Illumina Amplicon Sequencing Protocol

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Adapted from [https://github.com/EnvGen/LabProtocols/blob/master/Amplicon\_dual\_IDX-\_prep.rst](https://github.com/EnvGen/LabProtocols/blob/master/Amplicon_dual_index_prep.rst)

* This protocol is designed to help you perform a custom amplicon NGS experiment
* This protocol describes how to generate and multiplex amplicons for up to 384 samples. Note that not all barcodes need to be used, ie. you can multiplex fewer samples, however, more samples can be added by incorporating additional index sequences into the protocol.
* It is worth focusing on up to 96 samples at a time by working in 96-well plates

**Sample Setup for up to 96 wells**

* it is good practice to make a plate map with sample names clearly shown to ensure there is no confusion, particularly during barcoding/indexing steps
* I suggest you setup an excel document to keep track of samples and barcodes, as well as data collected along the way, eg. DNA concentrations. It also simplifies calculating sample volumes for pooling etc. I have a template for this if wanted.

**Initial optimization: amplification of target DNA**

* to enable your target of interest to be sequenced, it needs to be made “NGS-compatible” by adding the illumina sequencing primer and flow-cell adapters. Moreover, index sequences need to be added to allow your samples to be multiplexed. To do so, these sequences are added throughout two subsequent PCRs.
* The first round PCR involves designing and optimizing a primer set that (i) is specific to your target sequence, and (ii) contains a universal or common sequence for the 2nd round index primers to bind.
* The primer to be ordered should have the following sequence:
  + Primer1: Illumina adapter + forward target primer

5’- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG -forward-primer-3’

* + Primer2: Illumina adapter + reverse target primer

5’- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG -reverse-primer-3’

* Optimization of this PCR should be performed as you might for any normal PCR. I would recommend the following:
  + Dilute and determine the concentration of primers
  + Perform a gradient PCR to determine the optimal annealing temperature
  + Ensure that the product is specific (a single band) and the amplification is robust (solid band on a gel).
    - Poor amplification / non-specific products at this stage will cause problems later in the protocol and sequencing. Consider designing new primers if you are having problems at this stage.
* 

**STEP 1: First round PCR: Amplification of your target from genomic DNA**

1. proceed to amplify target sequences with optimized PCR conditions in a 96-well plate
   1. it is worth trying to standardize DNA template concentrations prior to PCR
   2. use limited cycles of PCR, i.e. 20X cycles, to limit the introduction of PCR-generated errors

**STEP 2: Clean first round PCR products**

* basic AmpureXP protocol with some in-house modifications.

1. add X μl of H2O to each well of the PCR plate to bring the total volume per well to 50 μl (ie. if initial PCR is 20 μl, add 30 μl of H20)
2. add 50 μl of AmpureXP beads to each well of a 96-well round bottom plate
3. transfer 50 μl of PCR product to the 96-well plate containing 50 μl AmpureXP mix and pipette up and down 10X to mix
4. incubate at least 10 mins on bench
5. place plate on 96-well plate magnet for 5 mins (make sure liquid appears clear)
6. Discard supernatant by pipette / aspirate
7. Wash samples by adding 190 μl of 70-80% Ethanol to each sample and wait 30 second**s**
8. Discard Ethanol by pipette / aspirate
9. Repeat steps 8 & 9 for total of two washes
10. Remove from plate from magnet and allow to air dry for 2-3 mins – make sure you cannot detect any ethanol
    1. NOTE: do not allow plate to dry for too long – beads will start to crack and become hard to resuspend
    2. Not a huge problem if this happens, but makes it more difficult as mixing steps following become more difficult – keep going though
11. Take the plate off the magnet and resuspend the beads in 22 μl of H2O, pipetting up and down 10X to mix thoroughly
12. incubate at least 10 mins on bench
13. place the plate back on the magnet 5 mins (make sure liquid appears clear)
14. transfer 20 μl of the supernatant to a new 96-well PCR plate

