due to endonuclease action (RNase A, RNase III, etc) or formalin fixation[1]. These modifications and the reduction in size of the RNA fragments favour- the incorporation of non-small RNA species (rRNA, mRNA, etc) that would otherwise be excluded in high quality samples.

In this study, the performance of the Element Biosciences® AVITI™ platform in combination with the NEXTFLEX™ Small RNA-Seq Kit v4 was evaluated for sequencing small RNA libraries from low-quality RNA derived from preserved mouse tissue. The aim was to assess library performance and sequencing output in a realistic use case involving difficult input material, as well as to explore the impact of different homogenization methods on small RNA profiles.



Methods

Eight tissue samples (skeletal muscle combined with adipose tissue) were collected from formalin-preserved specimens of wild mice by researchers at CSIRO (Australia). Four samples were homogenized using a bead-beating method, and the other four were processed using a cryogenic grinding approach. RNA was extracted with TRIzol® (Thermo Fisher Scientific) yielding an average RIN of 1.38 and average DV200 of 29.01, indicative of severely degraded material.

Libraries were prepared at the Garvan Genomics Platform (Garvan Institute). A total of 500 ng of input RNA per sample was used for library preparation using the NEXTFLEX Small RNA-Seq Kit v4, following the manufacturer's instructions with slight modifications; due to the poor RNA quality, 18 cycles of PCR were performed, and adapters were diluted to 1/4 concentration. Libraries were quantified using a Qubit® fluorometer (Thermo Fisher Scientific), pooled equimolarly, and loaded at 9 pM with 3% PhiX spike-in on an Element Biosciences® AVITI™ platform using 2 × 75 bp paired-end sequencing. Small RNA analysis was performed using a custom Revvity script, and alignment was carried out using mature miRNA sequences from miRBase v22.1.

