Title: CHANGEseq
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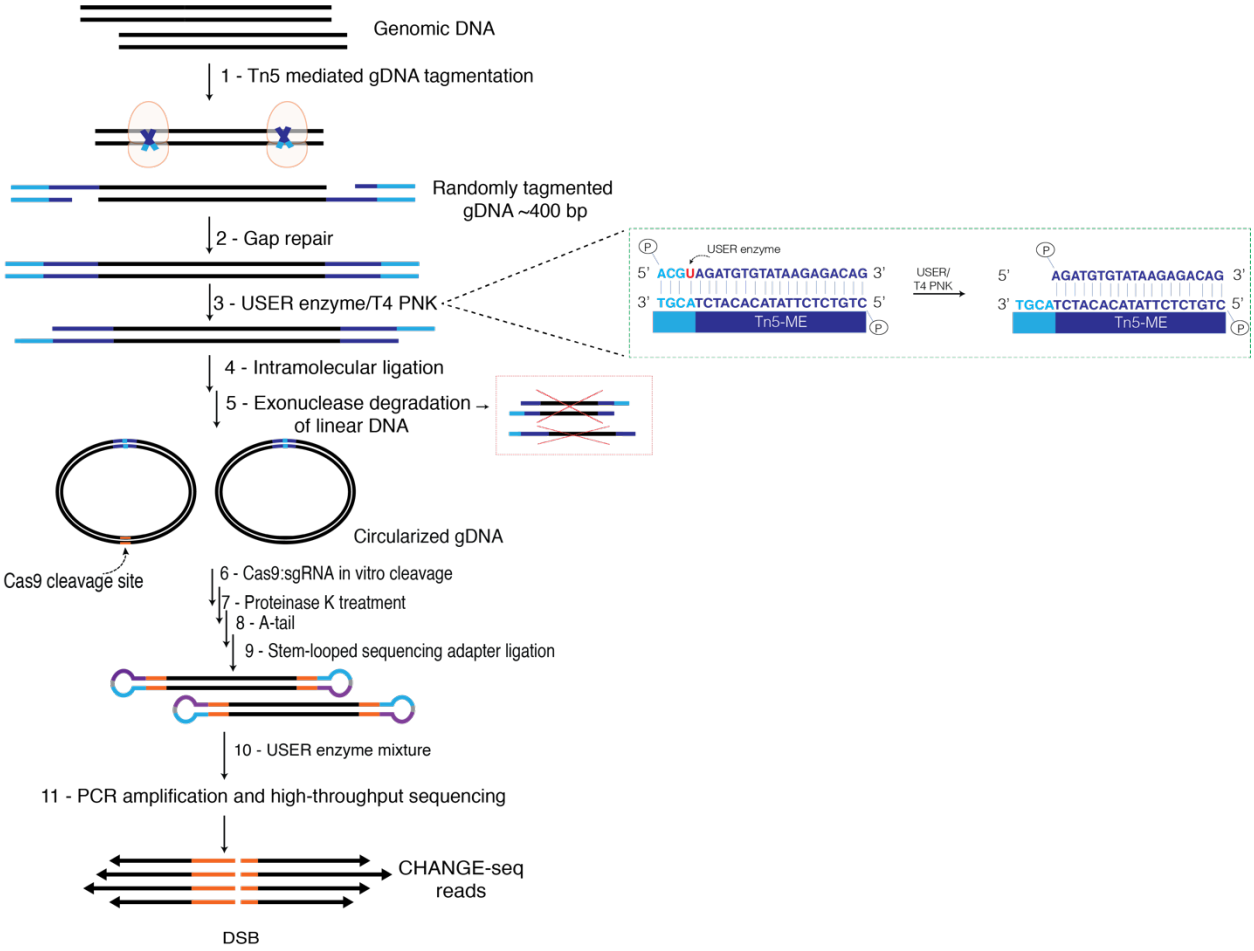

