Sep 11, 2025

Illumina Dual Index Amplicon Sequencing Sample Preparation 18sV4 (Piredda primer pair)

DOI
https://dx.doi.org/10.17504/protocols.io.4r3l21423g1y/v1

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Protocol Citation: Eivind Stensrud, Alexander Eiler 2025. Illumina Dual Index Amplicon Sequencing Sample Preparation 18sV4 (Piredda primer pair) . **protocols.io** https://dx.doi.org/10.17504/protocols.io.4r3l21423g1y/v1

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Protocol status: Working
We use this protocol and it's working

Created: July 23, 2025

Last Modified: September 11, 2025

Protocol Integer ID: 223067

Keywords: illumina dual index amplicon sequencing sample preparation, piredda primer pair, piredda et al 2017 primer set, preparation of pcr product, pcr product, primer pair, amplicon, primer, piredda et al, primer set

Abstract

Preparation of PCR products for amplicon sequencing using 18sV4 Piredda et al 2017 primer set.

Guidelines

	Index 2 (i5)	Index 2 (i5) Sequence	Index 1 (i7)	Index 1 (i7) Sequence
	Illu_N501F	TAGATCGC	Illu_N701R	TCGCCTTA
	Illu_N502F	CTCTCTAT	Illu_N702R	CTAGTACG
	Illu_N503F	TATCCTCT	Illu_N703R	TTCTGCCT
	Illu_N504F	AGAGTAGA	Illu_N704R	GCTCAGGA
	Illu_N505F	GTAAGGAG	Illu_N705R	AGGAGTCC
	Illu_N506F	ACTGCATA	Illu_N706R	CATGCCTA
	Illu_N507F	AAGGAGTA	Illu_N707R	GTAGAGAG
	Illu_N508F	CTAAGCCT	Illu_N708R	CCTCTCTG
	Illu_N521F	CTTGCTTT	Illu_N709R	AGCGTAGC
	Illu_N522F	GGCTTCAA	Illu_N710R	CAGCCTCG
	Illu_N523F	AATCGGCA	Illu_N711R	TGCCTCTT
	Illu_N524F	GGTTCAAA	Illu_N712R	TCCTCTAC

	Illu_N525F	ACTTCGAC	Illu_N733R	GGTATAAG
	Illu_N526F	TGACTTGC	Illu_N735R	CAGCTAGA
	Illu_N527F	TAGGACCT	Illu_N736R	CCATAGCA
	Illu_N528F	GGAGACTT	Illu_N738R	GGTATAGC
	Illu_N529F	AGGTTACG	Illu_N739R	GGTTATGC
	Illu_N530F	AATTCGCT	Illu_N740R	TAGGCAAG
	Illu_N531F	TCAGCTAA	Illu_N741R	TTGTCCAT
	Illu_N532F	GCGATATG	Illu_N743R	TCTAGGCA

Materials

Thermo Scientific, dNTP Mix (2 mM each): R0242
New England Biolabs, Q5 High-Fidelity DNA Polymerase: M0491LVIAL
New England Biolabs, Q5 Reaction Buffer: B9027SVIAL
Eurofins, NGSgrade Oligos (HPLC purified) Piredda Forward and reverse primers with illumina handles.
Thermo Fisher Scientific, Invitrogen, TE, pH 8.0, RNase-free: AM9858
Thermo Fisher Scientific, Invitrogen, UltraPure DNase/RNase-Free Distilled Water: 10977035

Abstract

1 Preparation of PCR products for amplicon sequencing using Piredda et al. 2017, 18sV4 primer set.

Introduction

2 Standard procedure for preparing libraries for metabarcoding using the 18sV4 Piredda primer set (Piredda et al., 2017) https://doi.org/10.1093/femsec/fiw200.

The primer set is regarded as near universal for protist (phytoplankton) and is the modified version of the Tareuk primer pair (**10.1111/j.1365-294X.2009.04480.x**), and amplifies the ecological important phytoplankton group of haptophytes better than the original primer set https://doi.org/10.1093/femsec/fiw200.

Safety warnings

3 The lab work should be conducted in designated rooms for DNA-extractions, pre-PCR and post-PCR.
Perform plate set-up in the pre-PCR lab, and all PCR products should only be worked with in the post-PCR.
These rooms should have a strict one-way flow, meaning no equipment or samples should be moved from post-PCR to pre-PCR.
Always include (a) positive control(s) with a known mock-community, and add (a) negative plate control(s) in addition to the negative control(s) from the DNA extraction.

