RNA Sample QC Guidelines

We expect that your samples will be supplied in the correct containers and at the required concentration and quality (requirements are stated below). Timely completion of your project depends on you following these guidelines.

To prevent delays to projects that are batch-processed using our automated systems, in the event that a sample does not meet our QC criteria, we will still proceed with the library preparation. Your project manager will contact you to inform you of the problems with sample QC. It will then be at your discretion whether you sequence the library. If you choose not to sequence the library and instead provide us with a replacement sample(s), drop that sample or cancel your project you will still be charged for library prep and sample QC. Additional costs for QC and library preparation will be levied for each replacement sample. All new samples will be added to the end of the queue for the next batch of samples to be processed.

For further details on packing and submitting your samples, please see the following document ‘Guidelines for Preparing and Submitting Samples’.

Sample QC

The following sample QC guidelines are broken down into requirements for

1. Standard input RNA library preparations (for library types including 3’ mRNA, RNA-Seq No Isolation, RNA-Seq PolyA, RNA-Seq Ribozero and Small RNA)
2. Low input RNA library preparations (for the ultra low library type)

Unless you have a quote for both library types, you only need to read one of these sections not both. Please refer to your quote to select and read the relevant section and ensure that your samples meet our QC criteria. If you are unsure please contact your project manager prior to sending the samples. If your submitted sample(s) do not meet our QC criteria we will still proceed to make a library. A sample of poor quality is likely to produce a poor, or biased, library.

1. Standard Input RNA Library Requirements

This section applies when your quote lists one or more of the following library types: 3’ mRNA (3mRNA), RNA-Seq No Isolation, RNA-Seq PolyA, RNA-Seq Ribozero and Small RNA.

a. General Considerations and Suitable QC Methods
Ensure that RNase-free tubes, plates, lids and tips are used throughout. Take all additional precautions suitable for RNA work. All RNA samples should be DNase treated with a PCR-grade DNase. Preferably this would be done as part of the extraction process (i.e., on-column DNase step) but if samples must be DNase treated subsequently then the enzyme should be inactivated afterwards if necessary for the kit being used and purification should be carried out using columns or phenol/chloroform. All RNA samples must be in ultrapure water when submitted.

Quantification should be done using the Qubit RNA Broad-Range Assay kit. Nanodrop is a viable alternative to give a rough quantification of RNA samples but please be aware that any reading will likely be an overestimate.

Samples should be run on a RNA bioanalyzer chip to determine the quality, RIN values should be 8 or above. If that is not possible an estimate of integrity can be obtained using a 1% RNA gel, below is an example of good quality RNA (Figure 1).

![Image of RNA gel](http://www.norgenbiotek.com)

Please check the OD ratios by nanodrop. It is known that some extraction kits routinely produce samples with low 260/230 ratios and this could have a negative impact on the library preparation. If you see low 260/230 ratios, we recommend that you perform an ethanol precipitation. If this is not possible or you have concerns then please contact your project manager.

b. Concentration and Volume Required

The concentrations and volumes required for each library preparation type are given in the table below, along with some library-type specific notes in numbered bullets underneath the table. These concentrations are based on measurement by ribogreen. Please note that non-fluorescence-based method tend to overestimate RNA quantity, leading to actual concentrations being lower than expected. There is also likely to be some instrument to instrument variation even with ribogreen.

Please note samples should be normalized. If the amounts of RNA are variable across a project we will have to consider input options and will normalise as we think is appropriate. If you have specific normalization requirements please talk to your project manager.

As standard we will normalize input amounts to 100ng of total RNA for both polyA and ribodepletion protocols. If in-house QC indicates low RIN values then input requirements may need to be increased accordingly. Please provide sufficient material to accommodate for this
and also to repeat a library preparation if considered necessary. For samples <100ng we will use the appropriate maximum input. The absolute minimum is 10ng. In all cases where we have 20ng or more, we will aim to keep 10ng back in case a repeat is needed.

Please note it is easier, cheaper and faster for you to arrange for leftover material to be retrieved than it is to resend top up material for additional rounds of QC, resulting in project delays and additional cost.

