Ultra-long read sequencing protocol for RAD004 Version 3

Josh Quick

Abstract

The intention of this protocol is to isolate high molecular weight DNA. This means you should avoid any pipetting without using a wide-bore or cut off pipette tip, vortexing, mixer shakers or anything else which generate a velocity gradient which may shear the DNA. In addition you should be very careful not to introduce nucleases by making up buffers with nuclease-free water. Avoid unnecessary heating and do not freeze, isolated DNA should be stored in the fridge, a good extraction will be stable for months. Currently tested on *E. coli* and human cell lines, however it is likely to work with many gram-negative bacteria and mammalian cells.

Citation: Josh Quick Ultra-long read sequencing protocol for RAD004. **protocols.io**
dx.doi.org/10.17504/protocols.io.mrxc57n
Published: 22 Jan 2018

Guidelines

Acknowledgements:

This protocol was developed by Josh Quick for the Nanopore WGS Consortium (https://github.com/nanopore-wgs-consortium). Updating this protocol to keep it working with each new version of the rapid kit would not be possible without the contributions of Nick Loman, Matt Loose and John Tyson. We would also like to thank David Stoddart, Simon Mayes and Daniel Turner at Oxford Nanopore for their support. Please follow on Twitter for latest updates and results:

@Scalene

@pathogenomenick

@mattloose

@DrT1973

Citation:

https://doi.org/10.1101/128835.

Protocol Reagents:

TLB:

100 mM NaCl

10 mM Tris-Cl, pH 8.0

25 mM EDTA, pH 8.0

0.5% (w/v) SDS

20 µg/ml Qiagen RNase A (add fresh just before use)

EB+Triton-X100:

10 mM Tris-Cl pH 8.0, 0.02% Triton X-100

Expected Results for Human:

The NA12878 data in rel4 was generated using RAD002 kits. We put a lot of effort into reproducing these run metrics using the updated RAD003 kits and although we discovered many optimisations to the standard protocol, read lengths in particular fell short of what we had previously generated. The RAD004 kit featured changes to the tethering chemistry and we have observed a significant improvement in the performance of the kit. The yield for ultra-long read flowcells is expected to be lower than those with standard libraries, we attribute this to membrane damaged caused by the very high concentrations of DNA. We have been able to generate 50-100,000 reads per flowcell (1-2 Gb) although when working with HMW DNA we expect some variability due to DNA heterogeneity. A read length N50 of 100 Kb is considered an excellent result. The longest read sequenced with this protocol is 1.2 Mbp by Matt Loose.

Example run metrics for RAD004:

General summary:

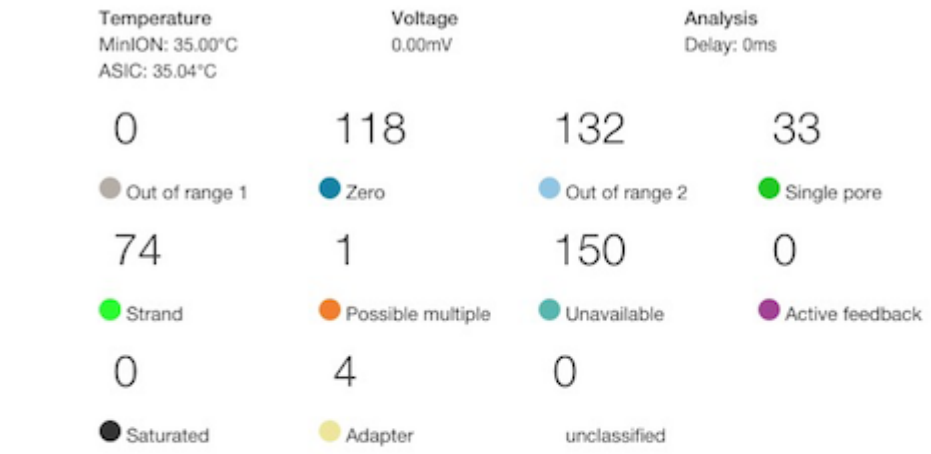
Number of reads: 33,907
Total bases: 1,324,105,498
Median read length: 17,472
Mean read length: 39,051.1
Read length N50: 90,923
Active channels: 492