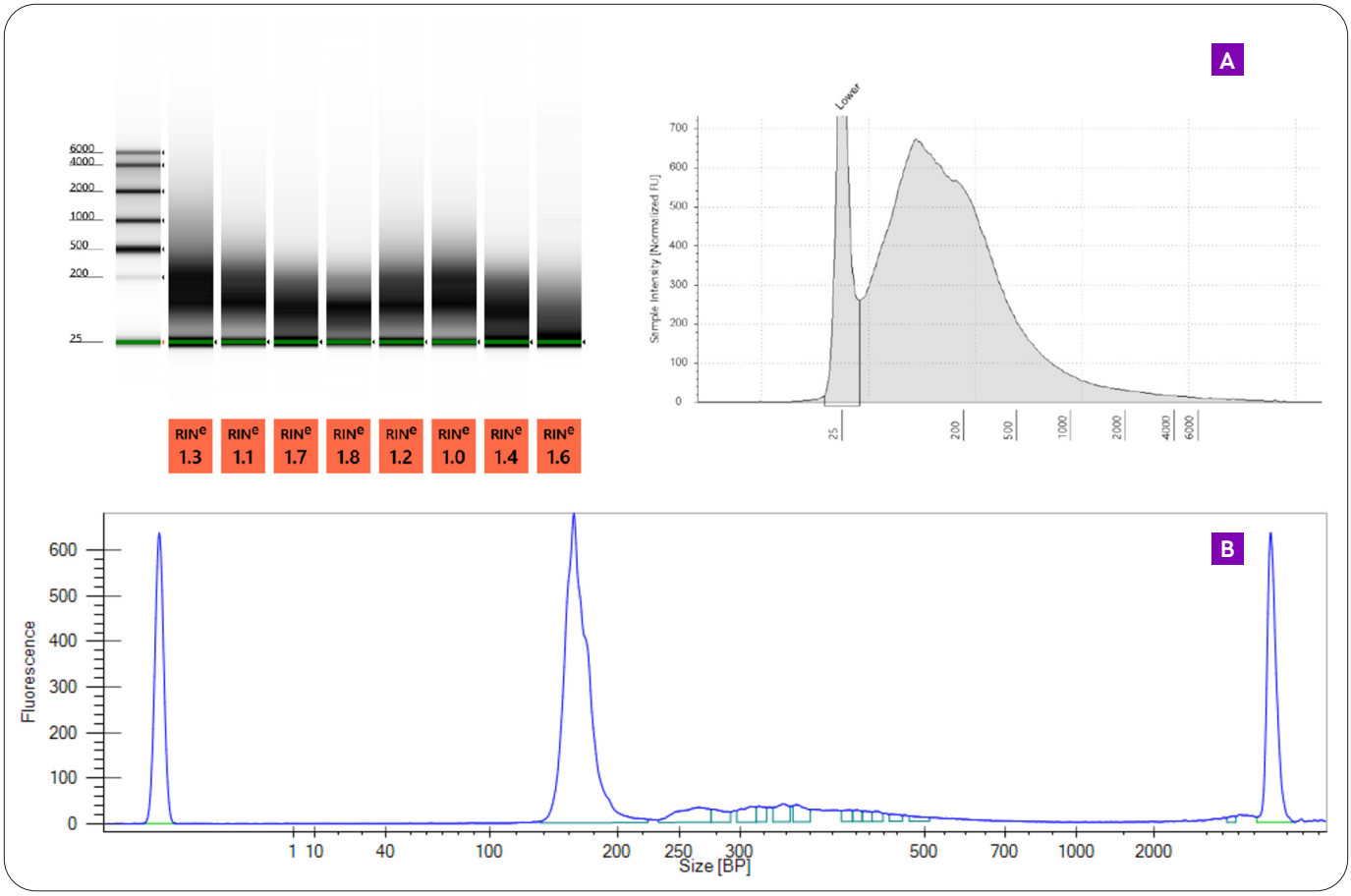


Figure 1: A. Representative Tapestation trace of the starting RNA samples B. LabChip™ GX Touch™ profile of a representative small RNA library.

Results

The NEXTFLEX Small RNA-Seq Kit v4 successfully generated libraries from all eight samples. A prominent peak was observed at 155 – 164 bp in each case, as expected for small RNA libraries (Figure 1).

Sequencing yielded an average of ~ 64.5 million paired end reads per sample, with average quality score of 43.68. 93% of reads surpassed Q40, reflecting an error rate of less than 0.01% consistent with the high accuracy reads from the

AVITI and intact library input. Initial read assignment revealed that ~21% of inserts were shorter than 15 nt and could not be aligned ("too short"), consistent with the extensive RNA fragmentation typically seen in degraded samples. Approximately 10% of reads were >15 nt but failed to align.

Importantly, the two homogenization methods produced highly comparable results in terms of insert length distribution and read alignment metrics. Variability across samples was more strongly associated with individual sample differences than with the homogenization approach used (Figures 2 and 3B).

