Sample requirements for Illumina Sequencing

DNA isolation.

Ensure that your DNA sample:

- Is double-stranded. Single-stranded DNA will not be adapted in this template preparation process and can interfere with quantitation and polymerase binding.
- Has undergone a minimum of freeze-thaw cycles.
- Has not been exposed to high temperatures (> 65°C for 1 hour can cause a detectable decrease in sequence quality), pH extremes (< 6 or > 9).
- If purifying DNA from a gel fragment, DO NOT use ethidium bromide for staining. We recommend SYBR Safe DNA Gel Stain with visualization on a blue light box (https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/dna-stains/sybr-safe.html). UV will damage. Even a few seconds of UV irradiation appears to render DNA non-sequenceable.
- Has an OD260/280 ratio of approximately 1.8 to 2.0 (Nanodrop).
- Does not contain insoluble material.
- Does not contain RNA contamination.
- Has not been exposed to intercalating fluorescent dyes or ultraviolet radiation.
- Does not contain chelation agents (i.e. EDTA), divalent metal cations (i.e.,Mg^{2+}), denaturants (Guanidinium salts, Phenol), or detergents (SDS,Triton-X100).
- Does not contain carryover contamination from the starting organism/tissue (heme, humic acid, polysaccharides, polyphenols, etc.). Some DNA isolation procedures might contain some contaminant(s) that can affect the quality of the sequencing run.
- Quantification is done using a fluorometric method (QUBIT or PicoGreen). Nanodrop (absorbance) is not adequate for quantification, although it does provide valuable information about sample's purity.

Requesting library construction services:

The NGS core lab offers library construction services on a limited number of applications. Please see table provided below.

These guidelines apply to all libraries intended for sequencing on any of the Illumina instruments, regardless of the application (e.g., ChIP-seq,

ATAC-Seq, WGBS, RRBS, RAD-Seq, GBS, RNA-Seq, etc.).

For Library Construction	Amount required	Input Quality and Amount, Comments
Standard DNA-Seq	0.2-1.0 µg per sample	If DNA is <5kb (e.g., FFPE), call Core lab
PCR-free DNA-Seq	>250 ng	Requires qPCR library quantification
Low-input DNA-Seq	10-50 ng	Sample will be processed using low-input protocol
Ultra-low input DNA-Seq	0.2-5.0 ng	Sample will be processed using ultra-low input protocol
Whole-genome-bisulfite converted (WGBS)	>200 ng	If sample is <5kb or amount is <200ng, call Core lab
Reduced Representation BS (RRBS)	>200 ng	If sample is <5kb or amount is <200ng, call Core lab
16S Metagenomics (V3 and V4)	50 ng per sample	Submit samples to the GeneExpression/GT core
RNA-Seq	>100 ng total RNA of oligo dT capture. >500 ng for ribodepletion	Submit samples to the GeneExpression/GT core (GE/GT) Best if RIN>7. Call GE/GT core if RIN<7, or amount is <100 ng
10X Genomics, Linked-reads	>100 ng	DNA must be >30 kb. Core offers size selection services if desired (will need ~1 µg). 10X Controller runs are done on a minimum of 8 samples.
10X Genomics, ATAC-seq single nuclei	Pre-isolated, single nuclei	DNA must be >30 kb. Core offers size selection services if desired (will need ~1 µg). 10X Controller runs are done on a minimum of 4 samples.

Pre-constructed libraries:

These guidelines apply to all libraries intended for sequencing on any of the Illumina instruments, regardless of the application (e.g., ChIP-seq, ATAC-Seq, WGBS, RRBS, RAD-Seq, GBS, RNA-Seq, etc.).

1. Construct your library and use appropriate size selection conditions based on the application and/or run configuration that you will be requesting. Please remember that the size of your target is the library size, minus the size of the adaptors (the latter being typically ~130 bp). For example, if you intend to sequence using a 2x300 cycles configuration, your insert must be at least 300 bp.
2. Quantify each library using a fluorometric method (QUBIT or PicoGreen). Nanodrop (absorbance) is not adequate for quantification, although it does provide valuable information about sample's purity. For best results for pooling and clustering, qPCR quantification may be necessary. This method quantifies "functional" library molecules only (those containing the illumina adaptors). We always recommend

qPCR quantification for 16S library pools and for PCR-free DNA-Seq libraries.

3. Check the average size of your libraries with TapeStation, Bioanalyzer or Fragment Analyzer. Regardless of the sequencing application, your library size should not be >1000 bp. Fragments above this size will not cluster well and will interfere with proper quantification and sizing. Likewise, the library size must be larger than the size of the adaptors. For blunt-end ligation protocols, adaptors show up at ~65 bp (single) or ~130 bp (ligated). These must be removed by AMPure XP beads at 0.85:1.00 (bead to sample ratio). For libraries in which adaptors are added by PCR, make sure that primer dimers are removed from the prep.
4. Calculate molarity (https://support.illumina.com/bulletins/2016/11/converting-ngl-to-nm-when-calculating-dsdna-library-concentration-.html).
5. If sequencing barcoded libraries in multiplex, pool in the appropriate proportions as dictated by the desired number of reads per sample. For RNA-Seq experiments pooling is typically done equimolarly. However, this may not be the case for other applications or for your experimental goals. The NextGen Core provides services for library QC and pooling for a fee.
6. Request sequencing services. To use our facility, your lab will need to have a CrossLab account (PI and lab members). From our website (www.biotech.ufl.edu), use your account credentials to login CrossLab and select UF-ICBR core. Navigate to the NextGen Sequencing core> Illumina sequencing services> MiSeq, etc. to fill out service request form.
7. Submit your library (or pool) to the ICBR NextGen DNA sequencing core. We feel that it is best practice to request independent library pool evaluation services before sequencing, including quantification of "functional" library molecules by qPCR. You can choose to decline these services, in which case you will need to provide the core with all QC data for your pool, plus detailed conditions for sequencing run set up. We need at least 15 ul at 10 nM (~3-5 ng/ul).
8. If your protocol requires custom sequencing primers, please provide at least 10 ul of 100 uM primers in individual 1.5-ml tubes clearly labeled with the name of the primer and concentration. Please indicate in the iLab submission form that custom sequencing primers are needed for the run.
9. Indicate in the iLab submission form if your libraries have low nucleotide diversity (i.e. amplicon libraries, 16S, ITS). If this is the case, the lab needs to be notified so more PhiX is added to the run for higher sequence diversity. This is important to generate high-quality data on Illumina platforms. For more information, please refer to the Illumina support bulletins: MiSeq (https://www.illumina.com/documents/products/technotes/technote_low_diversity_rta.pdf), HiSeq (https://www.illumina.com/documents/products/technotes/technote-hiseq-low-diversity.pdf), NextSeq (https://support.illumina.com/bulletins/2016/03/best-practices-for-lowdiversity-sequencing-on-the-nextseq-and-miniseq-systems.html), How much PhiX spike-in is recommended? (https://support.illumina.com/bulletins/2017/02/how-much-phix-spike-in-is-recommended-when-sequencing-low-divers.html).