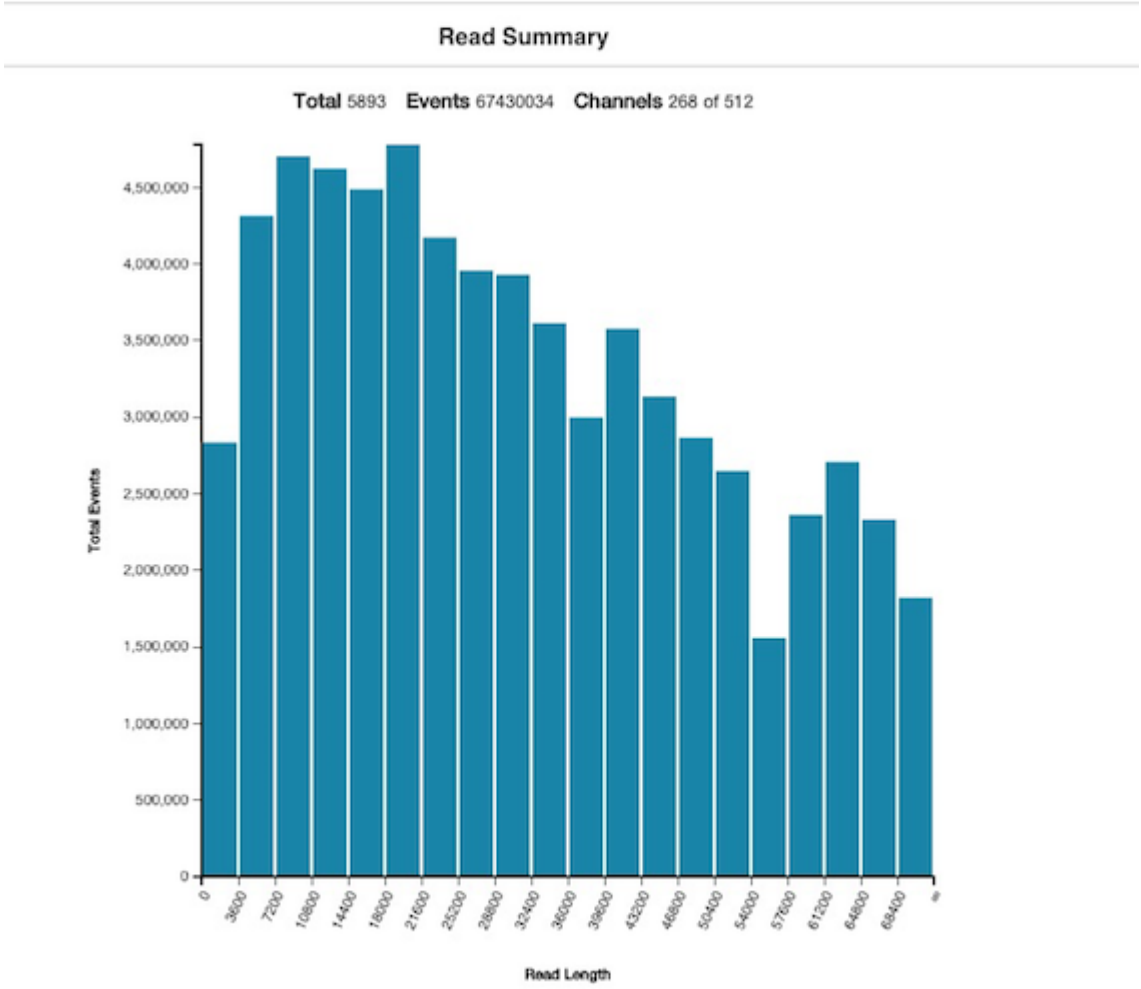
Top 5 longest reads and their mean basecall quality score:

1: 975,623 (10.26)
2: 862,356 (10.28)
3: 722,772 (7.62)
4: 709,230 (10.19)

5: 699,282 (9.88)

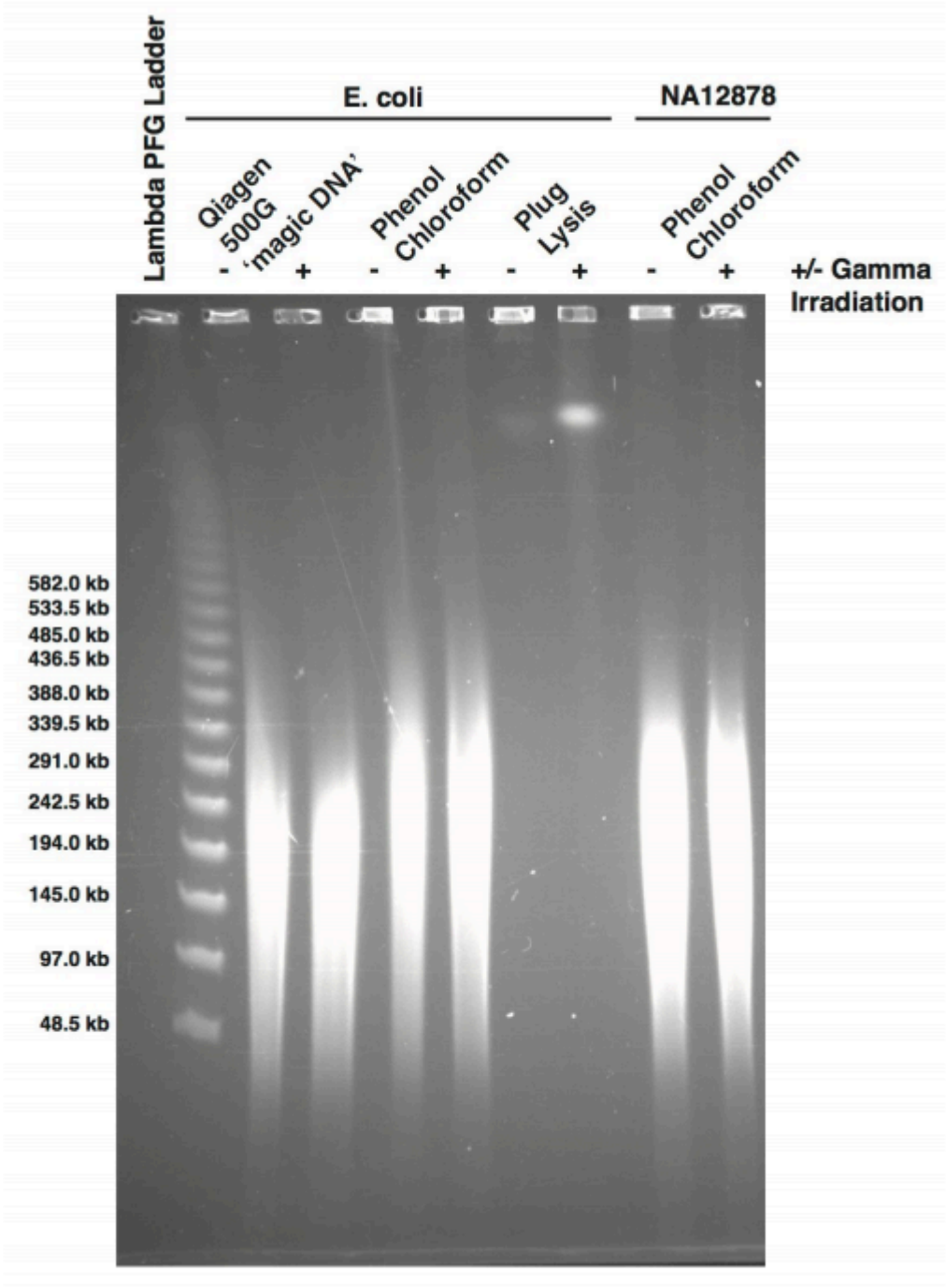
Run metrics from NanoStats.txt in Guppy output.