**STEP 3: Quantify and normalize sample concentrations**

* this is an optional step, however, it will likely improve your sequencing outcome if you follow it, especially if your starting DNA concentration and/or quality is variable.
* The point of this step is to make sure you have the same amount of DNA going into the second round PCR
* To do this, I measure the PCR product concentration using a Qubit
  + this could be done spectrophotometrically, however, Qubit is better.
  + I use 2 μl per sample, and follow the Qubit protocol
* Once the DNA concentration has been determined for each sample, I calculate the required volume of H2O to add to each well of a new plate, so that by transferring a defined amount of PCR product, a normalized DNA concentration is achieved
  + I make an excel spreadsheet and use formulas to calculate the H2O required for each sample based on transferring 2 μl of PCR product to achieve a final concentration of 0.5 ng/μl
  + Eg. If [PCR product] = 17 ng/μl
    - C1V1=C2V2 => 17 ng/μl x 2 μl = 0.5 ng/μl x V2
    - V2 = 17x2/0.5 = 68 μl total volume
    - >> 66 μl H20 + 2 μl PCR product = 0.5 ng/μl PCR product
  + the reason for using 0.5 ng/μl is that by adding 2 μl in the second-round PCR, there will be a total of 1 ng of DNA per well
    - I guess this could be any number, it is just what I do.
* You should finish this step with a plate of DNA samples in the same orientation as the initial setup for which the DNA concentration has been adjusted to 0.5 ng/μl.
* This new plate and the original plate can be stored at -20°C if required

**STEP 4: Second round amplification: addition of barcodes and adapters by PCR**

* in this step, the adapters are extended to include the index sequences and flow-cell attachment regions.
* This is a limited cycle PCR simply to add these additional sequences, rather than to provide significant amplification / increase in DNA concentration
* The index primers in this protocol are based on the NexteraXT indices, and provide enough combinations to multiplex 384 samples
  1. If more samples are required to be multiplexed, come see me and we can design additional index primers.
* The index primers are labeled to aid setup in a 96-well plate, with the i7(N7XX) sequences labeled 1-24 which align in columns, and the i5(S5XX) sequences labeled A-P which align with rows (based on a 384 well plate). Use the plate map below to keep track of your samples and the index combinations in the PCR.



**Reaction mix per well**

(note: this just gives an indication of what is in each reaction, if individual reactions need to be made, and is not how it is prepared below)

|  |  |
| --- | --- |
| **Component** | **1x reaction** |
| KAPA HIFI 2x master mix | 10 |
| Primer i7(N7XX) (10 uM) | 0.5 (0.25 μM) |
| Primer i5(S5XX) (10 uM) | 0.5 (0.25 μM) |
| H2O | 7 |
| DNA (0.5 ng/μl) | 2 |
| ***TOTAL*** | ***20 μl*** |

\*\*\* note: we use KAPA HiFi DNA polymerase mix, however, this could be substituted for your favorite enzyme. Just follow the manufacturers instructions for setup.

**Preparation**

1. aliquot PCR primers to be used into 8-well strips, with primers in numerical/alphabetical order, and the strips orientated so that the i7(N7XX) primers are horizontal and the i5(S5XX) primers vertical in a PCR rack
   1. primers should be at **1.25 uM** concentration and have sufficient volume for 4 μl per well
2. aliquot sufficient polymerase mastermix in each well of an 8-well strip for all wells in your PCR plate and have sufficient volume for 10 μl per well

**Setup**

1. working on ice, add primers using a multichannel pipette:
   1. add 4 μl from your VERTCIAL i5(S5XX) primer strip to the COLUMNS of you plate
   2. add 4 μl from your HORIZONTAL i7(N7XX) primer strip to the ROWS of you plate
2. multichannel from your polymerase 8-well strip 10 μl of polymerase into each well
3. multichannel from your DNA plate, 2 ul of 0.5 ng/μl DNA into their corresponding wells.
4. Seal plate, vortex, and spin in a plate centrifuge (2 mins at 2000 g)
5. Proceed with PCR cycling conditions below
   1. I typically use a qPCR machine when using the KAPA HiFi mix for PCR as it contains SYBR green and is therefore possible to monitor how each sample is amplifying in real time. This is not necessary and can be performed in a standard PCR machine.

**Cycling conditions**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Temperature** | **Time** | **Cycles** |
| Denaturation | 98 | 2 mins | 1X |
| Denaturation | 98 | 30 |  |
| Annealing | 60 | 30 | 10-12 cycles |
| Extension | 72 | 30 |  |
| Final | 72 | 5 mins | 1X |
| Hold | 4 |  |  |

**STEP 5: Clean second round PCR products**

* basic AmpureXP protocol with some in-house modifications.

