Accurate representation of small RNAs using NEBNext Low-bias Small RNA Library Prep Kit

Heather M Raimer Young, Deyra N Rodriguez, Alec Monovich, Chen Song, Brittany S Sexton, Gautam Naishadham, Bradley W. Langhorst, Louise Williams | New England Biolabs, Inc.

Introduction

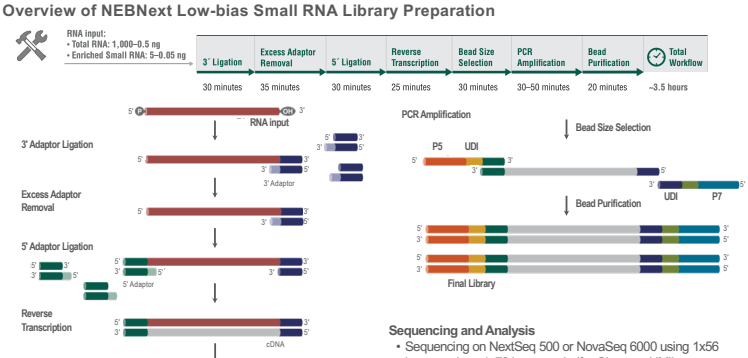
Changes in expression levels of small non-coding RNAs (sncRNAs) have been implicated in the development and progression of cancers as well as neurological and cardiovascular diseases. The accurate detection of sncRNAs is essential for using them as disease biomarkers. Next generation sequencing (NGS) is a powerful tool for the comprehensive detection and sequence characterization of sncRNAs. However, sncRNA library preparation methods often limits the accuracy and sensitivity of sncRNA detection. Biases are often introduced in many ligation-based methods, which obscures the true sncRNA composition. Improvements in sncRNA library preparation methods are essential for using sncRNAs as clinical biomarkers.

We have developed a novel ligation-based small RNA library preparation method that is characterized by reduced bias in addition to enhanced sensitivity and accuracy of sncRNA detection. sncRNA libraries can be made in one day using a streamlined protocol that uses bead-based size-selections and cleanups. The robustness of this method is demonstrated across a broad input range as well as multiple challenging sample types such as FFPE RNA and cfRNA.

Even representation of sncRNAs using this low bias sncRNA library preparation method was confirmed using a pool of synthetic miRNAs. ~90% of miRNAs were within 2-fold of the expected abundance, compared to < 30% for other methods. Additionally, miRNA detection is consistent using 0.5 ng to 1000 ng of total RNA from human brain. This low bias sncRNA method also works well on challenging sample types. To explore these sample types further, low bias sncRNA libraries were made using samples from human FFPE RNA and cfRNA. Regardless of input or sample, this method shows a robust capability to generate high quality libraries with an increased confidence in the types of small RNAs detected and therefore the potential identification of small RNA biomarkers across diseases.

Methods

Overview of NEBNext Low-bias Small RNA Library Preparation



RNA Input Types:

- 1000 ng – 0.5 ng of human brain total RNA
- 100 ng human testis total RNA
- 100 ng – 1 ng FFPE RNA (breast and lung)
- 100 ng Plant RNA (Arabidopsis, soy, maize)
- 0.5 ng – 0.25 ng
- 0.3 ng of a synthetic miRNA mix (100x miRNA-like sequences

Vendor Kits:

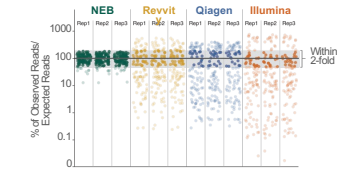
- 3 Ligation-based competitor kits. Manufacturers' RNA input recommendations and protocols were followed:
  - Revvity, NEXTFLEX Small RNA Sequencing Kit V4
  - Qiagen, QIAseq miRNA Library Kit
  - Illumina, TruSeq Small RNA Library Preparation Kit

Sequencing and Analysis

- Sequencing on NextSeq 500 or NovaSeq 6000 using 1x56 base reads or 1x72 base reads (for Qiagen + UMI)
- 30 million reads to 5 million reads were used in a down-sampling analysis
- 15 million reads were typically used in analysis
- Reads were adaptor trimmed (Flexbar) and aligned to respective genomes (human – hg38; Arabidopsis – Arabidopsis thaliana TAIR 10; rice - IRGSP-1.0; soy - Glycine max v2.1) the hg38 genome with STAR (v2.7.8a)
- The STAR reference was built using gencode v35 main annotations (contains 1,881 miRNAs) supplemented with gencode tRNA annotations, rRNA annotations for subunits not included in gencode, and piRNA annotations from piRNAdb v1.7.6 that did not overlap other annotations

