**Telomere Capture Sequencing Protocol** / 10/2023 / Santiago E Sanchez, Artandi Lab, Stanford University

Consumables

* -  rCutsmart buffer (NEB B6004S)
* -  100 mM rATP (ThermoFisher R0441)
* -  EcoRV-HF (NEB R3195S)
* -  NEBNext® dA-Tailing Module (NEB E6053)
* -  Promega ProNex Size Selection Beads (Promega #NG2001)
* -  InvitrogenTM UltraPureTM BSA (50 mg/mL) (Fisher AM2616)
* -  NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing (NEB E7180S). Alternatively, you can use the three NEBNext products below:
* -  NEBNext FFPE Repair Mix (NEB M6630)
* -  NEBNext Ultra II End repair/dA-tailing Module (NEB E7546)
* -  NEBNext Quick Ligation Module (NEB E6056)
* -  1.5 mL Eppendorf DNA LoBind tubes
* -  0.2 mL thin-walled PCR tubes
* -  Nuclease-free water (ThermoFisher AM9937)
* -  Freshly prepared 80% ethanol in nuclease-free H2O
* -  Qubit Assay Tubes (ThermoFisher Q32856)
* -  Qubit dsDNA BR Assay Kit (ThermoFisher Q33266)
* -  5 M NaCl
* - NEB Monarch HMW DNA extraction kit for cells and blood (NEB # T3050L)
* For R10 chemistry runs: ONT LSK 114 + NA expansion + Sequencing Auxillary Vials V14
* For R9 chemistry runs: ONT LSK 110 + AMII expansion + Sequencing Auxillary Vials V12
* R9 or R10 MinION or PromethION flow cells

Canonical Sequence Barcoded Capture Oligos

|  |  |
| --- | --- |
| 5' --> 3' | |
| seqTether | AACCTTGGAGATGCACGGAGCA |
| Barcoded Canonical Telomere Capture (Mod: 5' Phosphate) | |
| p-t3-nb01 | TGCTCCGTGCATCTCCAAGGTTCACAAAGACACCGACAACTTTCTTCCTAACC |
| p-t3-nb02 | TGCTCCGTGCATCTCCAAGGTTACAGACGACTACAAACGGAATCGACCTAACC |
| p-t3-nb03 | TGCTCCGTGCATCTCCAAGGTTCCTGGTAACTGGGACACAAGACTCCCTAACC |
| p-t3-nb04 | TGCTCCGTGCATCTCCAAGGTTTAGGGAAACACGATAGAATCCGAACCTAACC |
| p-t3-nb05 | TGCTCCGTGCATCTCCAAGGTTAAGGTTACACAAACCCTGGACAAGCCTAACC |
| p-t3-nb06 | TGCTCCGTGCATCTCCAAGGTTGACTACTTTCTGCCTTTGCGAGAACCTAACC |
| p-t3-nb07 | TGCTCCGTGCATCTCCAAGGTTAAGGATTCATTCCCACGGTAACACCCTAACC |
| p-t3-nb08 | TGCTCCGTGCATCTCCAAGGTTACGTAACTTGGTTTGTTCCCTGAACCTAACC |

1. Perform HMW DNA extraction as directed by NEB Monarch HMW DNA kit protocol. Use 1800 RPM for proteinase + RNAse digestion step.
   1. Optional: quality control HMW DNA by Agilent Tapestation (60 kb ladder) or pulse field gel electrophoresis. Most DNA should be >> 60 kb
2. Ligate telomere capture oligos onto HMW DNA in 100 uL reaction

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock Conc | Final Conc | Volume (uL) |
| HMW gDNA |  | 3-15 ug |  |
| NucFree H2O |  |  | Up to 79 uL |
| rCutsmart | 10x | 1x | 10 |
| rATP | 100 mM | 1 mM | 1 |
| p-t3-nbXX | 10 uM | 500 nM | 5 |
| Quick T4 DNA Ligase | 2000 U/uL | 50 U/uL | 5 |