<table>
<thead>
<tr>
<th>Type of Library Preparation</th>
<th>Amount of total RNA required</th>
<th>Concentration</th>
<th>Volume</th>
<th>OD 260/280 and 260/230</th>
<th>Bioanalyzer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PolyA&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>100ng-1μg</td>
<td>3.3-33ng/μl</td>
<td>30μl</td>
<td>~2</td>
<td>RNA 6000 Nano</td>
</tr>
<tr>
<td>Ribodepletion&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>100ng-1μg</td>
<td>10ng-100ng/μl</td>
<td>17μl</td>
<td>~2</td>
<td>RNA Pico</td>
</tr>
<tr>
<td>No selection&lt;sup&gt;4&lt;/sup&gt;</td>
<td>10-100ng</td>
<td>2-20ng/μl</td>
<td>5μl</td>
<td>~2</td>
<td>RNA 6000 Nano</td>
</tr>
<tr>
<td>Small RNA&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.8μg</td>
<td>~200ng/μl</td>
<td>9μl</td>
<td>~2</td>
<td>RNA 6000 Nano</td>
</tr>
<tr>
<td>PolyA and smallRNA</td>
<td>1.9-2.8 μg</td>
<td>~200ng/μl</td>
<td>10-14μl</td>
<td>~2</td>
<td>RNA 6000 Nano</td>
</tr>
<tr>
<td>3' mRNA&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.4 μg</td>
<td>~200ng/μl</td>
<td>12μl</td>
<td>~2</td>
<td>RNA 6000 Nano</td>
</tr>
</tbody>
</table>

1- For our standard polyA RNA prep, we prefer 100ng-1μg made up to 30μl in ultrapure water, but we can start from as little as 10ng if required, please discuss with your project manager if you expect low amounts of RNA.

2- If you request that a previous kit is used, we will need more material from you.

3- Please note we can only put a maximum of 14μl into Ribodepletion preps.

4- We will not QC the samples in-house so please make sure you have performed your own QC prior to sending the samples.

5- For small RNA library preps we need 1.5-3μg of total RNA in 7μl of ultrapure water, please note that we can only put a maximum of 5μl into the prep. Although you do not need to enrich for small RNA, please make sure that you use an RNA extraction kit that isolates large and small RNA molecules.

6- For 3' mRNA preps, we prefer to use 2μg in 5μl but we can start with varying quantities (we always require 5 μl for the prep itself) down to as little as 500pg if required. Please discuss with your project manager.

N.B. When optimal sample concentrations cannot be obtained it may still be possible to proceed, please discuss this with your project manager.
c. Plating and layout

For plating and layout of standard input samples, please refer to the document entitled “Guidelines for preparing and submitting samples for library prep or libraries for pooling” NOT the section below.

2. Low Input RNA Library Requirements

This section applies when your quote lists RNA-Seq Ultra Low library preparation and is suitable both for single cell or up to 100 cell bulks and low yield RNA samples.

We will not QC low input samples in-house so please make sure you have performed your own QC prior to sending the samples. We will perform amplification across your entire sample set based on the lowest input you advise us of. If a cell type is known to contain particularly low levels of RNA, for example naïve T-cells, please inform the project manager.

a. General Considerations

1. **Ensure that RNase-free tubes, plates, lids and tips are used.** Take all additional precautions suitable for RNA work. If submitting RNA, samples should be DNase treated with a PCR-grade DNase. Preferably this would be done as part of the extraction process (i.e. on-column DNase step) but if samples must be DNase treated subsequently then the enzyme should be inactivated afterwards if necessary for the kit being used and purification should be carried out using columns or phenol/chloroform. If submitting RNA, all samples should be in ultrapure nuclease-free water (please see section d for details on lysis buffer if submitting cells).

2. **It is strongly recommended that regardless of the types of plates or samples being sent that you send additional back-up plates** - This/these backup plate(s) could be used as replacement if, for any reason, the library preparation failed and having them in-house means that we can proceed immediately and without delay. NB, backup plates will be disposed of if unused at the end of the project unless you arrange to collect them.

b. Plating and layout considerations

**NB this is for plating of samples for the “ultra low” preparation only. For standard preps there is a separate document, referenced above.**

1. The 384-well 4titude Framestar skirted PCR plate is essential for automation compatibility, wells must be colourless and transparent. (catalogue number 4ti-0384/C [https://www.brookslifesciences.com/products/framestar-384-well-skirted-pcr-plate#ordering])

The plate should be well sealed with Thermo Scientific Adhesive PCR Seals (catalogue number #AB-0558).
2. 384-well plates should be filled one quadrant at a time, starting with Quadrant (Q) 1 and progressing to Q2, Q3, Q4 in that order. Quadrants should be filled by column from top to bottom, see plate map and well orders below.