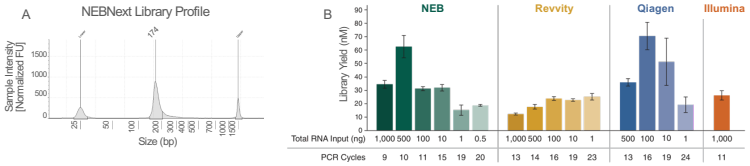
Results

Even Representation of Synthetic miRNAs



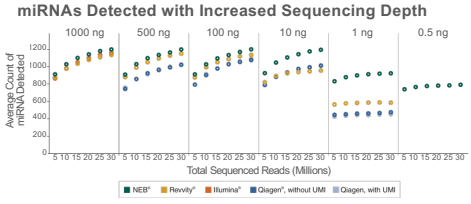
An equimolar mix of synthetic control miRNAs were used to assess library bias in the NEBNext Low-bias Small RNA Library Prep Kit and in libraries generated using small RNA kits from Revvity, Qiagen and Illumina. These synthetic miRNAs included five with 3′ 2′-O-methyl modifications. Expected reads were calculated using total reads mapped to the synthetic controls divided by the total control reads (black line at 100%). NEBNext libraries had 90% of reads within 2-fold of expected. Competitor kits had between 19-30% and had more underrepresented sequences (50-65%) than overrepresented (15-24%).

Robust Small RNA Library Prep



Small RNA libraries were made using 1000 ng – 0.5 ng of human brain total RNA. (A) Representative TapeStation profiles for Low-bias Small RNA libraries. (B) NEBNext Low-bias Small RNA library yields were similar or higher when compared to yields for competitor kits from Revvity, Qiagen and Illumina when using the same RNA input amounts. NEBNext libraries typically used 2-5 less PCR cycles. Library yields were determined using the Agilent TapeStation with High Sensitivity D1000 reagents. Yields shown represent three technical replicates and error bars are standard deviation.

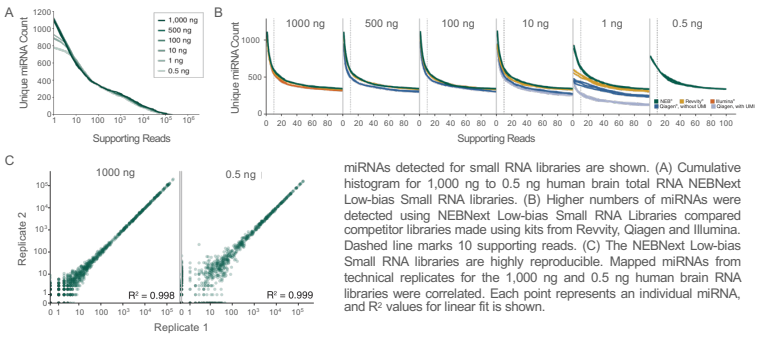
miRNAs Detected with Increased Sequencing Depth



miRNAs detected at increasing sequencing depths for small RNA libraries made using 1,000 ng – 0.5 ng of human brain total RNA. The Low-bias Small RNA Libraries consistently detected more miRNAs at all inputs and sequencing depths when compared to competitor kits from Revvity, Qiagen and Illumina. Reads were progressively down-sampled from 30 to 5 million reads. The number of miRNA observed with at least one read were averaged among three technical replicates.

