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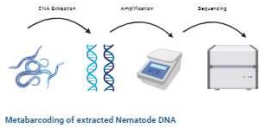
Nematode DNA Illumina Amplicon Sequencing using two step PCR



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We use this protocol and it's working

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Abstract

This document is designed to provide the researcher with all of the information required to undertake the metabarcoding of DNA from Nematodes which have already been physically separated from soil. Here we outline our methodology for two step amplicon sequencing using the Illumina MiSeq platform.

The aim is to provide a framework upon which the researcher may choose to add their own modifications to suit novel applications.

Much of the protocol has been adapted for use from the methodology outlined in :

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol. 2013 Sep;79(17):5112-20. doi: 10.1128/AEM.01043-13. Epub 2013 Jun 21. PMID: 23793624; PMCID: PMC3753973.

Primers selected are based upon those referenced in the text and in reference section.

All kits and equipment are given as recommendations based upon resources available to UKCEH laboratories and are not intended to be discriminatory against alternatives or emerging methodologies. However, we would recommend researchers perform their own validation of any changes.

Nextera index plates- Prepared prior to Step 1 PCR

1 Order indexing primers direct from oligo manufacturer (For example IDT, Sigma genosys or MWG) suspended in water 0.5 µM scale, Desalt purification and 10 µM concentration. These indexing primers consist of: an Illumina Nextra adapter i5 (Forward primer) AATGATACGGCGACCACCGAGATCTACAC or i7 (reverse primer) CAAGCAGAAGACGGCATACGAGAT, Unique 8bp barcode sequence and pre-adapter i5 TCGTCGGCAGCGTC (F) or i7 GTCTCGTGGGCTCGG (R). Barcode design when demultiplexed allows for the unique assignment of an individual sequence to sample. Figure 1 gives a graphical representation of how 16 Forward, and 24 Reverse barcodes sequences are allocated to give 384, unique barcode combinations. Full barcode sequence including an additional 384 barcode combinations (named arrays A-H) is given in attached document.

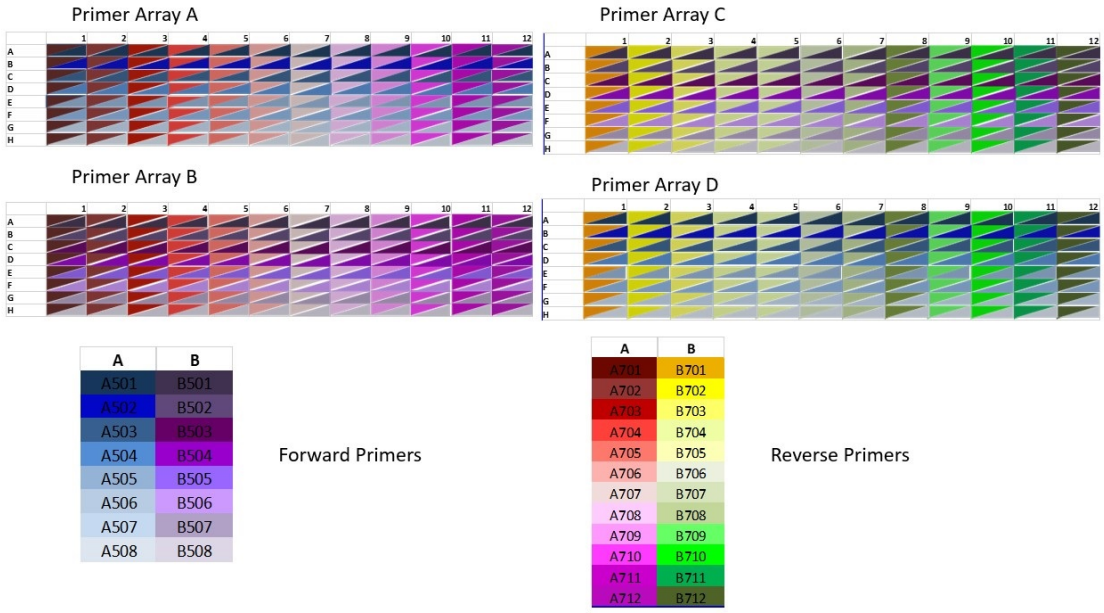


Figure 1: An illustration of Dual-index primer pair allocation

Array Plate description.xlsx

2 Prepare index plate 'masters' mannually allocating 250 µL of each diluted primer stock [M] 10 micromolar (µM) per well as per plate design detailed above into Deep-Well microtiter plates. Alternatively this can be done using a liquid handling Robot.

