* Oligo sequences are listed at the end. The directions for making the wash buffers are given in the Meyer paper (http://www.nature.com/nprot/journal/v8/n4/full/nprot.2013.038.html).
** Note that we dilute Circligase II and its accompanying reagents with respect to the original protocol. This is to make the library preparation similarly affordable to standard Illumina preps.
*** Always include 1uL of positive control (see Appendix) at 100 nM as a sample, and a negative control (sterile water).

1. Use eluted DNA as sample input. Add the above in a PCR strip tube and vortex.

Input	**Volume (uL) per sample**
Water	36-(sample_volume)
Sample	(up to 29)
CircLigase II Buffer	4
MnCl2 (comes with CircLigase)	2

2. Add 1 uL of FastAP (1 Unit) to each mixture and mix by flicking the tube with your finger. Spin the tubes down in microcentrifuge.
3. Incubate the reactions in a thermal cycler with heated lid (**105 C**) for:
    a. **10 min** at **37 C**
    b. **2 min** at **95 C**
    c. Immediately remove from thermocycler and quench the tubes in an **ice** water bath for **1 min**.
4. Spin down tubes in microcentrifuge.
5. Mix the following very well and add to each reaction mixture:

Input	**Volume (uL) per sample**
Water	2.5
PEG-4000 (50%)	32
Adapter oligo CL78 (10 uM)	1

*** Make sure you use fresh PEG-4000 (50%), do not UV treat.

6. Vortex the tubes and briefly spin down in microcentrifuge.
7. Add **1.5 uL of Circligase II enzyme** to reaction mixtures, seal the tubes, and mix by flicking. Briefly spin down in microcentrifuge.
8. Incubate the reactions in a thermal cycler with heated lid for **2 hours** at **60 C**.

9. Add 2 uL of Stop solution to each reaction mixture, you can freeze the samples at **-20 C** for up to a few weeks before moving forward.

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10. Resuspend stock of MyOne Streptavidin coated bead and transfer 20 uL for each sample into a 1.5 mL tube.
11. Add appropriate amount to SDS from stock Bead Binding Buffer (**3B**) solution (should have a total of (n/4 +1) mL).
12. Pellet magnetic beads on magnetic rack and discard supernatant.
13. Wash beads **twice** with 500 uL of **3B**. Then resuspend beads in 250*n uL of **3B** where n is the number of samples.
14. Per samples, transfer an aliquot of 250 uL of beads suspended in **3B** to a 1.5 mL tube.
15. Prepare an **ice** water bath.
16. Incubate the ligation product for step 7 for **1 min** at **95 C** with a heated lid. Immediately remove at the end of the minute and quench in **ice** water bath for at least **1 min**.
17. Transfer samples into appropriate tubes with 3B.
18. Rotate tubes for at least **20 minutes** at room temperature. When finished, spin tubes in microcentrifuge, briefly.
19. Place tubes on magnetic rack, pellet the beads, and discard the supernatant.
20. Wash each sample with 200 uL of Wash Buffer A (**WBA**), discard, and then was with Wash Buffer B (**WBB**).
21. With **WBB** still in the tubes make the following components for Mix 1 (**M1**):

Input	**Volume (uL) per sample**
Water	40.5
Isothermal Amplification Buffer (10x)	5
dNTP mix (25 mM each)	0.5
Oligo CL9 (100 uM)	1

22. Discard **WBB** and add 47 uL of **M1** to each sample.
23. If you do not have a thermal mixer, set fresh PCR strip tubes in a thermal cycler with lid open and hold at **15 C**.
24. Resuspend bead by vortexing. Incubate tubes in a thermal shaker for **2 min** at 65 C. Place the tubes in an **ice** water bath for **1 min**. Immediately transfer contents of tubes to the pre-cooled **15 C** tubes.
25. Add 3 uL of Bst 2.0 Polymerase to each of the reaction contents.
26. Incubate (with heated lid) the reaction mixtures from **15 C** to **37 C** by ramping up at **1 C** per **minute** and hold incubation at **37 C** for **5 min.** If you don't have a thermal mixer. Pause the thermocycler every **5 minutes** and briefly vortex tubes to keep beads suspended. The total time for this should be **26 minutes**.

