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Multiplexed Strand-specific RNA-Seq Library Preparation for Illumina Sequencing Platforms

Important Things to know before you start:

- This protocol generates strand-specific reads, but may lead to slightly reduced coverage at the 5'-end of mRNA transcripts if used for single-end sequencing. This is because fragmented RNA molecules are only sequenced from the 3'-end and complete sequencing to the 5'-end may not occur. This bias can be partially negated by generating longer reads or completely overcome with paired-end sequencing, where sequencing starts from both ends.

- This protocol is suitable with various amounts of mRNA input (1 to 100 ng). When a high amount of input is used, the number of PCR cycles needs to be reduced for the final amplification. Optimization is required to calibrate the starting material amount and number of PCR cycles.

- The fragmentation step should be optimized in your own lab. You will want to start with RNA fragments that peak at around 200 bps.

- The multiplexing design uses PCR primers to introduce the 6-bp index sequence to the final library product, which is compatible with the HiSeq2500 and TruSeq protocol from Illumina.

- All procedures can be carried out in 100ul thin-wall PCR tubes to save time and increase throughput. We use a custom-made magnetic stand that fits the PCR tubes, however many commercially available magnetic stands will work.

- **It is strongly encouraged to perform mRNA purification and *ds*DNA production in one day. Do not use mRNA samples stored in -80°C longer than 2 weeks.**

- Actinomycin D dissolved in dimethylsufoxide (DMSO) to 1 µg/ µL, stock at -20°C.

- Buffer for mRNA purification

  . Binding Buffer (20mM Tris-Hcl PH7.5, 1M LiCl and 2mM EDTA)
  . Washing Buffer (10mM Tris-Hcl PH7.5, 150mM LiCl and 2mM EDTA)
  . Elution Buffer (10mM Tris-Hcl PH 7.5)

Things to be aware of when working with Sera-mag Speedbeads Carboxylate-modified

- Sera-mag Speedbeads can replace SPRI beads. It is not necessary to use at the original concentration. Dilute the Sera-mag speedbeads 50 times. The process to do this is described in the Supplemental Information section 1.

- When using Sera-mag Speedbeads, adding 0.5x volumes of 100% of ethanol (EtOH) helps the recovery of RNA and DNA fragments smaller than 100 bps.

- When not being used, the Sera-mag Speedbeads suspension should be kept at 4°C or on ice. Every time, right before use, vortex the suspension and make sure the beads are well mixed. Do this whenever you need to aliquot beads from stock tubes, even when the bead suspension looks uniform.

- Do not over-dry Sera-mag Speedbeads, as this leads to difficult re-hydration and elution. If beads appear as black chunks in the elution liquid, continue to vortex or pipette up and down, make sure the elution mixture is uniform, and increase the elution time to 5 minutes (usually 2 minutes).

- Elution efficiency with water is very high. There is no need to elute Sera-mag Speedbeads more than once as this will not increase your yield but rather dilute your elution concentration.

- **Method for using homemade Sera-mag Speedbeads** (NOT Sera-mag Oligo(dT)) is as follows:

    1) Add indicated amount of Sera-mag Speedbeads (usually at 1.6X of the sample volume, unless specified otherwise) to each sample. Vortex thoroughly.
    2) Incubate at room temperature for at least **7** minutes to ensure sufficient binding, then briefly spin down.
    3) Place the tubes on the magnetic stand and allow beads to separate from liquid.
    4) When the supernatant is clear, typically after 2 minutes, remove and discard the supernatant (DNA is bound with Sera-mag Speedbeads).
    5) Add 120µL of 75% EtOH into each tube without disturbing the bead pellet while tubes are still sitting on the magnetic stand.
    6) Let tubes sit on the magnetic stand at RT for 30 seconds, then use 200 µL pipette to remove and discard the EtOH without disturbing the beads.