1. add 30 μl of H2O to each well of the PCR plate to bring the total volume per well to 50 μl
2. add 50 μl of AmpureXP beads to each well of a 96-well round bottom plate
3. transfer 50 μl of PCR product to the 96-well plate containing 50 μl AmpureXP mix and pipette up and down 10X to mix
4. incubate at least 10 mins on bench
5. place plate on 96-well plate magnet for 5 mins (make sure liquid appears clear)
6. Discard supernatant by pipette / aspirate
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8. Discard Ethanol by pipette / aspirate
9. Repeat steps 8 & 9 for total of two washes
10. Remove from plate from magnet and allow to air dry for 2-3 mins – make sure you cannot detect any ethanol
    1. NOTE: do not allow plate to dry for too long – beads will start to crack and become hard to resuspend
    2. Not a huge problem if this happens, but makes it more difficult as mixing steps following become more difficult – keep going though
11. Take the plate off the magnet and resuspend the beads in 22 μl of H2O, pipetting up and down 10X to mix thoroughly
12. incubate at least 10 mins on bench
13. place the plate back on the magnet 5 mins (make sure liquid appears clear)
14. transfer 20 μl of the supernatant to a new 96-well PCR plate

**STEP 5: Pool PCR products and quantify library**

* optional – you could determine the concentration of products again at this step before pooling, however, it is probably not necessary – I don’t do it.

1. transfer 10 μl of each second round PCR product from your plate into a single microcentrifuge tube
   1. transferring 10 μl of your 20 μl total allows you to have a backup if necessary
   2. if this is too much volume for a single microcentrifuge tube (ie. 384 products x 10 μl wont fit), transfer all into a larger tube first, vortex well to mix, and take a suitable aliquot.
2. Determine the concentration of DNA using the Qubit.
3. Adjust the DNA concentration to 2 nM
   1. You will need to take the size of the amplicon and the Qubit value to determine this.
   2. I have a calculator on the Grant Lab website to help with this.
      1. <https://nematodegenetics.files.wordpress.com/2014/05/miseq-library-concentration-dilution-calculator-aug-2014.xlsx>

**STEP 6: Sequencing**

* Follow the MiSeq loading protocol as per Illumina instructions
* Some tips
  + it is worth using ~10-20% PhiX as a spike in to increase the diversity of the library
  + don’t load the sequencer with more than 10 pM final library concentration
    - a lower than recommend loading concentration can help significantly with amplicon/low diversity libraries, and you will still get plenty of reads from your library.