1. Flick tube to mix and spin down
2. Incubate @ 37 C x 16 h / overnight
3. Heat inactivate ligation reaction at 65 C x 10 mins.
4. Add 5 uL of 20 U/uL EcoRV to inactivated ligation reaction mixture
   1. You can use your favorite **blunt** cutter, but it *must* be a **blunt** cutter.
5. Incubate @ 37 C x 30 minutes and then heat inactivate at 65 C x 10 mins.
6. Perform 3’ dA-tailing of non-telomeric DNA with the following 125 uL reaction:

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock Conc | Final Conc | Volume (uL) |
| Blunt Digested DNA |  |  | 105 |
| NucFree H2O |  |  | 2.5 |
| NEB dA-tailing buffer | 10X | 1X | 12.5 |
| NEB Klenow Exo- | 100 mM | 1 mM | 5 |

1. Flick tube to mix and spin down.
2. Incubate @ 37 C x 30 mins
3. Add 1.6X volume of Promega ProNex size selection beads (200 uL)
4. Incubate on a rotating mixer for 5-10 mins at room temperature
5. Spin down tube and pellet beads on a magnet
6. While avoiding the pellet, aspirate and waste supernatant.
7. Add 300 uL of Promega Wash Buffer or 80% Ethanol and gently pipette to wash beads
8. Repeat the previous two steps
9. Air-dry pellet on the magnet for 3-5 mins
10. Aspirate any remaining EtOH / Wash Buffer from tube while on magnet
11. Resuspend pellet in 99 uL of nuclease free H2O / low TE / Promega elution buffer
12. Incubate @ 37 C x 15 mins
13. Pellet beads on a magnet and allow supernatant to clear
14. Collect eluate containing telomere capture oligo ligated DNA and use 1 uL to quantify with Qubit BR dsDNA assay kit.
    1. The optimal amount of input DNA from telomere capture libraries is still a moving target. For now, I always use the entirety of the DNA I collect in the following steps. That said, quantification is an important step to verify recovery of DNA from clean-up step
15. Anneal seqTether to DNA with the following 100 uL reaction

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock Conc | Final Conc | Volume (uL) |
| Telocaptured DNA |  |  | 94 |
| NaCl | 5 M | 50 mM | 1 |
| seqTether | 10 uM | 500 nM | 5 |

1. Flick tube to mix and spin down
2. Incubate @ 50 C x 1 h
3. Add 1X volume of Promega ProNex size selection beads (100 uL)
4. Incubate on rotating mixer at room temperature for 5-10 mins
5. Spin down tube and pellet beads on a magnet
6. While avoiding the pellet, aspirate and waste supernatant.
7. Add 200 uL of Promega Wash Buffer or 80% Ethanol and gently pipette to wash beads
8. Repeat the previous two steps
9. Air-dry pellet on the magnet for 3-5 mins
10. Aspirate any remaining EtOH / Wash Buffer from tube while on magnet
11. Resuspend pellet in 32 uL of nuclease free H2O / low TE / Promega elution buffer
12. Incubate @ 37 C x 15 mins
13. Pellet beads on a magnet and allow supernatant to clear
14. Collect eluate containing telomere capture oligo ligated DNA and use 1 uL to quantify with Qubit BR dsDNA assay kit.
    1. **If Multiplexing:** After quantification, pool approximately equimolar amounts of DNA from each barcoded sample into the same tube for a total of 1 ug of barcoded DNA. The most accurate way to do this is to use a fragment analyzer to assess the size of the barcoded library and calculate the fmols of DNA given by X ng of each sample depending on its average length. The quicker (but less accurate) way is to simply assume all of the samples are more or less the same number of fmols / ng and pool the same # ng of each sample. If you expect there to be very stark differences in telomere length per sample, I suggest doing the accurate way to avoid having a highly unbalanced library. Otherwise, loading the same number of ng of each sample is adequate. We have successfully run 8-plex libraries and obtained 300+ telomeres per sample after 48 h of sequencing. We believe higher plexity is possible but more optimization will be required as a large number of reads in telomere capture libraries are “unclassified”, meaning there is no detected barcode in the read; nevertheless, there are certainly telomere reads in the unclassified majority. In single-plex runs, unclassified telomere lengths can be pooled with the barcoded measurements but in multiplex runs it is currently impossible to know the sample of origin.
    2. **After pooling, proceed as if performing a single-plex run.** You can select “barcoding” in the MinKNOW GUI when setting up the sequencing run and select the kit with the appropriate barcodes (NB01-12) to monitor barcode count during sequencing. It is also possible to demultiplex the raw data after sequencing is completed so live demultiplexing is not required.
15. Perform sequencing adapter ligation with the following reaction

