Submission of samples for preparation of 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 -80°C 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 in 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 our other QC measures will not necessarily 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 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 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.

Guidance on Sample Randomisation

To ensure reliable sequencing data, it is important to address batch effects as part of your experimental design. Batch effects are caused by processing samples in multiple batches and can introduce biases that can confound biological effects. Randomising sample layouts by distributing replicates and experimental groups across plates and positions within plates helps to mitigate batch effects. We do not provide a sample layout randomisation service, but we strongly recommend considering this when designing your sample layout, prior to submission. Effective randomisation approaches include:

- **Interleaving replicates**: Spread replicates across plates.
- **Balancing groups**: Represent all conditions on each plate.
- **Avoiding positional bias**: Randomly assign wells.

Sample submission requirements

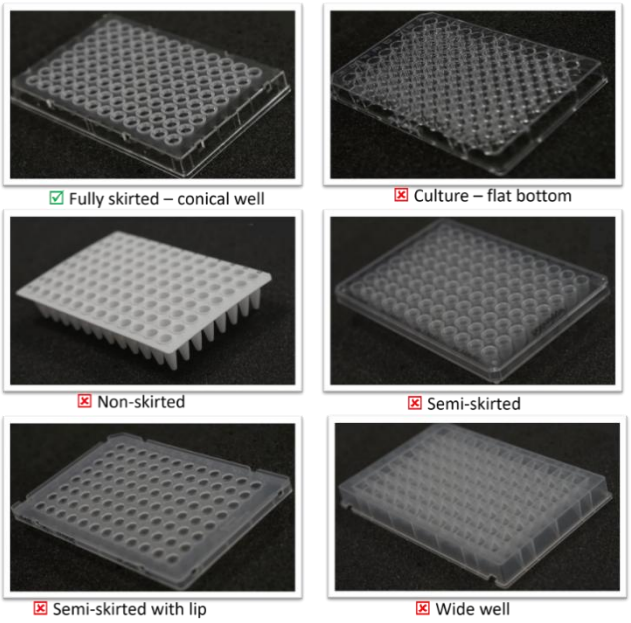
The concentrations required will depend on the type of libraries being generated. The table below outlines the minimum input for each type of library.

Type of RNA	DNAse treatment	Pre-library treatment	RNA per sample	Volume
Total RNA	Before submission	Miniaturised RNAseq	50-100 ng	10-50 µl
		NEB polyA selection	≥20-1000 ng	10-50 µl
		NEB rRNA depletion	≥10-1000 ng	10-20 µl
		Illumina Ribo-Zero plus depletion	≥20-500 ng	10-20 µl
		ZymoSeq RiboFree depletion	≥20-500 ng	10-20 µl
		QIAseq FastSelect depletion	≥150-1000 ng	10-20 µl
		riboPOOLs depletion	≥150-5000 ng	10-20 µl
		NEB cDNA synthesis	≥5-250 ng	10-20 µl
		NEB cDNA synthesis – low input	≥0.002-5 ng	10-20 µl
	After submission	Miniaturised RNAseq	150-200 ng	10-50 µl
		NEB polyA selection	≥200-2000 ng	10-50 µl
		NEB rRNA depletion	≥30-2000 ng	10-50 µl
		Illumina Ribo-Zero plus depletion	≥150-600 ng	10-50 µl
		ZymoSeq RiboFree depletion	≥50-5000 ng	10-50 µl
		QIAseq FastSelect depletion	≥250-1000 ng	10-50 µl
		riboPOOLs depletion	≥300-5000 ng	10-50 µl
		NEB cDNA synthesis	≥15-750 ng	10-50 µl
		NEB cDNA synthesis – low input	≥1-15 ng	10-50 µl
PolyA-enriched or rRNA-depleted RNA	Before submission	Not applicable	≥10-100 ng	10-20 µl
	After submission	Not applicable	≥30-250 ng	10-50 µl

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

If you are unable to meet the stated requirements for your library type, please contact us at CGR_Lab@liverpool.ac.uk and we will be happy to offer further advice. Please note that we cannot QC samples with concentrations below 0.5 ng/µl or a total quantity of RNA below 5 ng.

For samples that do not meet these requirements, we will not be able to guarantee successful library preparation, and we will have to charge for any work undertaken, regardless of the quality of the results.



For projects that involve ≥24 samples, we require samples to be submitted in a 96-well, fully-skirted plate. Please arrange your samples down the plate in a column-wise fashion, leaving 2 empty wells per plate so that we can add internal controls, as shown in the diagram below.

Sample position is very important for our workflows. If the submitted samples are not arranged as in the diagram below, you will be charged an additional £50 per plate to cover the cost of re-ordering the samples. It may also take longer for us to complete your project.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41	Sample 49	Sample 57	Sample 65	Sample 73	Sample 81	Sample 89
B	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42	Sample 50	Sample 58	Sample 66	Sample 74	Sample 82	Sample 90
C	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43	Sample 51	Sample 59	Sample 67	Sample 75	Sample 83	Sample 91
D	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44	Sample 52	Sample 60	Sample 68	Sample 76	Sample 84	Sample 92
E	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45	Sample 53	Sample 61	Sample 69	Sample 77	Sample 85	Sample 93
F	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46	Sample 54	Sample 62	Sample 70	Sample 78	Sample 86	Sample 94
G	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39	Sample 47	Sample 55	Sample 63	Sample 71	Sample 79	Sample 87	Empty
H	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40	Sample 48	Sample 56	Sample 64	Sample 72	Sample 80	Sample 88	Empty

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.

For projects involving <24 samples, submission in a 96-well plate is still recommended but we will also accept tubes. We request that samples submitted in tubes 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).

If you have any further questions, please contact us at CGR_Lab@liverpool.ac.uk .