3 Make PCR plate clones of the ‘master’ plates, each well containing 5ul of mixed primer, label, seal and store at -20 for future use.

Step I- Amplicon PCR with modified primers

4 Make a standardised dilution plate of template DNA from DNA extraction process. DNA concentration is determined through Qubit BR or HS assay of raw extract, and then each extract is diluted to a standardised concentration (this is subject to extraction success, but shouldn't be below 2ng/ul to allow for effcient PCR), using the equation:

$$\frac{\text{Desired Concentration (ng/ul)}}{\text{Raw extract Concentration (ng/ul)}} \times \text{Final desired volume} = \text{Amount of DNA to add}$$

To work out the amount of water its : Final desired volume - Amount of DNA to add

If DNA extract concentration is below 2ng/ul we would recommend that DNA is either concentrated or if feasible 2ul of template DNA used in subsequent PCR steps.

5 Step 1 Amplicon primers consist of a pre adapter, sequencing primer and specific locus primer. Target primers are designed as per Table 1:

	A	B	C	D	E	F	G	H
	Source reference	Universal primer name	Target 18S Hypervariable region	pre-adapter	Sequencing primer sequence	Specific locus primer	Combined sequence	Primer pair group
	Mangot et al (2012) https://doi.org/10.1111/1462-2920.12065	NSF563	V4	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CGCGGTAATTCCAGCTCCA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGCGGTAATTCCAGCTCCA	F
	Mangot et al (2012) https://doi.org/10.1111/1462-2920.12065	NSR951	V4	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	TTGGYRAATGCTTTCGC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTGGYRAATGCTTTCGC	F
	Blaxter et al (1998) https://www.nature.com/articles/32160	SU18A	V1-V3	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	AAAGATTAAGCCATGCATG	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAAGATTAAGCCATGCATG	A

	A	B	C	D	E	F	G	H
	Blaxter et al (1998) https://www.nature.com/articles/32160	SSU9R	V1-V3	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	AGCTGGAATTACCGCGGCTG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGCTGGAATTACCGCGGCTG	A
	Kawanobe et al (2021) https://doi.org/10.1016/j.apsoil.2021.103974	F548_A	V4	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	AGAGGGCAAGTCTGGTGCC	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGGGCAAGTCTGGTGCC	C
	Hadziavdic et al (2014) https://doi.org/10.1371/journal.pone.0087624	R915	V4	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	TCCAAGAATTTCACCTC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCAAGAATTTCACCTC	C
	Porazinska et al (2009) https://onlinelibrary.wiley.com/doi/10.1111/j.1755-0998.2009.02611.x	NF1	V7-V8	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	GGTGGTGCATGGCCGTTCTTAGTT	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGTGGTGCATGGCCGTTCTTAGTT	E
	Porazinska et al (2009) https://onlinelibrary.wiley.com/doi/10.1111/j.1755-0998.2009.02611.x	18Sr2b	V7-V8	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	TACAAAGGGCAGGGACGTAAT	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTACAAAGGGCAGGGACGTAAT	E

Table 1: Step I amplicon primers employed during for the amplification of Nematode communities

6 Using column ‘Combined sequence’ given in Table 1, order primers direct from oligo manufacturer (For example IDT, Sigma genosys or MWG) suspended in water 0.5 µM scale, Desalt purification and 100 µM concentration.

6.1 Choice of primer set and region should be selected based upon project requirements - see Defra report for project CN115 (Taylor, Newbold and Cortez, 2024)- as differing hypervariable regions may influence sequence community composition results.

7

	A	B	C
	Reagent	Per Sample	Per 100
	Molecular Grade Water	27.3 ul	2730 ul
	5 X Buffer	10 ul	100 ul
	High GC Buffer	10 ul	100 ul
	10 mM dNTP's	1 ul	100 ul
	Q5 Taq Polymerase	0.5 ul	50 ul
	Primer F (100 mM)	0.1 ul	10 ul
	Primer R (100 mM)	0.1 ul	10 ul
	DNA Template (at ~5 ng/ml)	1 ul	Added separately

Table 2: 1 Step PCR Reagents in the order in which they should be added. *Note that Template is added separately after PCR Mastermix plate has been prepared.*

8 All reagents from above Table 2 (except template DNA) are added to a 5ml Tube (or equivalent) to form a PCR Mastermix.
Mastermix is prepared on ice, using filter tips and under sterile conditions in the PCR hood.