Before starting

4 Ensure that metadata files are finalised before starting library preparations in the lab.

Necessary equipment:
Pipettes P10, P100/P200 + P1000
Pipette tips (w + w/o filters)
Eppendorf tubes
96-well plate / PCR strips w/ lids
96-well plate seal
Seal applicator
Microplate rack / PCR strip rack
Racks
Microcentrifuge
Vortex

Clean bench and equipment (ex. pipettes and racks) with 10% chlorine, wait 10 minutes, then proceed to clean with 70% Ethanol and wait additional 10 minutes.

If working in an UV-hood, to sterilize the hood, turn on the UV for 30 minutes.
Avoid using chlorine on metal, and ethanol on hood plastics.

DNA extraction

5 Conduct the DNA extraction, we recommend using the DNeasy PowerWater Sterivex Kit (remember to add negative DNA extraction(s)) for water samples. Follow the manufacturer recommendation with the exception of a dual elution of 100 µl volume. (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/microbial-dna/dneasy-powerwater-sterivex-kit)

Quantify the extracted DNA using PicoGreen.
DOI: dx.doi.org/10.17504/protocols.io.dm6gpdbr1gzp/v1

Primers

6 **Piredda primer set (V4 18S Next.For and V4 18S Next.Rev primers)**
NGSgrade Oligos (HPLC purified) (Eurofins), the primer set has attached Illumina adapters from Sinclair et al. 2015. https://doi.org/10.1371/journal.pone.0116955 and Juottonen et al. https://doi.org/10.1111/1462-2920.15058 incorporate random nucleotides to reduce amplification bias in the forward primer.

Resuspend the primers with TE Buffer or Nuclease-Free water to obtain a 100 µM stock solution (store in -20 °C).
To reduce risk of cross-contamination and primer degradation, we recommend to make several aliquots of 10 µM primers by diluting with either TE-buffer or Nuclease-Free Water.

Forward primer (59mer):
V4 18S Next.For (5'-3'):
ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNN CCA GCA SCY GCG GT A ATT CC
Reverse primer (48mer):
V4 18S Next.Rev (5'-3'):
AGA CGT GTG CTC TTC CGA TCT NNN NNN ACT TTC GTT CTT GAT YRA TGA

Illumina adapters and spacers are marked in bold, while the taxa specific primers are in roman.

First PCR reactions

7 **Work in pre-PCR room**

Calculate the volumes of each reagent needed to create the Mastermix for the number of samples you are running (+10% extra to account for pipetting lost and potential errors). Remember to run the samples in duplicates/triplicates to reduce PCR-stochasticity.

	A	B	C	D	E
	Reagent	Stock conc.	Mastermix conc.	Volume 1 reaction (µl)	Volume X reactions (µl)
	5X Q5 Reaction Buffer	5X	1X	5	
	Forward primer	10 µM	0,2 µM	0,5	
	Reverse primer	10 µM	0,2 µM	0,5	
	dNTPs	10 mM	200 µM	0,5	
	Q5 High-Fidelity DNA Polymerase	0,25			
	Nuclease-Free Water	16,25			
	Template DNA			2*	
	Total			25	

Table 1:
PCR cycling conditions has been optimized with an Applied Biosystems QuantStudio 3. Add up to 2 µl DNA template from environmental samples. For positive control, add 1 µl mock community template and 1 µl Nuclease-Free water. For negative control, add 2 µl Nuclease-Free water.

7.1 **Remember to wear gloves and lab coat. Change gloves whenever necessary.**

7.2 Clean the working area and equipment with 10% chlorine, wait 10 minutes before continuing.

7.3 Clean the working area and equipment with 70% ethanol, wait 10 minutes before continuing.

7.4 Briefly vortex and spin down all reagents in a microcentrifuge, except the polymerase. Mix the polymerase by pipette mixing 10 times and spin briefly down in a microcentrifuge.

7.5 Prepare a Mastermix according to Table 1. Remember to make 10% extra to account for pipetting loss and potential errors.

