Metro South Health

PRINCESS ALEXANDRA HOSPITAL
Procedure

Section: Clinical
PROCEDURE TITLE: Illumina Sequencing Library Prep

Review Officer: Consultant, Department of Gastroenterology and Hepatology

Review Summary: v1

Applicable To: Gastroenterology and Hepatology Staff

Date Introduced or Last Review Date: 10/2019

Next Review Date: 10/2023

Authority: Head of Clinical Function Testing, Gastroenterology and Hepatology

Replaces: New procedure

Key Words: DNA sequencing

PURPOSE

To test tissue samples with low bacterial load/high level of human DNA (e.g. intestinal biopsies).

OUTCOME

Accurate sequencing of bacterial DNA

AUTHORISED TO UNDERTAKE THE PROCEDURE

Gastroenterology and hepatology staff trained in DNA sequencing

INDICATIONS OR CONTRAINDICATIONS

N/A

RISKS AND PRECAUTIONS

PPE must be worn

STEPS OF THE PROCEDURE

Amplicon PCR of gDNA

Note – it is best to first do a test amplicon PCR using 917F-1392R primers (without adapters), as below however in a smaller reaction volume, to make sure you can get product from your gDNA samples. DO NOT do this step if it means you will run out of gDNA template.

PCR components:

2 µL Primer 917F with Illumina adapter (10 µM)
2 µL Primer 1392R with Illumina adapter (10 µM)
25 µL Q5 enzyme
} *Put these in the master mix*
21 µL gDNA template and H_2O
50 µL TOTAL

PCR cycle components:

98 °C		for 3 mins
25 cycles of	95° C	for 20 sec
	51° C	for 20 sec
	72° C	for 20 sec
72° C		for 2 mins

Protocol:

Set up and label a numbering system for a 96 well plate. NB – if sequencing with ACE, it is best to number your samples across rows first:

	1	2	3	4	5	6	7	8	9	10	11	12
A	AR1	AR2	AR3	AR4	AR5	AR6	AR7	AR8	AR9	AR10	AR11	AR12
B	AR13	AR14	AR15	AR16	AR17	AR18	AR19	AR20	AR21	AR22	AR23	AR24
C	AR25	AR26	etc									
D												
E												
F												
G												
H												

1. Make master mix for the number of reactions (plus a few more) needed
2. Add 29 µL of master mix to each PCR reaction tube (PCR microplate if >24 samples)
3. Add gDNA

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gDNA sample requirement – approximately 100–200 ng gDNA template (should be around 1-2 µL) based on the nanodrop concentration. You must use the same amount of template for every sample. For Mock sample, use the same volume as that used for your lowest concentration sample

4. Use E. coli gDNA 100 ng for the positive control; use water only for negative (no template) control
5. Add appropriate amount of water (total of template DNA and water should be 21 µL)
6. Run PCR as above
7. Divide PCR product into 2 x 25 µL lots – do PCR clean up on one, store the other for safekeeping. Label as “amplicon PCR 917F, 1392R”
8. Run Gel to check result (2% agarose, 2 µL per well and 3 µL of loading dye)
9. Do PCR clean up

PCR clean-up:

Reagents needed:

AMPure XP beads (bring to room temp)	20 µL per sample
80% ETOH (prepare fresh)	400 µL per sample
10 mM Tris pH 8.5	52.5 µL per sample
Magnetic bead stand	
Dispensing trough (for multichannel pipette)	

1. Use 25 µL of the amplicon PCR product, in PCR tubes/96-well PCR plate for the clean-up
2. Centrifuge the amplicon PCR plate at 1000 g for 1 min (can use little green benchtop spinner; or large benchtop centrifuge if using plate)
3. Vortex AMPure beads for 30 sec. Add to dispensing trough
4. Add 20 µL of beads to each PCR sample (use multichannel). Gently pipette up and down 10 times to mix
5. Incubate at room temp for 5 mins
6. Place in magnetic stand for 2 mins, or until supernatant has cleared
7. While still in the magnetic stand, remove and discard supernatant (use multichannel)
8. While still in the stand, wash the beads with 80% ETOH.
   a. Add 200 µL of ethanol to reaction PCR tube (use multichannel)
   b. Incubate for 30 seconds
   c. Carefully remove and discard supernatant
9. Repeat a second ETOH wash as above. After this one, use a P20 pipette to remove residual ETOH
10. While still in the magnetic stand, let beads air dry for 10 min (e.g. in biosafety cabinet)
11. Remove amplicon PCR tubes from stand. Add 52.5 µL of 10 mM Tris to each PCR tube (use multichannel). Gently pipette up and down 10 times to mix. Make sure beads are fully resuspended
12. Incubate at room temp for 2 mins
13. Place in magnetic stand for 2 mins, or until supernatant has cleared
14. Carefully transfer 50µl of the supernatant into a clean labelled tube. Label this “cleaned up amplicon PCR 917F, 1392F”