Once Mastermix has been made add 49 µL of Mastermix to each well of a 96 well PCR plate using a multichannel pipette (accurate for volumes between 100 µL - 300 µL).

We recommend the use of :

Axygen™ 96-Well Low Profile PCR Microplates (SKU AXY-PCR-96-LP-FLT-C)
https://www.thistlescientific.co.uk/product/96-well-polypropylene-flat-top-pcr-microplate-low-profile-no-skirt-clear-nonsterile/

and

Equipment

Research Plus Multichannel pipette	NAME
Multichannel Pipette	TYPE
Eppendorf	BRAND
3125000052	SKU
https://online-shop.eppendorf.co.uk/UK-en/Manual-Liquid-Handling-44563/Manual-Pipetting--Dispensing-44564/Eppendorf-Research-plus-PF-534798.html	LINK

Add 1 µL ***of standard dilution from step 4 to respective position in the mastermix plate. This ensures full traceability when samples are demultiplexed during the bioinformatics steps performed post sequencing. It is therefore imperative that experimenters make a note of which sample is added to each well of the PCR mastermix plate.***

9 Seal plates with PCR film, and centrifuge, to concentrate sample.

We recommend the use of:

https://www.thermofisher.com/order/catalog/product/AB0558

10 Initiate PCR reaction on Thermal cycler
We recommend the BIORAD C1000 touch thermal cyler https://www.bio-rad.com/en-uk/product/c1000-touch-thermal-cycler?ID=LGTW9415

PCR program:

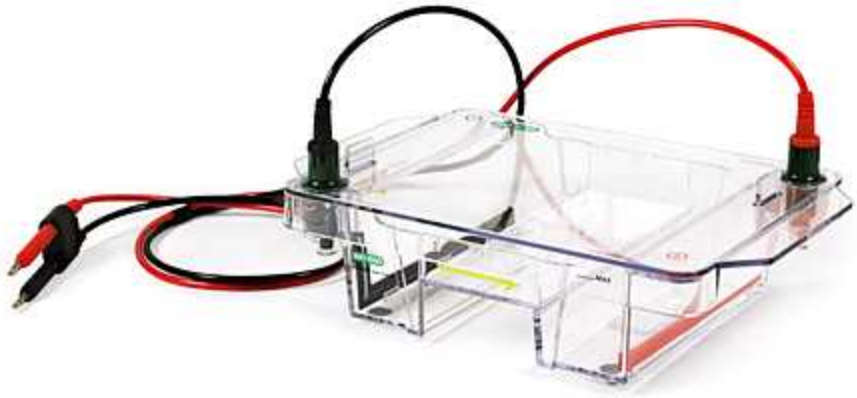
- Pre-denature 95 °C for 10:00m
- Denature 95 °C 00:20s
- Anneal 52 °C (SU18A-SSU9R), 62°C (F548_A-R915), 51°C(NF1-18Sr2b) and 55°C (NSF563-NSR941) for 00:30s
- Extension 72 °C for 00:30s
- Repeat ii. – iv. 35 cycles
- Final extension 72 °C for 10:00m

Gel Electrophoresis

11 Verify PCR products using gel electrophoresis.

11.1 Prepare the appropriate electrophoresis tank and casting tray. The number and type of gels required will depend on the number of samples.

A Mini-sub Cell GT Horizontal Electrophoresis Tank. Can run up to 28 samples + two ladder lanes. The 7 x 10 cm casting tray requires 50 ml agarose mix.

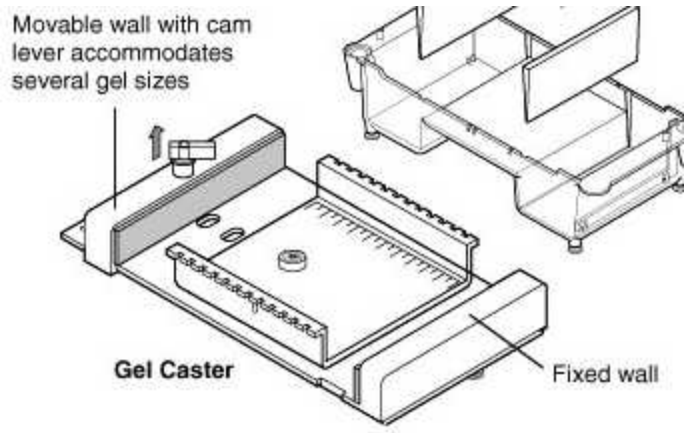


A Wide Mini-sub Cell GT Horizontal Electrophoresis Tank (Midi Tank). Can run up to 58 samples + two ladder lanes. The 15 x 10cm casting tray requires 100ml agarose mix.

