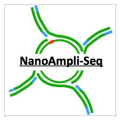

NanoAmpli-Seq - Sample processing and sequencing library preparation workflow

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ABSTRACT

TAGS

sequencing

amplicon

Show tags

EXTERNAL LINK

https://www.biorxiv.org/content/early/2018/01/07/244517

PROTOCOL STATUS

Working

We use this protocol in our group and it is working very well.

GUIDELINES

Workflow was designed and optimised for sample preparation of 16S rRNA gene amplicons with the use of random hexamer-free Rolling Circle Amplification followed by MinION MK1b sequencing.

However, this protocol can be customised for amplification of any prokaryotic and eukaryotic gene from ~200 to ~2000bp.

MATERIALS

NAME	CATALOG #	VENDOR
T7 Endonuclease I - 250 units	M0302S	New England Biolabs
Blunt/TA Ligase Master Mix - 50 rxns	M0367S	New England Biolabs
NEBNext FFPE DNA Repair Mix - 24 rxns	M6630S	New England Biolabs
Magnetic stand for micrcentrifuge tubes	12321D	Life Technologies
ethanol		
NEBNext Ultra II Q5 Master Mix - 50 rxns	M0544S	New England Biolabs
NEBNext End repair / dA-tailing Module (E7546)		
Corning® Filtered Pipette Tips, 1000 µL 1000 Tips	38031	Stemcell Technologies
Corning® Filtered Pipette Tips, 200 µL 960 Tips	38032	Stemcell Technologies
Corning® Filtered Pipette Tips, 10 µL 960 Tips	38034	Stemcell Technologies
Qubit dsDNA HS Assay Kit	Q32851	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
DNA LoBind Tubes	#022431021	Eppendorf
PCR tubes, strips or plates		
HighPrep™ PCR	AC-60050	
Plasmid-SafeTM ATP-Dependent DNAse	E3101K	Epicentre
Sygnis TruePrime™ RCA Kit	SYG390100	Lucigen
g-TUBE	520079	Covaris
1D² Sequencing Kit	SQK-LSK308	
Flow Cell R9.5	View	
Molecular Grade Water	60-2450	ATCC

MATERIALS TEXT

Equipment required:

1. PCR thermocycler from any vendor
2. PCR cabinet/hood with UV sterilization
3. Thermal mixer with appropriate blocks from any vendor
4. Pipettes with varying volumes range from any vendor
5. Centrifuge for 2 ml and 0.2 ml tubes from any vendor
6. MinION^{TM} MK1b device and compatible personal computer

Primers for PCR amplification of 16S rRNA gene can be ordered from any provider.

SAFETY WARNINGS

BEFORE STARTING

Make sure you have all necessary equipment, reagents and PCR primers before begining the protocol.

For preparation of multiple samples please, use 'START EXPERIMENT' then 'SCALE PROTOCOL'.

PCR amplification of 16S rRNA gene

1 Master Mix - combine the following reagents using volumes below:

- **9.9 µl Molecular Grade Water**
- **12.5 µl NEBNext Ultra II Q5 Master Mix**
- **0.8 µl of 10µM primer: Forward_PHO+**
- **0.8 µl of 10µM primer: Reverse_PHO+**

Total volume: **24 µl of reagents** in **2ml tube**.

Aliquot **24µl of Master Mix** reagents in **0.2ml PCR tubes** then add **1µl of DNA** to each tube.
Final volume: **25µl of reagents** in **0.2ml PCR tubes**.

Incubate the reaction at the thermocycler according to the following conditions:

- **00:00:30 sec** — **98 °C Initial denaturation**
- **00:00:05 sec** — **98 °C Denaturation**

00:00:10 sec | 62 °C Annealing
00:00:35 sec | 72 °C Extension
00:02:00 min | 72 °C Final extension
00:00:00 | 8 °C Hold

This PCR assay requires **20 cycles** of Denaturation, Annealing and Extention.

During the PCR assay running go to Step 2 (prepare 70% ethanol) then to Step 3 (prepare Qubit reagents).
Finally remove tubes from PCR machine and continue with Step 2.

NOTE
Amplification of samples can be performed in duplicates or triplicates as use of replicates will help reduce PCR biases.

Make sure both primers are 5' PHO positive. If protocol is used for the first time or was modified to amplify different gene/s than 16S rRNA, we recommend verifying the results of the PCR assay. To confirm the correct size of the amplicons, we advise using standard agarose gel or automated capillary electrophoresis.

PCR product clean up

2 Prepare **5-10ml of 70% ethanol** depending on the number of samples, e.g. **3.5ml of 100% ethanol** with **1.5ml of Molecular Grade Water** and keep it on ice.

