Submission of samples for preparation of amplicon 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
Tighe *et al.*, Journal of Biomolecular Techniques 2017
Kim *et al.*, Microbiome 2017
Debelius *et al.*, Genome Biology 2016
D'Amore *et al.*, Genome Biology 2016
Pollock *et al.*, AEM 2018
Frau *et al.*, Scientific Reports 2019

Amplicon design

We recommend an overlap of at least 30 bases between forward and reverse reads to enable generation of stitched reads for downstream analysis. Base quality is reduced towards the end of a read, and this is particularly prominent for longer reads (2x250 or 2x300 sequencing). Your experimental design should take this into account.
For amplicons targeting the V4 region of 16S rRNA, we use the following primers, which include annealing sites for the second-round PCR (shown in red):

- Forward primer:
  5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA3'
- Reverse primer:
  5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT3'

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 16S V4 amplicon generation protocol includes a total of 25 PCR cycles (10 first-round PCR cycles to amplify the target region and 15 second-round PCR cycles to incorporate indexed Illumina adaptors). 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 (see sequence above for the 16S V4 region). 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

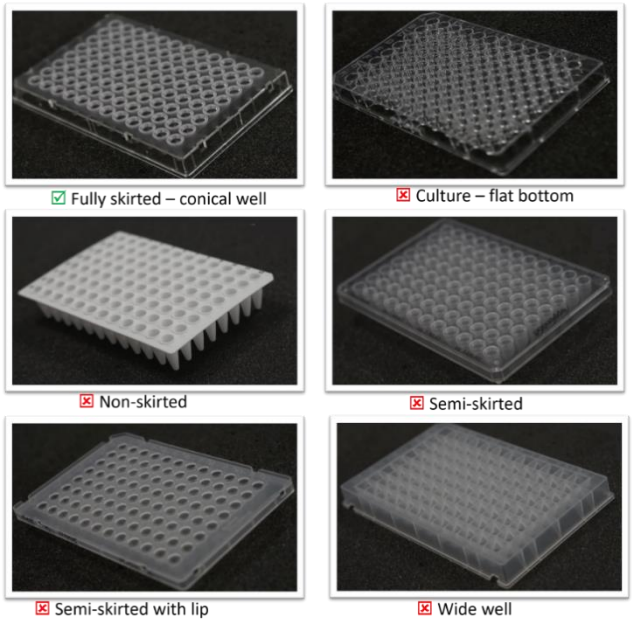
For collaborators submitting first-round PCR products of their region of interest (rather than gDNA), we require that the first-round PCR products are generated using target-specific primers that incorporate the annealing sites for our second-round PCR primers as overhangs. The annealing site sequences are shown in red text in the "Amplicon design" section above.
If the pre-submission amplification test described above 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 a nested, second-round PCR to add the indexed adaptors, without the risk of over-amplification. If more cycles will be required, we ask that you make us aware of this in advance.

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.

Sample submission requirements

- Amplicons should be free from contaminants unless sample clean-up is included in your quote.
- For submission of DNA, please quantify your samples using a dye-based method (e.g. Qubit), rather than a spectrophotometric one (e.g. NanoDrop).
- We use the KAPA HiFi Hotstart ReadyMix for PCR reactions and, if possible, we recommend that this is also used in the pre-submission amplification test (see above), as alternative polymerases may perform differently.
- We require that a maximum of 94 samples are included in each 96-well plate. This will then enable us to include positive and negative controls for each plate (at no additional cost).

For projects that involve data analysis at the CGR, we ask that information relating to sample metadata is uploaded to the 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 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

For projects involving <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).

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.