

## Supplementary Protocol: CIRCLE-seq Library Preparation

| Reagent  | Vendor              |
|--|---------------------|
| Genra Puregene Tissue Kit  | Qiagen              |
| Qubit dsDNA BR Assay Kit   | Thermo Fisher       |
| Agencourt AMPure XP magnetic beads                                 | Beckman Coulter     |
| High throughput, “with bead”, PCR-free Library Preparation Kit     | KAPA Biosystems     |
| Enzymes and buffers  | New England Biolabs |
| – Lamda exonuclease  |                     |
| – Exonuclease I ( <i>E.coli</i> )                                  |                     |
| – USER enzyme  |                     |
| – T4 polynucleotide kinase   |                     |
| – T4 DNA Ligase  |                     |
| – Cas9 nuclease, <i>S. pyogenes</i>                                |                     |
| Plasmid-Safe™ ATP-Dependent DNase                                  | Epicentre           |
| MEGAscript™ Kit  | Thermo Fisher       |
| NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers Set 1) | New England Biolabs |
| KAPA HiFi HotStart ReadyMix  | KAPA Biosystems     |
| ddPCR™ Library Quantification Kit for Illumina TruSeq              | Bio-Rad             |
| KAPA Library Quantification Kit for NGS (Universal)                | KAPA Biosystems     |

## CIRCLE-seq Hairpin Adapter

oSQT1288 /5Phos/CGGTGGACCGATGATC /ideoxyU/ ATCGGTCCACCG\*T

Annealing Program: 95°C for 5 min, -1°C/min for 70 cycles, hold at 4°C.

### Overview Notes

- Approximately 25 µg of starting DNA is required for each gRNA (or control) sample.
- 1 control sample, where Cas9:gRNA is not added to cleavage step, should be run for each unique genomic DNA source

### Input Quantification and Shearing

1. Genomic DNA is sheared to an average length of 300 bp according to the standard operating protocol for the Covaris S2.
2. Sheared DNA is cleaned up with 1.8X Ampure XP SPRI beads according to manufacturer's protocol, and eluted in 35 µl of 1X TE buffer.

### End-repair

3. For each end-repair reaction:

| Component                                | Volume       |
|--|--------------|
| Nuclease-free H <sub>2</sub> O           | 8 µl         |
| KAPA End Repair Buffer (10X)             | 7 µl         |
| KAPA End Repair Enzyme Mix               | 5 µl         |
| <b>Total Master Mix</b>                  | <b>20 µl</b> |
| Sheared genomic DNA (5 µg) (from step 2) | 50 µl        |
| <b>Total</b>                             | <b>70 µl</b> |

End Repair Program: 20°C for 30 min, hold at 4°C.

4. 1.7X SPRI cleanup (120 µl of Agencourt Ampure XP beads), elute in 42 µl of 1X TE buffer.

### A-tailing

5. For each A-tailing reaction:

| Component                                 | Volume       |
|---|--------------|
| KAPA A-tailing Buffer (10X)               | 5 µl         |
| KAPA A-tailing Enzyme                     | 3 µl         |
| <b>Total Master Mix</b>                   | <b>8 µl</b>  |
| End Repaired DNA with beads (from step 4) | 42 µl        |
| <b>Total</b>                              | <b>50 µl</b> |

A-tailing Program: 30°C for 30 min, hold at 4°C.

6. 1.8X SPRI cleanup (90  $\mu$ l of PEG/NaCl SPRI Solution), elute in 30  $\mu$ l of 1X TE buffer.

### Adapter Ligation

7. For each ligation reaction to annealed adapter oSQT1288:

| Component                                      | Volume                      |
|--|-----------------------------|
| KAPA Ligation Buffer (5X)                      | 10 $\mu$ l                  |
| KAPA T4 DNA Ligase                             | 5 $\mu$ l                   |
| Annealed Hairpin Adapter oSQT1288 (40 $\mu$ M) | 5 $\mu$ l                   |
| <b>Total Master Mix</b>                        | <b>20 <math>\mu</math>l</b> |
| A-tailed DNA with beads (from step 6)          | 30 $\mu$ l                  |
| <b>Total</b>                                   | <b>50 <math>\mu</math>l</b> |

Ligation Program: 20°C for 1 hr, hold at 4°C.