7.6 Vortex and spin down Mastermix in a microcentrifuge.

7.7 Add 23 µl Mastermix to each well according to plate layout.

7.8 Add 2 µl template DNA according to plate layout.

7.9 Add 2 µl Negative control according to plate layout.

7.10 Add 1 µl mock community + 1 µl PCR water according to plate layout.

7.11 Seal the plate, vortex and spin the plate down with a microcentrifuge.

7.12 Run PCR using the cycling conditions described in Table 2.

7.13 **PCR cycling conditions (first round of PCR)**

	A	B	C	D
	Step	CYCLES	TEMP	TIME
	Initial Denaturation		98°C	2 minutes
	Denaturation	10 Cycles*	98°C	10 seconds

	A	B	C	D
	Annealing		44°C	30 seconds
	Extension		72°C	20 seconds
	Denaturation	15 Cycles**	98°C	10 seconds
	Annealing		62°C	30 seconds
	Extension		72°C	20 seconds
	Final Extension		72°C	5 minutes
	Hold		4°C	∞

Table 2:
PCR cycling conditions have been optimized with an Applied Biosystems QuantStudio 3. **Aim to use as few cycles as possible**, we recommend to run a few test samples with the reaction conditions showed above.
*If you need to increase the number of cycles, do not increase the number of cycles at 44 degrees annealing temperature.
** If you need to increase the number of cycles, you can increase up to 30 cycles at 62 degrees annealing temperature.

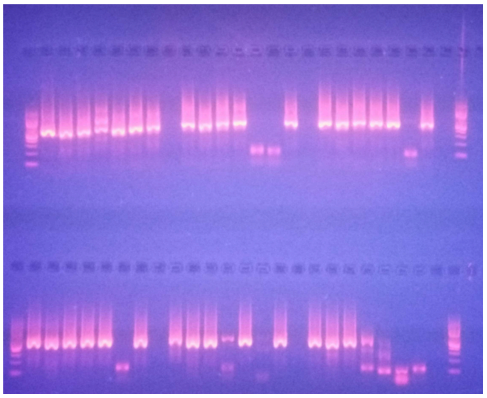
PCR visualization

8 **Conduct in post-PCR lab**

After the first PCR reaction, pool the replicated samples and inspect the PCR products using Agarose gel electrophoresis (1%).

Successful amplification should give bands at ~530bp (See picture below).

Note that some primer dimers may occur, and are removed in the purification step.



Purification

9 **Conduct in post-PCR**
Perform purification with magnetic beads (Cytiva Sera-Mag Select) using 0.8x beads concentration.
DOI: dx.doi.org/10.17504/protocols.io.n2bvj393wlk5/v1

Second PCR reactions

10 A second PCR is conducted for attaching standard illumina handles (bold) and index primers
Multiplex_fwd:
AATGATACGGCGACCACCGAGA{TCTACAC}-[i5 index] ACACTCTTTCCCTACACGACG
Multiplex_rev:
CAAGCAGAAGACGGCATACGAGAT-[i7 index]-
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

We use 20 different forward index/barcode primers and 20 different reverse index/barcode primers.
Combining both primers (20X20) makes it possible to generate 400 unique tags to run 400 samples of each unique primer set in one final pool for sequencing.
(See Guidelines for index sequences)

Second PCR cycling conditions

11 Prepare Mastermix and mix with forward and reverse indices according to plate layout in the pre-PCR lab.

Bring the plate(s) with Mastermix and primers to the post-PCR lab, where samples from the 1st PCR reaction is added according to plate layout.

Calculate the volumes of each reagent needed to create the Mastermix for the number of samples you are running (+10% extra to account for pipetting lost and potential errors) (Table 3).

Note: Not necessarily to run the samples in duplicates

	A	B	C	D	E
	Reagent	Stock conc.	Mastermix conc.	Volume 1 reaction (µl)	Volume X reactions (µl)
	5xQ5 Reactio Buffer	5X	1X	4,00	
	Forward index (i5, illu-N5xx)	5µM	0,25 µM	1,00	
	Reverse index (i7, illu-N7xx)	5µM	0,25 µM	1,00	
	dNTPs	2mM	200 µM	2,00	
	Q5 HF DNA polymerase	2 U/µl	0.02 U/µl	0,20	
	Template from 1st PCR			2,00	
	Nuclease-Free water			9,80	
	Σ			20,00	

Table 3:
PCR cycling conditions has been optimized with an Applied Biosystems QuantStudio 3. Add 2 µl of purified PCR product of both environmental samples, positive and negative controls.

11.1 **Remember to wear gloves and lab coat. Change gloves whenever necessary.**

11.2 Clean the working area and equipment with 10% chlorine, wait 10 minutes before continuing.

11.3 Clean the working area and equipment with 70% ethanol, wait 10 minutes before continuing.

11.4 Briefly vortex and spin down all reagents in a microcentrifuge, except the polymerase. Mix the polymerase by pipette mixing 10 times and spin briefly down in a microcentrifuge.

