GARVAN INSTITUTE OF MEDICAL RESEARCH

Sequins with RNA sequencing

~Laboratory Protocol~

Sequins are synthetic reference RNA standards that are added at a fractional concentration to your RNA sample, and undergo concurrent library preparation, sequencing and analysis. This protocol describes the laboratory steps required to dilute, store and spike the sequins into your RNA sample prior to library preparation for RNA sequencing.

Further detail on bioinformatic analysis is available from www.sequin.xyz and for further technical questions, please contact: sequin@garvan.org.au.

For a detailed background on the design, validation and use of sequins, please see 'Spliced synthetic genes as internal controls in RNA sequencing experiments' by Hardwick et al., (2016) *Nature Methods* DOI: 10.1038/nmeth.3958

This protocol adds sequins at a relative fraction according to the amount of RNA in the sample. However, sequins can alternatively be added to your RNA sample at an absolute amount to enable absolute measurements of gene expression, fold-changes between samples and improved normalisation. For further information, please refer to 'Measuring Absolute RNA Copy Numbers at High Temporal Resolution Reveals Transcriptome Kinetics in Development.' by Owens et. al., (2016) *Cell Reports* Jan 26;14(3):632-47.

Receive sequins 1

Upon receipt of RNA sequins, first check to ensure they have not thawed during shipment. Please contact us immediately if you have any concerns. Immediately transfer the RNA sequins to frozen storage at -80°C (sequins should not be stored in a -20°C frost-free freezer).

Each tube contains RNA sequins in 10 µL solution, which is sufficent for ~100 typcial RNAseq libraries. On first thaw, spin the tube down to collect the contents at the bottom of tube, and prepare smaller single-use aliquots to minimize subsequent freeze-thaw cycles.

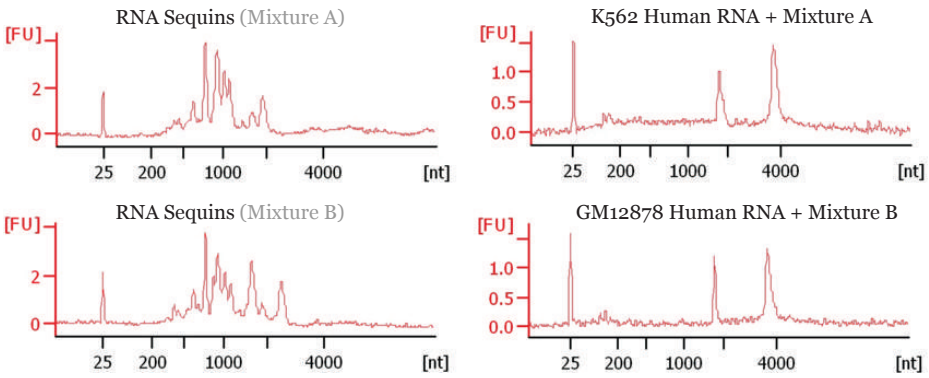

Figure 1. *Example traces of RNA sequins using an 2100 BioAnalyzer with the RNA Nano Kit (Agilent Technologies) for (left upper) neat Sequins Mixture A and (left lower) neat Sequins Mixture B. Also shown are example traces for (right upper) K562 RNA with Sequins Mixture A and (right lower) GM12878 with Sequins Mixture B.*

Sequins are designed, validated and manufactured at the Garvan Institute of Medical Research, Sydney Australia.

Revision History | Publication Number 4.1 | Revision Date October 2018

Dilute sequin stock 2

RNA sequins are provided in solution in nuclease-free water at a concentration of 15 ng/µL. The RNA sequins should be first be prepared to the correct dilution, before spiking in the appropriate amount into your RNA sample. We recommend users prepare a fresh dilution of the RNA sequins from single-use aliquots.

Please use the table below to determine the amount and dilution of sequins that should be used according to the sample RNA amount:

Sample RNA	Input RNA sequin mass	RNA Sequin (Dilution from 15ng/µL stock)
20 ng	0.2 ng	1 µL (1:75)
50 ng	0.5 ng	1 µL (1:30)
100 ng	1.0 ng	1 µL (1:15)
500 ng	5.0 ng	1 µL (1:3)
1000 ng	10.0 ng	1 µL (2:3)

Table 1. *Guidelines for diluting RNA sequins according to sample RNA amounts (recommended 1% spike-in).*

Spike into RNA sample 3

The diluted RNA sequins should then be added directly to the sample RNA.

RNA sequins can be added to RNA samples prior to processing step, such as poly-A enrichment or rRNA depletion. Whilst this enables an assessment of these processing steps, the amount and dilution of RNA sequins added may need to be modified accordingly.

RNA sequins are provided in two mixture formulations - Mix A and B - that contain the same sequin transcripts, but at different molar ratios, thereby emulating fold-change differences in gene expression and alternative splicing between the two mixtures. If you are performing a gene-profiling RNAseq experiment to identify differences in gene expression and splicing between two conditions, we suggest that Mix A and Mix B are alternatively added to seperate samples from each condition being compared (ensure that you do not add both mixtures to a single sample). This enables the use of RNA sequins to assess the detection of fold-change differences between samples.

Add only one freshly diluted RNA sequin preparation (either Mixture A or B) to each single

Library preparation 4

Use the combined sample as input according to the protocol of your preferred library preparation kit.

The downstream library preparation workflow may require user's to concentrate the sample RNA after the addition of the RNA sequins. Sequin-containing RNA samples can be concentrated by either ethanol precipitation, SPRI® bead purification (e.g. RNAClean® XP, Beckman Coulter), column-based methods (e.g. RNA Clean & Concentrator™ Kit, Zymo Research), or using vacuum centrifugation (e.g. Eppendorf Vacuum Concentrator Plus) at room temperature.

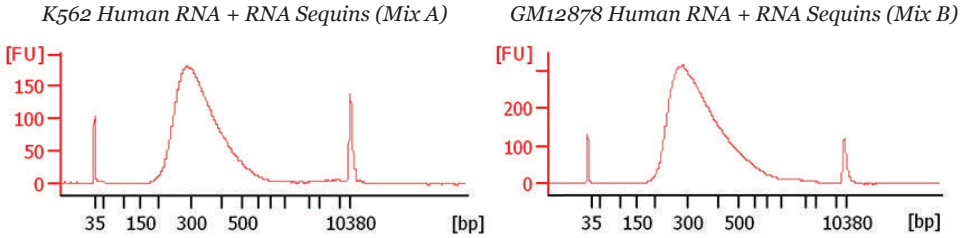

Figure 2*. Successful sequin-containing (total) RNA Libraries. A, K562 with Sequins Mix A. B, GM12878 with Sequins Mix B. Samples analysed by Agilent 2100 BioAnalyzer trace (size distributions sequenced on an Illumina® HiSeq 2500 Instrument).*

Sequencing 5

The library that is generated from the combined RNA sample and sequins is then sequenced according to manufacturer's instructions.