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Amplicon PCR & Library Preparation for Illumina Sequencing

 [Environmental DNA](#)

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We use this protocol and it's working

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Abstract

This metabarcoding protocol is for generating amplicon sequences from multiple samples in preparation for Illumina Next Generation Sequencing.

Amplicons are generated for each sample (PCR1) then cleaned using magnetic beads. Unique sample identifying tag sequences are attached to each sample (PCR2), pooled, cleaned again and finally quantified using qPCR.



Materials

- Amplicon-specific primers with Illumina sequencing primer sites (see Step 1)
- 0.2ml 96-well PCR plates
- 1.5ml microcentrifuge tubes
- Meridian Bioscience MyTaq HS Mix (BIO25046)*
- Thermocycler
- Electrophoresis gel equipment and reagents
- Promega ProNex[®] Bead Kit (NG2003)*
- 96-well and microcentrifuge tube magnetic racks
- Fi5 & Fi7 IDT Primers (see Step 3)
- Agilent D1000 ScreenTapes (5067-5582) and buffer (5067-5602)*
- Promega QuantiFluor dsDNA System (E2670)*
- Real-time PCR thermal cycler
- KAPA Library Quantification (KK4873)*
- Low TE (Tris10mM,EDTA0.1mM) recipe for 500ml:
 - 5ml 1M Tris-HCl (pH 8.0)
 - 100µl 0.5M EDTA (pH8.0)
 - 495ml ddH₂O
 - Autoclave.

*Can be substituted.

Safety warnings

- ! Use Good Lab Practice and wear protective equipment. Avoid contact with skin.
All chemicals can be disposed of down the sink with copious amounts of water to dilute the ethanol down to >20% of the waste.

PCR1

- 1 Using amplicon-specific primers (XX) tailed with Illumina sequencing primer sites (F – small RNA primer; R – read 2 primer), e.g.:

F 5'-3';
 ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNXXXXXXXXXXXXXXXXXXXXX
 R 5'-3'; GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTXXXXXXXXXXXXXXXXXXXXX

PCR1 adds 72bp to your product size due to adapters.

1.1 Controls

When running any PCR amplification reaction, positive and negative controls should be added to determine if the PCR is working as expected (+ve control) and to make sure no contamination is being introduced (-ve control). Negative controls from the DNA extraction may also be included. You may also choose to add replicates of either/both extraction and PCR.

1.2 PCR the Amplicon/s from your DNA

A	B
μL/reaction	Reagent
10	Meridian Bioscience MyTaq HS Mix
2	F primer (at 1 to 5 μM)*
2	R primer (at 1 to 5 μM)*
5	Sterile ddH2O
1♦	DNA (at ~10 ng/μl)

Table 1: PCR1 Recipe

* You may need to optimise the amount of primer used to avoid excess/unincorporated/dimer being carried through to the next step, while still getting good/strong amplification.

♦ If your DNA concentration is particularly low (which can be the case in studies on diet & eDNA) increase the amount of DNA you use and decrease the amount of ddH2O accordingly.

PCR program:

95°C for 5 min



Followed by 25-35 cycles of:

94°C for 30 sec

Tm°C* for 30 secs to 1min 30sec *this depends on the Tm of your specific primers

72°C for 30 secs to 2 min (depending on length)

Then:

72°C for 10 min

1.3 Electrophoresis Agarose Gel

Run 4µl of PCR product on a 1% agarose gel along with a 100bp ladder to make sure samples have amplified, the amplicon is the correct size and that there is a minimal amount of primer dimer.

Bead Cleaning

- 2 Prior to PCR2 we need to bead clean the samples to remove short extraneous DNA fragments and reagents left over from PCR1.

2.1 Purification with Promega Pronex® Beads

1. Allow an aliquot of beads to reach RT (30 min), and vortex to resuspend them.
2. Add a ratio of resuspended Pronex beads appropriate for your amplicon size (see Table 2). Mix well by pipetting up and down 10 times and incubate at RT for 5-10 min.
3. Place on a magnetic rack to separate beads from the solution. When the liquid is completely clear, aspirate the supernatant and keep in a new plate (just in case your target amplicon is accidentally removed). Do not disturb the pellet of separated magnetic beads and do not remove the samples from the magnetic plate.
4. With the samples still on the magnetic place, add **100 µl** of ProNex Wash Buffer (or 80% ethanol). Incubate at RT for 30-60 seconds, then carefully aspirate out and discard.
5. Repeat step 4.
6. Allow the beads to dry at RT for 5-10 min, while on the magnetic stand. Drying will allow traces of ethanol to evaporate, but over-drying the beads (if the pellet cracks) can significantly decrease elution efficiency.
7. Remove samples from the magnetic plate and elute with **15 µl** of Low TE (get a fresh sterile aliquot to ensure no contamination). Mix well by pipetting up and down 10 times (ensure that the beads are fully immersed & mixed with the Low TE). Incubate at RT for 5-10 min.
8. Place on a magnetic rack to separate beads from the solution (~ 1 min).
9. Remove the solution containing your DNA to a fresh plate without carrying over any beads.

