

cpn60 metagenomic amplicon library preparation for the Illumina Miseq platform

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Method Article

Keywords: metagenomics, microbial ecology, Miseq, cpn60, barcode, amplicon sequencing, PCR, microbiome, marker gene

Posted Date: March 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.pex-1438/v1>

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Abstract

This protocol can be applied to determine the composition of a microbial community. The *cpn60* gene (also known as *groEL*, *hsp60*) is present in almost all bacteria and a 552-558 bp region of the gene has been established as a barcode for species level identification of bacteria. The primer cocktail used in this protocol amplifies *cpn60* barcode sequences from bacteria with a wide range of G+C content. Some species of *Mycoplasma* lack the *cpn60* gene and therefore this method is not recommended to detect *Mycoplasma*. DNA sequences generated from this method could be compared to *cpnDB*, a public database of *cpn60* sequences, for identification. Library preparation involves *cpn60* amplicon generation, PCR clean-up, index PCR, index PCR clean-up, library quantification, normalization, pooling, library denaturation and loading. Time taken to complete depends on the number of samples included. If using 96 samples, the procedure takes 8 hours but there are several stages where the samples could be stored and continued the next day. Specific instructions are provided for the Illumina MiSeq platform, but the protocol could easily be adapted for other sequencing platforms.

Introduction

High throughput sequencing of amplified marker gene sequences (barcodes) is a simple and rapid method of assessing the taxonomic composition of a complex microbial community. The “meta-barcoding” approach is often based on amplification of one or more variable regions of the 16S rRNA gene, however, this target often fails to provide species level resolution. The *cpn60* gene (also known as *groEL* or *hsp60*) contains a 552-558 bp variable region (the *cpn60* “universal target”) that can be amplified with “universal” PCR primers and this sequence has been demonstrated to meet the criteria as a barcode for Bacteria [1]. The sequence provides species level resolution and has been shown to be a good predictor of whole genome sequence similarities [2–6]. Since *cpn60* is conserved in Bacteria, Eukaryotes and some Archaea, *cpn60* barcode sequencing can be used for simultaneous detection of multiple Domains of life [7]. A public database of *cpn60* sequences, *cpnDB* (www.cpnDB.ca), facilitates the identification of *cpn60* barcode sequences [8]. As a result of these features, *cpn60* meta-barcode sequencing has been applied to the characterization of a wide variety of host-associated and environmental microbiomes [7, 9–14].

Amplicon sequencing methods are subject to a variety of sources of bias, including amplification primer bias. To minimize this effect of *cpn60* amplification, we developed a primer “cocktail” approach to ensure amplification of *cpn60* sequences from genomes with a wide range of G+C content [15]. Use of a single annealing temperature of 60 °C is appropriate for most samples, although practitioners may want to consider pooling PCR products produced across an annealing temperature gradient in some situations [16].

The protocol presented here is a modification of the Illumina 16S Metagenomic Sequencing Library Preparation (Part #15044223) [17] and describes the production of cpn60 amplicon libraries for sequencing on the MiSeq platform. The length of the amplicon prevents assembly of complete barcode sequences, but we have shown that as little as 150 bp from the 5' end of the barcode sequence is generally sufficient for species level identification [18]. We routinely use a 500 cycle sequencing kit but run it as 401x101 rather than 251x251 and use only the Read 1 sequences in downstream analysis.

Reagents

1. Platinum Taq polymerase (Invitrogen 10966-034)
2. 100 mM dNTPs (Invitrogen 10297-018)
3. Primers M279, M280, M1612, M1613, each at 100 μ M (any supplier)

Forward primers (Illumina adapter sequences are underlined):

M279 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAIIIIIGCIGGIGAYGGIACIACIAC-3'

M1612 5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAIIIIIGCIGGYGACGGYACCSACSAC-3'

Reverse primers (Illumina adapter sequences are underlined):

M280 5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGYKIYKITCICCRAAICCIGGIGC-3'

M1613 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGRCGRTCRC CGAAGCCSGGIGCCTT-3'

(I = inosine, R = A or G, Y = C or T, K = G or T, S = G or C)