Sub-Cell 96 GT Horizontal Electrophoresis Tank. Can run up to 100 samples + two ladder lanes. The 25 x 15 cm casting tray requires 250ml agarose mix.

11.2 To make a [M] 1 % (v/v) agarose gel : For a Midi Gel, measure 1g of molecular grade agarose powder into a glass pyrex beaker and make up to 100 mL using 1 X TBE buffer. Heat in microwave to dissolve for approx 00:02:00 . When no agarose crystals are visable, cool the gel in a sink filled with a little water, to just above room temperature,. Cooling in this way will take atleast 00:02:00 . As a rule of thumb if you're able to hold the base of the beaker without discomfort the gel is ready. 4m

11.3 To a 100ml mix add 1 µl ethidium bromide, or 5 µl Gel Red
Swirl to mix and pour into gel casting tray set up as below.



Biorad gel caster. To use push two casting walls together and tighten using lever.

11.4 Place combs directly into the casting tray (in above tray line up comb ends with 2nd notch and push down). If bubbles are present these can be pushed out of gel using combs before final positioning and tightening lever. 45m

Leave gel to set for at least 00:45:00 .

11.5 Whilst gel is setting, prepare samples using 5 µL PCR amplicon to 1 µL Bromophenol blue gel loading buffer.
https://www.thermofisher.com/order/catalog/product/10816015#/10816015

11.6 When set load loosen casting lever and place tray and gel into Gel tank filled with 1X TBE buffer to fill line. Remove combs (doing this in the buffer prevents bubbles forming in wells). Add 5 µL Hyper ladder 1KB https://www.bioline.com/hyperladder-1kb.html (or similar) to first well of agarose gel by placing tip into well and gently depressing pipette control, whilst slowly removing tip from well. Repeat proceedure with samples, each being placed in its an individual well.

11.7 When all samples are loaded put on gel tank lid, and plug in cables (red to red, black to black). Set gel to run for 00:45:00 at 90 Volts. 45m

11.8 When run complete image gel using gel image system (eg https://www.bio-rad.com/en-uk/product/gel-doc-xr-gel-documentation-system?ID=O494WJE8Z).

A successful PCR should look similar to below.