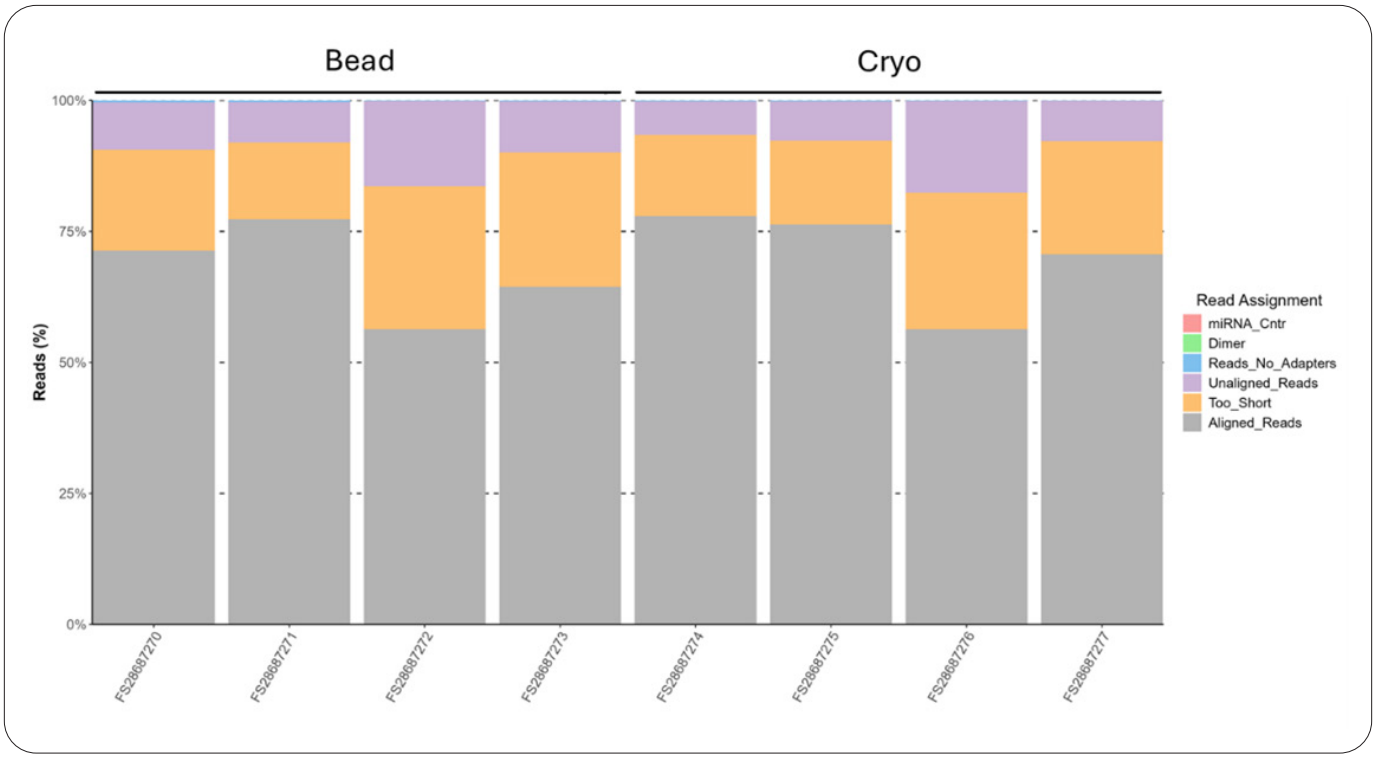


Figure 2: Percentage of read assignments for each of the eight samples (FS28687270 to FS28687277).

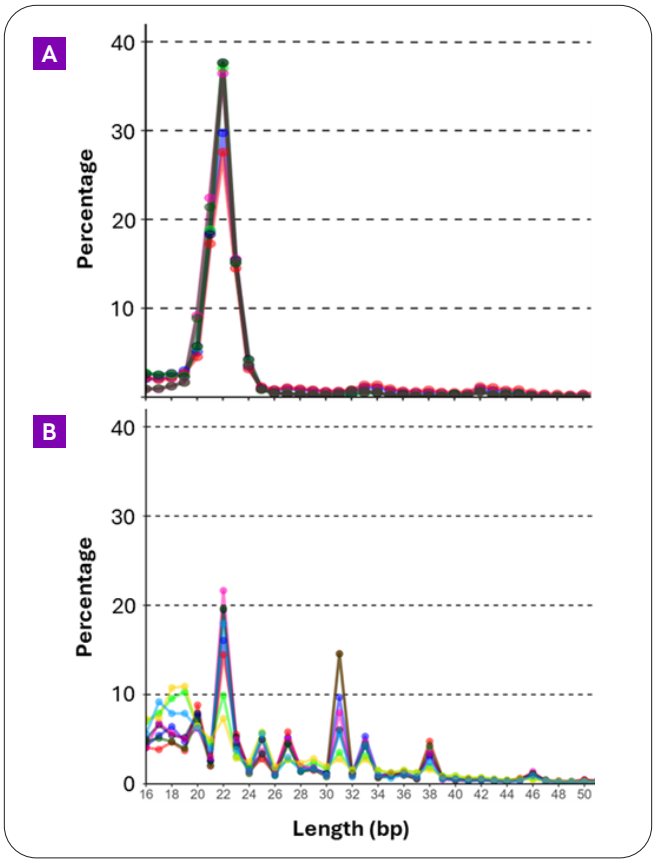


Figure 3: A. Insert length distribution in libraries generated from high quality brain tissue. B. Insert length distribution of mapped reads in the samples studied. Please note that the samples coming from preserved tissue are enriched in inserts with size shorter than a miRNA, and a prominent peak of ~31 bp appears.

The breakdown by RNA class showed that rRNA fragments were the most abundant species, accounting for ~35.7% of the mapped reads. This is likely due to the degraded nature of the samples, allowing rRNA fragments to be incorporated into the libraries. Remarkably, ~31.5% of the reads were classified as putative piRNAs. Given that piRNAs are typically restricted to germline cells and expressed at very low levels in somatic tissues like muscle or adipose[2], this proportion is almost certainly an artifact. This phenomenon may result from mis-annotation of multimapping reads or the classification of degradation-derived fragments as piRNAs[3]. Additionally, reference piRNA annotations in mouse are less uniform than other species (e.g. miRNA), with variable definitions and annotation quality across resources.

Only ~ 0.3% of reads mapped to miRNAs. This is approximately 100-fold lower than typical values observed in fresh mouse or human tissues (~ 40–60%). This result likely reflects the poor RNA integrity, rather than any limitation of the library preparation kit or sequencing platform. As indicated previously, extensively degraded RNA is enriched in small fragments with 5′-phosphate, that enter library prep, reducing the contribution of miRNA to the total percentage of mapped reads. The RNA type composition was consistent across both homogenization methods (Figure 4).

