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 library types including SMARTer and SMART-Seq2)

Please read the relevant sections 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 (ie 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).

![RNA gel image](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^1,2</td>
<td>100ng-1μg</td>
<td>3.3-33ng/μl</td>
<td>30 μl</td>
<td>~2</td>
<td>RNA 6000 Nano</td>
</tr>
<tr>
<td>Ribodepletion^2,3</td>
<td>100ng-1μg</td>
<td>10ng-100ng/ μl</td>
<td>17 μl</td>
<td>~2</td>
<td>RNA Pico</td>
</tr>
<tr>
<td>No selection^4</td>
<td>10-100ng</td>
<td>2-20ng/ μl</td>
<td>5 μl</td>
<td>~2</td>
<td>RNA 6000 Nano</td>
</tr>
<tr>
<td>Small RNA^5</td>
<td>1.8μg</td>
<td>~200ng/μl</td>
<td>9 μl</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>10-14 μl</td>
<td>~2</td>
<td>RNA 6000 Nano</td>
</tr>
<tr>
<td>3' mRNA^6</td>
<td>2.4 μg</td>
<td>~200ng/μl</td>
<td>12 μl</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.

2. Low Input RNA Library Requirements

This section applies when your quote lists one or more of the following library types: SMARTer and SMART-Seq 2.

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

a. General Considerations

Ensure that RNase-free tubes, plates, lids and tips are used. 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 (ie 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 water (please see section d for details on lysis buffer if submitting cells).

It is known that glycogen is not compatible with SMARTer preps and so it should not be used as a carrier.

Our preferred plates have been detailed in our "guidelines for preparing and submitting samples". For low input preps especially, due to the small volumes handled and the automation of the library preparation it is absolutely ESSENTIAL to use plates with the following characteristics:
- Fully skirted
- Low profile
- Thin walls
- Transparent wells

We have validated the following plates
- Thermo Fisher PCR Plates #AB0800 (formerly ABgene)
- 4Titude FrameStar® 96 Well Skirted PCR Plates # 4ti-0960
- 4titude 96 Well Standard, Skirted PCR Plates # 4ti-0740

We understand that in some cases you will be sorting in small batches and will not be able to sort directly into our validated plates. However, you should be aware that
failure to re-plate into one of the plates listed above can lead to failures and contamination during the earlier parts of the library prep. If you are unable to re-plate and provide samples in the plates listed above then it is essential that you sort back-up plates in case of any problems and you should be aware that there may be additional incurred costs if we do need to repeat. We would also ask you to avoid strip caps and to use plate seals, we recommend Thermo Scientific Adhesive PCR Seals #AB-0558.

Please contact your project manager if you don’t have access to any of the validated plates mentioned. We reserve the right to refuse samples not provided in suitable plates.

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

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.

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
- 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

b. Suitable QC Methods

Quantification should be done using an RNA Pico kit on the bioanalyzer. If samples will be 5ng/μl or above then the Qubit RNA High-Sensitivity Assay kit can be used for quantification.

The RNA Pic bioanalyzer chip can also be used to determine the quality, RIN values should be 8 or above.

c. Concentration and Volume of RNA 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.
All 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 your lowest input sample.

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>Amount required</th>
<th>Concentration</th>
<th>Volume</th>
<th>OD 260/280 and 260/230</th>
<th>Bioanalyzer</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMARTer&lt;sup&gt;1&lt;/sup&gt;</td>
<td>100pg-10ng</td>
<td>12pg-3ng/μl</td>
<td>3-9 μl</td>
<td>~2</td>
<td>RNA Pico</td>
</tr>
<tr>
<td>SMART-Seq2</td>
<td>10pg-10ng</td>
<td>5pg-5ng/μl</td>
<td>2-2.3 μl</td>
<td>~2</td>
<td>RNA Pico</td>
</tr>
</tbody>
</table>

1- For standard SMARTer RNA preps, we require 100pg-10ng of total RNA in 9μl ultrapure water. If you have samples below 100pg please contact your project manager to discuss the options.

d. Requirements for Cells in Lysis Buffer

If providing cells for SMARTer, please freeze in a maximum of 5 μl lysis buffer with RNase inhibitor. If you have requested the Smart-Seq 2 protocol please freeze in a maximum of 2.3μl lysis buffer with RNase inhibitor.

The required lysis buffer 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.