4. Molecular biology grade water
5. 80% ethanol, freshly prepared
6. NucleoMag Beads (Macherey-Nagel, 744970.50)
7. Tris 10 mM pH 8.5 (any supplier)
8. Qubit ds DNA BR kit (Invitrogen Q32850)

9. High sensitivity DNA kit (Agilent, 5067-4626)
10. Nextera XT index kit (Illumina, FC-131-2001 or FC-131-2002 or FC-131-2003 or FC-131-2004)
11. KAPA taq (Roche KK2601)
12. 96 well 0.8 mL deepwell plate (Thermo Fisher Scientific, AB0859)
13. 96 well PCR plate (appropriate for PCR machine used)
14. Foil Plate sealers (VWR 60941-126)
15. Pipette tips (filter tips recommended, any supplier)
16. Reagent reservoir (Any supplier)
17. Thin-walled PCR tubes for Qubit (Axygen, PCR-05-C)
18. 1.7 ml microcentrifuge tubes (Axygen, MCT-175-L-C)
19. 2x KAPA HiFi Hot Start Ready Mix (Roche, KK2602)

Equipment

1. Pipettors (P2, P20, P200 and P1000)
2. Multi-channel pipettor
3. 96-well plate magnet (Thermo Fisher, AM10027)
4. Qubit Fluorometer (Invitrogen, Q33216)
5. Bioanalyzer 2100 (Agilent)
6. Plate shaker (VWR, 13500-890)
7. Bench-top centrifuge with rotor to hold 96-well plates
8. Microcentrifuge
9. Thermocycler
10. Water bath
11. MiSeq (Illumina) and Illumina Experiment Manager software
12. TrueSeq Index Plate Fixture (Illumina, FC-130-1005)

Recommended: PCR clean-spot

Procedure

The procedure is presented in 5 steps:

- A. cpn60 PCR
- B. cpn60 PCR clean-up
- C. Index PCR
- D. Index PCR clean-up
- E. Quantification, Normalization and Pooling

A. cpn60 amplicon PCR:

1. Prepare primer cocktail (1:3 molar ratio). Three hundred microliters primer cocktail is sufficient to amplify 300 samples.

3 μ L M279 (100 μ M)

3 μ L M280 (100 μ M)

9 μ L M1612 (100 μ M)

9 μ L M1613 (100 μ M)

276 μ L ultrapure water

(Total volume 300 μ L)

2. Prepare PCR master mix considering one PCR reaction per sample. When calculating the number of samples, include no template control (PCR control), extraction controls (reagent control for DNA extractions) and the positive control.

Per reaction (assuming 2 μ L template volume):

38.1 μL ultrapure water

5 μL 10x PCR buffer

2.5 μL MgCl_2 (50 mM)

0.4 μL Platinum Taq Polymerase

1 μL dNTP mix (10 mM)

1 μL primer cocktail

3. Dispense 48 μL of master mix into the required number of wells of 96-well PCR plate. Add 2 μL template DNA.

4. Seal the PCR plate with foil and centrifuge at 1000 x g for 1 minute at room temperature.

5. Place the sealed PCR plate into the thermocycler.

6. Amplify the cpn60 library following the cpn60 PCR program in a thermocycler:

5 min at 95 °C

40 cycles of (30 sec at 95 °C, 30 sec at 60 °C, 30 sec at 72 °C)

2 min at 72 °C

7. Evaluate the cpn60 PCR products by running the no template control and the positive control amplicons in a 1% agarose gel containing ethidium bromide and visualize using an imaging system. If there is a ~680 bp amplicon observed in the positive control and no amplification in the no template control (Fig. 1), proceed to amplicon clean-up step or store the amplicon PCR plate at 4 °C and proceed to PCR clean up the next day.

B. cpn60 PCR clean-up:

This step is carried out to remove primer-dimers and un-incorporated dNTPs and primers.

1. Bring the amplicon PCR plate and the NucleoMag beads to room temperature.

2. Centrifuge the amplicon PCR plate at 1000 x g at room temperature for 1 minute and remove the plate seal very carefully.