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SAFE TO STORE at -30 °C for 1 month

Index (barcoding) PCR

PCR components:

5 µL (per sample)	Nextera XT index 1 primer (N7XX)	NB: need 480 µL for 96 wells
5 µL (per sample)	Nextera XT index 2 primer (S5XX)	
25	Q5 enzyme	Need 2,400 µL for 96 wells
5	PCR water	
10	DNA template (from cleaned amplicon PCR)	
50	Total	

PCR cycle components:

95 °C		for 3 mins
25 cycles of	95° C	for 30 sec
	55° C	for 30 sec
	72° C	for 30 sec
72° C		for 5 mins

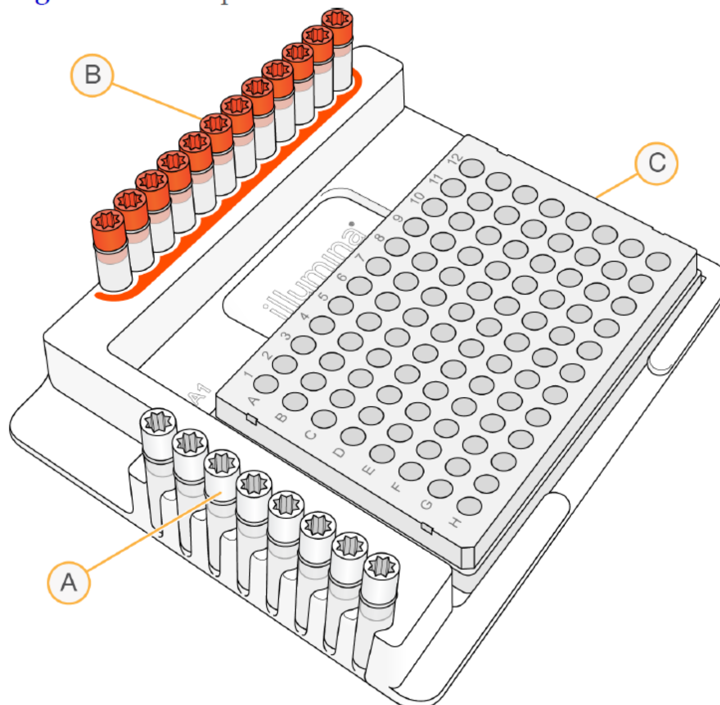
Protocol:

1. Set up and label a numbering system for a 96 well plate, for example (easiest if this is the same as your amplicon DNA layout, if you did 96 wells for that one): NB – if sequencing with ACE, it is best to number you samples across rows first:

	Index1 (I7)	**N701**	**N702**	**N703**	**N704**	**N705**	**N706**	**N707**	**N710**	**N711**	**N712**	**N714**	**N715**
Index2 (I5)		1	2	3	4	5	6	7	8	9	10	11	12
S502	A	AR1	AR2	AR3	AR4	AR5	AR6	AR7	AR8	AR9	AR10	AR11	AR12
S503	B	AR13	AR14	AR15	AR16	AR17	AR18	AR19	AR20	AR21	AR22	AR23	AR24
S505	C	AR25	AR26	etc									
S506	D												
S507	E												
S508	F												
S510	G												
S511	H												

2. Lay out your new 96 well PCR plate and the barcode primers (N7 horizontally along top, S5 vertically alongside) as per the diagram (Figure 4 of page 11 of 16S Metagenomic Sequencing Library Preparation)