Read length histogram view from MinKNOW 1.23 in playback mode after one hour. A lot of the data is off the scale which only extends to 72,000 events. Reads longer than this are not counted in the yield value which means the standard scaling factor of bases = events * 1.4 will not apply.

Channel states view from MinKNOW 1.23 in playback mode from the start of the run. Pore occupancy (strand / strand + single pore) of 0.75 should be achievable with this method. Overall numbers in strand with RAD004 are often >200 but fall quickly, regular restarts are needed to achieve the maximum yield.



Pulsed-field gel, taken from http://biorxiv.org/content/biorxiv/early/2017/04/20/128835.full.pdf showing different extractions, this method is in lanes 8 and 9. Smear extends to about 350 kb but closely matches E. coli DNA extracted using the same method demonstrating it is the extraction method not the starting size of the chromosomes driving the size.

Materials

- 1X PBS (Phosphate-buffered saline) by Contributed by users

RNase A 19101 by Qiagen

✓ Nuclease-free Water by Contributed by users

AccuGene molecular biology water 51200 by Lonza

Tris-HCl, pH 8.0 (UltraPure) 15568025 by Thermo Fisher Scientific

100ml Ammonium Acetate [5M] R012 by G-Biosciences

Ethanol absolute 107017 by Merck Millipore

Sodium Dodecyl Sulfate, 500gm H5114 by Promega

Sodium chloride 5M solution SB8889.SIZE.500ml by Bio Basic Inc.

✓ AccuGENE 0.5 M EDTA Solution (1L) 51234 by Contributed by users

Proteinase K (2 ml) 19131 by Qiagen

Protocol

DNA Extraction

Step 1.

Take 5x10^{7} human cell pellet fresh or stored at -80°C in a 50 ml Falcon tube (for *E. coli* I used an overnight culture of *E. coli* growing in 50 ml LB broth spun down at 4500 x g for 10 minutes)

NOTES

Josh Quick 13 Dec 2017

DNA extraction protocol adapted from Molecular Cloning by Sambrook and Russell (third edition) Chapter 6 protocol 1.

DNA Extraction

Step 2.

Resuspend by pipette mixing in 200 µl sterile PBS.

AMOUNT

200 µl Additional info: PBS

REAGENTS

✓ 1X PBS (Phosphate-buffered saline) by Contributed by users

DNA Extraction

Step 3.

Add 10 ml TLB and vortex at full speed for 5 seconds.

AMOUNT

10 ml Additional info: TLB

NOTES

Josh Quick 19 Jan 2018

Vortexing here is to thoughly resuspend the cells to achieve a homogenous DNA solution once cells are lysed, DNA will not be damaged as it is still packaged inside the cell.

DNA Extraction

Step 4.

Incubate at 37°C for 1 hour.

TEMPERATURE

37 °C Additional info:

NOTES

Josh Quick 19 Jan 2018

Solution will turn transparent as the cells lyse.

DNA Extraction

Step 5.

Add 100 µl Qiagen Proteinase K or other stock solution to a final concentration of 200 µg/ml. Mix by slowly rotating end-over-end 3 times.

AMOUNT

100 µl Additional info: Proteinase K

REAGENTS

Proteinase K (2 ml) 19131 by Qiagen

DNA Extraction

Step 6.

Incubate at 50°C for 2 hours, mix every 30 minutes by slowly rotating end-over-end 3 times.

TEMPERATURE

50 °C Additional info:

DNA Extraction

Step 7.

Add light phase-lock gel to 2 x 15 ml Falcons. If it is only available in 2 ml tubes, transfer it by cutting the lid off 3 x 2 ml tubes and spinning it out into each 15 ml Falcon.