3. Transfer 50 μl of PCR product from the amplicon PCR plate to the deepwell plate using a multi-channel pipettor.
4. Calculate the total volume of NucleoMag beads needed (32.5 μl per sample).
5. Vortex NucleoMag beads for 1 minute to resuspend, and aliquot the required total volume of NucleoMag beads to a reagent reservoir.
6. Add 32.5 μl of NucleoMag beads to each well of the deepwell plate using a multi-channel pipette, changing tips after each transfer to prevent cross-contamination between samples.
7. Seal the deepwell plate with a foil sealer and shake it in the plate-shaker at 1800 rpm for 2 minutes. (If a plate-shaker is not available, pipetting up and down 10 times using a multi-channel pipet is an alternative method for mixing)
8. Incubate the plate at room temperature for 5 minutes.
9. Put the deepwell plate on the magnetic stand for 2 minutes until the supernatant is clear.
10. Using a multi-channel pipette, remove the supernatant while the PCR clean-up plate is sitting on the magnet. Change the tips in between the samples. If the beads get disturbed during the pipetting process, wait until they re-settle. Never throw away beads.
11. Add sufficient volume of freshly prepared 80% ethanol (200 μl /well) to a reagent reservoir.
12. While the plate is sitting on the magnet, add 200 μl of ethanol to each well of the PCR clean-up plate using a multi-channel pipette.
13. Incubate the PCR clean-up plate on the magnetic stand for 30 seconds.
14. While the PCR clean-up plate is still sitting on the magnet, remove the supernatant very carefully using a mutichannel pipette.
15. Perform a second wash by repeating steps 12-14.
16. Using a P20 tip, remove all the residual ethanol while the PCR clean-up plate is sitting on the magnet. Change tips between wells.
17. Leave the PCR-clean up plate on the magnet for 10 minutes to dry the beads.
18. Add sufficient volume of 10 mM Tris pH 8.5 to a reagent reservoir (52.5 μL /well).
19. Elute the amplicon DNA from the beads by removing the PCR clean-up plate from the magnet and adding 52.5 μl of 10mM Tris pH 8.5 to each well using a multi-channel pipette.
20. Seal the PCR clean-up plate using a foil plate sealer.

21. Shake the PCR clean-up plate using the plate shaker at 1800 rpm for 2 minutes. (If a plate-shaker is not available, pipetting up and down 10 times using a multi-channel pipet is an alternative method for mixing).
22. Incubate the PCR clean-up plate at room temperature for 2 minutes.
23. Return the PCR clean-up plate to the magnet for 2 minutes until the supernatant is clear.
24. Label a new PCR plate as “NucleoMag bead cleaned cpn60 amplicon”.
25. While the PCR clean-up plate is sitting on the magnet, use a multi-channel pipette to transfer 50 µl of supernatant from each well to the newly labelled plate. Change the tips in between the samples. If the beads get disturbed during the pipetting process, wait until they settle.
26. Evaluate the cpn60 amplicons by running a randomly selected subset of NucleoMag cleaned amplicons in a 1% agarose gel. If there is a ~680 bp amplicon observed in the samples with the absence of primer dimers (Fig. 2), proceed to the index PCR step or store the plate at -20 °C for up to 1 week.

C. Index PCR

(From the Illumina *16S metagenomics sequencing library preparation protocol*, Part #15044223)

1. Assign the indexes against the samples using Illumina Experimental Manager software and print the template (Fig. 3)
2. Bring the index primers, KAPA HiFi HotStart Ready Mix and the NucleoMag cleaned amplicon plate from Step B-25 to room temperature.
3. Vortex the index primers and centrifuge briefly to settle. Arrange the index primers in the True Seq Index Plate Fixture as shown in the template in Fig. 3.
4. Label a fresh 96-well PCR plate as “Index PCR”.
5. Transfer 5 µl of each cleaned cpn60 PCR product to the index PCR plate using a multi-channel pipet.
6. Store the remaining NucleoMag bead cleaned indexed cpn60 amplicons at -20 °C.
7. Add 5 µl of Nextera XT Index primer 1 (Nxxx) to the appropriate wells according to the Illumina Experimental Manager Template (Fig. 3). Re-cap the index primer tube with a new orange cap provided with the index kit.
8. Add 5 µl of Nextera XT Index primer 2 (Sxxx) to the appropriate wells according to the Illumina Experimental Manager Template (Fig. 3). Re-cap the index primer tube with a new white cap provided with

the index kit.

