SUPPLEMENTARY INFORMATION

CHANGE-seq reveals genetic and epigenetic effects on CRISPR-Cas9 genome-wide activity

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Supplementary Protocol: CHANGE-seq

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.

Tn5 expression and purification Recombinant Tn5 is expressed and purified according to a protocol previously described by Piccelli et al[49]. Tn5 can also be obtained commercially (Lucigen) although we have not tested the commercial enzyme in CHANGE-seq.

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

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

CHANGE-seq library preparation

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

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

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

3| 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.

4| 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).

5| 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.

6| 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 (500 ng)	*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.

Note: One negative control is required for each unique source of gDNA. The negative control is processed in exactly the same manner as other samples but with water substituted for CRISPR–Cas RNP at step 16.

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.

21| 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.

22| 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.

23| 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.

24| 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).

25| 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	

26| 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	*Variable*
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		

34| 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	$1.2x10^2$

35| 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.

36| 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.

37| 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)).

38| 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.

39| 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.

40| 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.

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

Supplementary Table 1. Table of optimization areas.

Test	Description	Samples
Test 1	DNA purification and DNA gap repair	9
Test 2	DNA purification conditions	15, 18
Test 3	Looped adapter	1, 2, 13, 14
Test 4	DNA gap repair	3, 11, 12, 22, 23, 28
Test 5	Shifting the size distribution of tagmented DNA	17
Test 6	Exonuclease treatment (gap selection)	16, 31
Test 7	Oligoduplex concentration for transposome assembly	21
Test 8	Exonuclease treatment after circularization	24, 25, 29
Test 9	NGS adapter titration	20, 26
Test 10	Proteinase K treatment after IVC	33
Test 11	Proteinase K treatment after IVC and exonuclease treatment after circularization	38, 42
Test 12	Proteinase K treatment after IVC, exonuclease treatment after circularization (one-step) and NGS adapter titration	37, 41
Test 13	Proteinase K treatment after IVC, exonuclease treatment after circularization (two-steps) and NGS adapter titration	40
Test 14	Guanidine-based beads for DNA purification and amount of gDNA for tagmentation	30, 36, 39
Test 15	NGS adapter ligation followed by USER enzyme treatment	32, 34, 35
Test 16	SPRI-beads for DNA purification followed by Proteinase K treatment	19, 27
Test 17	End-repair after IVC	4, 5, 6, 7, 8, 10

Supplementary Table 2. sgRNAs and NGS primers list.

See attached file.

Supplementary Table 3. Complete list of CHANGE-seq detected sites.

See attached file.

Supplementary Table 4. List of CHANGE-seq target sites specificity ratio.

See attached file.

Supplementary Table 5. List of on-target site targeted sequencing counts.

See attached file.

Supplementary Table 6. Complete list of GUIDE-seq detected sites.

See attached file.

Supplementary Table 7. List of off-target sites targeted sequencing counts (standard targeted sequencing).

See attached file.

Supplementary Table 8. List of off-target sites targeted sequencing counts (rhAmpSeq).

See attached file.

Supplementary note

CHANGE-seq optimization

Restriction-enzyme independent genomic DNA circularization with the original CIRCLE-seq method relied on the ligation of stem-loop adapters to end-repaired, A-tailed, sheared genomic DNA, followed by exonuclease selection of adapter-ligated DNA, with each enzymatic step followed by required DNA purifications. To substantially streamline this process, in CHANGE-seq we replaced the first 10 library preparation steps with a single reaction mediated by Tn5. We designed a custom 23-bp Tn5 transposon sequence comprised of 19-bp required for transposition and 4-bp containing an uracil required for subsequent overhang generation. First, we tested the feasibility of using this sequence for circularization in a PCR amplicon containing the required sequences in both ends, followed by USER enzyme and T4 PNK treatment to release the palindromic overhangs, and successful circularization was evaluated by capillary electrophoresis (**Supplementary Figure 1**).