Combine replicates (3x20µl) into single **2ml tube**, then add **30µl of magnetic beads** (0.5x ratio) to the **60µl of PCR product** and incubate for **00:02:00 min at room temp**. The place tube in the magnetic rack, allow beads to set then discard the liquid. Subsequently, wash the pellet twice with **200µl of freshly prepared ice-cold 70% ethanol**. Discard the ethanol and briefly centrifuge the tube at low speed, place the tube back on the magnetic rack, remove residuals of the ethanol and place the tube at the heat block for around **00:00:10 sec at 50°C**. Be careful not to overdry the beads. Use **20µl Molecular Grade Water** kept at the heat block **50 °C** to resuspend the beads, incubate the tube for **00:02:00 min at room temp**. Then place the tube back on the rack, allow beads to set and transfer the **20µl PCR product** into a fresh **2ml tube**.

NOTE
To expand the lifespan of HighPrep™ reagents and reduce the chance of contaminating 50ml stock reagents, we recommend to aliquot the beads in 2ml tubes, e.g. 1500µl. That will decrease a need for moving the stock reagents from the fridge every time a small volume of magnetic beads is used and reduce a time needed for the reagents to reach room temperature.

PCR product concentration estimation

3 Prepare Qubit™ dsDNA HS reagents and related standards, according to the vendor instructions (1).
i.e., **199µl dsDNA HS Buffer** with **1 µl dsDNA HS Reagent**
e.g., **995µl dsDNA HS Buffer** and **5µl dsDNA HS Reagent** for 5 reactions

To determine the concentration of cleaned amplicons combine:
1µl of PCR product with **199µl Qubit reagents**.

EXPECTED RESULT
The concentration of samples should be ~50ng in a total (depending on assay efficiency) of cleaned amplicons per sample.
1) https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_HS_Assay_UG.pdf

Self-ligation for the formation of plasmid-like structure

4 If multiple amplicon pools are being used, normalized their concentration to 2ng/µl and transfer 45µl of each sample to new **0.2ml PCR grade tube**. Then add **5µl of Blunt/TA Ligase Master Mix** to the **0.2ml PCR** containing purified amplicons. Gently mix the reagents by pipetting up and down and incubate the tube for **00:15:00 min at 10°C** and **00:10:00 min at 25°C** in a PCR thermocycler (make sure lid heating is turned off).

Initate Step 5 while the ligation reaction is ongoing in the thermocycler.

Reverse phase cleanup

5 Vortex HighPrep™ reagents and transfer **150µl of magnetic beads** into a clean **2ml tube**. Place the tube on a magnetic rack for **00:02:00 min** or until beads separate from the liquid. While the tube is on the magnetic rack, remove **90µl of clear buffer** without disturbing the beads and place it inside the lid of the **2ml tube**. Then discard remaining **60µl** of the buffer without disturbing the beads. Subsequently, transfer exactly **75µl of clear buffer** (concentrate beads by 50%) from the lid into the tube and discard the remaining wastes from the tube's lid. Remove the tube from magnetic rack and gently vortex to resuspend the beads. Keep 50% **concentrated beads** at room temperature until needed.

Add precisely **17.5µl concentrated beads** (0.35x ratio) to **50µl of a self-ligation mix** from Step 4. Gently mix the tube by pipetting up and down then, incubate the mixture for **00:02:00 min at room temp**. Place the tube on a magnetic rack and allow the beads to separate. The beads will bind to long linear amplicons (i.e., chimeric amplicons) while the liquid contains short linear amplicon and plasmid-like structures. Finally, transfer **67.5µl of clear liquid** to the new **2ml tube**.

Remaining **57.5µl of concentrated beads** will be used at Step 11 and 13 until that time keep the beads at room temp.

NOTE

Concentration of beads by 50% described for Step 5 has to be done every time reaction is performed. We do not recommend preparing a high volume of concentrated beads and storing them. Magnetic beads stored at high-concentration (i.e.-50%) for a long time clump and lose their efficiency.