Supplementary Fig. 1 | Detailed overview of CHANGE-seq method. Genomic DNA is randomly tagmented to an average of ~400 bp with a custom Tn5-transposome with an uracil-containing adapter. 9-nt Tn5-generated gaps in the DNA are filled in with a high-fidelity uracil-tolerant U+ polymerase and sealed with Taq DNA ligase. 4 bp overhangs are released with a mixture of USER enzyme and T4 PNK. DNA molecules are circularized at low concentrations that favor intramolecular ligation. Unwanted linear DNA is degraded with an exonuclease cocktail (comprised of Exonuclease I, Lambda exonuclease and Plasmid-Safe ATP-dependent DNase). Purified circular DNA is treated with Cas9:sgRNA RNP and cleaved DNA ends at on- and off-target sites are released for NGS library preparation, PCR amplification, and pair-end high-throughput sequencing.

CHANGE-seq protocol

REAGENTS

- Gentra Puregene Tissue Kit (Qiagen, cat.no. 158667)
- IDTE pH 8.0 (1X TE Solution) (Integrated DNA Technologies, cat.no. 11050204)
- HTP Library Preparation Kit PCR-free (96rxn) (Kapa Biosystems, cat.no. KK8235)
- PEG/NaCl SPRI solution, supplied with HTP Library Preparation Kit PCR-free (96rxn) (Kapa Biosystems)
- 2X Kapa HiFi HotStart Ready Mix (Kapa Biosystems, cat.no. KK2602)
- T4 Polynucleotide Kinase (PNK) (New England BioLabs, cat.no. M0201L)
- T4 DNA Ligase (New England BioLabs, cat.no. M0202L)
- 10X T4 DNA ligase Buffer (New England BioLabs), supplied with T4 DNA Ligase
- USER Enzyme (New England BioLabs, cat.no. M5505L)
- Exonuclease I (*E. coli*) (New England BioLabs, cat.no. M0293L)
- Lambda Exonuclease (New England BioLabs, cat.no. M0262L)
- Plasmid-Safe ATP-dependent DNase (Epicentre, cat.no. E3110K)
- Plasmid-Safe 10X Reaction Buffer (Epicentre), supplied with Plasmid-Safe ATP-dependent DNase
- 25mM ATP solution (Epicentre), supplied with Plasmid-Safe ATP-dependent DNase
- Cas9 nuclease *S. pyogenes* (New England BioLabs, cat.no M0386M)
- 10X Cas9 buffer (New England BioLabs), supplied with Cas9 nuclease *S. pyogenes*
- NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers Set 1) (New England BioLabs, cat.no. E7600S)
- NEBNext adapter for Illumina (New England BioLabs), supplied with NEBNext® Multiplex Oligos for Illumina®
- Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, cat.no. Q32853)
- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, cat.no. Q32854)
- Qubit assay tubes (Thermo Fisher Scientific, cat.no. Q32856)
- MiSeq® Reagent Kit v3 (600 cycle) (Illumina, cat.no. MS-102-3003)
- Flow Cell, supplied with MiSeq® Reagent Kit v3 (600 cycle)
- Hyb Buffer, supplied with MiSeq® Reagent Kit v3 (600 cycle)
- PhiX Control V3 KIT (Illumina, cat.no. FC-110-3001)
- Sodium hydroxide solution, volumetric, 1 M NaOH (1N) (Sigma-Aldrich, cat.no. 71463-1L
- North Alcohol Wipes (Dynarex, cat.no. 19-014-855)
- VWR Lens Cleaning Tissue (VWR, cat.no. 52846-001)
- EDTA 0.5 M (Thermo Fisher Scientific, cat.no. 15575020)
- Ethanol (Sigma, cat.no. E7023)
- Tween-20 (Sigma-Aldrich, cat.no. P7949)
- Sera-Mag Magnetic Beads; Carboxyl, Speedbeads; hydrophobic; 5 solids (Fisher/GE, cat.no. 9981123)
- Guanidine thiocyanate (Sigma, cat.no. G9277)
- Sodium Chloride 5M Sterile (Fisher, cat.no. 50146927)
- TRIS Buffer 1.0 M solution, pH 8.0 (Fisher, cat.no. 50146868)
- Polyethylene Glycol 8000 (Fisher, cat.no. 507516674)
- Taq DNA ligase (NEB, cat.no. M0208L)
- KAPA HiFi HotStart Uracil+ ReadyMix (250 x 50 µl reactions) (Kapa Biosystems, cat.no. KK2802)
- Proteinase K (NEB, cat.no. P8107S)
- Lib Quant Kit (Illumina/Uni) (Kapa Biosystems, cat.no. KK4824)
- N,N-Dimethylformamide (Sigma, D4551-250ML)