NOTES

Josh Quick 20 Jan 2018

15 ml Falcons are used as they are narrower decreasing the surface area of the interface/gel. Using two means they balance each other in the centrifuge and gives phenol space to move which improves the emulsion.

DNA Extraction

Step 8.

Split the viscous lysate into the two 15 ml Falcon tubes prepared with phase-lock gel, this is easiest using a 10 ml serological pipette at slow speed.

DNA Extraction

Step 9.

Add 5 ml recently opened BioUltra TE-saturated phenol to each Falcon tube containing lysate.

AMOUNT

5 ml Additional info: TE-saturated phenol

REAGENTS

BioUltra TE-saturated phenol 77607 by Sigma Aldrich

DNA Extraction

Step 10.

Place on a HulaMixer at 20 rpm for 10 minutes, if a fine emulsion has not formed after a minute gradually increase the rotation speed.

ANNOTATIONS

Max Stammnitz 12 May 2018

Hi Josh,

During these mixing steps on the Hula (#10 and #14), do you use 20 rpm end-over-end rotation, or instead gentle tilting/vortexing, or a combination of these?

Thanks a lot,
Max

DNA Extraction

Step 11.

Spin in a centrifuge at 4500 rpm for 10 minutes.

DNA Extraction

Step 12.

Pour the aqueous phases into two new 15 ml Falcon tubes containing phase-lock gel, try to avoid transferring any protein which may form a white layer above the phase-lock gel.

DNA Extraction

Step 13.

Add 2.5 ml buffer saturated phenol and 2.5 ml chloroform-isoamyl alcohol 24:1 to each tube.

AMOUNT

2.5 ml Additional info: TE-saturated phenol

AMOUNT

2.5 ml Additional info: Chloroform-Isoamyl Alcohol

REAGENTS

BioUltra TE-saturated phenol 77607 by Sigma Aldrich

Chloroform-Isoamyl Alcohol 25666 by Sigma Aldrich

ANNOTATIONS

Lisa Mehner 24 Apr 2018

Can you also take Phenol-Chloroform-Isoamyl instead of Phenol and then Chloroform-Isoamyl

DNA Extraction

Step 14.

Place on a HulaMixer at 20 rpm for 10 minutes, if a fine emulsion has not formed after a minute gradually increase the rotation speed.

DNA Extraction

Step 15.

Spin in a centrifuge at 4500 rpm for 10 minutes.

DNA Extraction

Step 16.

Combine the aqueous phases from the two tubes by pouring slowly into a new 50 ml Falcon tube.

DNA Extraction

Step 17.

Add 4 ml 5 M ammonium acetate.

AMOUNT

4 ml Additional info: Ammonium Acetate

REAGENTS

100ml Ammonium Acetate [5M] R012 by G-Biosciences

DNA Extraction

Step 18.

Add 30 ml ice-cold ethanol and watch the DNA precipitate, bubbles will over time pull the mass of DNA to the surface so it looks like a Jellyfish with tentacles hanging down.

AMOUNT

30 ml Additional info:

REAGENTS

Ethanol absolute 107017 by Merck Millipore

ANNOTATIONS

Shruti Iyer 15 Mar 2018

100% ethanol at this step?

Josh Quick 16 Mar 2018

Yep should be 2 volumes of absolute ethanol.

DNA Extraction
Step 19.

Make a hook by melting the tip of glass capillary in a blue flame so it curls over.

DNA Extraction
Step 20.

Hook out the DNA in one-piece if possible lift up and allow the excess liquid to drip off.

DNA Extraction
Step 21.

Submerge the DNA in a 50 ml Falcon tube containing 70% ethanol.

NOTES
Josh Quick 13 Dec 2017

It should have fully tightened up into a whitish opaque pellet.

DNA Extraction
Step 22.

Carefully work the pellet off the glass rod using the rim of an Eppendorf tube, let the pellet drop into the tube.

DNA Extraction
Step 23.

Go back for the rest of the DNA if it broke apart and repeat.

DNA Extraction
Step 24.

Add 1 ml 70% ethanol to the Eppendorf.