SAFETY INFORMATION

This approach of concentrating magnetic beads was optimised to remove long DNA structures i.e. >2000bp. However, the beads need to be prepared fresh and mixed with an **exact volume of PCR products**. That is why we recommend **using a pipette wheel** to measure the volume of liquids and **recalculate the number of reagents** added to the tube when necessary. This approach of concentrating beads is an improved version of (Additional file 6) https://doi.org/10.1186/1471-2164-15-645

Plasmid and short amplicon clean-up

6 Add **33.75µl of magnetic beads** (0.5x ratio) to **67.5µl of clear liquid** from Step 5, gently vortex and incubate the tube for **00:02:00 min at room temp**. Place the tube on the magnetic rack and allow beads to set, discard the liquid. Subsequently, wash the pellet twice with **200µl of freshly prepared ice-cold 70% ethanol**. Discard the ethanol and briefly centrifuge the tube at low speed, place tube back in the magnetic rack, remove residuals of the ethanol and place the tube at the heat block for around **00:00:10 sec at 50°C**. Remember to do not overdry the beads. Use **15µl of Molecular Grade Water** kept at the heat block **50 °C** to resuspend the beads, incubate the tube for **00:02:00 min at room temp**. Then place the tube back on the rack, allow beads to set and transfer the **15µl of PCR product** into a fresh **2ml tube**.

Removal of linear molecules from plasmid mix

7 Combine the following reagents using volumes below:

15µl of **self-ligated and purified amplicons**
2µl of **Molecular Grade Water**
2µl of **25mM ATP**
5µl of **10x Reaction Buffer**
1µl of **Plasmid-Safe DNase (10U)**

Total volume: **25µl** of **reagents** in **0.2ml PCR grade tube**.

For mini-preparation, incubate the reaction in a thermocycler according to the following conditions:

37 °C Incubation **00:15:00 min**

After incubation, clean the products as described in Step 6 (elute in **10µl of Molecular Grade Water**) and determine the concentration of DNA in the cleaned product as described in Step 3.

NOTE

Do not deactivate the Plasmid-safe DNase enzyme with 30min incubation at 70°C as recommended in the protocol. This process would take additional 30min. The subsequent magnetic bead cleanup will remove ATP, Reaction Buffer, nucleotide debris and active DNase enzyme.

Reagent volumes are followed according to a mini-preparation protocol (2), for higher volumes, please check Lucigen and Epicentre instructions.
2) https://www.lucigen.com/docs/manuals/MA044E-PlasmidSafe-DNase.pdf

Rolling Circle Amplification (RCA)

8 Perform RCA in triplicate for each pooled sample and include negative controls using **Molecular Grade Water** instead of cleaned product from Step 7. All reagents are included in the TruePrime™ RCA kit and should be kept on ice unless specified otherwise.

#1 Below are conditions and volumes for a 1 and 5 RCA reactions:
Combine **3µl of cleaned product** from Step 7 with **2.5µl of Buffer D** (provided with TruePrime™ RCA kit) in **0.2ml PCR grade tube.** Pipette up and down to mix and incubate at room temperature for **3-5 minutes.**

#2 While the sample is being incubated, prepare the amplification mix and keep on ice until needed.

1 sample	5 samples	Reagents
9.3µl	**46.5µl**	of **Molecular Grade Water**
2.5µl	**12.5µl**	of **Reaction buffer**
2.5µl	**12.5µl**	of **dNTPs**
2.5µl	**12.5µl**	of **Enzyme 1**
0.7µl	**3.5µl**	of **Enzyme 2**
Total volume: **17.5µl**	**87.5µl**	of **Amplification Mix**

#3 After **3-5 minutes**, add **2.5µl of Buffer N** to **5µl of incubated sample #1** and pipette up and down to mix.

Finally, add **17.5µl of amplification mix #2** to the **7.5µl of DNA mix #3**, pipette up and down to homogenise the reaction then incubate the **0.2ml PCR grade tube** at **29.5°C** on a heat block or thermocycler for **120-150 minutes** - depending on assay efficiency.

After **90min**, the efficiency of the assay can be tested according to Step 3. If RCA resulted in appropriate concentration of amplification product, then the amplification mix can be followed by enzymatic fragmentation (Step 9). However, if the concentration of the RCA products is lanow, then reagents c incubated for another **30-60min** and quantified according to Step 3.

Incubation time takes around 2 hours. Take a break!

NOTE

Reagent volumes are used according to TruePrime™ RCA kit (3). An increase of incubation temperature will boost the efficiency of phi29 polymerase, however, that in turn will trigger an increase of unspecific product formation in the negative controls. Higher incubation temperatures (i.e. 32-36°C) may be investigated to reduce incubation time (by 40-60%) and shorten overall workflow. However, we recommend to perform reaction at ~29.5°C that makes the RCA protocol slightly longer (120-150min) but more reliable as negative control sample will have no unwanted amplification.

