# Quick-guide for ExiSEQ NGS Spike-in kit - small RNA/microRNA (800100)

A vial containing a mix of 52 synthetic 5' phosphorylated microRNAs of different concentrations. The ExiSEQ NGS Spike-ins are dried down. The vial contains sufficient spike-ins for up to 500 samples. A vial containing nuclease-free water is provided for re-suspension of the spike-ins.

**Before starting the experiment**

Prior to use, the ExiSEQ NGS Spike-ins must be dissolved in 500 µL nuclease-free water. Vortex for 5 seconds and leave the suspension on ice for 30 minutes to dissolve. Vortex for 5 seconds again and spin to collect tube contents. Exiqon recommends to aliquot the dissolved ExiSEQ NGS Spike-ins, and to avoid repeated freeze/thawing. The re-suspended ExiSEQ NGS Spike-ins can be stored at -20 °C short term. For long-term storage, keep the vials at –80 °C after re-suspension.

**Recommended usage**

It is recommended to add the ExiSEQ NGS Spike-ins during RNA isolation to monitor the comparability and reproducibility of the whole process from RNA isolation to sequencing. Alternatively the ExiSEQ NGS Spike-ins can be added directly to the RNA, prior to library preparation. However, by adding the ExiSEQ NGS Spike-ins after RNA isolation, any information on RNA isolation, especially with regard to the small RNA fraction, will be lost.

The small RNA content of samples can differ significantly from sample to sample and source to source. For more accurate ratios of spike-ins *vs* endogenous small RNAs in the samples, experimental titration of ExiSEQ NGS Spike-ins is recommended. Consider the table as a guide for ExiSEQ NGS Spike-ins amounts.

| Sample type | Approximate amount of ExiSEQ NGS Spike-ins |
|---|---|
| Serum/plasma | 1 µL per isolation from 500 µL serum/plasma* |
| Cells or tissue | 1-10 µL per isolation, corresponding to ~1-2 µL spike-ins per volume of RNA eluate to be made into a library. Note that results in Tissue/Cell RNA isolation will vary depending on the isolation method and use of mechanical tissue homogenization. |
| RNA from serum/plasma | 1 µL per RNA eluate of 25-50 µL (originating from 500 µL serum/plasma)* |
| RNA from cells or tissue | 1-2 µL per 100 ng RNA to be made into a library |

*The recommendations above are adjusted to elution volumes from biofluid isolations around 20-30 µL. Input material from biofluids is 6-10 µL into library preparation.

**Evaluation of the ExiSEQ NGS Spike-in data**

The 52 ExiSEQ NGS Spike-ins added during RNA isolation are present at different concentrations and if used in the right amounts will represent microRNAs ranging from very low to very high endogenous levels (with ExiSEQ NGS Spike-ins accounting for approximately 1 - 3 % of total reads).

ExiSEQ NGS Spike-ins are very useful for evaluating NGS performance and can be used to assess the technical reproducibility across samples and verify the linearity of the NGS reads mapped to these exogenous sequences across samples.

This protocol will provide sufficient reads derived from the ExiSEQ NGS Spike-ins for post-sequencing analysis using a standard small RNA NGS analysis pipeline. At www.exiqon.com/mirna-NGS-spike-ins the sequences for the 52 ExiSEQ NGS Spike-ins can be downloaded as a single FASTA file. Reads should be mapped to the ExiSEQ NGS Spike-in sequences (using Bowtie2 or similar mapping algorithm) and spike-in reads should be filtered out from the rest of the data. We recommend "perfect match" settings when mapping, filtering and counting ExiSEQ NGS Spike-in reads in a dataset (FASTQ files). Following counting of the ExiSEQ NGS Spike-in reads, they should be normalized to the total number of reads per sample. If TPM (tags per million reads) are to be used then use the following formula.

For each ExiSEQ NGS Spike-in, calculate TPM based on each sample:

$$\mathbf{TPM} = \frac{\mathbf{\#\ Spike\text{-}in\ reads}}{\mathbf{Total\ reads}} \mathbf{*1000000}$$

After this simple normalization to individual sample reads has been done for all spike-ins in all samples, correlation matrices should be plotted for all sample-to-sample comparisons. This is done to evaluate the sample-to-sample correlation in the sample set. Expected correlation should be $R^2$ of 0.95-0.99. If comparing day-to-day correlation, the correlation is usually weaker than within a batch of samples purified on the same day. If samples deviate from these values they could be technical outliers and should potentially be excluded from downstream analysis. Note that a few of the ExiSEQ NGS Spike-ins are present at low concentrations to represent very low TPM counts and will therefore give low numbers of reads, especially if the sequencing depth is not high. They should be excluded from the correlation analysis. A rule of thumb would be to exclude ExiSEQ NGS Spike-in data lower than 1 TPM if read depth is approximately 10 Million reads per sample.





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