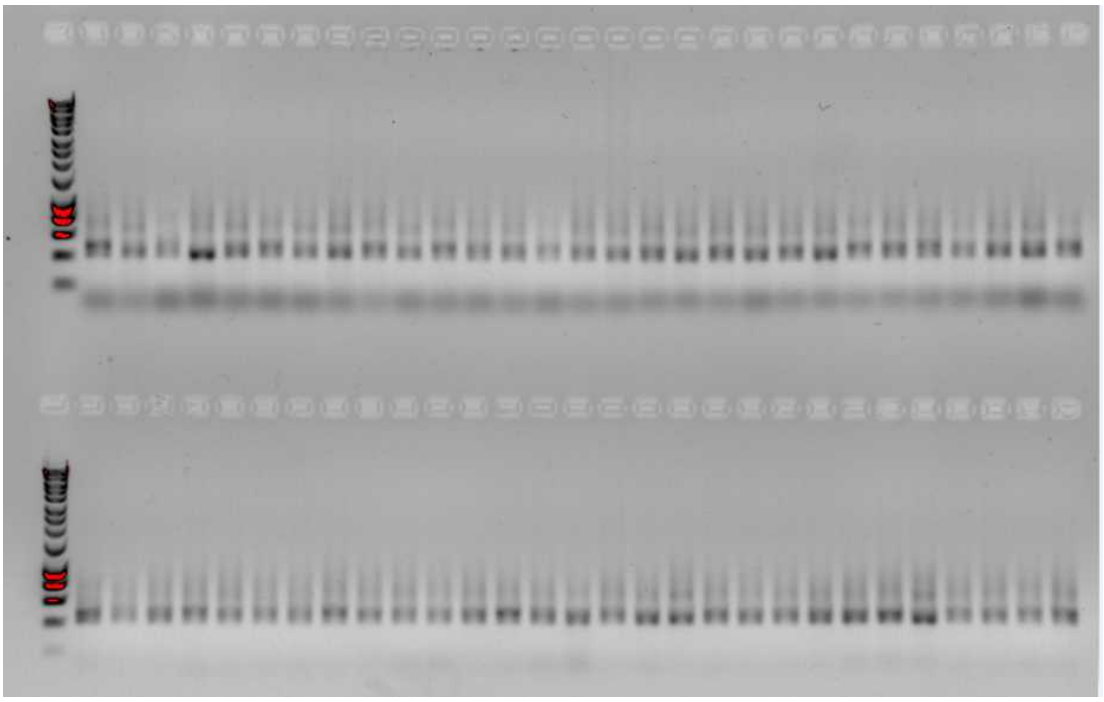


Figure 4: Result of ITS2 PCR.

PCR Clean-up

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Clean-up Amplicon PCR step I using ZymoZR-96 kit (standard Manufacturers protocol), again using filter tips and upmost care throughout as samples are not indexed.
https://zymoresearch.eu/collections/dcc-pcr-purification-kits/products/zr-96-dna-clean-up-kit

Alternatively use Multiscreen-PCR$_{96}$ Filter plate under standard manufacturers protocol if liquid handling robot and vacuum manifold are available.

https://www.merckmillipore.com/GB/en/product/MultiScreen-PCR96-Filter-Plate,MM_NF-MSNU03050

Step II - Nextera indexing PCR

13

Use 1-10 µL Cleaned PCR product as template for Step II- Indexing PCR (Table 3). Template input should be driven by PCR concentrations observed in during step 11. Water added to PCR master mix is adjusted to make each reaction total 25 µL .

	A	B	C
	A	B	C

	A	B	C
	Reagent	Per Sample	Per Plate
	Molecular Grade Water	7.25 ul	725 ul
	5 X Buffer	5 ul	500 ul
	High GC Buffer	5 ul	500 ul
	10 mM dNTP's	0.5 ul	50 ul
	Q5 Taq Polymerase	0.25 ul	25 ul
	Primer Array mix	5 ul	Added separately
	PCR Template	2 ul	Added separately

Table 3: Step II PCR Reagents in the order in which they should be added. *Note that Template and primer mix are added separately after PCR Mastermix plate has been prepared.*

14 PCR programme :

- Denature 95 °C for 02:00m
- Denature 95 °C for 00:15s
- Anneal 55 °C for 00:30s
- Extension 72 °C for 00:30s
- Repeat to total 10 cycles

Normalisation

45m

15 Resultant PCR products are normalised using the Norgen NGS Normalization 96-Well Kit – 61900 under standard manufacturers protocol:
UK distributers
https://geneflow.co.uk/product/ngs-normalization-96-well-kit-61900/

16 Use ~ 5 µL normalised PCR product from each sample to form a pooled library.

17 Vacuum concentrate to a volume of approx 30 µL pooled normalised plate library.
We reccomend:
https://www.eppendorf.com/gb-en/eShop-Products/Centrifugation/Concentrator/Concentrator-plus-p-PF-25748

17.1 Whilst Vacuum concentration is occuring prepare a 50ml 1% Agarose gel, with a wide comb.

18 Add 5 µL loading buffer to concentrate and run for 00:45:00 at 90 Volts. 45m

18.1 Excise resultant band using sterile scapel, and visualised on blue light Led trans illuminator

https://www.smobio.com/shop/product/ve0100-b-boxtm-blue-light-led-epi-illuminator-419

19 Extract DNA from agarose Gel using **QIAquick Gel Extraction Kit - QIAGEN** under manufactuers standard protocol. Samples eluted in 30 µL buffer EB.

Quantification

20 Gel Purified libraries are quantified using **Invitrogen™ Qubit™ 1X dsDNA High Sensitivity (HS) quantification kit**
https://www.fishersci.co.uk/shop/products/qubit-1x-dsdna-hs-br-assay-kits/15850210
Under manufacturers protocol and companion qubit fluorometer
https://www.fishersci.co.uk/shop/products/qubit-4-fluorometer-wifi/16223001#?keyword=Qubit%204%20flurometer

21 Calculate Molar concentration using the https://www.bioline.com/media/calculator/01_07.html

	A	B
	Amplicon	Amplicon bp (inc illumina adapters)
	SU18A-SSU9R	624
	F548_A-R915	452
	NF1-18Sr2b	447
	NSF563-NSR941	533

Note: The above lengths are calculated by using mean amplicon specific primer fragment length (determined through in silico analysis), plus additional illumina specific tags. This is important as library run concentration is based upon molarity.