For tagmentation, we expressed and purified the Tn5 transposase according to a protocol previously described by Picelli *et. al*[1] and optimized tagmentation conditions using U2-OS genomic DNA (**Supplementary Figure 1**). Genomic DNA tagmentation was performed with the custom transposome containing Tn5 transposase complexed with the uracil-containing oligonucleotide duplex (**Fig. 1b**). Following tagmentation, DNA gap repair is required, as Tn5 has a staggered nicking activity on the DNA, generating a 9-nt gap flanking the integrated transposon. We tested several strategies for DNA gap repair (**Supplementary Table 1, test 4**), and improvements were measured as on-target site enrichment after Cas9 cleavage and high-throughput sequencing (**Fig. 1c**). We found that the combination of an error-correcting polymerase with Taq DNA ligase resulted in the best condition for DNA gap repair and included Taq DNA ligase in subsequent tests for developing the technique.

Stringent DNA purification was required after tagmentation and gap repair steps to completely eliminate Tn5 and the error-correcting polymerase used in gap repair, respectively. Initially, these stringent purifications were performed using QIAquick PCR purification kit (Qiagen), however, to streamline these steps and make them high-throughput and automation compatible, we developed an alternative: SPRI guanidine based beads for purification.

For selection of covalently closed circular DNA molecules over the residual linear DNA background, we initially used Plasmid-Safe as previously described. However, CHANGE-seq sensitivity was slightly reduced when compared to original CIRCLE-seq. By quantifying the Plasmid-Safe treated circularized DNA with fluorescent and absorbance methods, we observed residual single-stranded DNA remaining after Plasmid-Safe treatment (which explained a discrepancy in DNA concentration measured by both methods). We hypothesized that this remaining linear DNA contributed to the increased background of these samples. We then enzymatically selected for covalently-closed DNA molecules with a modified cocktail of exonucleases, comprised of Plasmid-Safe, Lambda exonuclease and Exonuclease I, further reducing sequencing background and enriching on-target site read counts.

Additionally, as Cas9 remains tightly bound to the DNA ends after cleavage[2], treatment of Cas9-cleaved DNA with proteinase K to remove bound nuclease further improved read-count enrichment, presumably by releasing additional cleaved sites that were not previously available for A-tailing. Finally, we optimized and reduced the ratio of sequencing adapter:gDNA circles, which reduced the rate of primer dimer formation and also increased sensitivity.

The majority of the DSBs generated by Cas9 in the DNA produces blunt ends, however, in a sgRNA target-dependent context, a mixture of blunt and 1-nt 5' overhangs can be created after cleavage. To retrieve these potential off-target sequences, we tested an additional DNA end repair step after Cas9 *in vitro* cleavage. However, the addition of an end repair step substantially reduced the sensitivity of CHANGE-seq, suggesting the persistence of some linear DNA, presumably in a very low concentration. Further optimization of this end repair step will be required to adapt CHANGE-seq to CRISPR-Cas nucleases such as Cas12a (Cpf1) that leave 5' staggered DNA overhangs.

We performed all tests for CHANGE-seq development in U2-OS genomic DNA on a well characterized *EMX1* target site. Conditions tested are listed on **Supplementary Table 1** and results are shown on **Fig. 1c**.

CHANGE-seq automation

We developed CHANGE-seq methods for the Beckman Coulter Biomek FX^P Dual Hybrid system by dividing the protocol in three parts: (1) genomic DNA circularization; (2) selection of circularized DNA library; and (3) *in vitro* cleavage of circularized DNA with CRISPR-Cas9, followed by standard next-generation sequencing library preparation. Each part consisted of optimized enzymatic reactions followed by magnetic bead-based purification steps, associated with optimized distribution of viscous enzymatic master mixes to ensure appropriate mixing parameters for all reactions. Enzymatic incubations were automated on an on-deck Applied Biosystems Automated Thermal Cycler (**ATC**).