7) Repeat 75% EtOH washing one more time. The EtOH washing is done on the magnetic stand at all times.
8) At the end of second washing, use 10 µL pipette to remove any residual water/EtOH.
9) Let samples sit on magnetic stand and air dry the samples (a gentle stream of air can be used to accelerate the drying process), but DO NOT over-dry the beads. Over-dried Sera beads are hard to rehydrate and may reduce elution efficiency. If beads are accidently over-dried, vortex tubes until beads are well suspended and increase rehydration time to 5 minutes.
10) Remove tubes from magnetic stand and re-suspend samples in indicated volume of elution buffer (nuclease-free water or EB buffer), then vortex thoroughly.
11) Let samples sit at room temperature for 2 minutes to ensure sufficient elution.
12) At this step, whether beads are removed from samples or not are indicated in the protocol. If separation of beads is needed, place the samples on magnetic stand and wait until the supernatant is clear. Transfer the supernatant (now DNA is in the supernatant) to a new tube and proceed to the next step. If separation of beads is not needed, directly proceed to the next step with beads in the samples.
13) Samples are now purified and ready for down-stream procedures.
14) For size selection (use 0.8X to get rid of products over 400bp); follow the procedures in the protocol for this step.

Protocol 1: Preparation of mRNA fragments

1.1 mRNA purification

1. Use 15 µg (can use as low as 1 µg) of purified total RNA and top with Nuclease-free H_2O to 50 µL.
2. Heat RNA sample at 65°C for 2 min (thermocycler) and immediately chill on ice before use.
3. To prepare beads for binding, wash 15 µL of Sera-mag Olgio (dT) beads with 120 µL of Binding Buffer by vigorously pipetting. Place on magnetic stand and after liquid clears, remove supernatant.
4. Repeat step 3 once, then suspend washed Sera-mag Olgio (dT) in 50 µL of Binding Buffer.
    - *If processing more than one sample, this step can be combined: e.g. wash 180µL beads at once for 12 samples.*
    - *You can use as low as 5 µL or as high as 50 µL Sera-mag Oligo(dT) for this step depending on the nature of the input total RNA and the required mRNA quantity. In general, 15µL of Sera-mag Oligo(dT) is good for purification of mRNA from 15ug total RNA.*
5. Add 50 µL of Sera-mag *Olgio (dT)* to the 50 µL of total RNA, incubate with vortexing at RT for 15 min. If using Fisher Scientific Digital Vortex Mixer, use speed setting 1200-1675.
6. Put on magnetic stand (wait until the liquid is clear and beads are pulled to the side of the tube) and then remove liquid without disturbing beads.
7. Take tubes off the magnetic stands and wash Sera-mag Olgio (dT) by pipetting vigorously with 120 µL of Washing Buffer.
8. Repeat step 6 and 7 once.
9. Remove Washing Buffer as in step 6 once more, and then add 50 µL of Elution buffer to the washed and reasonably dry Sera-mag Olgio (dT) beads.
10. Place tubes at 80°C in hot incubator/shaker with mild agitation or in thermocycler for 2 minutes to elute the mRNA.
11. **Immediately** place on the magnet collector and let the liquid clear.
12. Pipette the eluted 50 µL of mRNA to a new Lo-bind® tube and keep on ice during the following bead washing steps.
13. Wash the original beads twice with water by repeating the following: add 120 µL of water, mix vigorously off magnetic stand, place tubes with mixed beads on stand until liquid clears, and then remove liquid.
14. After removing wash water for the 2nd time, suspend beads in 50 µL of Binding Buffer.

15. Combine 50 µL of washed beads with the 50 µL of mRNA elution in the new tube.
16. Mix well, and incubate at RT for 15 mins with vortexing. If using Fisher Scientific Digital Vortex Mixer, use speed setting 1200-1675.
17. Put on magnetic stand (wait until the liquid is clear and beads are pulled to the side of the tube) and then remove liquid without disturbing beads.
18. Take tubes off the magnetic stands and wash Sera-mag Olgio (dT) by pipetting vigorously with 120 µL of Washing Buffer.
19. Repeat step 17 and 18 once.
20. Remove Washing Buffer as in step 19 once more.
21. Add 16 µL of Elution buffer and heat at 80°C in incubator/shaker with mild agitation or in thermocycler to elute the mRNA from the beads.
22. Quantify mRNA on Qubit® to normalize mRNA samples for fragmentation.