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock Conc | Final Conc | Volume (uL) |
| seqTether’d DNA |  |  | 30 |
| AMII (R9) or NA (R10) |  |  | 5 |
| NEB Quick Ligation Buffer | 5X | 1X | 10 |
| Quick T4 DNA Ligase | 2000 U/uL | 200 U/uL | 5 |

1. Flick to mix and spin down
2. Incubate @ room temperature x 30 mins
3. Add 1X volume Promega ProNex size selection beads
4. Incubate on rotating mixture for 5-10 mins at room temperature
5. Spin down tube and pellet beads on a magnet
6. While avoiding the pellet, aspirate and waste supernatant.
7. Add 200 uL of ONT Long Fragment Buffer (LFB)
8. Take tube off of magnet, flick to resuspend the pellet in LFB, and spin down
9. Return tube to magnet and re-pellet
10. Repeat the previous three steps
11. Aspirate any remaining LFB from the tube while on magnet
12. Allow the pellet to air dry for 3-5 mins
13. Aspirate any remaining LFB from the tube while on magnet
14. Resuspend pellet in 26 uL of ONT EB (Elution Buffer)
15. Incubate @ 37 C x 15 mins
16. Pellet beads on a magnet and allow supernatant to clear
17. Collect eluate containing telomere capture oligo ligated DNA and use 1 uL to quantify with Qubit BR dsDNA assay kit.
18. Load the entire eluate onto the flow cell according to the appropriate ONT sequencing

*Loading an R10 library onto a MinION flow cell*

1. Thaw the Sequencing Buffer (SB), Library Solution (LIS), Flush Tether (FLT), InvitrogenTM UltraPureTM BSA (50 mg/mL), and one bottle of Flow Cell Flush (FCF) at room temperature.
2. Mix the Sequencing Buffer (SB), Library Solution (LIS), Flow Cell Flush (FCF) and Flush Tether (FLT) tubes thoroughly by vortexing and spin down at room temperature.
3. To prepare the flow cell priming mix, add 30 μL of thawed and mixed Flush Tether (FLT), and 5 μL of InvitrogenTM UltraPureTM BSA (50 mg/mL) directly to 1,170 μl of thawed and mixed Flow Cell Flush (FCF), and mix by vortexing at room temperature.
4. Flush the MinION flow cell by drawing back a small volume to remove any bubbles and then flush 800 μL via the priming port. Wait 5 minutes.
5. During the incubation, prepare the library for loading:

|  |  |
| --- | --- |
|  | Volume (uL) |
| SB / Sequencing Buffer | 75 |
| LIS / Library Solution | 51 |
| Library Eluate | 25 |
| Total |  |

1. Complete the flow cell priming by loading 200 μL of priming mix into the flow cell priming port (not the SpotON sample port).
2. Load your library by pipetting dropwise 75 μL of library to the flow cell via the SpotON sample port. Wait 30 minutes and load the remaining 75 μL of the library.
3. Close the ports and start sequencing:
   1. Select SQK-LSK114 without expansion packs or barcoding.
   2. Select 48 h run duration and 1000 bp minimum read length.
   3. HAC basecalling.
   4. Either Pod5 or Fast5 can be selected (pod5 is more space-efficient)