<table>
<thead>
<tr>
<th>Quadrant</th>
<th>First Well</th>
<th>Quadrant Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>Upper Left</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>Upper Right</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>Lower Left</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>Lower Right</td>
</tr>
</tbody>
</table>

3. Include at least 2 blanks in each quadrant. One of the blanks in each quadrant will be used to add our internal control so take this into account if you require more than 1 blank for your own analysis.

4. Use an appropriate layout for your samples. We recommend that you organise your samples on each plate so that:
   - the distribution of your different experimental groups (WT vs. Mut, Control vs. Treatment, ...) is even within and between plates and quadrants, where multiple quadrants are used.
   - if you submit more than one plate, the blanks are placed at different positions so the identity of each plate can be confirmed during data analysis
   - the overall pattern of each plate is asymmetrical (position of blanks and potentially bulks) so the orientation of the plate can be confirmed during data analysis

5. If sorting has been carried out into other types of plate, we recommend resorting using the above guidelines. If resorting is not possible then you must transfer samples to these 384-well plates, this introduces the risk that material will not be transferred, this will be at your own risk. Samples plated in non-compatible plates will be refused and returned to you at your cost.

To transfer samples from a 96-well to a 384-well plate, use a multi-channel pipette to transfer the first plate into Q1 in the diagram above (and so on). Eg, if pipetting using an 8-channel pipette your first column needs to go in as below.
It is important that you plate each quadrant in columns. If you have full quadrants and only a 12-channel pipette then your first row needs to go in as below.

c. **Suitable QC Methods for RNA**

Quantification should be done using a low range bionalyzer or tapestation. These systems will allow you to quantify the samples and to check their quality.
d. Concentration and Volume of RNA Required

The concentration and volume required is given in the table below.

All RNA samples must be normalised as the PCR cycling conditions in this protocol are determined based on input amount. We will therefore increase PCR cycling to match the information you give for your lowest sample.

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>Amount required</th>
<th>Concentration</th>
<th>Volume</th>
<th>Bioanalyser</th>
<th>OD 260/280 and 260/230</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA-Seq ultra low</td>
<td>&lt;1ng</td>
<td>5pg-0.5ng/µl</td>
<td>2 µl</td>
<td>~2</td>
<td>RNA Pico</td>
</tr>
</tbody>
</table>

d. Requirements for Cells in Lysis Buffer

Cells can be submitted in either 0.8% (vol/vol) Triton X-100 or in NEB 1x lysis mix, with NEB lysis mix being the preference. **Lysis efficiency in either buffer should be checked** prior to the final sort using a visual check under a microscope/cell counter. We know from other users that certain cell types may not lyse properly, such as keratinocytes. NK cells and potentially macrophages will contain a lot of native RNAses which may require harsher conditions. **We will not check lysis efficiency.**

Triton-X can be made by combining 0.8% (vol/vol) Triton X-100 and 2 U/µl RNase inhibitor. This buffer can be stored at 4 degrees for 6 months. **Cells in this buffer should be in a final volume of 2µl.**

NEB lysis mix is available to purchase from NEB and can be used as detailed in the table below, again, the **final volume should be 2µl**. The catalogue number for the lysis mix is E5530 [https://www.neb.uk.com/products/neb-catalogue/ngs-sample-prep/nebnext-reg:-single-cell-lysis-module?returnurl=/SearchResults%3Fs%3Dlysis%20module&pn=1&ps=12&b=true#jump](https://www.neb.uk.com/products/neb-catalogue/ngs-sample-prep/nebnext-reg:-single-cell-lysis-module?returnurl=/SearchResults%3Fs%3Dlysis%20module&pn=1&ps=12&b=true#jump)

<table>
<thead>
<tr>
<th>Input type</th>
<th>Single cell</th>
<th>&lt;100 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10x Lysis buffer</td>
<td>0.2µl</td>
<td>0.2µl</td>
</tr>
<tr>
<td>Murine RNase Inhibitor</td>
<td>0.1µl</td>
<td>0.1µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>1.7µl</td>
<td>&lt;1.7µl (to final volume 2µl)</td>
</tr>
<tr>
<td>Total volume</td>
<td>2.0µl</td>
<td>2.0µl</td>
</tr>
</tbody>
</table>
It is our observation that with 100 cells, the overall volume of cells, lysis buffer and RNase inhibitor is already 2 µl so no nuclease -free water is required. This will depend on the chip used in the cell sorter but if the volume is lower and no nuclease-free water is added it won’t affect the prep.

Once cells are lysed, they should be briefly centrifuged then stored at -80°C until they are being shipped to us.