    Brutnell Lab:

    a. Make a master mix (MM) with 1ul Qubit® RNA HS reagent and 199 ul Qubit® RNA HS buffer per sample **at RT**, and vortex.
    b. Add 199ul MM and 1ul mRNA sample to Qubit® tube, vortex 2-3 seconds, wait at least 2 mins, and measure.

       * Qubit® reagents are very sensitive to temperature and should be measured at RT.

       *Do not expose stock Qubit® RNA HS reagent to light.

       *Your sample with reagent can be measured accurately for at least 15 minutes after vortexing.

1.2 mRNA fragmentation

1. Assemble the following mix
   - 5X First Strand Buffer 4 µL
   - mRNA (50 ng or variable) x µL
   - Nuclease free water (8-x) µL
2. Incubate at 94°C for 5 min (thermocycler), then put the tubes on ice
   - *Incubation time should be experimentally determined in your own lab. Your fragmented mRNA should peak around 200-250 bps.*

Protocol 2: cDNA synthesis

2.1 Reverse Transcription

1. Assemble the following mix
   - mRNA 12 µL
   - Random Primer 0.5 µL
   - SupeRase-In 0.25 µL
   - DTT (100 mM) 1 µL
2. Heat at 65°C for 3 min (thermocycler), place on ice and add
   - Nuclease free water 4 µL
   - DTT (100 mM) 1 µL
   - dNTPs (25 mM) 0.1 µL
   - SupeRase-In 0.5 µL
   - SuperScript III Reverse Transcriptase 0.5 µL
   - Actinomycin D(1 µg/ µL) 0.12 µL
3. Perform reverse transcription reaction using following PCR program:
   - 25°C 10min
   - 50°C 50min
   - 70°C 15min
   - 4°C Hold
4. Bind the RNA/cDNA hybrid using 40 µL of Sera-mag Speed beads with 20 µL of EtOH (100%) for 7 mins and wash twice with 75% EtOH (see **Method for using homemade Sera-mag Speedbeads)**.
5. After removing 75% EtOh wash, elute the RNA/cDNA hybrid with 16 µL of Nuclease Free Water and transfer to a new tube.

2.2 Second-strand synthesis with dUTPs

1. Prepare the 2nd strand reaction on ice as follows:
   - RNA/cDNA hybrid (w/o beads) 16 µL
   - 10x Blue Buffer (or NEB buffer 2) 2 µL
   - **dUTP mix** (10mM dA, dC, dG and 20 mM dU) 1 µL
   - RNase H 0.5 µL
   - DNA polymerase I 1 µL
   - DTT (100mM) 0.5 µL
2. Incubate at 16°C for 2.5 hours (thermocycler).
3. Bind the *ds*DNA using 34 µL of Sera-mag Speed beads and wash twice with 75% EtOH (see **Method for using homemade Sera-mag Speedbeads)**.
4. After removing 75% EtOh wash, add 16 µL of Nuclease Free Water (eluting the product, do not transfer to a new tube) and proceed to the next step.

Protocol 3: Library construction

3.1 End-repair

1. Prepare the reaction on ice as follows:
   - *ds*DNA (w/ beads) 16 µL
   - 10x End Repair Buffer 2 µL
   - 10 mM dNTP mix 1 µL
   - End Repair Mix LC 1 µL
2. Incubate at 20°C for 30 min (thermocycler).
3. Bind for 7 mins the end-repaired DNA by adding 32 µL of XP buffer, and the wash twice with 75% EtOH (see **Method for using homemade Sera-mag Speedbeads)**.
4. After removing 75% EtOh wash, add 17 µL of Nuclease Free Water (eluting the product, do not transfer to a new tube) and proceed to the next step.