9. Add 25µl of 2x KAPA HiFi HotStart Ready Mix each well.
10. Add a sufficient volume of molecular biology grade water to a reagent reservoir (10 µL/well).
11. Add 10 µl of molecular biology grade water to each well using a multi-channel pipette, pipetting up and down 10 times to mix.
12. Cover the plate with a PCR plate sealer.
13. Centrifuge the plate at 1000 x g for 1 minute at room temperature.
14. Place the PCR plate in the thermocycler.
15. Run the Index PCR program:
5 min at 95 °C
8 cycles of 30 sec at 95 °C, 30 sec at 55 °C and 30 sec at 72 °C
5 min at 72 °C
Hold at 4°C

D. Index PCR clean-up

1. After completion of the index PCR program, centrifuge the index PCR plate at 1000 x g for 1 minute at room temperature.
2. Transfer 50 µl of indexed PCR product from the index PCR plate to a fresh 96-well deepwell plate (PCR clean-up plate) using a multi-channel pipette.
3. Calculate the total volume of NucleoMag beads needed (32.5µl per sample).
4. Vortex the NucleoMag beads for 1 minute to resuspend and then add the required amount of NucleoMag beads to a reagent reservoir.
5. Add 32.5µl of NucleoMag beads to each well using a multi-channel pipette, changing tips between samples.
6. Seal the PCR clean-up plate with a foil sealer and shake it in the plate shaker at 1800 rpm for 2 minutes. (Pipetting up and down 10 times using a multi-channel pipet is the alternative method in the absence of a plate shaker)

7. Incubate the plate at room temperature for 5 minutes to allow the amplicons to bind to the beads.
8. Put the PCR clean-up plate on the magnetic stand for 2 minutes until the supernatant is clear.
9. While the PCR clean-up plate is sitting on the magnet, use a multi-channel pipette to remove the supernatant. Change tips between samples. If the beads get disturbed during the pipetting process, wait until they settle. Never throw away beads.
10. Add sufficient volume of freshly prepared 80% ethanol to a reagent reservoir (200 μ L/well).
11. While the PCR clean-up plate is sitting on the magnet, add 200 μ l of 80% ethanol to each well using a multi-channel pipette.
12. Incubate the PCR clean-up plate on the magnetic stand for 30 seconds.
13. While the PCR clean-up plate is sitting on the magnet, carefully remove the supernatant using a multi-channel pipette.
14. Perform a second wash by repeating steps 11-13.
15. While the PCR clean-up plate is sitting on the magnet, use a P20 tip remove all the residual ethanol. Change tips between wells.
16. Leave the PCR-clean up plate on the magnet for 10 minutes to dry the beads.
17. Add sufficient volume of 10 mM Tris pH 8.5 to a reagent reservoir (27.5 μ L.well).
18. To elute the indexed amplicons from the beads, remove the PCR clean-up plate from the magnet and add 27.5 μ l of 10mM Tris pH 8.5 to each well using a multi-channel pipette.
19. Seal PCR clean-up plate using a foil plate sealer.
20. Shake PCR clean-up plate using the plate shaker at 1800 rpm for 2 minutes. (If a plate-shaker is not available, pipetting up and down 10 times using a multi-channel pipet is an alternative method for mixing).
21. Incubate PCR clean-up plate at room temperature for 2 minutes.
22. Place the PCR clean-up plate on the magnet for 2 minutes until the supernatant is clear.
23. Label a new PCR plate as "NucleoMag bead cleaned indexed cpn60 amplicon" and the date.
24. While the PCR clean-up plate is sitting on the magnet, transfer 25 μ l of supernatant to the newly labelled plate using a multi-channel pipette and changing tips between samples. If the beads get disturbed during the pipetting process, wait until they settle.

25. Evaluate the indexed cpn60 PCR product by running a randomly selected subset of NucleoMag cleaned indexed cpn60 amplicons in a 1% agarose gel (see Fig. 2). Alternatively, dilute selected amplicon libraries 1:50 and assess on a Bioanalyzer DNA High Sensitivity DNA chip (Fig. 4). If there is a ~730 bp amplicon observed with the absence of primer dimers (~200 bp), proceed immediately to the library quantification and pooling step (E) or store the cleaned-up indexed PCR products at -20 °C for up to 1 week.