- TAPS (Sigma, T5130)
- Magnesium chloride hexahydrate (Sigma, M9272-500G)

REAGENT SETUP

Resuspend the CHANGE-seq custom transposon oligonucleotides (oCRL225 and oCRL226) Resuspend the oligonucleotides to 100 µM in TE pH 8.0. Keep the resuspended oligonucleotides at -20°C.

oCRL225 /5Phos/ACG/ideoxyU/AGATGTGTATAAGAGACAG

oCRL226 /5Phos/CTGTCTCTTATACACATCTACGT

Anneal CHANGE-seq custom transposon (oCRL225 and oCRL226) Mix the oligonucleotides as follows:

Component	Volume (µl)
oCRL225 100 µM	50
oCRL226 100 µM	50
Total	100

On a thermocycler, set up the follow annealing program: 95°C for 5 min, -1°C/min for 70 cycles, hold at 4°C.
After annealing, add 100 µl of TE pH 8.0 to bring the concentration of the annealed oligonucleotides to 25 µM. Keep the annealed oligonucleotides at -20°C. The annealed adapters will be used for transposome assembly.

2X Tn5 dialysis buffer 100 mM Hepes-KOH, pH 7.2, 0.2 M NaCl, 0.2 mM EDTA, 2 mM DTT, 0.2% Triton X-100, 20% glycerol.

Transposome assembly Perform the transposome assembly as follows:

Component	Volume (µl)
Tn5 (1.85 mg/ml)	360
Annealed oCRL225/oCRL226 (25 µM)	150
2X Tn5 dialysis buffer	520
Total	1030

Incubate at room temperature for 1 hour and then store at -20 °C.

5X TAPS-DMF buffer 50mM TAPS-NaOH pH 8.5, 25mM $MgCl_2$, 50% v/v DMF.

SPRI-guanidine binding buffer 4M guanidine thiocyanate, 40mM TRIS, 17.6mM EDTA, pH 8.0. TRIS 1M pH 8 and EDTA 0.5M pH 8 can be added to the 4M guanidine (after the guanidine is solubilized in water – add the proper volume for getting the right final concentration) and then the pH will be very close to 8. Bring the pH to 8 with HCl.

Sera-Mag Magnetic Beads preparation Add 1 ml of Sera-Mag Magnetic Beads (Fisher/GE) to a 1.5 ml Eppendorf tube. Place in a magnetic rack. Remove the liquid. Remove the tube from the rack. Add 1 ml of TE and homogenize. Place back in the magnetic rack and remove the liquid. Repeat this step for a total of

two TE pH 8.0 washes. Then, add 1 ml of TE pH 8.0. Note: this beads preparation step is required for preparing SPRI-guanidine beads and SPRI-beads.

SPRI-guanidine beads preparation Add 10 ml of 5M NaCl to 9 g of PEG 8000 and then add SPRI-guanidine binding buffer (prepared as described above) up to 49 ml. Homogenize during 5 min. Add 1 ml of Sera-Mag Magnetic Beads in TE (prepared as described above) and homogenize. Keep at 4°C.

SPRI-beads preparation Add 10 ml of 5M NaCl, 500 µl of 1M TRIS and 100 µl of 0.5M EDTA to 9 g of PEG 8000. Complete the volume to 49 ml with ultra-pure water. Add 1 ml of Sera-Mag Magnetic Beads in TE (prepared as described above) and homogenize. Add 27.5 µl of Tween-20 and homogenize. Keep at 4°C.

PROCEDURE

Genomic DNA Isolation

1l Perform genomic DNA isolation with Gentra Puregene Kit (Qiagen), following the manufacturer's instructions.