3.2 dA-tailing

1. Prepare the reaction as follows:
   - DNA(w/ beads) 17 µL
   - 10x Blue Buffer (NEB buffer 2) 2 µL
   - 10 mM dATP mix 1 µL
   - Klenow 3'-5' exo$^-$ 0.5 µL
2. Incubate at 37°C for 30 min (thermocycler).

3. Bind for 87 mins the A-tailed DNA by adding 33 µL of XP buffer and wash twice with 75% EtOH (see **Method for using homemade Sera-mag Speedbeads)**.
4. After removing 75% EtOh wash, add 10 µL of Nuclease Free H_2O and transfer the elution to a new tube.

3.3 Universal Y- Shaped Adapter Ligation

1. Prepare the reaction on ice as follows:
   - DNA (w/o beads) 10 µL
   - Universal adapter (5 µM) 0.5 µL
   - Nuclease free water 0.5 µL
   - 2x Ligation Buffer 12 µL
   - T4 DNA Ligase 1 µL
2. Incubate at 20°C for 20 min (thermocycler).
3. Take 12 µL (half) and save in -80°C freezer with date and sample details.
4. Take the other half (12 µL) of the ligation product and mix with 8 µL of Nuclease-free water and 16ul of Sera-mag Speedbeads (this makes a 0.8X Speedbead solution for large fragment removal).
5. Incubate at RT for 7 min, place on magnetic stand, and after liquid clears, transfer the supernatant (37 µL) to a new tube and discard the beads that are bound to the large fragments.
6. Mix the supernatant with 37 µL of Sera-mag Speed beads, incubate at RT for 7 min, wash twice, and elute in 20 µL of Nuclease Free Water, do not transfer to a new tube.
5. Mix with 20 µL of XP buffer, incubate at RT for 7 min.
6. Wash twice with 75% EtOH (see **Method for using homemade Sera-mag Speedbeads).**
7. After second EtOH removal, add 30 µL of EB and mix well.
8. Set on magenetic stand, and wait until liquid clears.
9. Transfer the elution to a new tube.
7. Save 15 µL of elute in -80°C freezer with date and sample details and proceed to the next step with the other 15 µL for 18 cycles of PCR to test library amplification, discard the beads. The 18 cycle PCR will help validate the fragment size, quantity, and quality of your library. This information will help inform how many cycles of PCR are needed to amplify your final library without over-cycling. Over-cycling will bias reads toward highly abundant reads.

Protocol 4: Library amplification and multiplexing

4.1 dUTP excision and amplification

1. Digest 2nd strand DNA with Uracil DNA Glycosylase at 37°C for 30 min (thermocycler)
   - dsDNA (w/o beads) 15 µL
   - Uracil DNA Glycosylase 1 µL
2. Prepare the PCR reaction on ice as follow:
   - UDG digested DNA 16 µL
   - Nuclease-free water 1.5 µL
   - Index Primer (10 µM) 0.5 µL
   - Universal Primer (10 µM) 0.5 µL
   - 5x Fidelity Buffer 5 µL
   - 10 mM dNTPs 1 µL
   - KAPA HiFi Hot Start DNA Polymerase 0.5 µL
3. PCR cycle
   - 95°C 2 min
   - 98°C 20 sec
   - 65°C 30 sec
   - 72°C 20 sec

   } **Variable Cycles (8-14)**

   - 72°C 5 min
   - 4°C
4. Bind the dsDNA by adding 28 µL of Sera-mag Speed beads and wash twice with 75% EtOH.
5. Elute with 12 µL Qiagen EB, store in -80°C freezer with date and sample details

4.2 Prepare indexed library

1. Measure each indexed library concentration using 1 µL of purified library DNA in an Invitrogen Qubit® instrument with the DNA HS protocol. (*Nanodrop should never be used for quantification of library, as it leads to unevenness among indexed samples*).
2. Combine equal amount of indexed libraries, depending on the concentrations of each library and the desired final volume (no less than 10 µL). Many protocols call for factoring library size into the equation for multiplexing libraries. Given the high degree of

fragment size uniformity between samples, the Brutnell lab pools samples based on concentration and desired final library concentration and volume.