If using multiple libraries mix equal concentrations of each arrays: e.g. Array plate A library + Array plate B library.

Your library is now ready for sequencing!

Sequencing on V2 or V3 Miseq 7m

22 Please note that much of the following is an ammended version of the Schloss lab SOP, therefore their work should always be referenced.

Remove a V3 600 cycle/V2 500 cycle reagent cartridge from the -20 °C freezer. Place in room temperature water bath for one hour. Place Hyb buffer tube in 4 °C fridge. While reagent cartridge is thawing, perform steps 23-27.

23 Prepare fresh [M] 0.1 Molarity (M) NaOH.

24 To a 1.5 mL tube, add 10 µL of library and 10 µL of [M] 0.1 Molarity (M) NaOH. To a separate tube add 2 µL PhiX, 3 µL PCR grade water, and 5 µL of [M] 0.1 Molarity (M) NaOH. Pipette to mix. Note: NaOH concentration on the flow cell must remain under 0.001M.

25 Allow the tubes to incubate at room temperature for 00:05:00 . Immediately add 980 µL of ice-cold Hybridisation buffer (Hyb) to the library tube, and 990 µL Hyb to the PhiX tube. 5m

Note: the resulting 20pM PhiX can be frozen and used for subsequent runs.

26 Use HTI to further dilute both the library and PhiX to 7.5 pM.
See example below:

a. (1.78 nM library x 10 ul) + (0.1N NaOH x 10 ul) + 980 ul HTI = 17.8 pM Lib, 0.001N NaOH

b. 17.8pM lib/7.5 =2.3733333333

c. 1000/2.373333333 =421.35ul (Denatured Library to add to 578.65 HTI Buffer)

d. 20pM PhiX /7.5 =2.666666

e. 1000/2.66666 = 375ul (Denatured Library to add to 625 HTI Buffer)

27 For a 10% PhiX run, combine 900 µL of [M] 7.5 picomolar (pM) Library and 100 µL 7.5 pM PhiX in a final tube. 2m
Vortex. At this final stage heat to 96 °C for 00:02:00 . Then cool immediately on ice.

28 When the reagent cartridge has thawed, dry bottom with paper towel. Invert the cartridge repeatedly to check each well is thawed. This also serves to mix the reagents. Place in Fridge

29 Load 600 ul of the final Libary/PhiX solution into well 17 on the reagent cartridge.

30 Unbox flow cell and PR2 bottle.Thoroughly rinse the flow cell with Milli-Q water. Carefully dry by blotting with lint free wipes (Kimwipes). Give special attention to the edges and points of intersection between the glass and plastic. Wet a new wipe with 100% alcohol and wipe the glass on both sides avoiding the rubber intake ports.Visually inspect the flow cell to ensure there are no blemishes, particles, or fibers on the glass. Follow on screen instructions to load the flow cell, reagent cartridge, and PR2 bottle. Empty and replace the waste bottle.

31 Transfer reagent cartridge, flow cell, PR2 bottle, and flash drive with the sample sheet to the MiSeq. Open Sample Sheet from the flash drive in the MiSeq Local run manager software and queue run. Ensure the machine recognizes the correct sample sheet and the run parameters are correct.

32 Wait for the MiSeq to perform its pre-run checks, and press start. NOTE: If the pre-run check fails, try wiping down the flow cell again.

32.1 Ideal parameters for a 90% run:
a. Cluster density 1000-1200k/mm2 for V2 kits 1200-1400k/mm2 for V3 kits,
b. >85% clusters passing filter
c. 10% aligned (amount of PhiX)
d. No spikes in corrected intensity plot
e. All indices identified following index reads
f. Final >Q30 score of >70%

32.2 When run complete, perform post run wash as per manufacturers recommendations.

Protocol references

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol. 2013 Sep;79(17):5112-20. doi: 10.1128/AEM.01043-13. Epub 2013 Jun 21. PMID: 23793624; PMCID: PMC3753973.

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Porazinska, D.L., Giblin-Davis, R.M., Faller, L., Farmerie, W., Kanzaki, N., Morris, K., Powers, T.O., Tucker, A.E., Sung, W.A.Y. and Thomas, W.K., 2009. Evaluating high-throughput sequencing as a method for metagenomic analysis of nematode diversity. *Molecular Ecology Resources*, 9(6), pp.1439-1450.