Figure 4 TruSeq Index Plate Fixture



A Index 2 primers (white caps)
B Index 1 primers (orange caps)
C 96-well plate

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3. Make a master mix of Q5 enzyme (25 µL per sample) and water (5 µL per sample). Add 30 µL of Q5/water to all the wells (use multichannel; place total volume (include at least 4 samples worth extra) of Q5 enzyme/water mix required in reagent trough)
4. Add 5 µL of each barcode primer to the well (use multichannel to go across and down)
5. Add 10 µL of DNA template (cleaned amplicon PCR) and pipette up and down 10 x to mix
6. Centrifuge at 1000 g (large benchtop centrifuge for plate; or use small green benchtop spinner) for 1 min
7. Perform PCR as above

Amplicon PCR of gDNA

Use the protocol from Promega (Wizard SV Gel and PCR Clean-Up System)

<u>Reagents needed:</u>

Membrane binding solution 140 µL per gel band
Membrane wash solution 1,200 µL per gel band

Assumptions: Weight of gel = 140–150 mg
Volume of buffer to dissolve gel = 140 µL
Otherwise: Pre-weigh Eppendorf tube. Place excised gel in tube and re-weigh.
Calculate weight of gel. Add equivalent volume of buffer to dissolve gel (1 mg = 1 µL)

Protocol:

1. Gel slice and PCR product preparation:
    a. Excise DNA band from gel and place in 1.5ml microcentrifuge tube
    b. Add 140 µL of membrane binding solution. Vortex and incubate at 50-60 °C until gel is completely dissolved.
2. Binding of DNA
    a. Insert SV minicolumn into the collection tube.
    b. Transfer dissolved gel mixture into minicolumn tube. Incubate at room temp for 1 min
    c. Centrifuge at 16,000 x g for 1 min. Discard flow through and reinsert minicolumn into collection tube
3. Washing
    a. Add 700 µL membrane wash solution (ethanol added). Centrifuge at 16,000 g for 1 min. Discard flow through and reinsert minicolumn into collection tube
    b. Repeat step 3.a. with 400 µL of membrane wash solution. Centrifuge at 16,000 g for 5 mins
    c. Empty the collection tube and re-centrifuge the column assembly for 1 min with the microcentrifuge lid open to allow evaporation of any residual ethanol
4. Elution
    a. Carefully transfer the minicolumn to a clean 1.5ml microcentrifuge tube.
    b. Add 50 µL of nuclease-free water to the minicolumn. Incubate at room temp for 1 min. Centrifuge at 16,000 g for 1 min
    c. Discard the minicolumn and store the DNA at 4 °C or -20 °C

PCR clean-up:
<u>Reagents needed:</u>
AMPure XP beads (bring to room temp) 45 µL per sample
80% ETOH (prepare fresh) 400 µL per sample
10 mM Tris pH 8.5 22.5 µL per sample
Magnetic bead stand
Dispensing trough (for multichannel pipette)

1. Use 40 µL of the gel extracted PCR product, in PCR tubes/96-well PCR plate for the clean-up
2. Centrifuge the amplicon PCR plate at 1000 g for 1 min (can use little green benchtop spinner; or large benchtop centrifuge if using plate)
3. Vortex AMPure beads for 30 s. Add to dispensing trough
4. Add 45 µL of beads to each PCR sample (use multichannel). Gently pipette up and down 10 times to mix
5. Incubate at room temperature for 5 min
6. Place in magnetic stand for 2 min, or until supernatant has cleared
7. While still in the magnetic stand, remove and discard supernatant (use multichannel)
8. While still in the stand, wash the beads with 80% ETOH
    a. Add 200 µL of ethanol to reaction PCR tube (use multichannel)
    b. Incubate for 30 s
    c. Carefully remove and discard supernatant
9. Repeat a second ETOH wash as above. After this one, use a P20 pipette to remove residual ETOH
10. While still in the magnetic stand, let beads air dry for 10 min (e.g. in biosafety cabinet)
11. Remove amplicon PCR tubes from stand. Add 22.5 µL of 10mM Tris to each PCR tube (use multichannel). Gently pipette up and down 10 times to mix. Make sure beads are fully resuspended.
12. Incubate at room temp for 2 min
13. Place in magnetic stand for 2 min, or until supernatant has cleared
14. Carefully transfer 20 µL of the supernatant into a clean labelled tube. Label this “clean gel extracted index PCR)

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Quantification of DNA using Quantus

Advantage of Quantus – can measure concentrations <2 µg/mL

Reagents:

1 x TE buffer } Quantifluor DNA Working solution (need 100 µL per sample)
Quantifluor dsDNA dye }
DNA standard