Supplemental information 1: Making RNase-free Sera speed beads and XP buffer

1. To make 5M NaCl solution, add 147g of NaCl to a Nuclease-free treated bottle , fill to 500mL using Nuclease-free water, mix and store at room temperature

2. To make 40% PEG-8000, add 100g of PEG-8000 to a nuclease-free treated bottle, fill to 250mL using Nuclease-free water, vortex vigorously to dissolve all of the PEG-8000. 40% PEG-8000 solution should dissolve at room temperature, but if not heat at 80°C until dissolved. Store at room temperature.

3. How to make homemade Sera Beads (18% PEG,1M NaCl):

1) Vortex to re-suspend Sera-mag SpeedBeads and transfer 1 mL to a 1.5 mL Lo-Bind tube
2) Briefly spin down, place Sear Beads on the magnet and wait until beads are drawn to the magnet
3) Wash twice with Nuclease-free water following these steps: Add 1.5 mL Nuclease-free water, remove the tube from magnet, vortex to mix, brief spin down, return to magnet and then repeat.
4) Add 1ml nuclease-free water to washed beads, remove the tube from magnet, resuspend beads with nuclease-free water and put on ice.
5) Add 10mL 5M NaCl, 16.5mL Nuclease-free water and 1mL washed Sera beads from step 4 to a new 50 mL conical tube, mix well.
6) Add 22.5mL 40% PEG to the conical tube prepared in step 5, vortex until beads are well suspended
7) Aliquot/divide into 2 mL Lo-Bind tubes and store at 4 degree, test it using 100bp ladder

4. to make XP buffer (20% PEG, 2.5M NaCl), mix equal parts 5M NaCl and 40% PEG-8000, e.g. 1mL of 5M NaCl and 1 mL of 40% PEG-8000 to make 2mL of XP buffer. Store at RT.

Supplemental information 2:

1. Hybridization protocol for universal adaptor:

- Mix 25 µL of each of the two 100µM universal adapter oligos with a final volume of 50 µL (50µM) in 1x TE
- Heat to 75°C for 5 minutes
- Ramp down to 25°C by 1° per 30 second.
- Hold at 25° for 30 minutes.
- Freeze at -20°C, or place on ice for immediate use after diluting to working concentration (5µM).

Supplemental information 3: List of Adaptors (*See notes below* °) :