GUIDE-seq optimization in CD4$^+$/CD8$^+$T-cells

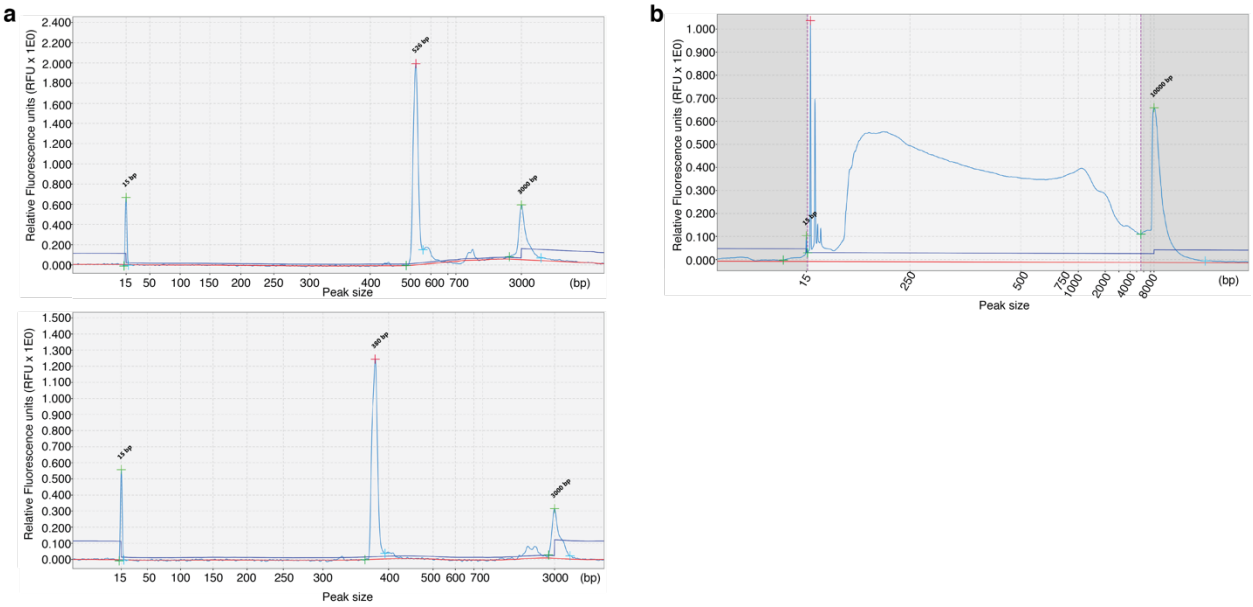
To optimize GUIDE-seq on human primary CD4$^+$/CD8$^+$T-cells, we first tested variations of GUIDE-seq double-stranded oligodeoxynucleotide (**dsODN**) tags with 5'-, 3'-, and both ends modified with two terminal phosphorothioate linkages for their ability to integrate into Cas9-induced DSBs. CD4$^+$/CD8$^+$ T-cells were nucleofected with Cas9:sgRNA RNP complexes with 100 pmol of dsODN with phosphorothioate modifications. sgRNAs targeting *CCR5* (*CCR5* site 8) and *PDCD1* (*PDCD1* site 13) were used for the optimization experiments. Three days post nucleofection cells viability was determined by FACS analysis with DAPI staining and cells were harvested for genomic DNA extraction and PCR with primers flanking the on-targeted site to measure indel frequency and dsODN integration by NGS (**Extended Data Figure 4, b, c**). dsODN with 3' end-protecting modifications showed higher rates of integration, while keeping similar levels of cell viability and indel frequency as the control nucleofected with Cas9:sgRNA only. Next, we determined the optimal amount of dsODN by nucleofecting different concentrations (25, 50, 100, 150 or 200 pmol) and measured cell viability, indel frequency and dsODN integration as mentioned above. We found that 100 pmol of 3'-modified dsODN was sufficient to achieve high integration rates with minimal impact on cell viability (**Extended Data Figure 4**).