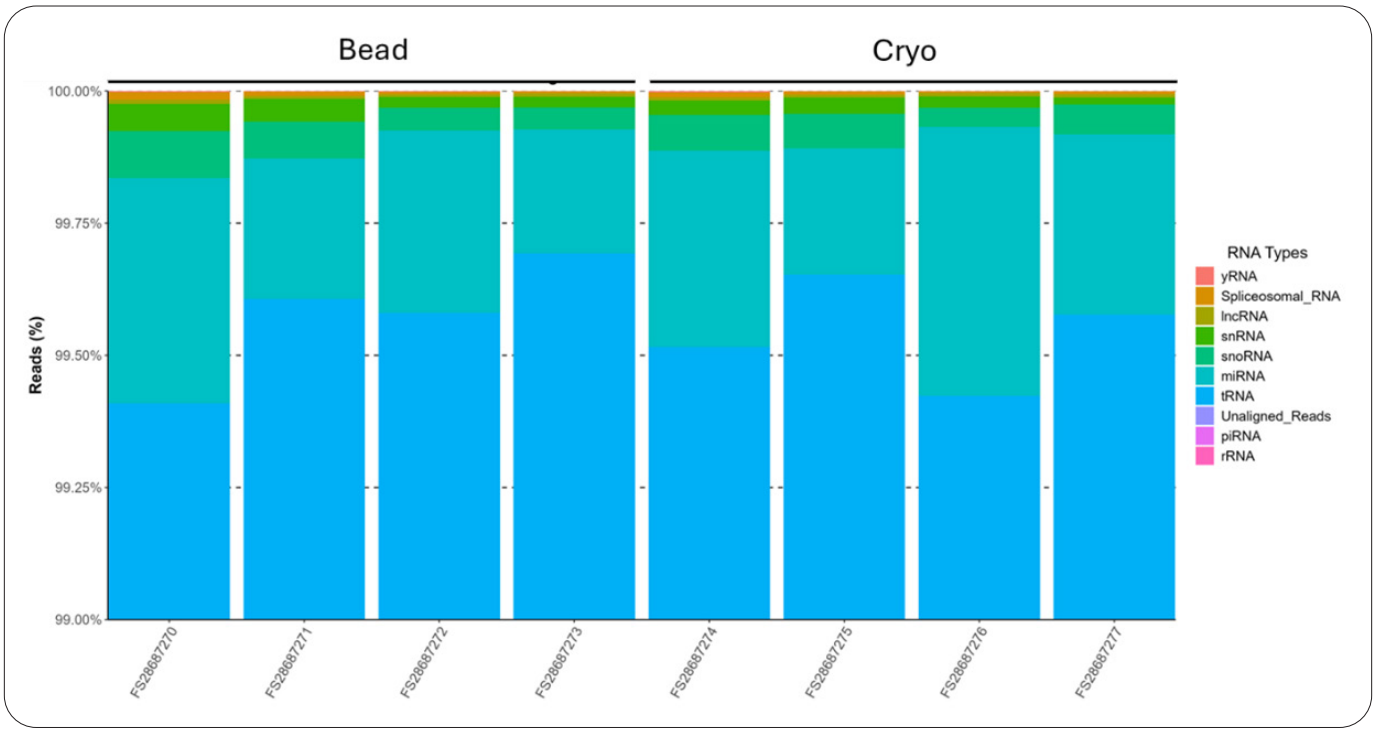


Figure 4: RNA type distribution for each of the eight samples (FS28687270 to FS28687277).

Focusing on the miRNA fraction, we identified and quantified known mature miRNAs. The top 10 most abundant miRNAs for each sample are shown in Figure 5. The most highly expressed species was mmu-mir-6240-1166, known to be expressed in skeletal muscle and adipose tissue. The second most abundant was mmu-mir-6236_53, with documented expression in adipose tissue and bone marrow. Together, these two miRNAs accounted for ~85% of all miRNA-aligned reads. Other species included mmu-mir-5100_853 and mmu-mir-1983_420, for which expression data is currently unavailable[4].