11.5 Prepare a Mastermix according to Table 3. Remember to make 10% extra to account for pipetting loss and potential errors.

11.6 Vortex and spin down Mastermix in a microcentrifuge.

11.7 Add 16 µl Mastermix to each well according to plate layout.

11.8 Add 1 µl Forward and Reverse primer (unique combination per sample) to each well on the 96-well plate or PCR strip.

11.9 **Move to Post-PCR**
Seal the plate while transfering to Post-PCR room.

11.10 Add 2 µl template DNA according to plate layout.

11.11 Seal the plate, vortex and spin the plate down with a centrifuge.

11.12 Run the second PCR using the cycling conditions described in **Table 4**:

11.13 PCR cycling conditions (second round of PCR)

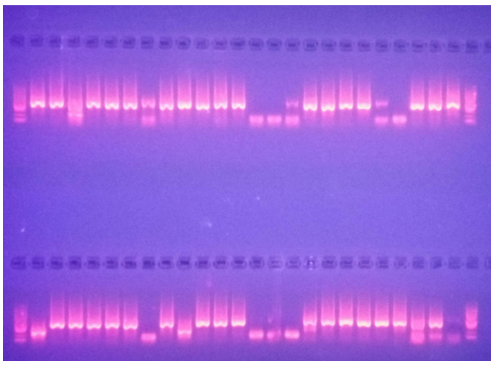
	A	B	C	D
	CYCLES	STEP	TEMP	TIME
		Initial Denaturation	98 C	30 sec
	15 cycles	Denaturation	98 C	10 sec
		Annealing	66 C	30 sec

	A	B	C	D
		Extension	72 C	30 sec
		Final Extension	72 C	2 min
		Hold	6 C	∞

Table 4:
PCR cycling conditions have been optimized with an Applied Biosystems QuantStudio 3. Number of cycles can be reduced to 10.

PCR visualization

12 **Conduct in Post-PCR**
After the second PCR reaction, pool the replicated samples and inspect the PCR products using Agarose gel electrophoresis (1%).
Successful amplification should give bands at ~600 bp (See picture below).
Note that some primer dimers may occur, and are removed in the purification step.



Purification

13 **Conduct in post-PCR**
Perform purification with magnetic beads (Cytiva Sera-Mag Select) using 0.75x beads concentration following the manufacturer recommendations.
DOI: dx.doi.org/10.17504/protocols.io.n2bvj393wlk5/v1

Quantify and pool in equimolar concentration

14 **Conduct in post-PCR:**
Quantify and pool the samples to create a final library using PicoGreen, if pooling samples from different marker lengths, see Sheet 3.
DOI: dx.doi.org/10.17504/protocols.io.dm6gpdbr1gzp/v1

Make sure to reach the requirements for illumina sequencing, with at least 20 µL of 10 nM library pool (Sheet 3).
Read more about the requirements here:
https://ngisweden.scilifelab.se/methods/illumina-user-provided/

14.1 **Conduct in post-PCR**
Calculation conducted in the Google Sheets.
Pool the PCR samples in equal DNA amount (ng) or for unequal length amplicons, in equal molecule amount (mol).

You will get one tube with a mix of all the samples in it.
To calculate the volume of each sample to be pooled (DNA amount mixing):

Use the lowest concentration sample to define the minimum amount of DNA (ng) that you have available from a single sample: the DNA concentration (ng/µL) of the lowest concentration sample multiplied with its volume (µL). This will be your target DNA amount for each sample.

Calculate how many µLs of each sample you need to achieve the target DNA amount: divide the target DNA amount with the concentration of each sample.

Pipette into one tube the calculated volume of each sample. Aim to use the same pipette for all samples (dilute or pipette multiple times) to avoid pipette calibration errors.

Quality control

15 Ensure that primer dimers have been removed from the final pool by using Agarose gel electrophoresis (1%). If primer dimers are still present, conduct a new bead purification as described above, or conduct gel extraction.

If the DNA concentration is too low, <10nM, you can either conduct another bead purification and elute with a lower elution volume (recommended).
Or use a SpeedVac to further up-concentrate the DNA (Remember that SpeedVac also up-concentrates salts from the elution buffer), which may be unfavorable for

sequencing.