CHANGE-seq library preparation

2l Genomic DNA tagmentation. Tn5 reactions are assembled as follows:

Component	Volume (µl)
5x TAPS-DMF buffer	20
Tn5 preassembled with oCRL225/oCRL226-MEDS	40
Genomic DNA (50 ng/µl)	20
H_2O	20
Total	100

Incubate in a thermocycler at 55°C for 7 minutes.

3l Dilute proteinase K 1:1 in water (2.5 µl of proteinase K and 2.5 µl of water) and add 5 µl of the dilution to the tagmented DNA. Incubate at 55°C for 15 minutes.

4l Add 1.8X volumes (189 µl) of SPRI-Guanidine beads to the tagmented DNA, mix thoroughly by pipetting 10 times. Incubate at room temperature for 10 minutes. Place the reaction plate onto a Magnum FLX magnetic rack for 5 minutes. Remove the cleared solution from the reaction plate and discard. Add 200 µl of 80% ethanol, incubate for 30 seconds and remove the supernatant. Repeat this step for a total of two ethanol washes. Remove ethanol completely and let the samples air dry for 3 minutes on the magnetic rack. Remove the plate from the magnetic rack and add 46 µl of TE pH 8.0, and pipette 10 times to mix. Incubate at room temperature for 2 minutes. Place the reaction plate back to the magnetic rack for 1 minute. Transfer the eluted DNA to a new plate, with 23 µl of sample in each well (each tagmentation reaction will be split in two reactions for gap repair Step 6, i.g. if you have 48 samples for tagmentation step 2, you will have 96 samples for gap-repair step 6).

5l Run 10 µl on a QIAxcel capillary electrophoresis instrument, in a 0.2 ml thin-walled 12-well strip tube with a QIAxcel DNA High Resolution Kit (Qiagen), QX Alignment Marker 15 bp – 10 kb (Qiagen) and QX Size Marker 250 bp – 8 kb (Qiagen), following manufacturer's instruction. Quantify by Qubit HS.

6l Gap repair. Perform the gap repair reaction as follows:

Component	Volume (µl)
2X Kapa HiFi HotStart Uracil+ Ready Mix	25
Taq DNA ligase (40 U/µl)	2
Purified Tagmented DNA (150-250ng)	23
Total	50

Incubate in a thermocycler at 72°C for 30 minutes.

7| Dilute proteinase K 1:1 in water (2.5 µl of proteinase K and 2.5 µl of water) and add 5 µl of the dilution to the gap-repaired DNA. Incubate at 55°C for 15 minutes.

8| Purify the gap repair reactions as previously described in step 4 by adding 1.8X volumes (99 µl) of SPRI-Guanidine beads to the gap repaired-DNA. Elute in 20 µl of TE pH 8.0. Combine every two eluted DNA samples for transferring to a new plate (each sample in the new plate will have 40 µl, meaning that if you have 96 samples for gap-repair n step 6, you will have 48 samples for USER/PNK treatment in step 9).

9| USER/PNK

Component	**Volume (µl)**
T4 DNA Ligase Buffer (10X)	5
USER Enzyme (1 U/µl)	3
T4 Polynucleotide Kinase (10 U/µl)	2
Gap-repaired DNA	40
Total	50

Incubate in a thermocycler at 37°C for 1 hour.

10| Add 1.8X volumes (90 µl) of SPRI-beads to the USER/T4 PNK treated DNA, mix thoroughly by pipetting 10 times. Incubate at room temperature for 10 minutes. Place the reaction plate onto a Magnum FLX magnetic rack for 3 minutes. Remove the cleared solution from the reaction plate and discard. Add 200 µl of 80% ethanol, incubate for 30 seconds and remove the supernatant. Repeat this step for a total of two ethanol washes. Remove ethanol completely and let the samples air dry for 3 minutes on the magnetic rack. Remove the plate from the magnetic rack and add 35 µl of TE pH 8.0, and pipette 10 times to mix. Incubate at room temperature for 2 minutes. Place the reaction plate back to the magnetic rack for 1 minute. Transfer the supernatant to a new plate. Transfer the supernatant to a new plate. Pool and quantify by Qubit dsDNA HS assay.