*Loading an R10 library onto a PromethION flow cell*

1. Thaw the Sequencing Buffer (SB), Library Solution (LIS), Flush Tether (FLT), InvitrogenTM UltraPureTM BSA (50 mg/mL), and one bottle of Flow Cell Flush (FCF) at room temperature.
2. Mix the Sequencing Buffer (SB), Library Solution (LIS), Flow Cell Flush (FCF) and Flush Tether (FLT) tubes thoroughly by vortexing and spin down at room temperature.
3. To prepare the flow cell priming mix, add 30 μL of thawed and mixed Flush Tether (FLT), and 5 μL of InvitrogenTM UltraPureTM BSA (50 mg/mL) directly to 1,170 μl of thawed and mixed Flow Cell Flush (FCF), and mix by vortexing at room temperature.
4. Flush the PromethIONflow cell by drawing back a small volume to remove any bubbles and then flush 500 μL via loading port. Wait 5 minutes.
5. During the incubation, prepare the library for loading:

|  |  |
| --- | --- |
|  | Volume (uL) |
| SB / Sequencing Buffer | 75 |
| LIS / Library Solution or LB / Library Beads | 51 |
| Library Eluate | 25 |
| Total |  |

1. Complete the flow cell priming by loading 500 μL of priming mix into the flow cell loading port.
2. Load your library by pipetting 150 μL of library to the flow cell into the loading port. Wait 10-15 mins before starting sequencing
3. Close the ports and start sequencing:
   1. Select SQK-LSK114 without expansion packs or barcoding.
   2. Select 48 h run duration and 1000 bp minimum read length.
   3. HAC basecalling.
   4. Either Pod5 or Fast5 can be selected (pod5 is more space-efficient).

*Loading R9 chemistry flow cells*

Everything above remains the same, EXCEPT the names of some reagents. R10 = R9 conversion below.

SB = SBII

LB = LBII

FCT = FLT

FCF = FB

AND for R9 runs you do not add any BSA to the flush buffer prior to priming the flow cell.

*Data Analysis with Telometer for R10 chemistry runs*

Dependencies: samtools, minimap2, pysam, python >=3.9, pandas

Once you have the pod5 or fast5, you will need to perform custom telomere basecalling with dorado (v0.3.4) according to: <https://github.com/nanoporetech/dorado>

Using the following basecalling model: [dna\_r10.4.1\_e8.2\_400bps\_sup@v4.2.0](mailto:dna_r10.4.1_e8.2_400bps_sup@v4.2.0)

**Note: superaccuracy basecalling models are extremely GPU-intensive. I recommend running this on a high-performance cluster or on a local computer with a GPU with at least 8GB of GDRR5 on-board RAM.**

After basecalling:

1. Align basecalled reads to t2t genome with minimap2 (if you would like our combined T2T v2.0 and Stong 2014 reference, let me know): (this is a one-liner, “\” separates flags but should not be entered into command line)  
  
minimap2 -ax map-ont \

-t 16 \

-N 5 \ -Y

-L \

/path/to/reference/t2t-and-subtel.fa \

/path/to/fastq\_dir/\*.fastq \

-o output.sam  
  
2. Convert output to bam and sort, index  
  
samtools view -bho output.bam output.sam  
samtools sort -o output-sort.bam output.bam  
samtools index output-sort.bam  
  
3. Run Telometer  
  
python telometer.py -b output-sort.bam -n telomere\_measurements.tsv

*(python3 telometer.py …. if running on machine with multiple python versions)*

*Data Analysis with Telometer for R9 chemistry runs*

Same as above, but there is no need to use the dorado basecalling model. The guppy HAC basecalling model used during sequencing is sufficient. Align those fastq files with minimap2 and proceed as above.