Name	Sequence to order from IDT (copy and paste into IDT website directly)
TruSeq Universal Adapter 1	A*A*TGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGAT*C*T
TruSeq Universal Adapter 2	/5Phos/G*A*TCGGAAGAGCACACGTCTGAACTCCAGTC*A*C
Universal PCR primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
Index primer 1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGT
Index primer 2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGT
Index primer 3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGT
Index primer 4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGT
Index primer 5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGT
Index primer 6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGT
Index primer 7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGT
Index primer 8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGT
Index primer 9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGT
Index primer 10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGT
Index primer 11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGT
Index primer 12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGT
Index primer 13	CAAGCAGAAGACGGCATACGAGATTTGACTGTGACTGGAGTTCAGACGTGT
Index primer 14	CAAGCAGAAGACGGCATACGAGATGGAACTGTGACTGGAGTTCAGACGTGT
Index primer 15	CAAGCAGAAGACGGCATACGAGATTGACATGTGACTGGAGTTCAGACGTGT
Index primer 16	CAAGCAGAAGACGGCATACGAGATGGACGGGTGACTGGAGTTCAGACGTGT
Index primer 17	CAAGCAGAAGACGGCATACGAGATCTCTACGTGACTGGAGTTCAGACGTGT
Index primer 18	CAAGCAGAAGACGGCATACGAGATGCGGACGTGACTGGAGTTCAGACGTGT
Index primer 19	CAAGCAGAAGACGGCATACGAGATTTTCACGTGACTGGAGTTCAGACGTGT
Index primer 20	CAAGCAGAAGACGGCATACGAGATGGCCACGTGACTGGAGTTCAGACGTGT
Index primer 21	CAAGCAGAAGACGGCATACGAGATCGAAACGTGACTGGAGTTCAGACGTGT
Index primer 22	CAAGCAGAAGACGGCATACGAGATCGTACGGTGACTGGAGTTCAGACGTGT
Index primer 23	CAAGCAGAAGACGGCATACGAGATCCACTCGTGACTGGAGTTCAGACGTGT
Index primer 24	CAAGCAGAAGACGGCATACGAGATGCTACCGTGACTGGAGTTCAGACGTGT
Index primer 25	CAAGCAGAAGACGGCATACGAGATATCAGTGTGACTGGAGTTCAGACGTGT
Index primer 26	CAAGCAGAAGACGGCATACGAGATGCTCATGTGACTGGAGTTCAGACGTGT
Index primer 27	CAAGCAGAAGACGGCATACGAGATAGGAATGTGACTGGAGTTCAGACGTGT
Index primer 28	CAAGCAGAAGACGGCATACGAGATCTTTTGGTGACTGGAGTTCAGACGTGT
Index primer 29	CAAGCAGAAGACGGCATACGAGATTAGTTGGTGACTGGAGTTCAGACGTGT
Index primer 30	CAAGCAGAAGACGGCATACGAGATCCGGTGGTGACTGGAGTTCAGACGTGT
Index primer 31	CAAGCAGAAGACGGCATACGAGATATCGTGGTGACTGGAGTTCAGACGTGT
Index primer 32	CAAGCAGAAGACGGCATACGAGATTGAGTGGTGACTGGAGTTCAGACGTGT
Index primer 33	CAAGCAGAAGACGGCATACGAGATCGCCTGGTGACTGGAGTTCAGACGTGT
Index primer 34	CAAGCAGAAGACGGCATACGAGATGCCATGGTGACTGGAGTTCAGACGTGT
Index primer 35	CAAGCAGAAGACGGCATACGAGATAAAATGGTGACTGGAGTTCAGACGTGT
Index primer 36	CAAGCAGAAGACGGCATACGAGATTGTTGGGTGACTGGAGTTCAGACGTGT
Index primer 37	CAAGCAGAAGACGGCATACGAGATATTCCGGTGACTGGAGTTCAGACGTGT
Index primer 38	CAAGCAGAAGACGGCATACGAGATAGCTAGGTGACTGGAGTTCAGACGTGT
Index primer 39	CAAGCAGAAGACGGCATACGAGATGTATAGGTGACTGGAGTTCAGACGTGT

