APPLICATION NOTE

AutoNorm™ of NEXTFLEX small RNA libraries using the iconPCR™ system.

Introduction

The distribution and abundance of microRNAs varies widely across sample types, reflecting their distinct physiological functions and regulatory needs. In human samples, most reports place microRNA at ~0.01% to 2% of total RNA, depending on cell type, tissue, and developmental stage. This biological variability complicates library preparation, since the same PCR conditions applied uniformly across different samples can result in over-amplification of libraries from miRNA-rich tissues and insufficient amplification of others, posing challenges for downstream normalization and risking uneven representation in multiplexed sequencing runs.

Normalization of small RNA libraries has traditionally been attempted by manipulating library preparation steps, such as adjusting adapter concentrations, modifying cleanup strategies, or altering cycle numbers empirically for each sample type. While these approaches can partially reduce variability, they often require additional optimization, introduce new sources of bias, and are difficult to scale when processing large sample sets.

An alternative is offered by n6's iconPCR™ system, which uses real-time monitoring during PCR amplification to balance libraries adaptively at the single well level. Instead of setting a fixed number of cycles in advance, the system terminates amplification of each sample when a user-defined fluorescence threshold is reached, equalizing library output across diverse input types. This feature, termed AutoNorm™ technology (n6), differs from conventional normalization in that it actively monitors DNA synthesis during each reaction rather than applying a fixed external adjustment. By defining a fluorescence threshold, the system brings all libraries to a comparable endpoint regardless of starting material or microRNA composition. In practice, this means that samples with abundant ligation products stop amplifying earlier, avoiding the accumulation of PCR duplicates, while samples with lower input or reduced complexity are allowed to continue until they reach the same normalized endpoint.

Methods

We evaluated the performance of the iconPCR™ system (n6) in combination with the NEXTFLEX™ Small RNA-Seq Kit v4. To cover the range of variability of microRNA content observed across human samples we used total RNA from three different samples: brain, skeletal muscle and placenta (BioChain). Brain tissue has among the highest microRNA content of any human tissue. In brain samples, roughly 1–2% of total RNA is microRNA, with miR-124 and miR-9 dominating the landscape. Skeletal muscle presents a lower overall proportion, typically 0.1–0.5% of total RNA, while placenta contains intermediate levels, often 0.5-1% of total RNA.

Libraries were prepared from the 3 sample types at two input amounts, 1 ng and 10 ng, following manufacturer's instructions. Amplification was performed on the iconPCR™ system (n6) either using standard conditions or with AutoNorm™ technology (n6), setting a fluorescence threshold of 6,000 relative fluorescence units. Duplicates of each condition were prepared. Final libraries were quantified using a Qubit® fluorometer (Thermo Fisher Scientific), pooled equimolarly, and loaded at 9 pM with 3% PhiX spike-in on an Illumina® Miseq™ platform using 1×50 bp paired-end sequencing, aiming for 1M reads/sample. Small RNA analysis was performed using a custom Revvity script, and alignment was carried out using mature miRNA sequences from miRBase v22.1.

Results

PCR cycles

Under standard conditions, 10 ng libraries were amplified for 20 cycles, whereas 1 ng libraries required 24 cycles. When the same samples were processed on an iconPCR™ system (n6), the number of cycles required to reach endpoint was reduced to an average of 18.7 cycles for 10 ng inputs and 23.3 cycles for 1 ng inputs. This adjustment brought the cycle number closer to the actual amplification needs of each sample type and minimized unnecessary extension in the miRNA-rich libraries (Figure 1).