8. 1X SPRI cleanup (50  $\mu$ l of PEG/NaCl SPRI Solution), elute in 30  $\mu$ l of 1X TE buffer.

### Enzymatic Treatments

#### Lambda Exonuclease/Exonuclease I (*E. coli*) Treatment

- 9.

| Component  | Volume                      |
|--|-----------------------------|
| Exonuclease I Reaction Buffer (10X)              | 5 $\mu$ l                   |
| Lambda Exonuclease (5 U/ $\mu$ l)                | 4 $\mu$ l                   |
| Exonuclease I ( <i>E. coli</i> ) (20 U/ $\mu$ l) | 1 $\mu$ l                   |
| <b>Total Master Mix</b>                          | <b>10 <math>\mu</math>l</b> |
| Adapter ligated DNA (1 $\mu$ g) (from step 8)    | 40 $\mu$ l                  |
| <b>Total</b>                                     | <b>50 <math>\mu</math>l</b> |

Incubation Program: 37°C for 1 hr, 75°C for 10 min, hold at 4°C.

10. 1.8X SPRI cleanup (90  $\mu$ l of Agencourt Ampure XP beads), elute in 40  $\mu$ l of 1X TE buffer.

### USER/T4 PNK Treatment

11.

| Component  | Volume                      |
|--|-----------------------------|
| T4 DNA Ligase Buffer (10X)   | 5 $\mu$ l                   |
| USER Enzyme (1 U/ $\mu$ l)   | 3 $\mu$ l                   |
| T4 Polynucleotide Kinase (10 U/ $\mu$ l)                               | 2 $\mu$ l                   |
| <b>Total Master Mix</b>  | <b>10 <math>\mu</math>l</b> |
| Lambda Exonuclease/Exonuclease I treated DNA with beads (from step 10) | 40 $\mu$ l                  |
| <b>Total</b>   | <b>50 <math>\mu</math>l</b> |

Incubation Program: 37°C for 1 hr, hold at 4°C.

12. 1.8X SPRI cleanup (90  $\mu$ l of PEG/NaCl SPRI Solution), elute in 35  $\mu$ l of 1X TE buffer.

### Intramolecular Circularization

13.

| Component                                       | Volume                       |
|---|------------------------------|
| Nuclease-free H <sub>2</sub> O                  | 8 $\mu$ l                    |
| T4 DNA Ligase Buffer (10X)                      | 10 $\mu$ l                   |
| T4 DNA Ligase (400 U/ $\mu$ l)                  | 2 $\mu$ l                    |
| <b>Total Master Mix</b>                         | <b>20 <math>\mu</math>l</b>  |
| USER/T4 PNK treated DNA (500 ng) (from step 12) | 80 $\mu$ l                   |
| <b>Total</b>                                    | <b>100 <math>\mu</math>l</b> |

Circularization Program: 16°C for 16 hrs.

14. 1X SPRI cleanup (100  $\mu$ l of Agencourt Ampure XP beads), elute in 38  $\mu$ l of 1X TE buffer.

### Plasmid-Safe ATP-Dependent DNase Treatment

15.

| Component  | Volume                      |
|--|-----------------------------|
| Plasmid-Safe Reaction Buffer (10X)               | 5 $\mu$ l                   |
| ATP (25 mM)                                      | 2 $\mu$ l                   |
| Plasmid-Safe ATP-Dependent DNase (10 U/ $\mu$ l) | 5 $\mu$ l                   |
| <b>Total Master Mix</b>                          | <b>12 <math>\mu</math>l</b> |
| Circularized DNA (from step 14)                  | 38 $\mu$ l                  |
| <b>Total</b>                                     | <b>50 <math>\mu</math>l</b> |

Incubation Program: 37°C for 1 hr, 70°C for 30 min, hold at 4°C.

16. 1X SPRI cleanup (50  $\mu$ l of Agencourt Ampure XP beads), elute in 15  $\mu$ l of 1X TE buffer.