E. Library Quantification, Normalization and Pooling

1. Bring the NucleoMag bead cleaned indexed cpn60 amplicon libraries from D to room temperature.
2. Determine the concentration of each amplicon library using the Qubit ds BR kit.
3. Determine the indexed PCR product size from the Bioanalyzer results from step D-25
4. Calculate the DNA concentration in nM using the below formula as shown in Illumina 16S metagenomics sequencing library preparation protocol:

library concentration in nM = [(DNA concentration or Qubit value in ng/μl) x 10⁶] / (660 g/mol x 748)

5. Adjust the concentration of each library to 4 nM with 10mM Tris pH 8.5 in 1.7 ml microcentrifuge tubes.
6. Store the remaining Indexed bead cleaned cpn60 amplicons at -20 °C.
6. Label a 1.7 ml microfuge tube as PAL (pooled amplicon library)
7. Add 5 μl from each diluted library to the PAL tube.
8. Follow the "Library Denaturation and MiSeq sample loading" section of the Illumina 16S metagenomics sequencing library preparation protocol. A 10 pM library with 5% phiX is recommended.

Troubleshooting

Amplification in the no template control: *Repeat cpn60 PCR.*

No amplification of positive control: *Check the PCR program on the thermocycler and mastermix composition before repeating cpn60 PCR.*

No visible cpn60 amplicon after PCR clean-up (step B): *Repeat cpn60 PCR and clean-up.*

Presence of primer-dimers after PCR clean-up (step B): *Repeat clean-up.*

Presence of primer-dimers after index PCR clean-up (step D): *Repeat clean-up.*

Time Taken

The complete protocol takes ~ 8hrs but there are several steps at which the procedure can be paused.

Anticipated Results

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Acknowledgements

The authors are grateful to all members of the Hill Lab past and present, and to our collaborators who have contributed to the ongoing development of cpn60-based methods.

Figures

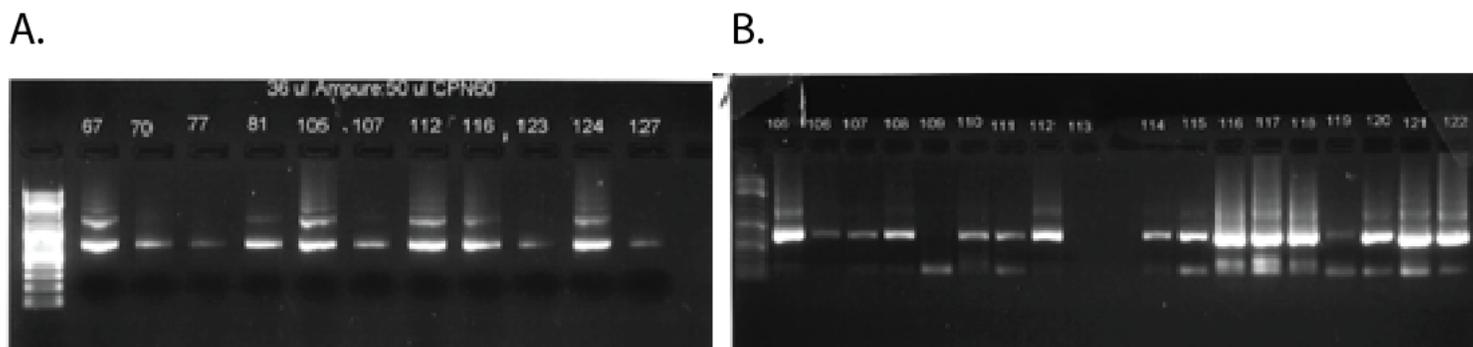


Figure 1

Results of cpn60 PCR with expected product of 650 bp. NTC = no template control.