27. Briefly spin down tubes in microcentrifuge. Pellet the beads on a magnetic rack and discard supernatant. Then do as follows:
   a. Wash once with 200 uL of **WBA** and discard.
   b. Add 100 uL of Stringency Wash Buffer (**SWB**) to the contents.
   c. Vortex tubes to resuspend bead.
   d. Incubate for **3 min** at **45 C** (I usually vortex the whole block at the **1 min** and **2 min** mark for **10 s** to keep beads suspended).
   e. Spin down contents in microcentrifuge.
   f. Pellet beads and discard excess **SWB**.
   g. Add 200 uL of **WBB**.

28. Mix the following contents for Mix 2 (**M2**):

Input	**Volume (uL) per sample**
Water	86.1
Buffer Tango (10x)	10
dNTP mix (25 mM each)	0.4
Tween 20 (1%)	2.5

29. Pellet the beads on magnetic rack, discard **WBB**, and add 99 uL of **M2** to each sample.
30. Vortex to resuspend beads in **M2**.
31. Add 1 uL of T4 DNA polymerase (5U) to each reaction and mix the tubes by vortexing.
32. Incubate reactions for **15 minutes** at **25 C** in a thermal shaker. If you do not have a thermal shaker, I would recommend vortexing samples briefly every **3-5 min**.
33. Add 10 uL of EDTA (0.5 M) to each reaction. Vortex to mix contents.
34. Repeat Step 27.
35. Mix the following contents for Mix 3 (**M3**):

Input	**Volume (uL) per sample**
Water	73.5
PEG-4000 (50%)	10
T4 DNA Ligase Buffer (10x)	10
Tween 20 (1%)	2.5
Double-Stranded Adapter (100 uM)	2

36. Pellet the beads on magnetic rack, discard **WBB**, and add 98 uL of **M3** to each sample.

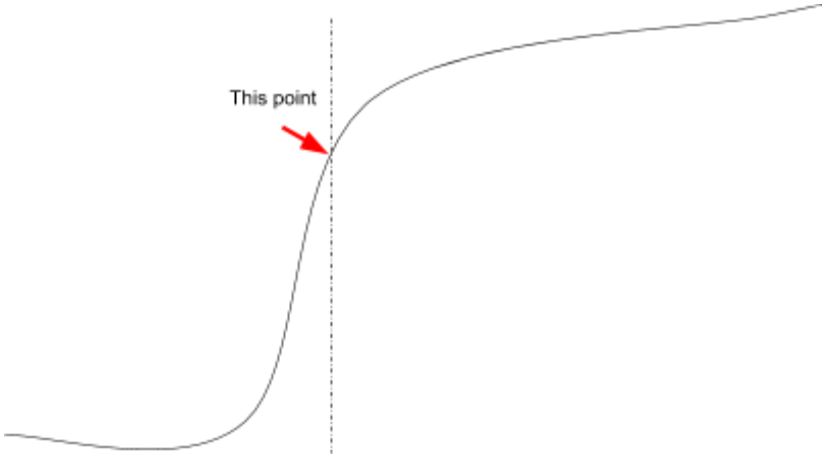
37. Vortex to resuspend beads in **M3**.
38. Add 2 uL of T4 DNA ligase (10U) to each reaction and mix the tubes by vortexing.
39. Incubate the reaction for **1 hour** at **room temperature**. Again if you don't have a thermal mixer, vortex tubes by hand every **3-5 min**.
40. At the end of the hour, repeat Step 27.
41. Pellet the beads on magnetic rack, discard WBB, and add 25 uL of Elution Buffer (I use **TET**) to each sample. Resuspend the beads by vortexing.
42. Transfer samples to PCR strip tubes.
43. Ready a 96 well plate magnet.
44. Incubate bead suspensions for **1 min** at **95 C** with heated lid. Immediately transfer beads to the magnetic rack and transfer the resulting supernatant to fresh 0.5 mL tubes.
45. In separate tubes, dilute the the supernatant product 1:20 in **TET** buffer. In addition, prepare a 1:500 dilution of the positive control stock in **TET** buffer.
46. Prepare a standard dilution (see the Original Paper) - I use 10^8, 10^6, 10^4, 10^2, and a negative control.
47. Prepare the following mixture for qPCR:

Input	**Volume (uL) per sample**
Water	13
Maxima Sybr Green qPCR master mix (2x)	10
For. primer (IS7 for assay A, CL107 for assay B)	0.5
Rev. primer (IS8 for assay A, CL108 for assay B)	0.5
Sample	1

48. The positive and negative controls that went through library prep, as well as the standards, should be used with both assays. The samples are only to be processed with assay A, and the positive control prepared in Step 45 should only be used in assay B. Assay A indicates molecules that have successfully been ds-adapted, while Assay B only target synthetic controls to determine the amount of DNA that had been ligated to beads. Using the calculated numbers of molecules from library prepped positive controls (call them LPA and LPB) and the number of molecules in the Step 45 positive control (S45B), you can determine the efficiency - LPA/S45B gives the overall efficiency of the reaction .
49. Once adding the PCR mix and samples to a 96 well plate, vortex to mix and spin down in a large centrifuge for **2 min** at 2500 g.
50. Run the following steps on the qPCR machine of choice:
    a. **95 C** for **10 min**
    b. **95 C** for **30 s**
    c. **60 C** for **1 min** (and capture fluorescence measurement)
    d. **72 C** for **1 min** (and capture fluorescence measurement)

e. Repeat b-d 39 times
f. Melt curve **60 C** to **95 C** at **0.5 C** per cycle
g. **Infinite** hold at **10 C**

51. When finished, look at the quantification curves:
    a. At **72 C**, your samples, unlike standards and controls, should have a downward sloping tail indicating short sequences at a variety of lengths.
    b. At **60 C**, find the cycle number at which the linear curves turns logarithmic.



    c. If you are proceeding with 12 uL of sample for indexing, subtract 9 cycles from the determined cycle to find the ideal number of cycles for indexing. If using all 24 uL for indexing, subtract 8 cycles.

52. Prepare the following PCR mix, make sure you use different indexing primers for each sample. If you want to use all 24 uL of product for indexing, just double below:

Input	**Volume (uL) per sample**
Water (to 50 uL)	28.5
AccuPrime Pfx reaction mix (10x)	5
AccuPrime Pfx polymerase (2.5 U/uL)	0.5
P7 indexing primer (10 uM)	2
P5 indexing primer (10 uM)	2
Library	12

53. Incubate the reactions in the thermal cycler with the following thermal profile:
    a. **95 C** for **2 min**
    b. **95 C** for **15 s**
    c. **60 C** for **30 s**
    d. **68 C** for **1 min**

    e. Repeat steps b-d for the number of steps determined in step 51 c.
54. Once finished, purify samples using a MinElute PCR purification kit or AMPure XP SPRI beads. Elute into 20 uL of TE buffer or provided EB.
55. Sequence!

We changed up the oligos used in the mixes discussed above, as well as the one used to make the double-stranded adapter. They are listed in the supplementary information of our paper (http://www.nature.com/article-assets/npg/srep/2016/160614/srep27859/extref/srep27859-s1.pdf):

CL9 Extension primer :
GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T*N*N* N*N

CL53 Double-stranded adapter 1 :
ACA CGA CGC TCT TC/3ddC/

CL57 Double-stranded adapter 2:
/5Phos/GGA AGA GCG TCG TGT AGG GAA AGA G*T*G* T*A*

CL78 Single-stranded adapter:
/5Phos/AGA TCG GAA GTT TTT TTT TT/3BioTEG/

All the other oligos and the reagents and equipment are listed in the Gansauge and Meyer paper (http://www.nature.com/nprot/journal/v8/n4/full/nprot.2013.038.html).