Centre for Genomic Research

UNIVERSITY OF LIVERPOOL

Submission of samples for Illumina RNA-Seq libraries

The CGR cannot guarantee good results from samples that do not meet the requirements set out in this document.

Maximising sample quality

To maximise quality, it is essential that your RNA samples:

- have been stored at -20/-80C and have not undergone multiple freeze-thaw cycles, which can affect RNA quality.
- have not been exposed to high temperatures or extremes of pH.
- have a 260:280 ratio and a 260:230 ratio of ≥1.8.
- do not contain insoluble material.
- are free from DNA contamination*.
- have been eluted and stored in nuclease-free water. Please do not use DEPC treated water, as this may interfere with enzymatic steps in the workflow.
- do not contain contaminating salts, metal ions, ethanol, phenol, polysaccharides or pigments.

Before extracting or treating your samples in any way, we recommend cleaning benches and equipment with an RNase decontamination solution and using fresh buffers/solutions that are free from RNase.

* DNA contamination of the RNA sample will greatly reduce the directionality of the sequenced library. Therefore, we recommend treatment of the RNA sample with DNase and removal of the enzyme prior to library preparation. In some cases, DNA digestion may have been incomplete due to sample type, level of gDNA contamination or efficiency of the kit. We recommend that prior to submission successful DNA removal is confirmed by PCR of all (or a subset) of the samples as well as a negative and a positive control sample. The PCR targeting gDNA is not part of the QC workflow at the Centre for Genomic Research and none of our other QC measures will conclusively indicate whether the samples are contaminated by DNA.

Assessing the quality and quantity of samples prior to submission

As part of the sample submission process, we will ask you to provide quantification data for your samples. It is important that the RNA is quantified accurately – we would recommend a dye-based, RNA-specific method, such as Qubit or a gel-based method such as the Agilent Bioanalyzer.

NanoDrop readings alone are not sufficient for accurate quantification but can help with assessing the quality of the sample. Submitted samples should have 260:280 and 260:230 ratios ≥1.8. If your samples require clean-up, the cost of this will be added to your invoice.

Please provide a gel image of all samples to confirm RNA integrity. We recommend working with intact total RNA with RIN values ≥ 7 as starting material for depletion or enrichment. This value is calculated by the Agilent Bioanalyzer software for most types of RNA. However, for some species the software cannot compute the RIN value. In those cases, RNA integrity can be estimated by the sharpness of the rRNA bands and a value close to zero in the 200-1200 bp range.

Depletion of specific RNA species (rRNA/globin mRNA)

Ribosomal RNA tends to represent 80-90% of purified total RNA samples. If this is not removed prior to library preparation, it will make up a significant proportion of the sequencing data, which is generally viewed as wasteful unless your project has a particular focus on rRNA sequences.

Please ensure you have informed the CGR enquiries team if your samples are likely to contain bacterial rRNA (e.g. gut/faecal material) or high levels of globin mRNA (i.e. RNA extracted from blood), as this may affect the library

preparation protocol that we would recommend. The CGR cannot take responsibility for failure to deplete bacterial rRNA or globin mRNA if it was not made clear that these RNA species were likely to be present in the sample/s before a quote was issued.

Please note that the baits used for rRNA or globin mRNA depletion have only been validated by the various manufacturers for certain organisms. We often achieve good results when using these kits with organisms that have not been validated by the manufacturers, but we have also observed poor performance with certain species. Unfortunately, we are unable to guarantee good performance when using these kits with samples taken from organisms that have not been validated by the manufacturers.

The ZymoSeq RiboFree RNA kit uses a non-probe-based method for depletion of high abundance RNA species within any given sample, which may result in better depletion for non-validated organisms. We have observed good performance with this kit when used by the CGR lab team but, unfortunately, we are also unable to guarantee the level of depletion that will be achieved with this kit.

The alternative to rRNA depletion is polyA selection, which will only enrich for mature polyadenylated transcripts, while depletion strategies will also generate sequence data for other non-polyadenylated transcripts. PolyA selection success depends on the integrity of total RNA extracted.

Please be aware that for samples that have been submitted after depletion/enrichment at an external site, the Centre for Genomic Research can take no responsibility for the level of mapping to rRNA, globin mRNA or any other undesired RNA species.

Sample submission requirements

The concentrations required will depend on the type of libraries being generated. The table below outlines the minimum input for each type of library.

Type of RNA	DNase Treatment	Pre-library treatment	RNA per sample	Volume
Total RNA	Before submission	PolyA selection	≥20 – 1000 ng	≤50 µl
		GLOBINclear and PolyA selection	≥1000 – 5000 ng	≤16 µl
		NEB rRNA depletion	≥10 – 1000 ng	≤15 µl
		Ribo-Zero Plus	≥20 – 500 ng	≤15 µl
		ZymoSeq RiboFree	≥200 – 5000 ng	≤10 µl
		FastSelect depletion	≥150 – 1000 ng	≤20 µl
		riboPOOL depletion	≥150 – 5000 ng	≤16 µl
		NEB cDNA synthesis	≥5 – 250 ng	≤9 µl
		NEB cDNA synthesis (low input)	≥0.002 – 5 ng	≤10 µl
	After submission	PolyA selection	≥50 – 2000 ng	≤50 µl
		GLOBINclear and PolyA selection	≥2000 – 5000 ng	≤50 µl
		NEB rRNA depletion	≥30 – 2000 ng	≤50 µl
		Ribo-Zero Plus	≥150 – 600 ng	≤50 µl
		ZymoSeq RiboFree	≥300 – 5000 ng	≤50 µl
		FastSelect depletion	≥250 – 1000 ng	≤50 µl
		riboPOOL depletion	≥300 – 5000 ng	≤50 µl
		NEB cDNA synthesis	≥15 – 750 ng	≤50 µl
		NEB cDNA synthesis (low input)	≥1 – 15 ng	≤50 µl
PolyA-enriched or rRNA-depleted RNA	Before submission	Not applicable	10 – 100 ng	≤7 µl
	After submission	Not applicable	30 – 250 ng	≤50 µl

Please note that we cannot QC samples with concentrations below 0.5 ng/ul or a total quantity of RNA below 5 ng. Therefore, we will not be able to guarantee successful library preparation, but we will charge for any work carried out on such samples.

As a general rule, the higher the amount of input RNA, the higher the quality of the resulting libraries will be.

We request that samples submitted in tubes (only for projects of <24 samples) are clearly labelled in numerical order for ease of sample identification. Please underline any numbers that could be misread upside-down (e.g. 6/9, 16/91).

For projects that involve ≥24 samples, please submit the samples in a 96-well plate, arranged down the plate in a column-wise fashion (sample 1 = well A1, sample 2 = B1, sample 3 = C1, etc.). Please pay careful attention to the sealing of 96-well plates prior to shipping: unfortunately, we do occasionally receive poorly sealed plates in which samples have leaked from their wells, leading to cross contamination.

If you are unable to meet the stated requirements for your library type, please contact us at CGR.Enquiries@liverpool.ac.uk and we will be happy to offer further advice.