**Telomere Capture Sequencing Protocol** / 11/2024 / Santiago E Sanchez, Artandi Lab, Stanford University

Consumables

* -  rCutsmart buffer (NEB B6004S)
* -  100 mM rATP (ThermoFisher R0441)
* -  HinfI / RsaI (NEB R0155M / R0167L)
* -  Promega ProNex Size Selection Beads (Promega #NG2001)
* -  NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing (NEB E7180S). Alternatively, you can use the three NEBNext products below:
* -  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
* R10 MinION or PromethION flow cells

Canonical Sequence Barcoded Capture Oligos

|  |  |
| --- | --- |
| 5' --> 3' | |
| seqTether | AACCTTGGAGATGCACGGAGCAAGCAAT |
| 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
   1. OPTIONAL: After DNA has been extracted, it is safe to pre-digest the DNA overnight with HinfI/RsaI in 1X rCutSmart buffer and perform an overnight ligation the next day. HOWEVER, because HinfI/RsaI can cut some ONT barcode sequences, either heat inactivate the digestion reaction at 65C for 15 minutes or perform a Promega bead clean up with a 1.6X bead to sample ratio to prevent barcode loss to restriction digestion. Overnight pre-digestion leads to more complete elimination of genomic DNA and is advised for multiplex runs with 10+ samples.
   2. OPTIONAL: You can pre-duplex the SeqTether and barcoded oligo by mixing equimolar amounts of 10 uM stock up to 10 uL in a PCR tube and then heating the mixture to 95 C for 5 min followed by cooling at RT for 30 mins. Add the full volume to the reaction below and use 74 uL of total DNA volume instead of 79 to adjust. This step saves you one hour on the second day of prep and leads to similar performance.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock Conc | Final Conc | Volume (uL) |
| HMW gDNA |  | 1-15 ug |  |
| NucFree H2O |  |  | Up to 79 uL (or 74 uL if pre-duplexing capture oligos, see above) |
| rCutsmart | 10x | 1x | 10 |
| rATP | 100 mM | 1 mM | 1 |
| p-t3-nbXX | 10 µM | 500 nM | 5 |
| Quick T4 DNA Ligase | 2000 U/µL | 50 U/µL | 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 each of 20 U/uL HinfI and RsaI to inactivated ligation reaction mixture
   1. Other telomere-sparing restriction enzymes are possible although we have found HinfI/RsaI perform well in our hands.
   2. OPTIONAL: Older versions of this protocol used a digestion with a single blunt cutter (e.g. 5 µL of 20U/µL EcoRV). Due to the cost of using the Klenow-Exo at scale, we have found that using multiple frequent cutters still allows for up to 12-sample multiplexing with good pore occupancy and throughput. If you do opt for a single blunt cutter, it is necessary to perform 3’ dA-tailing of non-telomeric DNA with the following 125 uL reaction in order to preserve telomere enrichment:

|  |  |  |  |
| --- | --- | --- | --- |
|  | 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. Incubate @ 37 C x 1h (optionally: with shaking at 400 RPM in a thermomixer)
2. Heat inactivate digestion reaction at 65 C x 15 mins.
3. Add 1.5-6X volume of Promega ProNex size selection beads (~160 µL)
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 µL 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 mins
10. Aspirate any remaining EtOH / Wash Buffer from tube while on magnet
    1. **IF YOU PRE-DUPLEXED: SKIP THE FOLLOWING STEPS, elute in 32 uL of nuclease free H2O / low TE / Promega elution buffer and proceed directly to step 35.**
11. Resuspend pellet in 94 uL of nuclease free H2O / low TE / Promega elution buffer
    1. **If multiplexing**, it is possible to multiplex samples from this step onwards by eluting each sample in 94 / N µL of elution buffer where N = 2-4 samples. You can then proceed with the rest of the prep using pools of 2-4 for up to 12 samples on a single flow-cell. While we believe multiplexing more samples on a single flow cell will be possible, we currently see optimal sequencing results with at most 12 barcoded samples.
12. Incubate @ 37 C x 10 mins
13. Pellet beads on a magnet and allow supernatant to clear
14. Collect eluate containing telomere capture oligo ligated DNA
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 (optionally: with shaking at 400 RPM in a thermomixer)
3. Add 1.5X volume of Promega ProNex size selection beads (150 µL)
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 µL 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 mins
10. Aspirate any remaining EtOH / Wash Buffer from tube while on magnet
11. Resuspend pellet in 32 µL of nuclease free H2O / low TE / Promega elution buffer
    1. **If multiplexing:** Resuspend pellet in 50 µL and after collecting the eluate, use the Qubit dsDNA BR kit to quantify DNA concentration in each single or pooled sample of post-SeqTether DNA.
12. Incubate @ 37 C x 10 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 2 µg 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 12-plex libraries and obtained hundreds 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 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 |
| Native Adapter / NA |  |  | 5 |
| NEB Quick Ligation Buffer | 5X | 1X | 10 |
| Salt-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 250 uL of ONT Long Fragment Buffer (LFB)
8. Take tube off 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 mins
13. Aspirate any remaining LFB from the tube while on magnet
14. Resuspend pellet in 33 uL of ONT EB (Elution Buffer)
15. Incubate @ 37 C x 10 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 up to 400 fmols onto a PromethION flow cell according to ONT flow cell loading instructions. Load up to 100 fmols onto a MinION flow cell according to ONT flow cell loading instructions.
    1. Abbreviated loading instructions (extended at the end of this protocol):
       1. Make priming mix (1170 µL Flush Buffer / ONT FB + 30 µL)
       2. Load flow cell onto sequencer. Set P1000 to 200 and carefully aspirate any free air in loading port (up to 230 µL).
       3. Load 500 µL of priming mix. Wait 5 minutes. Load second round of 500 µL of priming mix. Remaining can be stored at -20C and reused for a future run.
       4. Mix 34 µL of sequencing library with 100 µL of Sequencing Buffer (SB) and 68 µL of Library Beads (LB).
       5. Load all 200 µL of above mixture into flow cell using a P1000 pipette. Do not depress pipette fully. Instead, carefully and slowly turn the pipette’s volume adjusting knob or wheel to release mixture into flow cell loading port.

*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 | 100 |
| LB / Library Beads | 68 |
| Library Eluate | 32 |
| Total | 200 |

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 200 μL of library to the flow cell into the loading port. OPTIONAL: Wait 10-15 mins before starting sequencing to increase initial throughput.
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 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 | 24 |
| Total | 150 |

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)

*Data Analysis*

Please follow the step-by-step instructions at https://github.com/santiago-es/Telometer