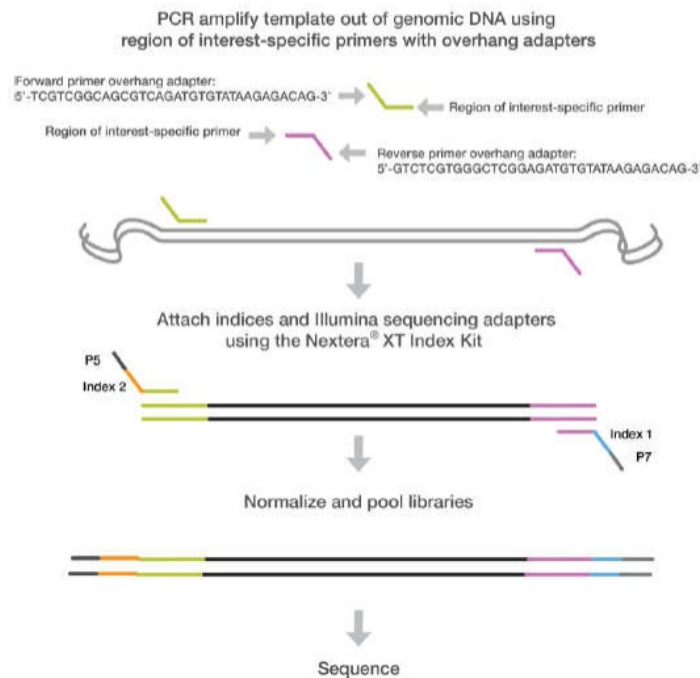




Tailed amplicon sequencing (aka 2-steps PCR strategy)

Workflow overview

The 2 PCR steps described in this guide must be performed by the users prior to libraries submission. Final libraries must have been purified (column or beads, see Illumina protocol mentioned below for beads protocol). See our website for libraries submission form and guidelines.



Scheme from Illumina

Detailed workflow

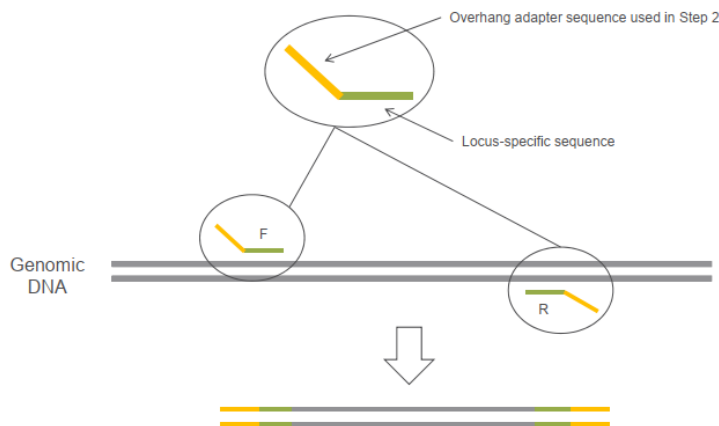
PCR1

Amplify the targeted region of interest (ROI) with primers bearing Illumina "anchor sequences" tails. These primers are ordered by user. The anchor tails are depicted in orange:

- FWD primer: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG - [locus specific sequence]
- REV primer: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG - [locus specific sequence]



Step 1: PCR to amplify regions of interest



Notes:

- If several ROIs are targeted on the same sample, PCRs are ideally performed separately, but these can also be done in a single tube if limiting the number of reactions is needed. If you go for this option, amplicons should have a similar size to avoid big difference in PCR and sequencing efficacy. In addition, they must not overlap.
- If the whole ROI is to be sequenced, the amplicons length should be consistent with the envisioned sequencing reads length. See below for more sequencing details.
- Contrary to Sanger sequencing, Illumina sequencing quality is good from 1st sequenced nucleotide, no need therefore to make the amplicon begin ahead of your ROI.
- Primers must be devoid of mutations (order HPLC or PAGE-purified only). Even more critical for sequencing on the Aviti for technical reasons related to its specific chemistry.
- Locus-specific portion of the forward and reverse primers (NOT including the overhang sequence) must have a melting temperature (T_m) of 60°C to 65°C. For this T_m calculation, use only the gene-specific portion in the calculation. For hairpin and dimer calculations though, use the full primer sequence including the overhang.

PCR2

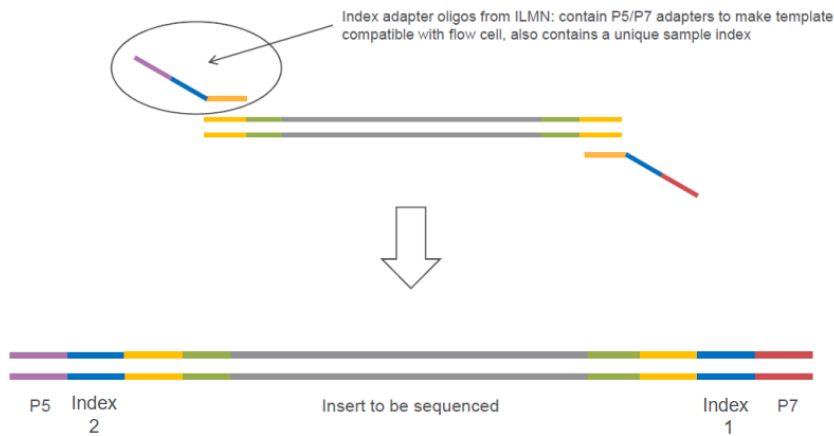
This second PCR serves to add the Illumina adapters, and indexes.