11| Intramolecular circularization

Component	**Volume (µl)**
T4 DNA Ligase Buffer (10X)	10
T4 DNA Ligase (400 U/µl)	2
USER/PNK treated DNA (500ng)	*variable*
H_2O	*variable*
Total	100

Incubate in a thermocycler at 16°C for 16 hours.

12| Add 1X volumes (100 µl) of SPRI-beads to the circularized DNA, mix thoroughly by pipetting 10 times. Incubate at room temperature for 10 minutes. Place the reaction plate onto a Magnum FLX magnetic rack for 3 minutes. Remove the cleared solution from the reaction plate and discard. Add 200 µl of 80% ethanol, incubate for 30 seconds and remove the supernatant. Repeat this step for a total of two ethanol washes. Remove ethanol completely and let the samples air dry for 3 minutes on the magnetic rack. Remove the plate from the magnetic rack and add 38 µl of TE pH 8.0, and pipette 10 times to mix. Incubate at room temperature for 2 minutes. Place the reaction plate back to the magnetic rack for 1 minute. Transfer the supernatant to a new plate.

13| Plasmid-Safe ATP-dependent DNase/Lambda Exo/ExoI treatment

Component	**Volume (µl)**
Exonuclease I Reaction Buffer (10X)	5
ATP (25 mM)	2
Plasmid-Safe ATP-Dependent DNase (10 U/µl)	2
Lambda Exonuclease (5 U/µl)	2
Exonuclease I (*E. coli*) (20 U/µl)	1
Circularized DNA	38
Total	50

Incubate in a thermocycler at 37 °C for 1 h, 70 °C for 30 min, hold at 4 °C.

14| Add 1X volumes (50 µl) of SPRI-beads to the circularized DNA, mix thoroughly by pipetting 10 times. Incubate at room temperature for 10 minutes. Place the reaction plate onto a Magnum FLX magnetic rack for 3 minutes. Remove the cleared solution from the reaction plate and discard. Add 200 µl of 80% ethanol, incubate for 30 seconds and remove the supernatant. Repeat this step for a total of two ethanol washes. Remove ethanol completely and let the samples air dry for 3 minutes on the magnetic rack. Remove the plate from the magnetic rack and add 15 µl of TE pH 8.0, and pipette 10 times to mix. Incubate at room temperature for 2 minutes. Place the reaction plate back to the magnetic rack for 1 minute. Transfer the supernatant to a new plate, pool and quantify by Qubit HS. Circularized DNA can be stored at -20°C.

***In vitro* cleavage of enzymatically purified, circularized gDNA**

15| sgRNA dilution and re-fold. Dilute the sgRNA to 9 µM in nuclease-free water and use the follow program on a thermocycler for sgRNA re-fold:

Step	**Temperature**	**Time**	**Cycles**
1	90 °C	5 min	1
2	90-25 °C	Ramp rate 2%	
Hold	4°C		1

16| *In vitro* cleavage with Cas9 and sgRNA. Setup *in vitro* cleavage master-mix:

Component	**Volume (µl)**
Cas9 Nuclease Reaction Buffer (10X)	5
Cas9 Nuclease, S. pyogenes (1 µM)	4.5
In vitro transcribed sgRNA (9 µM)	1.5

Total cleavage master-mix	11

Incubate at room temperature for 10 min.

Add circularized DNA, diluted to a total volume of 39 μl:

Cleavage master-mix	11
Plasmid-Safe DNase Treated DNA (125 ng)	39
Total	50

Incubate in a thermocycler at 37 °C for 1 h, hold at 4 °C.

17| Dilute proteinase K 1:4 in water (1 µl of proteinase K and 4 µl of water) and add 5 µl of the dilution to the *in vitro*-cleaved DNA and incubate in a thermocycler at 37 °C for 15 min.

18| Add 1X volumes (55 µl) of SPRI-beads to the *in vitro*-cleaved DNA, mix thoroughly by pipetting 10 times. Incubate at room temperature for 10 minutes. Place the reaction plate onto a Magnum FLX magnetic rack for 3 minutes. Remove the cleared solution from the reaction plate and discard. Add 200 μl of 80% ethanol, incubate for 30 seconds and remove the supernatant. Repeat this step for a total of two ethanol washes. Remove ethanol completely and let the samples air dry for 3 minutes on the magnetic rack. Remove the plate from the magnetic rack and add 42 µl of TE pH 8.0, and pipette 10 times to mix. Incubate at room temperature for 2 minutes. Keep the beads.