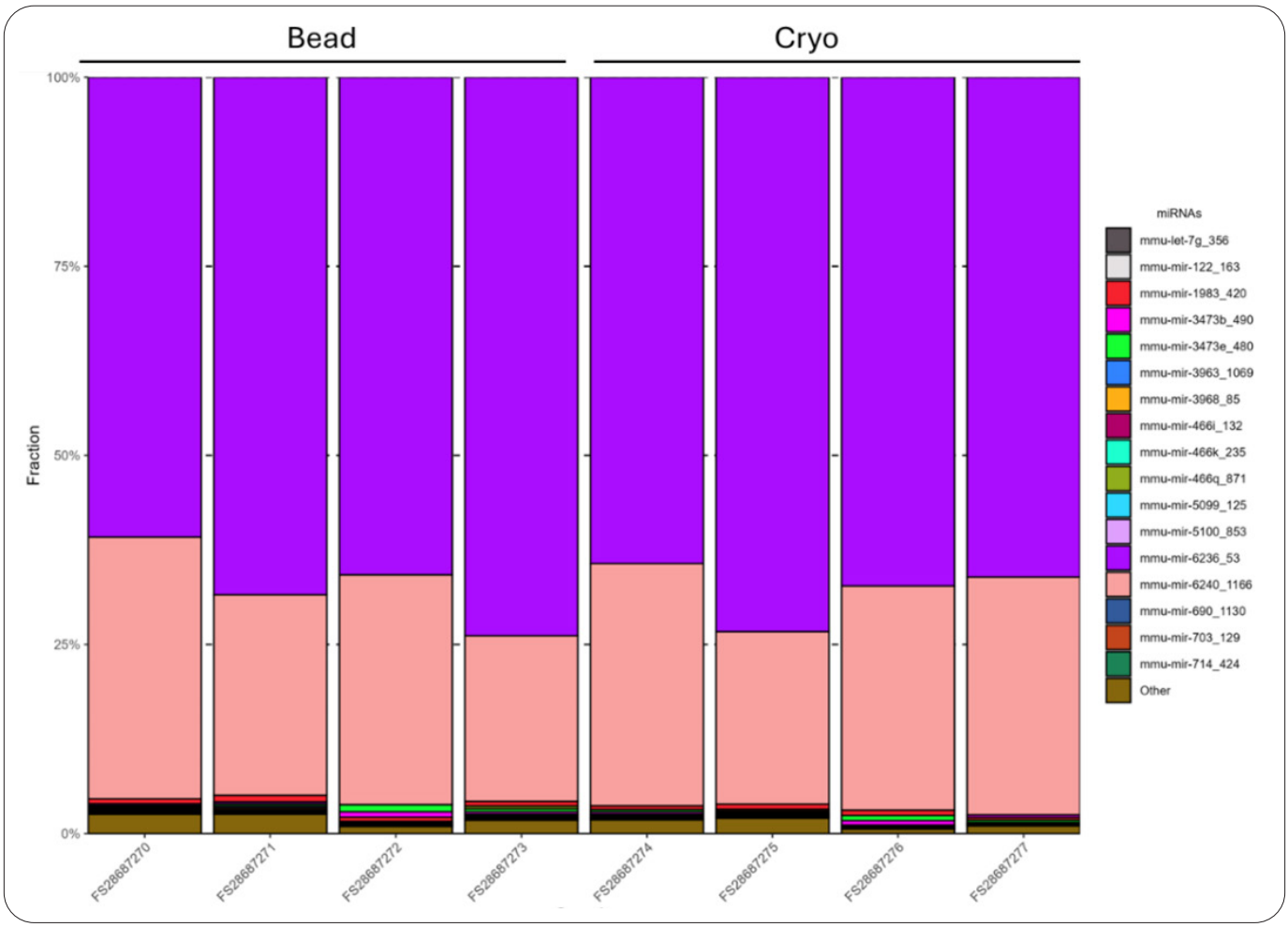


Figure 5: Top 10 most abundant miRNAs per sample.

Conclusions

This study demonstrates that the Element Biosciences® AVITI™ platform, in combination with the NEXTFLEX Small RNA-Seq Kit v4 enables successful sequencing of small RNA from highly degraded RNA samples. The high-quality scores obtained generally by AVITI™ platform can be relevant for isomiRNA discovery.

Despite RNA integrity being extremely low (RIN ~1.38), all libraries were successfully prepared and sequenced with high yield and quality metrics. While miRNA representation was reduced due to RNA degradation, this did not reflect limitations of the library kit or sequencer. Instead, the results point to the need for upstream depletion of abundant fragmented species such as rRNA, as well as removal of <15 nt fragments, in studies where degraded samples are used and miRNA yield (or other small RNA species) is a priority. These steps would likely also reduce false-positive piRNA annotations caused most likely by multimapping degraded fragments.

The two tested homogenization protocols produced comparable results, suggesting that either method can be effectively used for RNA preparation from preserved tissues.

References

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