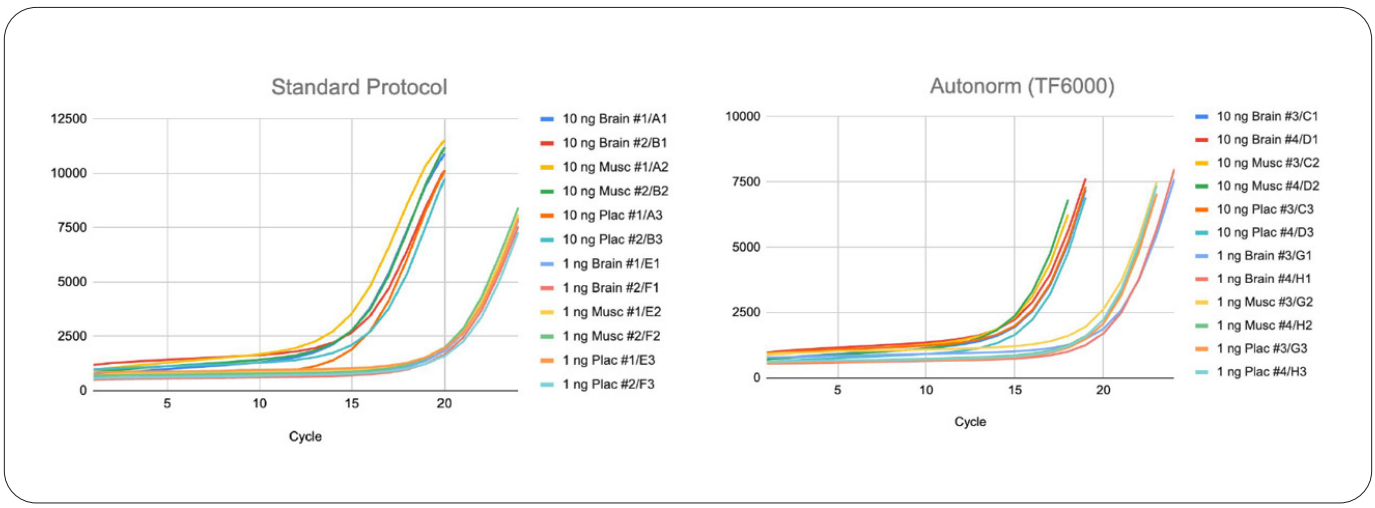

Figure 1: Amplification curves of small RNA libraries on the iconPCR™ system (n6) using standard protocol or with AutoNorm™ technology (n6).

Final library yield and average fragment size

Libraries generated with standard method had on average a concentration of 5.08 ng/µL and a CV =48.5% across different sample types and inputs. By contrast, libraries amplified with AutoNorm™ technology presented a concentration of 2.53 ng/mL and much lower variability across different sample types and inputs, with CV = 25.6% (Figure 2).