Commercial Nextera XT are strongly recommended (ref FC-131-1001 for 24 samples, or FC-131-2001 for 96 samples). The Illumina primers bind to the anchor/tail sequence added during PCR1, and serve to add the adapters needed for sequencing.

If relevant, pool the different PCR1 amplicons from the same sample before this second PCR (see above).



Step 2: 2nd round of PCR to add Illumina indices and sequencing adapters



Note: if you don't order commercial primers from Illumina, make sure you understand well in which orientation should be designed the indexes. In your primers, N701 for instance should be TCGCCTTA and N501 should be TAGATCGC.

Sequencing

Typically performed on the MiSeq, owing to its well-suited combination of low yield, long reads and lower sensitivity to low-diversity issues. Recently, the Aviti also became a good option since it also can do PE300 reads, especially when higher yields are needed (up to 300 mio for PE300). Here are a few considerations to make your choice:

- MiSeq yield for PE250/PE300 is 1-25 mio reads. Aviti yield for PE300 is 100-300mio.
- If insert is short-enough, single-end sequencing may be enough. Yet, since sequencing errors rate increases along the read, performing paired-end sequencing with an overlap will allow correcting mistakes during merging of overlapping reads.
- If paired-end sequencing is preferred, one should choose read length with sufficient overlap to allow for easy merging of the reads (15nt overlap at least).
- When determining the required reads length for sequencing, remember to also include your locus-specific primers, since it is also sequenced.

Primer design for especially low-diversity amplicons

When only a few amplicons are to be sequenced together, or when they contain a shared region, a “low diversity” issue occurs during sequencing (= all DNA clusters on the flow cell generate the same fluorescence signal, and the sequencer cannot identify clusters individually anymore). To mitigate this issue, the GECF will add some unrelated libraries during sequencing (typically 10-40%, to be discussed). A parallel strategy should be used by users when designing their ROI primers, to be chosen amongst these 2 strategies:



- the classical “wobble Ns strategy”, in which an increasing number of random nucleotides are added between the tails and the locus-specific regions:

- FWD primer: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG - [locus specific sequence]
- FWD primer: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG **N** - [locus specific sequence]
- FWD primer: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG **NN** - [locus specific sequence]
- FWD primer: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG **NNN** - [locus specific sequence]
→ pool these primers

- REV primer: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG - [locus specific sequence]
- REV primer: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG **N** - [locus specific sequence]
- REV primer: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG **NN** - [locus specific sequence]
- REV primer: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG **NNN** - [locus specific sequence]
→ pool these primers

With 4 primers (0N-3N), you will get enough diversity even in homopolymers of 3 nt, but not if longer stretches occur. Importantly, your amplicon must not have more than 3nt in a row in first 5nt of the first read (left side of the amplicon). If it is the case, more PhiX will have to be added to increase diversity (> 25%), please indicate if this is the case. Stretched >3nt elsewhere in the reads is less detrimental but please mention it anyway if this occurs.

- the cheaper “5Ns strategy”, specially useful for the Aviti, where you insure that the first 5 nucleotides of the run have perfect diversity (these 5 first nucleotides are the most critical ones), but where you leave to the added PhiX to take care of diversity issues downstream of these 5 first nucleotides. The advantage of this strategy is that you don’t need to order extra primer, just these 2:

- FWD primer: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG **NNNNN** - [locus specific sequence]
- REV primer: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG - [locus specific sequence]

Detailed experimental procedure

Illumina recommends following pages 6-15 of their protocol 15044223 Rev. B, simply replacing the input “Microbial Genomic DNA” by your relevant DNA input. You can decrease the AMPure beads:PCR ratio from 1.1 to 0.9x for the final cleanup, in order to get rid of putative primer dimers.

A few comments:

- Based on users experience, it seems that there is no need to clean-up or purify the 1st PCR product. Just transferring 2ul of PCR1 into 50ul final of PCR2 worked for several users.

- Use a proofreading polymerase for both PCRs. The first step may require a relatively robust one, while for the 2nd step a polymerase with the highest fidelity is probably the best choice. The Illumina-recommended 2x KAPA HiFi HotStart Ready Mix is a good starting point. In case you change this mastermix, changes in PCR conditions may also be required.

- Include a negative control PCR (just for PCR steps, not to be sequenced).



protocol v2.4

Versions log

- v2.0 (22.02.2021): Integration of diverse sources and protocols into a single document.
- v2.1: clarified that for creating amplicons in a single tube, they should have similar sizes. Added the "5Ns strategy".
- v2.2: clarified than pooled amplicons must not overlap.
- v2.3: indicated to decrease final beads ratio from 1.1 to 0.9x.
- v2.4: (09.12.2024): adapted design for aviti