Protocol:

NB: if the machine has been calibrated, you don't need to do steps 2, 3 and 7

1. Prepare the Quantifluor DNA Working solution:
   a. Dilute Quantifluor dsDNA dye 1:200 in 1 x TE buffer
2. Prepare blank:
   a. 100 µL of Quantifluor DNA Working solution and 100 µL 1xTE buffer to a 0.5 mL PCR tube
   b. Mix and protect from light
3. Prepare standard:
   a. Add 2 µL of DNA standard to 98 µL 1 x TE buffer
   b. Add 100 µL of Quantifluor DNA Working solution
   c. Mix and protect from light.
4. Prepare unknown:
   a. Dilute your "unknown" samples: 2 µL gDNA and 98 µL 1 x TE buffer
   b. Add 100 µL of diluted unknown sample to 100 µL of Quantifluor DNA Working solution to 0.5 mL PCR tube and mix. Protect from light
   c. Incubate for 5 min at room temperature
5. Reading samples:
   a. Select dsDNA protocol from Quantus Fluorometer
   b. Calibration:
      i. If need to calibrate, read the blank, then standard, then press "save"
   c. Samples:
      i. Enter the vol of unknown and the concentration units (this is the amount of volume added for quantification. Eg if 2 µL mixed with 98 µL of 1 x TE buffer, the volume entered is 2 µL)
      ii. Measure

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Library Quantification, Normalisatoin and Pooling

Calculate the DNA concentration, in nM, based on the size of the DNA amplicons (624 bp)

Formula:

$$\frac{Concentration\ (ng/\mu L)}{660\ g/mol\ \times\ average\ library\ size} \times 10^6 = concentration\ (nM)$$

For us:

$$\frac{Concentration\ (ng/\mu L)}{660\ g/mol\ \times\ 624} \times 10^6 = concentration\ (nM)$$

Protocol:

1. Illumina Instructions: Dilute the DNA to 4 nM, and aliquot 5 µL of this diluted DNA from each sample. i.e.:

$$\frac{Concentration\ (ng/\mu L)}{660\ g/mol\ \times\ 624} \times 10^6 = 4\ nM$$

$$Concentration = 1.65\ ng/\mu L$$

$$Therefore\ 1.65\ ng/\mu L = 4\ nM$$

$$Therefore\ a\ 5\ \mu L\ aliquot = 8.24\ ng$$

$$Therefore\ this\ protcol\ suggests\ 8.24\ ng\ of\ DNA\ for\ each\ sample\ added\ to\ the\ final\ pool$$

2. We have increased the amount to 10ng of DNA in ??l from each sample to the final pool as follows:

$$Sample\ volume + Top\ up\ (water) volume = 5\ \mu L$$

$$\frac{10\ ng}{x\ (concentration\ in\ ng/\mu L)} + y\ volume = 5$$

$e.g. if\ concentration\ of\ DNA\ was\ 2.5\ ng/\mu L, we\ \ add: 10/2.5 + y = 5, which\ \ is\ 4\ \mu L\ DNA\ +$ $1\ \mu L\ water$

3. Dilution and Pooling Instructions:
Based on adding 10 ng DNA (e.g. for 96 samples; change number of samples to suit your particular set up)

$5\ \mu L\ \times 96\ samples = 480\ \mu L\ total\ volume$

$Calculate\ volume\ of\ DNA\ to\ add\ for\ each\ sample\ using\ 10\ ng/x\ ng/\mu L$

$Add\ this\ up\ to\ get\ total\ DNA\ volume\ (it\ is\ okay\ if\ some\ samples\ require >$ $5\ \mu L, as\ long\ as\ the\ total\ volume\ is < 480\ \mu L)$

$Therefore\ add\ 10/x\ volume\ of\ DNA\ for\ each\ sample$

$Then\ top\ up\ with\ water\ i.e. 480\ \mu L - total\ DNA\ volume = \mu L\ of\ water\ to\ add$

$Gives\ room\ to\ add\ more\ than\ 5\ \mu L\ for\ some\ samples$

4. Store at 4 °C or on ice until submission for sequencing (library pooling should be done day before or on day of submission)

EVALUATION METHOD

The Head of Clinical Function Testing will keep the procedure updated.

SUPPORTING DOCUMENTS

Measuring bacterial density on biopsy samples using qPCR

REFERENCES

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