A	B	C
Approximate Size Cutoff (bp)	ProNex® Chemistry Ratio (v/v)	Estimated Equivalent AMPure® XP Ratio
100	3X	1.8X
150	2X	1.5X
250	1.5X	0.95X
350	1.3X	0.75X
475	1.2X	0.65X
550	1.15X	0.625X
650	1.1X	0.6X
800	1.05X	0.55X
1000	1X	0.5X

Table 2: Ratios for Size-Selective Purification of DNA Fragments

The ratios of **ProNex Chemistry** to sample are higher than those used in other methods with similar size-selective chemistries. If you have experience with another size-selective DNA purification product, such as AMPure® XP or SPRIselect®, do not use the same ratios of binding reagent to sample, as this will result in the loss of desired fragments.

PCR2

- Using tailed PCR to add unique identifier sequences (dual-plexed: Fi5 and Ri7 primers in unique combination for each sample) and Illumina sequencing sites to the amplicon products.

The general sequences of the forward and reverse primers with included **10bp barcode** are illustrated below.

Fi5_01

5'CAAGCAGAAGACGGCATAACGAGAT**TATCTTCTCGG**TGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T3'

Ri7_01

5'AATGATACGGCGACCAACGAGATCTACAC**CGTCGCCTAT**AACTCTTTCCCTACACGACGCTCTTCCGATC*T3'

PCR2 adds 73 bp to your overall product size.



3.1 PCR to Add Unique Identifying Sequences

	A	B
	μL/reaction	Reagent
	10	Meridian Bioscience MyTaq HS Mix
	0.5	Fi5 Primer (at 10 μM)*
	0.5	Fi7 Primer (at 10 μM)*
	1	Sterile ddH ₂ O
	8	Template from PCR1

Table 3: PCR2 Recipe

***IDT Illumina Unique Dual (UD) Indexes**, 1536 unique indexes available.

PCR program:

95°C for 5 min

Followed by ♦8-12 cycles of:

98°C for 10 sec

65°C* for 30 secs

72°C for 30 secs

Then:

72°C for 10 min

♦ Determine how many cycles you need from the concentration.

4 TapeStation

Run a selection (~4 per 96-well plate) of pre and post PCR2 samples on the Agilent TapeStation or BioAnalyser to check that the samples have increased in size from the addition of PCR2 primers.

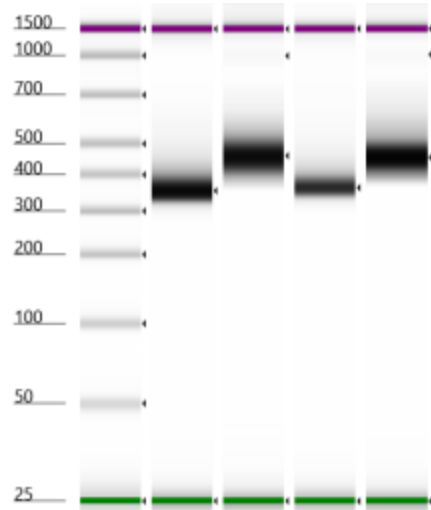


Figure 2: Example TapeStation image of two samples' PCR1 and PCR2 products.

Quantification and Pooling

5 Quantification

Quantify all PCR2 products using a fluorometer and the Promega Quantifluor dsDNA kit. Qubit and other fluorometers may be used, Nanodrop is not recommended.

1. Take **1ml** of 20X TBE from the Promega QuantiFluor dsDNA kit and add **19ml** ddH₂O.
2. Add **50μl** of the Quantifluor dsDNA dye and vortex. This is your Quantifluor mix.
3. Add **2μl** of a set of pre-made standards to a dry BMG black plate (0, 3.125, 6.25, 12.5, 25, 50, 100ng/μl)*.
4. Add **2μl** of PCR2 product to the black plate.
5. Add **200μl** of the Quantifluor mix to each well of your DNA and standards in the black plate.
6. Read the plate in the BioTek Fluorometer.
7. Leftover Quantifluor can be kept at 4°C, wrapped in foil, for future use.