3) https://p2v6h7b4.stackpathcdn.com/wp-content/uploads/2018/01/TruePrime%E2%84%A2-RCA-Kit.pdf

EXPECTED RESULT

After 120-150min, the concentration of samples should be ~30-40ng/µl that in turn gives:
25µl x triplicate x 30-50ng = 2250-3000ng in total and is sufficient for subsequent steps.
The bare minimum concentration of RCA product at this step is 2000ng before fragmentation.

** If the protocol is used for the first time or was modified to amplify different gene/s than 16S rRNA, we recommend verifying the results of the RCA assay. To confirm correct assay efficiency, we advise using standard agarose gel or automated capillary electrophoresis.

First step enzymatic de-branching

9 Combine all three **21µl RCA replicates** from Step 8 into a single **0.2ml PCR grade tube**. Mix **63µl of RCA product** with **2µl of T7 endonuclease I** and mix with use of wide bore tips then incubate for **00:05:00 min at room temp** .

Mechanical fragmentation

10 Transfer **65µl of RCA product** into a g-TUBE using wide bore pipette tips. Centrifuge the tube for **00:03:00 min at 1800rpm** or until the entire reaction mix passes through the fragmentation hole. Reverse the g-TUBE and centrifuge it for **00:03:00 min at 1800rpm** or until the entire reaction mix passes through the fragmentation hole.

Cleanup of fragmented RCA products

11 The **57.5µl of concentrated beads** from Step 5 is used for the cleanup of RCA fragmented products.

Gently vortex the concentrated beads (~50%) and add exactly **22.75µl of concentrated beads** (0.35x ratio) to **65µl of RCA fragmented products** (or recalculate the volume of concentrated beads if necessary). Mix the tube by pipetting up and down then, incubate the mixture for **00:02:00 min at room temp** . Subsequently, place the tube on a magnetic rack and allow the beads to separate. The beads contain long linear, concatemerized amplicons while the liquid contains short (<2000bp) fragments. Subsequently, wash the pellet twice with **200µl of freshly prepared ice-cold 70% ethanol**. Discard the ethanol and briefly centrifuge the tube at low speed, place back the tube at the magnetic rack, remove residuals of the ethanol and place the tube at the heat block for around **00:00:10 sec at 50°C** . Remember to do not overdry the beads. Use **65µl of Molecular Grade Water** kept at the heat block **50 °C** to resuspend the beads, incubate the tube for **00:02:00 min at room temp** . Then place the tube back on the rack, allow beads to set and transfer the **63µl of RCA product** into a fresh **2ml tube**. Remaining **2µl of RCA product** can be used for Qubit dsDNA HS assay.

Remaining **34.75µl of concentrated beads** will be used at Step 13 until that time keep the beads at room temp.

Secondary enzymatic de-branching

12 Mix **63µl of fragmented RCA products** with **2µl of T7 endonuclease I** and incubate for **00:05:00 min at 37°C** .

Cleanup of post fragmented RCA products

13 The **34.75µl of concentrated beads** from Step 11 is used for cleanup of RCA fragmented products at this stage.

Add precisely **29.25µl of concentrated beads** (0.45x ratio) to **65µl of RCA fragmented products** (or recalculate the volume of concentrated beads if necessary). Gently mix the tube by pipetting up and down then, incubate the mixture for **00:02:00 min at room temp** . Then, place the tube on a magnetic rack and allow the beads to separate. The beads contain long linear, concatemerized amplicons while the liquid contains short fragments. Subsequently, wash the pellet twice with **200µl of freshly prepared ice-cold 70% ethanol**. Discard the ethanol and briefly centrifuge the tube at low speed, place the tube back on the magnetic rack, remove residuals of the ethanol and place the tube at the heat block for sround **00:00:10 sec at 50°C** . Remember to do not overdry the beads. Use **55µl of Molecular Grade Water** kept at the

heat block 50 °C to resuspend the beads, incubate the tube for 00:02:00 min at room temp. Then place the tube back on the rack, allow beads to set and transfer the **53µl RCA product** into a fresh **0.2ml PCR grade tube**. Remaining **2µl of RCA product** can be used for **Qubit dsDNA HS assay** or verification of size fragments**.

NOTE
** If the protocol is used for the first time or was modified to amplify different gene/s than 16S rRNA, we recommend verifying the results of the PCR assay. To confirm the correct size of the amplicons, we advise using standard agarose gel or automated capillary electrophoresis.

