Submission of samples for preparation of amplicon libraries for PCR-barcoded Oxford Nanopore Technologies Libraries

QC of samples submitted for preparation of amplicon libraries is not included in our standard amplicon library preparation workflow, unless this has been specifically requested. Instead, samples are entered into the 1st round PCR at the submitted concentration.
The CGR cannot guarantee good results from samples that do not meet the requirements set out in this document.

Experimental design

No more than 94 samples should be included in any submitted 96-well plate – this allows us to process two PCR controls (negative and positive) for each plate. Importantly, we recommend inclusion of the following controls as part of your own samples:

- **Extraction kit negative control**: a mock nucleic acid extraction, using the reagents provided in the extraction kit and following the protocol, but without addition of any actual sample material. If applicable, lot-to-lot variation between extraction kits should be avoided. This may be easier to achieve if kits are purchased at the same time. If multiple lots of kits are used, this information should be incorporated into the statistical analyses.
- **Extraction kit positive control**: extraction from a mock community of known composition is recommended to determine the success of the extraction method.

For more recommendations on the design of metabarcoding studies, we suggest consulting the following publications:
Salter et al., BMC Biology 2014 - Reagent and laboratory contamination can critically impact sequence-based microbiome analyses
Kim et al., Microbiome 2017 - Optimizing methods and dodging pitfalls in microbiome research
Debelius et al., Genome Biology 2016 - Tiny microbes, enormous impacts: what matters in gut microbiome studies?
Pollock et al., AEM 2018 - The Madness of Microbiome: Attempting To Find Consensus
Frau et al., Scientific Reports 2019 - DNA extraction and amplicon production strategies deeply influence the outcome of gut mycobiome studies

Pre-submission amplification test

Samples are likely to contain a varying proportion of gDNA from non-target organisms, so it is difficult to normalise the actual input into the PCRs. Higher numbers of PCR cycles will introduce more biases during the amplicon generation. Our standard ONT amplicon generation protocol includes a total of 30 PCR cycles (15 first-round PCR cycles to amplify the target region and 15

second-round PCR cycles to incorporate ONT barcodes). These parameters work well for most samples, but we occasionally encounter samples which require a greater number of PCR cycles.

We require that you perform a test PCR before shipping the samples, using the same primers as in the first round PCR, targeting your region of interest. You should perform a PCR experiment with 25, 30 and 35 cycles for a representative subset (or all) of your samples, and check that these produce visible bands of the expected size on an agarose gel or an automated electrophoresis instrument (such as the Bioanalyzer, TapeStation or equivalent). We do not recommend diluting your samples to a standardised concentration before amplification – however, if your samples are very highly concentrated then it may be necessary to dilute them to some degree in order to observe successful amplification. The samples submitted to the CGR should be at the same concentration as those used in the pre-submission amplification test. We recommend that your test includes samples spanning the entire range of sample sites, conditions and concentrations obtained for your samples, as well as a positive and a negative control. Please upload an image showing the outcome of the test when submitting your project to the Samples Submission Portal (SSP). Please also indicate the total number of PCR cycles required to obtain visible products of the expected size. If you fail to provide this information, we will not be able to guarantee successful library preparation and/or sequencing performance. Any additional work required to rectify such issues may incur additional costs.

Submission of first-round PCR products

For collaborators submitting first-round PCR products of their region of interest (rather than gDNA), we require that the first-round PCR products are generated using target-specific primers that incorporate the annealing sites for our second-round PCR primers as overhangs.

- Forward primer: 5' **TTTCTGTTGGTGCTGATATTGC***[forward primer sequence]* 3'
- Reverse primer: 5' **ACTTGCCTGTCGCTCTATCTTC***[reverse primer sequence]* 3'

Highlighted in *blue and italics* is the part of the primer that anneals to the region of interest to allow amplification. Highlighted **red and bold** is the ONT overhang that is incorporated during the first PCR to allow barcoding during the second PCR.

If the pre-submission amplification test indicates that a total of 25, 30 or 35 PCR cycles were required to obtain visible products of the correct size, please carry out 10, 15 or 20 PCR cycles (respectively), for generation of the first-round PCR products to be submitted to the CGR. This will enable us to complete the library preparation with 15 cycles of second-round PCR to add the barcodes, and reduces the risk of over-amplification. If more cycles will be required, we ask that you make us aware of this in advance.

If amplicons have already been made without the ONT overhang but you still wish to use PCR barcoding, the overhang can be annealed onto submitted amplicons. The results of this method are more variable and we would strongly recommend incorporating the ONT overhang in the first PCR is possible.

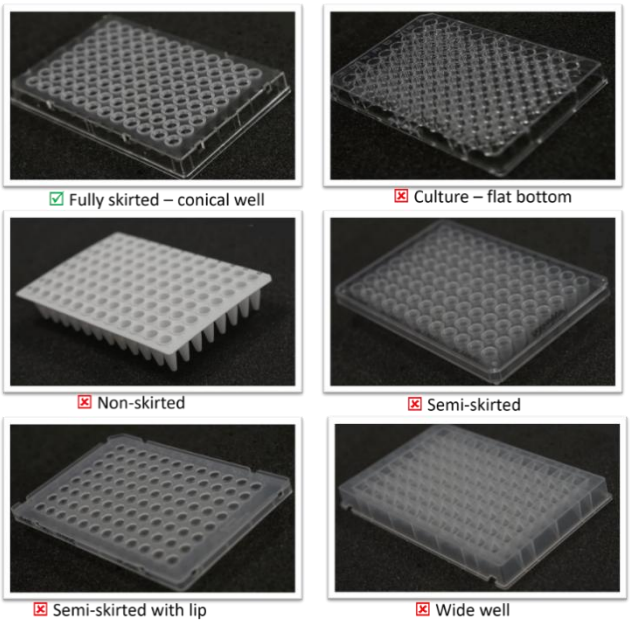
Sample submission requirements

- To maximise quality, it is essential that your submitted gDNA
    - have not been exposed to high temperatures or extremes of pH.
    - do not contain insoluble material.
    - are free from RNA contamination.
    - have been eluted and stored in a neutral, buffered solution.
    - have not been vortexed or shaken, as this can cause shearing of the DNA.
    - have not been exposed to intercalating fluorescent dyes or ultraviolet radiation. SYBR dyes do not damage DNA, but we would strongly advise against using ethidium bromide.
    - do not contain denaturants (such as guanidinium salts or phenol), divalent metal cations (such as Mg2+) or detergents (such as SDS or Triton-X100).
    - do not contain contamination from the original organism/tissue (haeme, humic acid, polyphenols, etc.)
- First-round amplicons should be free from contaminants as described above, unless sample clean-up is included in your quote.

Please be aware that if the library preparation fails due to the nature of the samples, we will charge for the work performed up until that point.

For projects that involve data analysis at the CGR, we ask that information relating to sample metadata is uploaded to the Sample Submission Portal at the time of sample submission to increase the speed at which the analysis component of a project can be completed. This information can be uploaded in the form of an Excel sheet/tab-delimited table in which the first column contains sample identifiers and subsequent columns contain different metadata for each sample.

For projects involving less than 24 samples, submission in a 96-well plate is still recommended but we will also accept tubes. We require 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).



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.

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.

	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

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.

If you are unable to meet the stated requirements or have any further questions, please contact us at CGR_Lab@liverpool.ac.uk and we will be happy to offer further advice.