Supplementary References

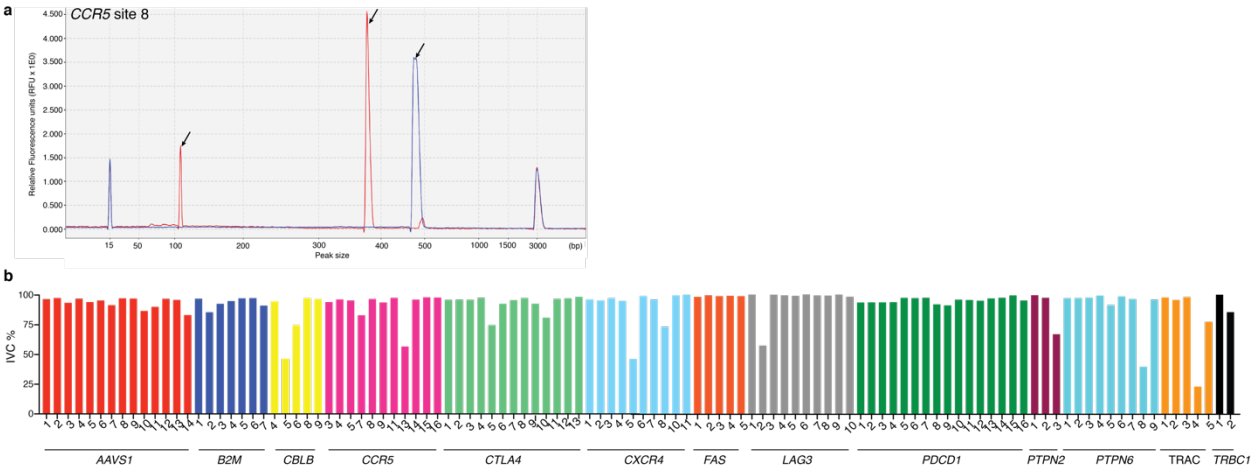
1. Picelli, S. *et al*. Tn5 transposase and tagmentation procedures for massively scaled sequencing projects. *Genome Res* **24,** 2033–2040 (2014).

2. Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C. & Doudna, J. A. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* **507,** 62 (2014).