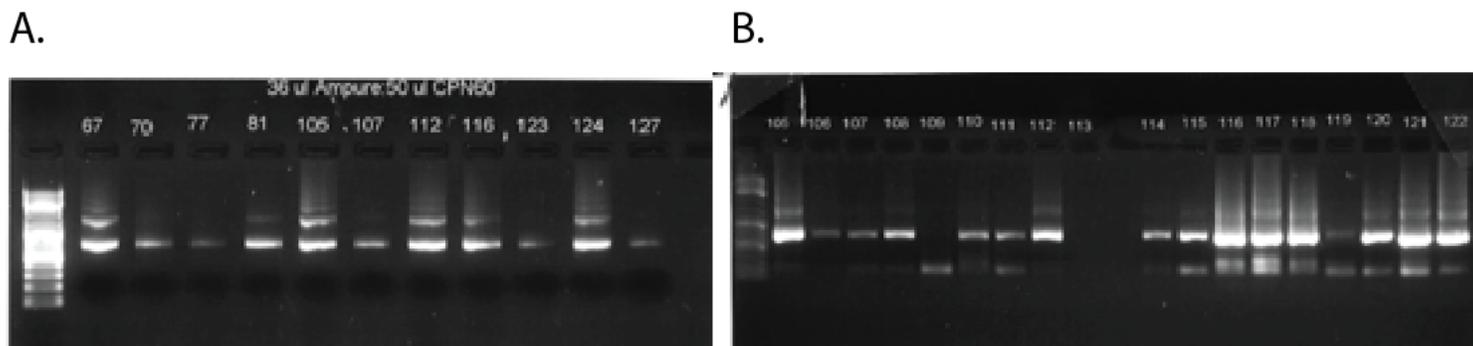


Figure 2

Results of cpn60 PCR with expected product of 650 bp. NTC = no template control.

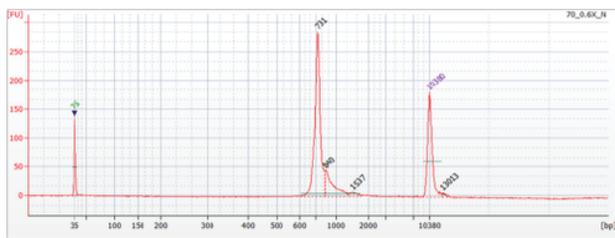
HPV-HIV-0380_0428

	1	2	3	4	5	6	7	8	9	10	11	12
A	332 S502 N701	333 S502 N702	334 S502 N703	335 S502 N704	336 S502 N705	337 S502 N706	338 S502 N707	339 S502 N710				
B	380 S503 N701	381 S503 N702	382 S503 N703	386 S503 N704	388 S503 N705	389 S503 N706	390 S503 N707	391 S503 N710	392 S503 N711			
C	396 S505 N701	397 S505 N702	398 S505 N703	399 S505 N704	400 S505 N705	401 S505 N706	402 S505 N707	403 S505 N710				
D	404 S506 N701	405 S506 N702	406 S506 N703	407 S506 N704	408 S506 N705	409 S506 N706	410 S506 N707	411 S506 N710				
E	372 S507 N701	413 S507 N702	376 S507 N703	415 S507 N704	416 S507 N705	417 S507 N706	418 S507 N707	419 S507 N710				
F	420 S508 N701	421 S508 N702	422 S508 N703	423 S508 N704	424 S508 N705	425 S508 N706	426 S508 N707	427 S508 N710	428 S508 N711			
G	NTC3 S510 N701	EC5 S510 N702	EC6 S510 N703	EC7 S510 N704								
H												

Figure 3

Example of Illumina index plate template.

A.



B.

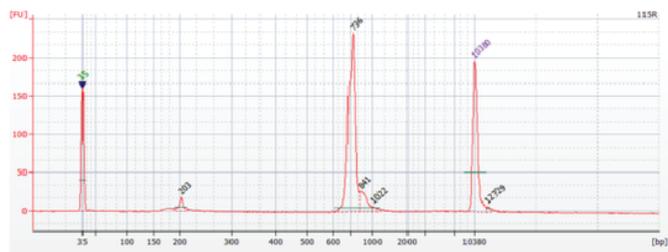


Figure 4

Bio analyzer results for a cpn60 amplicon library. (A) Successful library with expected major peak with apparent size of ~730 bp, and without primer dimers (B) Small peak at ~200 bp is indicative of primer dimer contamination. This library is not suitable for sequencing. Repeat clean-up.