## In Vitro Digestion with Cas9 and gRNA

17.

| Component                                     | Volume                      |
|---|-----------------------------|
| Cas9 Nuclease Reaction Buffer (10X)           | 10 $\mu$ l                  |
| Cas9 Nuclease, <i>S. pyogenes</i> (1 $\mu$ M) | 9 $\mu$ l                   |
| In Vitro Transcribed guide RNA (3000 nM)      | 3 $\mu$ l                   |
| <b>Total Master Mix</b>                       | <b>22 <math>\mu</math>l</b> |

Incubate at room temperature for 10 min.

|  |                              |
|--|------------------------------|
| Plasmid-Safe DNase Treated DNA (250 ng) (from step 16) | 78 $\mu$ l                   |
| <b>Total</b>   | <b>100 <math>\mu</math>l</b> |

Digestion Program: 37°C for 1 hr, hold at 4°C.

18. 1X SPRI cleanup (100  $\mu$ l of Agencourt Ampure XP beads), elute in 42  $\mu$ l of 1X TE buffer.

## A-tailing

19.

| Component  | Volume                      |
|--|-----------------------------|
| KAPA A-tailing Buffer (10X)                      | 5 $\mu$ l                   |
| KAPA A-tailing Enzyme                            | 3 $\mu$ l                   |
| <b>Total Master Mix</b>                          | <b>8 <math>\mu</math>l</b>  |
| Cas9/gRNA digested DNA with beads (from step 18) | 42 $\mu$ l                  |
| <b>Total</b>                                     | <b>50 <math>\mu</math>l</b> |

A-tailing Program: 30°C for 30 min, hold at 4°C.

20. 1.8X SPRI cleanup (90  $\mu$ l of PEG/NaCl SPRI Solution), elute in 30  $\mu$ l of 1X TE buffer.

## Adapter Ligation

21.

| Component                                   | Volume                      |
|---|-----------------------------|
| KAPA Ligation Buffer (5X)                   | 10 $\mu$ l                  |
| KAPA T4 DNA Ligase                          | 5 $\mu$ l                   |
| NEBNext Adaptor for Illumina (15 $\mu$ M) * | 10 $\mu$ l                  |
| <b>Total Master Mix</b>                     | <b>25 <math>\mu</math>l</b> |
| A-tailed DNA with beads (from step 20)      | 25 $\mu$ l                  |
| <b>Total</b>                                | <b>50 <math>\mu</math>l</b> |

\* NEBNext Adaptor for Illumina (#E7601A):

5'-/5Phos/GATCGGAAGAGC ACACGTCTGAACTCCAGTC/ideoxyU/ACACTCT TT CCTACACGACGCTCTTCCGAT C\*T-3

Ligation Program: 20°C for 1 hr, hold at 4°C.

22. 1X SPRI cleanup (50  $\mu$ l of PEG/NaCl SPRI Solution), elute in 47  $\mu$ l of 1X TE buffer.

## USER Enzyme Treatment

23. Add 3  $\mu$ l of USER Enzyme (1 U/ $\mu$ l) to the adapter ligated DNA with beads (from step 22).

24. 0.7X SPRI cleanup (35  $\mu$ l of PEG/NaCl SPRI Solution), elute in 20  $\mu$ l of 1X TE buffer.

## PCR

25.

| Component                                      | Volume                      |
|--|-----------------------------|
| Nuclease-free H <sub>2</sub> O                 | 5 $\mu$ l                   |
| KAPA HiFi HotStart ReadyMix                    | 25 $\mu$ l                  |
| <b>Total Master Mix</b>                        | <b>30 <math>\mu</math>l</b> |
| NEBNext i5 Primer (10 $\mu$ M)                 | 5 $\mu$ l                   |
| NEBNext i7 Primer (10 $\mu$ M)                 | 5 $\mu$ l                   |
| USER enzyme treated DNA (20 ng) (from step 24) | 10 $\mu$ l                  |
| <b>Total</b>                                   | <b>50 <math>\mu</math>l</b> |

PCR Program: 98°C for 45 s, 22 cycles of (98°C for 15 s, 65°C for 30 s, 72°C for 30 s), 72°C for 1 min, hold at 4°C.

26. 0.7X SPRI cleanup (35  $\mu$ l of Agencourt Ampure XP beads), elute in 30  $\mu$ l of 1X TE buffer.

## Library Quantification

27. Quantify the library using ddPCR Library Quantification Kit for Illumina TruSeq (Bio-Rad) on QX200 Droplet Digital PCR instrument, according to the manufacturer instructions. An alternative quantification method is using KAPA Library Quantification Kit for Next-Generation Sequencing (KAPA Biosystems), according to the manufacturer instructions.