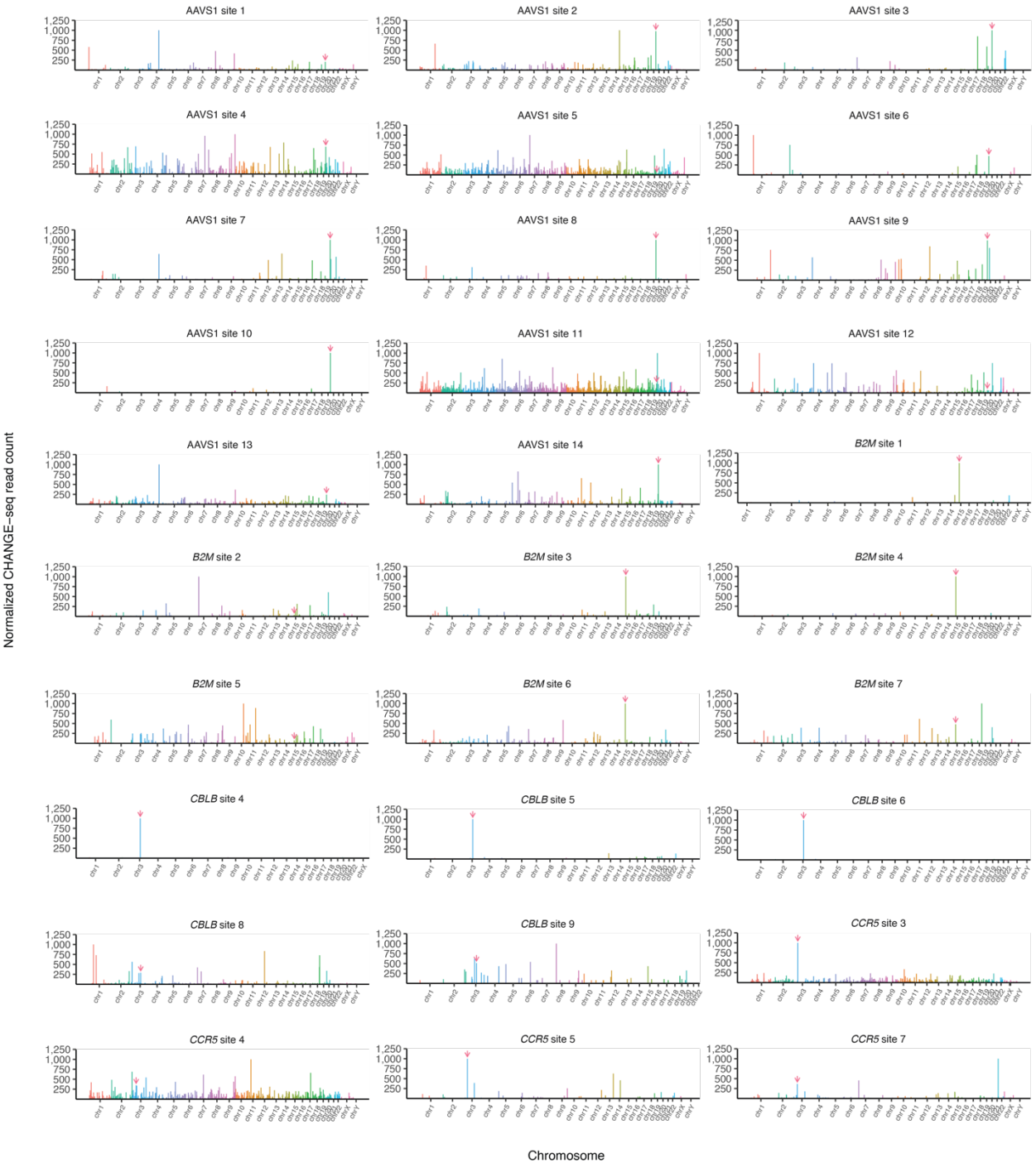
Supplementary Figures



Supplementary Figure 1 | Validation of the gDNA circularization process mediated by Tn5. a, QIAxcel capillary electrophoretic traces of the linear PCR amplicon with an uracil-containing Tn5-ME sequence in both ends (top) and circularized PCR amplicon (bottom). The shift in DNA mobility is consistent with the successful circularization of the PCR amplicon with the uracil-containing Tn5-ME adapter. **b,** QIAxcel capillary electrophoretic traces of genomic DNA tagmented with purified Tn5, with a median size distribution of 400 bp, using the same uracil-containing Tn5-ME adapter. (a-b) These experiments were performed at least two times with similar results.