Index primer 40	CAAGCAGAAGACGGCATACGAGATTCTGAGGTGACTGGAGTTCAGACGTGT
Index primer 41	CAAGCAGAAGACGGCATACGAGATTAGCGCGTGACTGGAGTTCAGACGTGT
Index primer 42	CAAGCAGAAGACGGCATACGAGATCGATTAGTGACTGGAGTTCAGACGTGT
Index primer 43	CAAGCAGAAGACGGCATACGAGATGCTGTAGTGACTGGAGTTCAGACGTGT
Index primer 44	CAAGCAGAAGACGGCATACGAGATATTATAGTGACTGGAGTTCAGACGTGT
Index primer 45	CAAGCAGAAGACGGCATACGAGATGAATGAGTGACTGGAGTTCAGACGTGT
Index primer 46	CAAGCAGAAGACGGCATACGAGATTCGGGAGTGACTGGAGTTCAGACGTGT
Index primer 47	CAAGCAGAAGACGGCATACGAGATCTTCGAGTGACTGGAGTTCAGACGTGT
Index primer 48	CAAGCAGAAGACGGCATACGAGATTGCCGAGTGACTGGAGTTCAGACGTGT
Index primer 49	CAAGCAGAAGACGGCATACGAGATGCTGGCGTGACTGGAGTTCAGACGTGT
Index primer 50	CAAGCAGAAGACGGCATACGAGATGCTTCTGTGACTGGAGTTCAGACGTGT
Index primer 51	CAAGCAGAAGACGGCATACGAGATGGACCAGTGACTGGAGTTCAGACGTGT
Index primer 52	CAAGCAGAAGACGGCATACGAGATGGATGCGTGACTGGAGTTCAGACGTGT
Index primer 53	CAAGCAGAAGACGGCATACGAGATGGCAGAGTGACTGGAGTTCAGACGTGT
Index primer 54	CAAGCAGAAGACGGCATACGAGATGTAAGTGTGACTGGAGTTCAGACGTGT
Index primer 55	CAAGCAGAAGACGGCATACGAGATGTCGTCGTGACTGGAGTTCAGACGTGT
Index primer 56	CAAGCAGAAGACGGCATACGAGATGTCTATGTGACTGGAGTTCAGACGTGT
Index primer 57	CAAGCAGAAGACGGCATACGAGATGTTCTTGTGACTGGAGTTCAGACGTGT
Index primer 58	CAAGCAGAAGACGGCATACGAGATGTTGCGGTGACTGGAGTTCAGACGTGT
Index primer 59	CAAGCAGAAGACGGCATACGAGATTAACTTGTGACTGGAGTTCAGACGTGT
Index primer 60	CAAGCAGAAGACGGCATACGAGATTACTCTGTGACTGGAGTTCAGACGTGT
Index primer 61	CAAGCAGAAGACGGCATACGAGATTATAGTGTGACTGGAGTTCAGACGTGT
Index primer 62	CAAGCAGAAGACGGCATACGAGATTCGATTGTGACTGGAGTTCAGACGTGT
Index primer 63	CAAGCAGAAGACGGCATACGAGATTGATCGGTGACTGGAGTTCAGACGTGT
Index primer 64	CAAGCAGAAGACGGCATACGAGATTGCGTTGTGACTGGAGTTCAGACGTGT
Index primer 65	CAAGCAGAAGACGGCATACGAGATTTATGAGTGACTGGAGTTCAGACGTGT
Index primer 66	CAAGCAGAAGACGGCATACGAGATTTCAGGGTGACTGGAGTTCAGACGTGT
Index primer 67	CAAGCAGAAGACGGCATACGAGATTTGCTGGTGACTGGAGTTCAGACGTGT
Index primer 68	CAAGCAGAAGACGGCATACGAGATTTGGCCGTGACTGGAGTTCAGACGTGT
Index primer 69	CAAGCAGAAGACGGCATACGAGATAAGCGGGTGACTGGAGTTCAGACGTGT
Index primer 70	CAAGCAGAAGACGGCATACGAGATAAGGTCGTGACTGGAGTTCAGACGTGT
Index primer 71	CAAGCAGAAGACGGCATACGAGATAATCAAGTGACTGGAGTTCAGACGTGT
Index primer 72	CAAGCAGAAGACGGCATACGAGATAATTCGGTGACTGGAGTTCAGACGTGT
Index primer 73	CAAGCAGAAGACGGCATACGAGATACCGTAGTGACTGGAGTTCAGACGTGT
Index primer 74	CAAGCAGAAGACGGCATACGAGATACCTGGGTGACTGGAGTTCAGACGTGT
Index primer 75	CAAGCAGAAGACGGCATACGAGATACGGCTGTGACTGGAGTTCAGACGTGT
Index primer 76	CAAGCAGAAGACGGCATACGAGATACGTACGTGACTGGAGTTCAGACGTGT
Index primer 77	CAAGCAGAAGACGGCATACGAGATACTAGAGTGACTGGAGTTCAGACGTGT
Index primer 78	CAAGCAGAAGACGGCATACGAGATAGAGACGTGACTGGAGTTCAGACGTGT
Index primer 79	CAAGCAGAAGACGGCATACGAGATAGCGCGGTGACTGGAGTTCAGACGTGT
Index primer 80	CAAGCAGAAGACGGCATACGAGATAGGCTTGTGACTGGAGTTCAGACGTGT
Index primer 81	CAAGCAGAAGACGGCATACGAGATAGTCGCGTGACTGGAGTTCAGACGTGT
Index primer 82	CAAGCAGAAGACGGCATACGAGATAGTTATGTGACTGGAGTTCAGACGTGT
Index primer 83	CAAGCAGAAGACGGCATACGAGATATATCTGTGACTGGAGTTCAGACGTGT
Index primer 84	CAAGCAGAAGACGGCATACGAGATATGAGCGTGACTGGAGTTCAGACGTGT