19| A-tailing. Setup the A-tailing master mix (reagents provided with HTP Library Preparation Kit PCR-free (96rxn) (Kapa Biosystems):

Component	**Volume (µl)**
Kapa A-tailing Buffer (10X)	5
Kapa A-tailing Enzyme	3
Total A-tailing master-mix	8

Add 8 µl of A-tailing master-mix to each eluted DNA sample with beads.

A-tailing master-mix	8
Cleaved DNA/beads	42
Total	50

Incubate on a thermocycler at 30 °C for 30 min, hold at 4 °C.

20| Add 1.8X volumes (90 µl) of PEG/NaCl SPRI solution (provided with HTP Library Preparation Kit PCR-free (96rxn) (Kapa Biosystems)) to A-tailed DNA, mix thoroughly by pipetting 10 times. Incubate at room temperature for 10 minutes. Place the reaction plate onto a Magnum FLX magnetic rack for 3 minutes. Remove the cleared solution from the reaction plate and discard. Add 200 µl of 80% ethanol, incubate for 30 seconds and remove the supernatant. Repeat this step for a total of two ethanol washes. Remove ethanol completely and let the samples air dry for 3 minutes on the magnetic rack. Remove the plate from the magnetic rack and add 25 µl of TE pH 8.0, and pipette 10 times to mix. Incubate at room temperature for 2 minutes. Keep the beads.

21l Adapter ligation. Setup the adapter ligation master-mix (reagents provided with HTP Library Preparation Kit PCR-free (96rxn) (Kapa Biosystems)):

Component	Volume (µl)
Kapa Ligation Buffer (5X)	10
Kapa DNA Ligase	5
NEBNext Adapter for Illumina (15 µM)	2.5
H_2O	7.5
Total master-mix	25

Add 25 μl of adapter ligation master-mix to each A-tailed DNA sample with beads.

Adapter ligation master-mix	25
A-tailed DNA/beads	25
Total	50

Incubate on a thermocycler at 20 °C for 1 h, hold at 4 °C.

Note: Prepare single-use aliquots of NEB adapters to avoid adapter dimer formation due to freeze–thaw hydrolysis of the 3′ T′.

22l Add 1X volumes (50 µl) of PEG/NaCl SPRI solution (provided with HTP Library Preparation Kit PCR-free (96rxn) (Kapa Biosystems)) to the adapter-ligated DNA and purify DNA as described in step 20. Elute in 47 µl of TE pH 8.0 and keep the beads.

23l USER enzyme. Add 3 µl of USER enzyme, provided with NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers Set 1) to the adapter ligated DNA with beads. Incubate at 37 °C for 15 min.

24l Add 0.7X volumes (35 µl) of PEG/NaCl SPRI solution (provided with HTP Library Preparation Kit PCR-free (96rxn) (Kapa Biosystems)) to the USER Enzyme treated DNA and purify as previously described in step 20. Elute in 20 µl of TE pH 8.0. Transfer the supernatant to a new semi-skirted PCR plate and quantify by Qubit dsDNA HS assay and proper Qubit assay tubes (usually about 2-5 ng/µl).

25l PCR. Setup a PCR master-mix for adding dual-index barcodes:

Component	Volume (µl)	Final concentration
Nuclease-free water	10	
2X Kapa HiFi HotStart Ready Mix	25	1X
Total master-mix	35	
Diluted PCR master-mix	35	
NEBNext i5 Primer (10 µM)	5	1 µM
NEBNext i7 Primer (10 µM)	5	1 µM
Total PCR mix	45	

26l Add 45 µl of PCR master-mix to each sample of purified, USER enzyme treated DNA (~20ng).