|  |  |  |  |
| --- | --- | --- | --- |
| **Primer name** | **NexteraXT code** | **Index sequence 1** | **Oligonucleotide sequence (5’ > 3’) 2** |
| F-[*target]-*illumina |  |  | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-target-forward-primer |
| R-[*target]-*illumina |  |  | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-target-reverse-primer |
|  |  |  |  |
| Amplicon\_IDX-N701\_1 | N701 | TAAGGCGA | CAAGCAGAAGACGGCATACGAGAT**TCGCCTTA**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N702\_2 | N702 | CGTACTAG | CAAGCAGAAGACGGCATACGAGAT**CTAGTACG**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N703\_3 | N703 | AGGCAGAA | CAAGCAGAAGACGGCATACGAGAT**TTCTGCCT**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N704\_4 | N704 | TCCTGAGC | CAAGCAGAAGACGGCATACGAGAT**GCTCAGGA**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N705\_5 | N705 | GGACTCCT | CAAGCAGAAGACGGCATACGAGAT**AGGAGTCC**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N706\_6 | N706 | TAGGCATG | CAAGCAGAAGACGGCATACGAGAT**CATGCCTA**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N707\_7 | N707 | CTCTCTAC | CAAGCAGAAGACGGCATACGAGAT**GTAGAGAG**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N710\_8 | N710 | CGAGGCTG | CAAGCAGAAGACGGCATACGAGAT**CAGCCTCG**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N711\_9 | N711 | AAGAGGCA | CAAGCAGAAGACGGCATACGAGAT**TGCCTCTT**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N712\_10 | N712 | GTAGAGGA | CAAGCAGAAGACGGCATACGAGAT**TCCTCTAC**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N714\_11 | N714 | GCTCATGA | CAAGCAGAAGACGGCATACGAGAT**TCATGAGC**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N715\_12 | N715 | ATCTCAGG | CAAGCAGAAGACGGCATACGAGAT**CCTGAGAT**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N716\_13 | N716 | ACTCGCTA | CAAGCAGAAGACGGCATACGAGAT**TAGCGAGT**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N718\_14 | N718 | GGAGCTAC | CAAGCAGAAGACGGCATACGAGAT**GTAGCTCC**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N719\_15 | N719 | GCGTAGTA | CAAGCAGAAGACGGCATACGAGAT**TACTACGC**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N720\_16 | N720 | CGGAGCCT | CAAGCAGAAGACGGCATACGAGAT**AGGCTCCG**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N721\_17 | N721 | TACGCTGC | CAAGCAGAAGACGGCATACGAGAT**GCAGCGTA**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N722\_18 | N722 | ATGCGCAG | CAAGCAGAAGACGGCATACGAGAT**CTGCGCAT**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N723\_19 | N723 | TAGCGCTC | CAAGCAGAAGACGGCATACGAGAT**GAGCGCTA**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N724\_20 | N724 | ACTGAGCG | CAAGCAGAAGACGGCATACGAGAT**CGCTCAGT**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N726\_21 | N726 | CCTAAGAC | CAAGCAGAAGACGGCATACGAGAT**GTCTTAGG**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N727\_22 | N727 | CGATCAGT | CAAGCAGAAGACGGCATACGAGAT**ACTGATCG**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N728\_23 | N728 | TGCAGCTA | CAAGCAGAAGACGGCATACGAGAT**TAGCTGCA**GTCTCGTGGGCTCGG |
| Amplicon\_IDX-N729\_24 | N729 | TCGACGTC | CAAGCAGAAGACGGCATACGAGAT**GACGTCGA**GTCTCGTGGGCTCGG |
|  |  |  |  |
| Amplicon\_IDX-S502\_A | S502 | CTCTCTAT | AATGATACGGCGACCACCGAGATCTACAC**CTCTCTAT**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S503\_B | S503 | TATCCTCT | AATGATACGGCGACCACCGAGATCTACAC**TATCCTCT**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S505\_C | S505 | GTAAGGAG | AATGATACGGCGACCACCGAGATCTACAC**GTAAGGAG**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S506\_D | S506 | ACTGCATA | AATGATACGGCGACCACCGAGATCTACAC**ACTGCATA**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S507\_E | S507 | AAGGAGTA | AATGATACGGCGACCACCGAGATCTACAC**AAGGAGTA**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S508\_F | S508 | CTAAGCCT | AATGATACGGCGACCACCGAGATCTACAC**CTAAGCCT**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S510\_G | S510 | CGTCTAAT | AATGATACGGCGACCACCGAGATCTACAC**CGTCTAAT**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S511\_H | S511 | TCTCTCCG | AATGATACGGCGACCACCGAGATCTACAC**TCTCTCCG**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S513\_I | S513 | TCGACTAG | AATGATACGGCGACCACCGAGATCTACAC**TCGACTAG**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S515\_J | S515 | TTCTAGCT | AATGATACGGCGACCACCGAGATCTACAC**TTCTAGCT**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S516\_K | S516 | CCTAGAGT | AATGATACGGCGACCACCGAGATCTACAC**CCTAGAGT**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S517\_L | S517 | GCGTAAGA | AATGATACGGCGACCACCGAGATCTACAC**GCGTAAGA**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S518\_M | S518 | CTATTAAG | AATGATACGGCGACCACCGAGATCTACAC**CTATTAAG**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S520\_N | S520 | AAGGCTAT | AATGATACGGCGACCACCGAGATCTACAC**AAGGCTAT**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S521\_O | S521 | GAGCCTTA | AATGATACGGCGACCACCGAGATCTACAC**GAGCCTTA**TCGTCGGCAGCGTC |
| Amplicon\_IDX-S522\_P | S522 | TTATGCGA | AATGATACGGCGACCACCGAGATCTACAC**TTATGCGA**TCGTCGGCAGCGTC |