Index primer 85	CAAGCAGAAGACGGCATACGAGATATGGAGGTGACTGGAGTTCAGACGTGT
Index primer 86	CAAGCAGAAGACGGCATACGAGATCAAGTAGTGACTGGAGTTCAGACGTGT
Index primer 87	CAAGCAGAAGACGGCATACGAGATCAATATGTGACTGGAGTTCAGACGTGT
Index primer 88	CAAGCAGAAGACGGCATACGAGATCATGCTGTGACTGGAGTTCAGACGTGT
Index primer 89	CAAGCAGAAGACGGCATACGAGATCCAACTGTGACTGGAGTTCAGACGTGT
Index primer 90	CAAGCAGAAGACGGCATACGAGATCCGCAGGTGACTGGAGTTCAGACGTGT
Index primer 91	CAAGCAGAAGACGGCATACGAGATCCTCGTGTGACTGGAGTTCAGACGTGT
Index primer 92	CAAGCAGAAGACGGCATACGAGATCGAGGTGTGACTGGAGTTCAGACGTGT
Index primer 93	CAAGCAGAAGACGGCATACGAGATCGCCATGTGACTGGAGTTCAGACGTGT
Index primer 94	CAAGCAGAAGACGGCATACGAGATCGGCGAGTGACTGGAGTTCAGACGTGT
Index primer 95	CAAGCAGAAGACGGCATACGAGATCGGTCTGTGACTGGAGTTCAGACGTGT
Index primer 96	CAAGCAGAAGACGGCATACGAGATCGTATTGTGACTGGAGTTCAGACGTGT
Index primer 97	CAAGCAGAAGACGGCATACGAGATCTACGGGTGACTGGAGTTCAGACGTGT
Index primer 98	CAAGCAGAAGACGGCATACGAGATCTCGGAGTGACTGGAGTTCAGACGTGT
Index primer 99	CAAGCAGAAGACGGCATACGAGATCTGACGGTGACTGGAGTTCAGACGTGT
Index primer 100	CAAGCAGAAGACGGCATACGAGATCTGGTTGTGACTGGAGTTCAGACGTGT
Index primer 101	CAAGCAGAAGACGGCATACGAGATCTTGACGTGACTGGAGTTCAGACGTGT
Index primer 102	CAAGCAGAAGACGGCATACGAGATGAATTGGTGACTGGAGTTCAGACGTGT
Index primer 103	CAAGCAGAAGACGGCATACGAGATGACGGTGTGACTGGAGTTCAGACGTGT
Index primer 104	CAAGCAGAAGACGGCATACGAGATGAGATAGTGACTGGAGTTCAGACGTGT
Index primer 105	CAAGCAGAAGACGGCATACGAGATGAGCCTGTGACTGGAGTTCAGACGTGT
Index primer 106	CAAGCAGAAGACGGCATACGAGATGATTACGTGACTGGAGTTCAGACGTGT
Index primer 107	CAAGCAGAAGACGGCATACGAGATGCAGTTGTGACTGGAGTTCAGACGTGT
Index primer 108	CAAGCAGAAGACGGCATACGAGATGCCGAGGTGACTGGAGTTCAGACGTGT

° *Illumina uses a green and red laser for sequencing. During each sequencing cycle, one of two nucleotides for each color channel must be registered. Therefore, it is important to maintain a balance between green-channel and red-channel indices for each base of the index, else index read sequencing may fail due to registration failure. This is especially important to check when sequencing libraries with low complexity (few indices pooled).*

- ° *Colored nucleotides indicate the indices.*
- ° *Red colored nucleotides indicate "red-channel" indices.*
- ° *Green colored nucleotides indicate "green-channel" indices.*