Submission of amplicons for preparation of native barcoded Oxford Nanopore Technologies libraries

As ONT platforms sequence single molecules, any defects (strand breaks, abasic sites, DNA adducts, cross-linking, etc.) can interfere with the library preparation and sequencing processes. The quality and quantity of the submitted DNA will determine the quality of the library, the number of flow cells the library can be sequenced over and the quality of the resulting sequence data. High quality DNA is crucial for optimal performance and 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 amplicons:

- are double-stranded. Single-stranded DNA is not compatible with ONT library preps.
- have not been exposed to high temperatures or extremes of pH.
- have a 260:280 ratio of 1.8-2.0 and a 260:230 ratio of 2.0-2.2.
- 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.).

Clean, target-specific PCR products are extremely important for obtaining high-quality sequence data. Non-specific products can represent a substantial percentage of the sequencing reads if they are not removed. To minimize the presence of non-specific products, consider the following recommendations for generating high-quality amplicons suitable for ONT library preparation and sequencing.

1. Begin with high-quality nucleic acids and work in a clean environment.
    a. If extracted nucleic acids must be stored, freeze at high concentrations in appropriately buffered solutions.
    b. To minimize possible contamination and degradation caused by multiple freeze/thaw cycles, aliquot DNA into smaller volumes for storage.

c. Set up PCR reactions in an environment free from sources of non-specific primer and template contaminants; ideally a laminar flow hood, using dedicated pre-PCR pipettor, tips and reagents.

2. Use PCR reagents and conditions for generating target-specific, full-length amplicons.
   a. Use the highest-fidelity polymerase compatible with your PCR amplification system.
   b. Use desalted or HPLC-purified oligos; damaged bases at the ends of the amplicons cannot be repaired by DNA damage repair enzymes.
   c. Optimize PCR conditions to minimize total time spent at high (>65ºC) temperatures, particularly during denaturation.
   d. PCR extension time should be long enough to ensure complete extension, taking into consideration the polymerase used and target amplicon size. For mixed samples with similar targets, it is important to complete extension at every step to avoid generating chimeric products in subsequent steps. As a general guideline, use extension times of one minute per 1000 base pairs (e.g., 3 minutes for a 3 kb product).

3. Use the lowest number of cycles required for obtaining adequate yields (ng) of PCR products to proceed with ONT library construction. Avoid over-amplification.

4. If non-specific products are present, optimize PCR conditions or perform bead-based size selection to enrich for PCR amplicons with the desired target size (see recommendations below).

We recommend purification of amplicons before library preparation to remove PCR reagents, buffers, primer dimers and short non-specific PCR products. Depending on the size of the target amplicon, the concentration of size selection beads required for purification varies. Refer to the table below for the appropriate concentration of size selection beads to use for purification. CGR offers this as a service if do not have the facilities to perform a bead clean prior to submission.

Amplicon size (bp)	**beads:sample ratio**
250 - 500	1.2 : 1
500 - 1000	0.8 : 1
1000 - 3000	0.5 : 1
>3000	0.45 : 1

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 DNA is quantified accurately – we would recommend a dye-based, dsDNA-specific method, such as Qubit. NanoDrop readings alone are not sufficient for accurate quantification but can help with assessing the quality of the sample.

Concentration measurements by Qubit and NanoDrop should not differ significantly. A significant difference in those values may indicate that the sample contains single-stranded DNA, gDNA, RNA and/or other contaminating compounds (which may not be reflected in reduced NanoDrop 260:280 and 260:230 ratios). Unfortunately, we do not have an exact acceptable difference between the readings.

Please provide a gel image or trace of all submitted samples confirming amplification of the intended target, including the type of ladder and an indication of fragment size(s). If there is more than one band or a smear, the sample may contain non-specific products or degraded DNA, be contaminated with RNA, or contain a contaminant that may affect the library preparation.

Please be aware that we do not recommend the inclusion of in-line barcodes prior to submission. Instead we would strongly recommend that each sample is given a unique barcode during our library preparation service. Please note that the CGR does not offer demultiplexing of ONT libraries for barcodes not incorporated by CGR, and we cannot guarantee the performance of such libraries.

Sample submission requirements

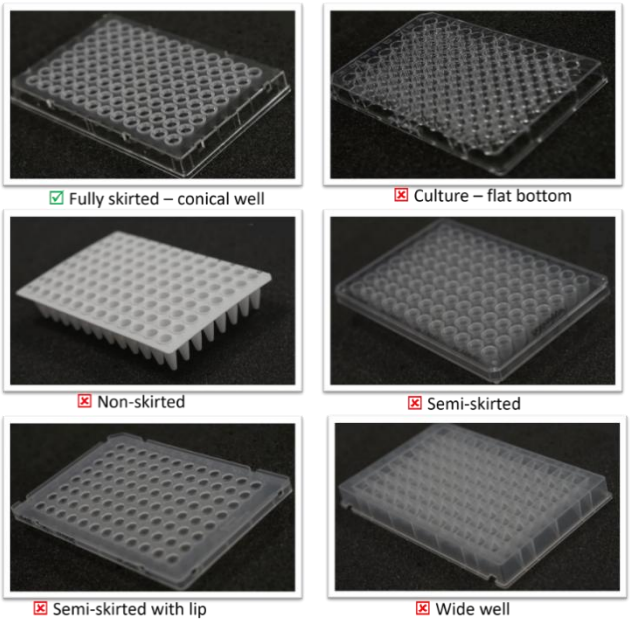
We request at least 300fmol of each amplicon for multiplexing. Refer to the table below for DNA input requirements for various amplicon sizes.

Mean amplicon size (bp)	Minimum input per sample
250	45ng per amplicon
500	90ng per amplicon
750	140ng per amplicon
1000	185ng per amplicon
3000	550ng per amplicon

We request that samples are submitted in volumes of 10-20 µl.

For projects that involve data analysis at the CGR, we ask that information relating to sample metadata is uploaded to the Sample Submission Portal (SSP) 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 for your library type, please contact us at CGR_Lab@liverpool.ac.uk and we will be happy to offer further advice.