Component	Volume (µl)	Final concentration
PCR mix	45	
USER enzyme treated DNA (~10-20ng)	5	~0.4 ng/µl
Total	50	

27| Perform the PCR using the following thermocycling conditions:

Step	Temperature	Time	Cycles
Denaturation	98 °C	45 s	1
Denaturation	98 °C	15 s	20
Annealing	65 °C	30 s	20
Extension	72 °C	30 s	20
Extension	72 °C	1 min	1
Hold	4°C		1

28| Purification Add 0.7X volumes (35 µl) of SPRI-beads to the PCR and purify as previously described in step 18. Elute in 30 µl of TE pH 8.0. Transfer the supernatant to a new semi-skirted PCR plate and run 3 µl in QIAxcel.

29| Make 1:10 serial dilutions of 50 µl from 10^{-1} to 10^{-5} dilution of each sample from the library (PCR), starting with 5 µl of DNA and 45 µl of nuclease-free TE pH 8.0, and mix well.

30| Assemble qPCR master-mix solution as follows:

Component	1 reaction (µl)	Final Concentration
KAPA SYBR FAST qPCR Master Mix (2X) + Primer Premix (10X)	12	1X
Nuclease-free water	4	
Total qPCR mix	16	

31| Assay 2 different dilution factors (4 µl) for each sample (10^{-4} and 10^{-5} from the library) in duplicate (in an appropriate 96-well plate). A standard curve (provided with Kapa Library Quantification Kit) and a non-template control (NTC) are required. Add 4 µl of each standard in duplicate, and nuclease-free water in the NTC. Add 16 µl of qPCR master-mix to each sample.

Component	Volume (µl)	Final concentration
qPCR mix	16	
Sample (add nuclease-free water into the NTC well)	4	*variable*
Total	20	

32| Seal the plate and spin down.

33| Run qPCR in appropriate thermocycler with the following program:

Cycling step	Temperature	Time	Cycles
Initial denaturation	95 °C	5 min	1

Denaturation	95 °C	30 s	35
Annealing/extension/data acquisition	60 °C	45 sec	35
Melt curve analysis	60-95 °C		

34l Add the appropriate DNA copies for each standard when setting up the qPCR plate in the qPCR program, as follows:

Standard	**dsDNA molecules/µl**
Standard 1	$1.2x10^7$
Standard 2	$1.2x10^6$
Standard 3	$1.2x10^5$
Standard 4	$1.2x10^4$
Standard 5	$1.2x10^3$
Standard 6	$1.2x10^2$

35l Analyze qPCR results. Multiply the average of duplicate values by the dilution factor and by the five-fold dilution factor of the qPCR reaction, as follows: Total copies/µl = # * dilution factor.

36l Pool library for MiSeq. Pool all the samples in one library at equimolar concentrations. 1X pooled library should be in a total volume of 5 µl, ~ 8×10^9 molecules.

37l Denature the pooled library (~ 8×10^9 molecules) by adding 5 µl of NaOH 0.2N and incubate at room temperature for 5 min. Then, add 940 µl of Hyb buffer (supplied with MiSeq® Reagent Kit v3 (600 cycle)).

38l Prepare the Phix control V3 (PhiX Control V3 KIT) as follows: mix 2 μl of 10 nM PhiX control with 3 µl of Tris-HCl 10 mM + 0.1% Tween-20, denature with 5 µl of NaOH 0.2N and incubate at room temperature for 5 min. Add 990 µl of Hyb buffer, to generate 20 pM PhiX. Then, make a 12.5 pM PhiX dilution, by mixing 375 µl of the 20 pM PhiX with 225 µl of Hyb buffer. Add 100 µl of the 12.5 pM Phix to the denatured library.

39l Clean the Flow Cell (supplied with MiSeq® Reagent Kit v3 (600 cycle)) with ultra-pure water, dry with lens tissues, followed by cleaning with alcohol wipes and lens tissue.

40l Load and sequence library using a MiSeq 600-cycle v3 kit according to manufacturer's instructions using MiSeq system. Sequencing is performed with 150 bp paired-end reads and 8 bp dual-index reads.

41l After sequencing, copy the demultiplexed output FASTQ files to a location accessible to CIRCLE-seq/CHANGE-seq analysis pipeline.