AMOUNT
1 ml Additional info: 70% ethanol

DNA Extraction
Step 25.

Spin down at 10,000 xg then remove as much of the 70% ethanol as possible.

DNA Extraction

Step 26.

Wash again with 1 ml 70% ethanol.

AMOUNT
1 ml Additional info: 70% ethanol

DNA Extraction

Step 27.

Spin down at 10,000 xg then remove as much of the 70% ethanol as possible.

DNA Extraction

Step 28.

Let the remaining ethanol evaporate by leaving at RT for 15 minutes.

TEMPERATURE
20 °C Additional info:

DNA Extraction

Step 29.

Add 100 µl EB + Triton-X100 and incubate without mixing at 5°C for 2 days to allow the pellet to fully resuspend into a translucent viscous gel.

AMOUNT
100 µl Additional info: EB+Triton-X100

TEMPERATURE
5 °C Additional info:

NOTES
Josh Quick 13 Dec 2017

We have found that adding Triton-X100 to a final concentration of 0.02% dramatically improves the transposase activity. If you don't want to add it to the stock DNA you can add it when you make the library.

ANNOTATIONS
Josh Quick 20 Jan 2018

We have found that optionally adding EDTA at a final concentration 0.1 mM of does not noticably affect the activity of the transposase.

QC DNA

Step 30.

Take a P2 pipette set to 1.5 µl with a cut-off tip and aspirate very slowly then try to work the DNA in the tip away from the DNA in the tube by continuously pulling and releasing the DNA to work it gradually away. Adjust the pipette volume to determine the volume of DNA you actually removed.

QC DNA

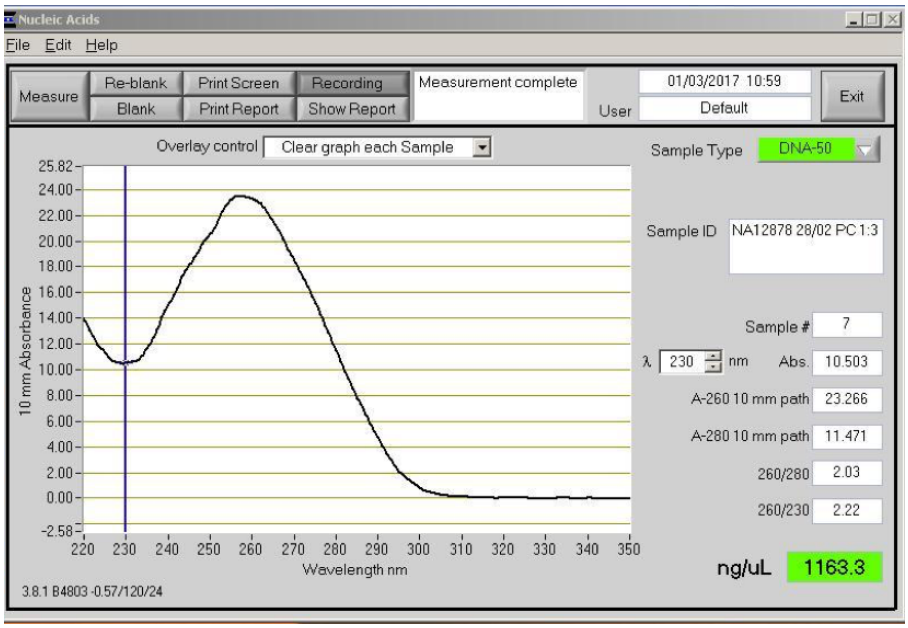
Step 31.

Quantify the DNA on the Qubit BR assay, the concentration should be >1 µg/µl, it is important to use BR as the buffer contains a detergent which appears to improve mixing.

QC DNA

Step 32.

Check the absorbance spectrum of the 1:10 diluted DNA on the NanoDrop, example trace below (ratios typically a little higher than other methods)



QC DNA

Step 33.

If necessary add more EB+Triton-X100 to the stock DNA to adjust concentration to 1 µg/ul but do not mix.