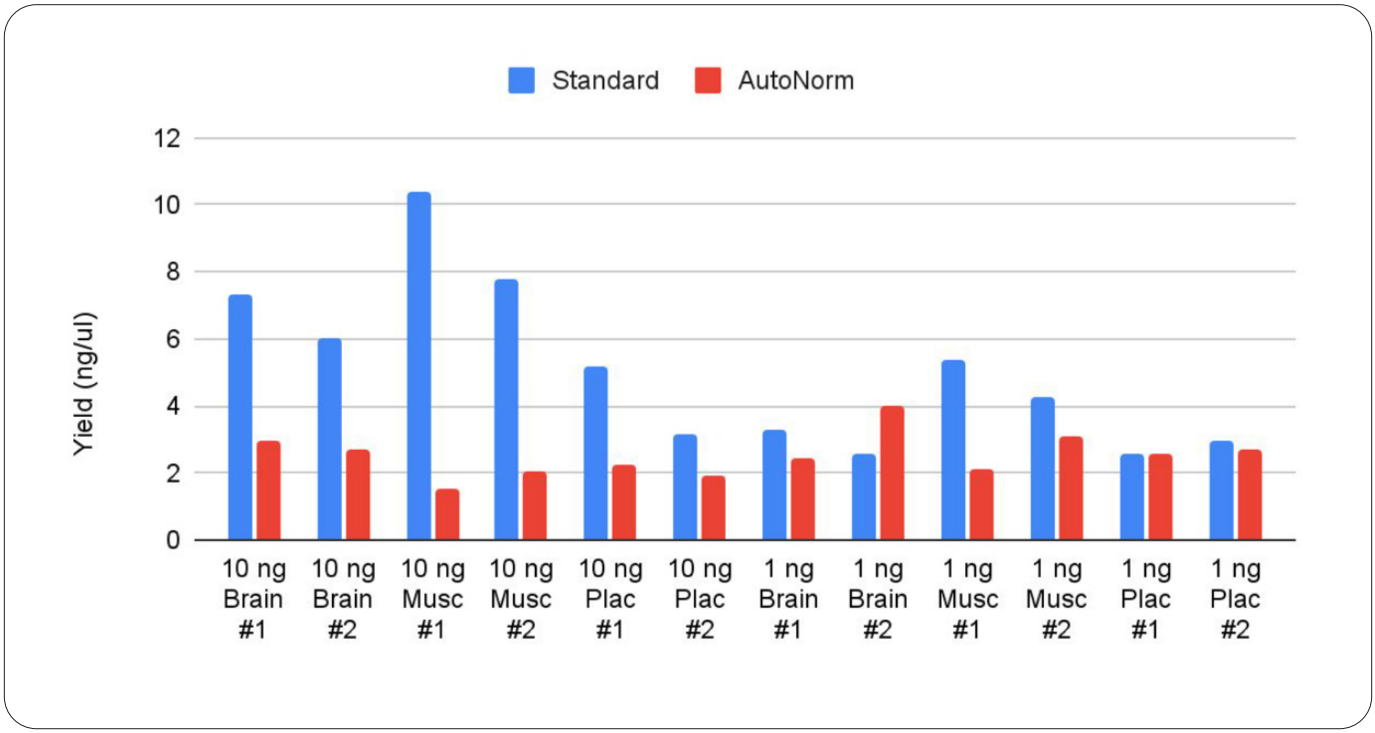

Figure 2: Final yield obtained for each of the conditions tested. Yield variation in libraries amplified with AutoNorm™ technology is lower than in those amplified with standard conditions.

Peak library size was similar, with 159 ±4 bp for standard group and 156±3 bp for AutoNorm™ group.

Sequencing data analysis

Mapped reads were annotated according to RNA class. Average values were calculated for both groups (standard and AutoNorm™) and the RNA composition profiles showed no significant differences (Figure 3).

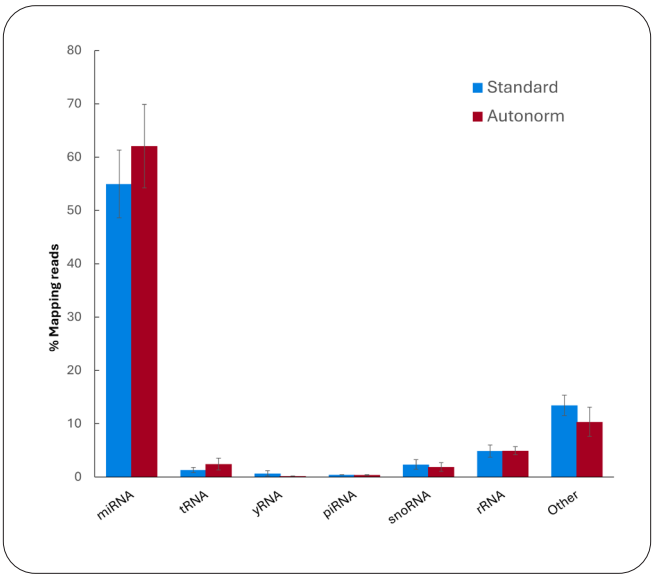

Figure 3: Average RNA type distribution for the two groups of samples (standard and AutoNorm™).

Conclusion

These results underline the advantages of combining NEXTFLEX Small RNA-Seq v4 chemistry with the iconPCR™ system (n6) for small RNA studies, particularly those involving different inputs and/or sample types. Unlike traditional normalization strategies that require empirical adjustments to the workflow, AutoNorm™ technology (n6) automates the process at the amplification step, reducing PCR cycles, improving reproducibility and streamlining pooling for sequencing, without introducing detectable bias in library composition.