* Mix well before use. Standards can be created by making a serial dilution of the DNA standard from the Quantifluor kit and Low TE.

6 Pooling

Using the formula below calculate the volumes of each sample so that each sample is evenly represented when pooling together. Add 8 - 24 samples into pools.



$$\frac{(\text{Final Volume } (\mu\text{l}) \times \text{Final Concentration } (\text{ng}/\mu\text{l}))}{(\text{Number of Samples} \times \text{Initial Concentration } (\text{ng}/\mu\text{l}))} = \text{Volume to Pool } (\mu\text{l})$$

Pool Bead Cleaning

- 7 Prior to sequencing we need to bead clean the samples to remove short extraneous DNA fragments and reagents left over from PCR2.

7.1 Purification with Promega Pronex® Beads

1. Allow an aliquot of beads to reach RT (30 min), and vortex to resuspend them.
2. Add a ratio of resuspended Pronex beads appropriate for your amplicon size (see Table 2). Mix well by pipetting up and down 10 times and incubate at RT for 5-10 minutes.
3. Place on a magnetic rack to separate beads from the solution. When the liquid is completely clear, aspirate the supernatant and save. Do not disturb the pellet of separated magnetic beads and do not remove the samples from the magnetic plate.
4. With the samples still on the magnetic rack, add **200µl** of ProNex Wash Buffer (or 80% ethanol). Incubate at RT for 30-60 seconds, then carefully aspirate out and discard.
5. Repeat step 4.
6. Allow the beads to dry at RT for a 5-10 min, while on the magnetic rack. Drying will allow traces of ethanol to evaporate, but over-drying the beads (if the pellet cracks) can significantly decrease elution efficiency.
7. Remove samples from the magnetic rack and elute with **25µl** of Low TE. Mix well by pipetting up and down 10 times (ensure that the beads are fully immersed & mixed with the low TE). Incubate at RT for 5-10 min.
8. Place on a magnetic rack to separate beads from the solution (~ 1 min).
9. Remove the solution containing your DNA to a fresh tube without carrying over any beads.

8 TapeStation

If there is no primer dimer present at Step 4, you may skip this step.

Run all pools on the Agilent TapeStation or BioAnalyser to check that no primer dimer is present, this will be a small peak shorter than your amplicon size (usually 50-150bp). If primer dimer is shown, repeat the bead clean and check again.

If the primer dimer contamination is large or cannot be removed efficiently with beads, the libraries may be run a Sage Science **BluePippin**.

Library Quantification

- 9 Due to the presence of unlabelled DNA from PCR2, quantification of the library by qPCR is most accurate. Using a commercial kit we can quantify products for Illumina sequencing on the a qPCR that only target DNA with i5 and i7 indexes attached.

9.1 KAPA qPCR

1. Make a serial dilution of each library: 100, 1000 and 10000 -fold.
2. For 100-fold dilution, Combine **99µl** of the dilution buffer with **1µl** of the library. Vortex.
3. For 1000-fold dilution, take out **1µl** from 1st dilution (100-fold) and add **9µl** of dilution buffer. Vortex.
4. For 10000-fold, take out **1µl** from 2nd dilution (100-fold) and add **9µl** of dilution buffer. Vortex.
5. Repeat steps 2-4 two times to produce three independent dilutions of the library.
6. Prepare KAPA Library Quantification Kit master mix with **6µl** of SYBR FAST primer mix and **2µl** of sterile ddH2O per well.
7. Add **2µl** of the kit standards, diluted sample libraries, or dilution buffer (no template control) to appropriate wells in the optical 96-well plate.
8. Dispense **8µl** of the master mix to the appropriate wells in the 96-well plate.
9. Spin plate and ensure that there are no bubbles at the bottom of wells or debris on the bottom of the plate. Cover plate with an optical adhesive film.
10. Run the plate on a qPCR machine with the following profile:
95°C for 5 min
Followed by 35 cycles of:
95°C for 30 sec
60°C for 45 sec

9.2 Pool into Final Library

Pool samples in equimolar amounts into one tube (per sequencing run).

The final library is now ready to be sequenced, you may wish to dilute the library and re-quantify using qPCR. Most Illumina sequencing protocols dilute from a concentration of **4nM** though commercial companies usually ask for **≥15nM** in **20-30µl**.



Protocol references

Campbell, N.R., Harmon, S.A. and Narum, S.R. (2014) 'Genotyping-in-thousands by sequencing (GT-SEQ): A cost effective SNP genotyping method based on custom amplicon sequencing', *Molecular Ecology Resources*, 15(4), pp. 855–867. doi:10.1111/1755-0998.12357.