QC DNA

Step 34.

Leave overnight at 5°C before use.

TEMPERATURE

5 °C Additional info:

Library preparation with RAD004

Step 35.

As slowly as you can pipette 16 µl DNA into a 0.2 ml PCR tube using a cut-off P20 pipette tip, retain the tip.

AMOUNT

16 µl Additional info: DNA

ANNOTATIONS
Sarah Stahl 23 Jan 2018

what is the input concentration? still ~ 400ng?

Library preparation with RAD004
Step 36.

Remove 1 µl from the tube as before and quantify using Qubit BR assay.

AMOUNT
1 µl Additional info:

NOTES
Josh Quick 13 Dec 2017

DNA may still be heterogenous in concentration and as such this is the most reliable way to quantify actually input.

Library preparation with RAD004
Step 37.

Add 1.5 µl FRA and 3.5 µl EB+Triton-X100. Using a P20 set to 18 µl and the tip from before, mix up and down as slowly as possible 8 times, retain the tip.

AMOUNT
1.5 µl Additional info: FRA
AMOUNT
3.5 µl Additional info: EB+Triton-X100

NOTES
Josh Quick 20 Jan 2018

Take care not to introduce bubbles as they are hard to remove.

Josh Quick 20 Jan 2018

We have found that 1.5 µl FRA is suffient to generate an efficient library and any more can lead to over-fragmentation.

Library preparation with RAD004
Step 38.

Using a thermocycler incubate at 30°C for 1 minute, 80°C for 1 minute then hold at 4°C.

TEMPERATURE
30 °C Additional info:
TEMPERATURE
80 °C Additional info:
TEMPERATURE

4 °C Additional info: hold

ANNOTATIONS

thidathip wongsurawat 06 Feb 2018

Should I change this to 50 degrees C for 10 minutes like you suggested previously?

Thank you.

Library preparation with RAD004

Step 39.

Add 1 µl RAP. Using a P20 set to 19 µl and the tip from before, mix up and down as slowly as possible 8 times, discard tip.

AMOUNT

1 µl Additional info: RAP

Library preparation with RAD004

Step 40.

Incubate at room temperature while you prime the flowcell.

Prime flowcell

Step 41.

Add 30 µl FLT to tube of FLB, vortex briefly and spin down. This is the flush mix.

AMOUNT

30 µl Additional info: FLT

Prime flowcell

Step 42.

Place new flowcell on MinION and run platform QC .

Prime flowcell

Step 43.

Using a P1000 remove a little storage buffer from the inlet port using the volume adjustment screw. Load 800 µl flush mix via the inlet port slowly using the plunger. Wait 5 minutes.

AMOUNT

800 µl Additional info: Flush mix

Prime flowcell

Step 44.

Lift the cover off the SpotON port. Load 200 µl flush mix via the inlet port slowly using the plunger, try to dispense at a speed where a bead of liquid becomes visible over the SpotON port which then gets siphoned back in.

AMOUNT

200 µl Additional info: Flush mix

Load library

Step 45.

Add 34 µl SQT and 20 µl NFW to the library tube.

NOTES

Josh Quick 20 Jan 2018

Loading beads are not used as they clump when mixed with the library.

ANNOTATIONS

David Catoe 07 Feb 2018

Should this be SQB? it seems like we only have about 9 uL SQT per kit.

Load library

Step 46.

Using a P100 set to 75 µl with a cut-off tip mix up and down as slowly as possible 5 times. On the final mix slowly pipette the diluted library onto the SpotON port as it gets siphoned in, this can take much longer than usual due to the viscosity.

NOTES

Josh Quick 13 Dec 2017

If it gets blocked abandon the siphon and load by holding the pipette vertically against the SpotON port and positively pipetting the library directly in.

Expected results for Human

Step 47.

See guidelines for expected results.

Warnings

When handling phenol always wear PPE, keep a solution of 50% (w/v) PEG-400 nearby to treat the burn in the case of accidental splashes.