Gap-filling and dA-tailing of fragmented RCA products

14 Combine the following reagents using volumes below:

53µl of **RCA product**
3.5µl of **FFPE DNA Repair Buffer**
3.5µl of **NEBNext Ultra II End Prep Buffer**
2µl of **NEBNext FFPE DNA Repair Mix**
3µl of **Ultra II End Prep enzyme mix**

Total volume: **65µl** in **0.2ml PCR grade tube.**

Mix the reaction mix by pipetting gently up and down (10-times) using wide bore tips then incubate the reaction at the thermocycler according to the following conditions:

20 °C 00:10:00 min
65 °C 00:10:00 min
4 °C 00:00:00 Hold

NOTE
Reagent volumes are followed according to NEBNext® FFPE DNA Repair Mix
https://www.neb.com/-/media/catalog/datacards-or-manuals/manualm6630.pdf - page 5

Cleanup of end-repaired and dA-tailed RCA products

15 Add **32.5µl of magnetic beads** (0.5x ratio) to **65µl of RCA product** from Step 14, gently vortex and incubate the tube for 00:02:00 min at room temp. After that time place tube on the magnetic rack and allow beads to set, discard the liquid. Subsequently, wash the pellet twice with **200µl of freshly prepared ice-cold 70% ethanol**. Discard the ethanol and briefly centrifuge the tube at low speed, place back the tube at the magnetic rack, remove residuals of the ethanol and place the tube at the heat block for around 00:00:10 sec at 50°C. Remember to do not overdry the beads. Use **35µl of Molecular Grade Water** kept at the heat block 50 °C to resuspend the beads, incubate the tube for 00:02:00 min at room temp. Then place the tube back on the rack, allow beads to set and transfer the **33µl of RCA product** into a fresh **2ml tube**.

Follow Step 3 to determine the concentration of DNA in samples and negative controls.

Library preparation and nanopore sequencing (Fig.1I)

16 Prepare **1D^2 libraries** for nanopore sequencing with **SQK-LSK308** kit by Oxford Nanopore Technologies (4).

Combine the following reagents using volumes below:

33µl of **~500ng RCA products** from Step 15
2.5µl of **1D^2 Adapter**
14.5µl of **Blunt/TA Ligase Master Mix**

Total volume: **50µl** in a **2ml tube**

Homogenise the reagents by pipetting gently up and down (10-times) using wide bore tips then incubate for 00:10:00 min at room temp. Then add **24µl of magnetic beads (0.4x ratio)** and incubate it for

00:02:00 **min at room temp** . Subsequently, place the tube on the magnetic rack and allow beads to set, discard the liquid. Wash the pellet twice with **200µl of freshly prepared ice-cold 70% ethanol**. Discard the ethanol and briefly centrifuge the tube at low speed, place back the tube at the magnetic rack, remove residuals of the ethanol and place the tube at the heat block for 00:00:10 **sec at 50°C** . Use **46µl of Molecular Grade Water** kept at the heat block 50 °C to resuspend the beads, incubate the tube for 00:02:00 **min at room temp** . Then place the tube back on the rack, allow beads to set and transfer the **45µl of RCA product** into a fresh **2ml tube**.

Combine the following reagents using volumes below:

45µl of **1D^2 adapted DNA**
5µl of **Barcode Adapter Mix (BAM)**
50µl of **Blunt/TA ligase**
Total volume: **100µl** in a **2ml tube**

Homogenise the reagents by pipetting gently up and down (10-times) using wide bore tips then incubate for 00:10:00 **min at room temp** . After that time add **40µl of magnetic beads (0.4x ratio)** and incubate it for 00:02:00 **min at room temp** . Subsequently, place the tube on the magnetic rack and allow beads to set, discard the liquid. Wash the pellet twice with **140µl of ABB buffe**r. Discard the ABB buffer and briefly centrifuge the tube at low speed, place the tube back in the magnetic rack, remove residuals of the ABB buffer. Use **15µl of Elution Buffer** kept at the room temperature to resuspend the beads, incubate the tube for 00:02:00 **min at room temp** . Then place the tube back on the rack, allow beads to set and transfer the **15µl of Elution Buffer** into a fresh **2ml tube**.

EXPECTED RESULT

Eluted 1D^2 libraries can be quantified with the use of Qubit reagents according to Step 3.
Expected concentration should be around 8-10ng/µl or 120-150ng in total.

Prime **R9.5 nanopore flow cell** (suitable for 1D^2 libraries) using protocols outlined by Oxford Nanopore Technologies (4). Load the libraries on the device and initiate 48h sequencing process.

NOTE

Some of the reagent volumes at Step 16 have been modified when compared to 1D^2 genomic protocol.
4) https://community.nanoporetech.com/protocols/1d%5E2-genomic-sequencing/v/lsd_9032_v11_revo_23mar2017/checklist-protocol