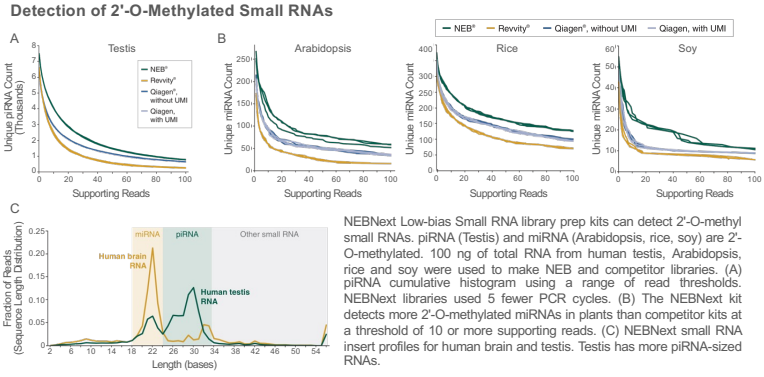
Results

NEBNext Low-bias Small RNA Libraries are Reproducible over a Wide Input Range



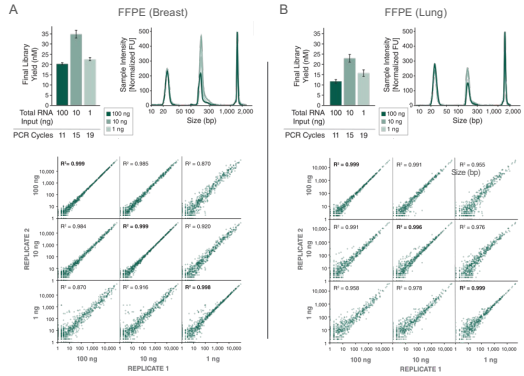
miRNAs detected for small RNA libraries are shown. (A) Cumulative histogram for 1,000 ng to 0.5 ng human brain total RNA NEBNext Low-bias Small RNA libraries. (B) Higher numbers of miRNAs were detected using NEBNext Low-bias Small RNA Libraries compared competitor libraries made using kits from Revvity, Qiagen and Illumina. Dashed line marks 10 supporting reads. (C) The NEBNext Low-bias Small RNA libraries are highly reproducible. Mapped miRNAs from technical replicates for the 1,000 ng and 0.5 ng human brain RNA libraries were correlated. Each point represents an individual miRNA, and R^2 values for linear fit is shown.

Detection of 2′-O-Methylated Small RNAs



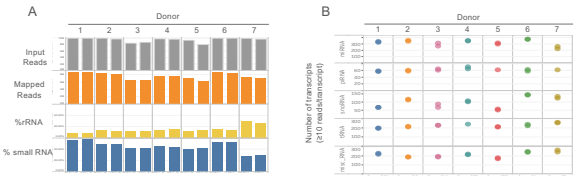
NEBNext Low-bias Small RNA library prep kits can detect 2′-O-methyl small RNAs. piRNA (Testis) and miRNA (Arabidopsis, rice, soy) are 2′-O-methylated. 100 ng of total RNA from human testis, Arabidopsis, rice and soy were used to make NEB and competitor libraries. (A) piRNA cumulative histogram using a range of read thresholds. NEBNext libraries used 5 fewer PCR cycles. (B) The NEBNext kit detects more 2′-O-methylated miRNAs in plants than competitor kits at a threshold of 10 or more supporting reads. (C) NEBNext small RNA insert profiles for human brain and testis. Testis has more piRNA-sized RNAs.

miRNA are Detected in FFPE Samples



NEBNext Low-bias Small RNA libraries were made using total RNA from human breast and human lung FFPE samples (100 ng – 1 ng). (A) Small RNA libraries were made using FFPE breast DNA. Library yields, TapeStation and correlations are shown for 100 – 1 ng inputs. (B) Small RNA libraries were made using FFPE lung DNA. Library yields, TapeStation and correlations are shown for 100 – 1 ng inputs. The correlations shown are for two replicates of each input for lung and breast FFPE libraries. Each point represents an individual miRNA, axes are shown in log scale, and R^2 values for linear fit is shown.

miRNA are Detected in cfRNA Samples



Low-bias Small RNA-seq metrics from cfRNA. (A) NEBNext Low-bias Small RNA have high mapping rates, low rRNA content, and high small RNA recovery. (B) Small RNAs detected with ≥10 reads, variability between donors collected with EDTA tubes are shown.

Conclusions

- The NEB Low-bias Small RNA Kit has significantly lower bias than competitor kits enabling accurate representation of small RNA abundance
- The kit is compatible with a wide input range (1,000 ng – 0.5 ng total RNA; 50 ng – 5 pg enriched small RNA) and utilizes bead-based size selection for all input amounts
- Consistent numbers of miRNAs detected across inputs
- Efficient capture of 2′-O-methylated small RNAs (piRNAs and plant miRNAs)
- Detects miRNAs from RNA isolated from FFPE samples
- Detects miRNAs from RNA isolated from cfRNA
- Single day workflow done in PCR strip tubes with minimal tube transfers

Acknowledgements

We thank Dora Posfai, Kristen Augulewicz, and Harry Bell for their technical support and members of NEBNext for productive discussions.