Supplementary Figure 2 | Validating *in vitro* cleavage efficiency of Cas9:sgRNA RNP complexes targeting 110 therapeutic target sites. a, Example electropherogram showing *in vitro* cleavage of a PCR amplicon containing the intended target site by Cas9:sgRNA RNP complex (red) versus an untreated control PCR product (blue). This experiment was performed at least two times with similar results. **b,** Barplot of *in vitro* cleavage efficiency assessment on PCR amplicons for 110 target sites (n=1) across 13 *loci* in human T-cells, quantified by DNA capillary electrophoresis.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B2M site 1
B2M site 2
B2M site 3
B2M site 4
B2M site 5
B2M site 6
B2M site 7
CBLB site 4
CBLB site 5
CBLB site 6
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CCR5 site 3
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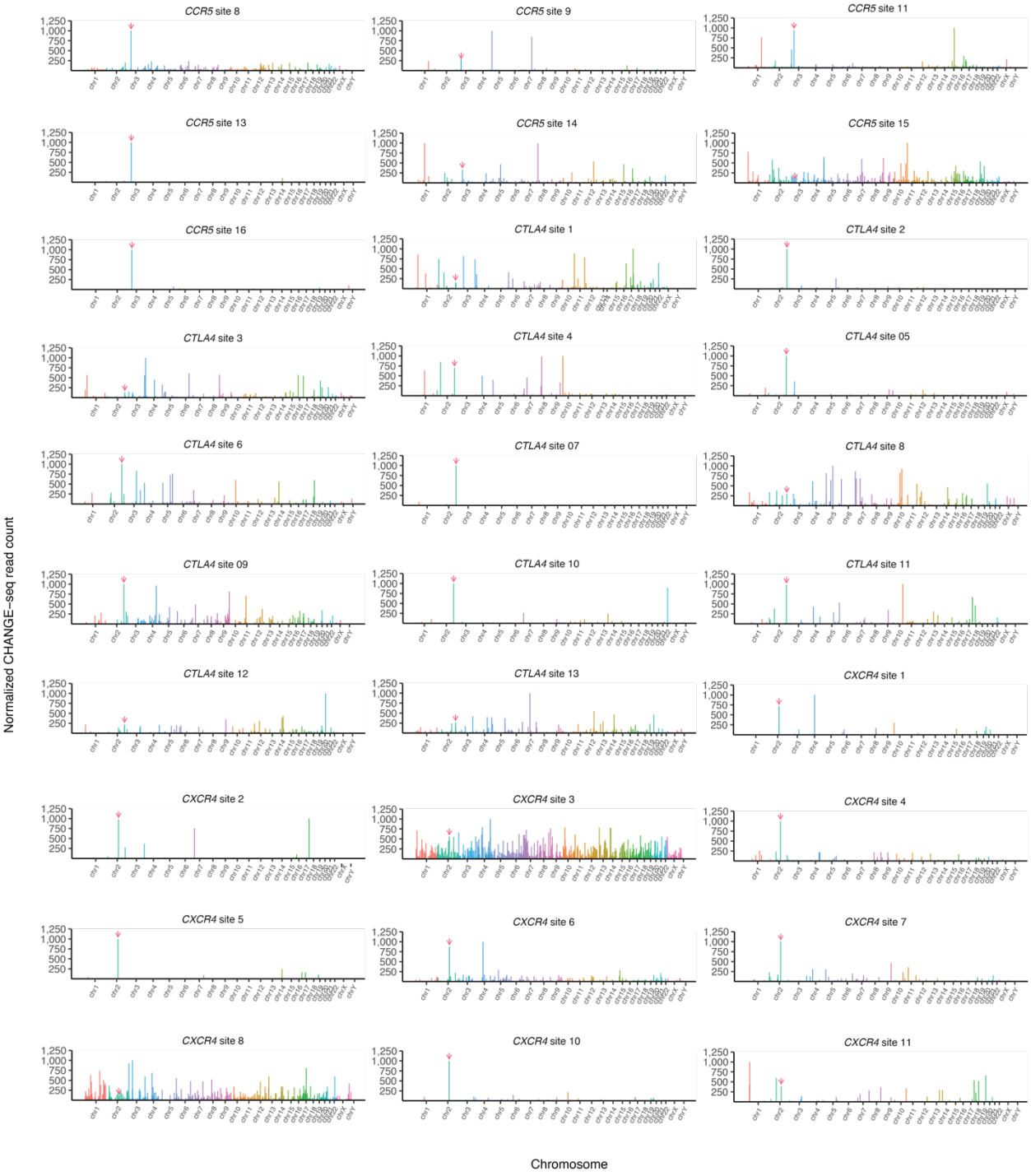
Normalized CHANGE–seq read count
Chromosome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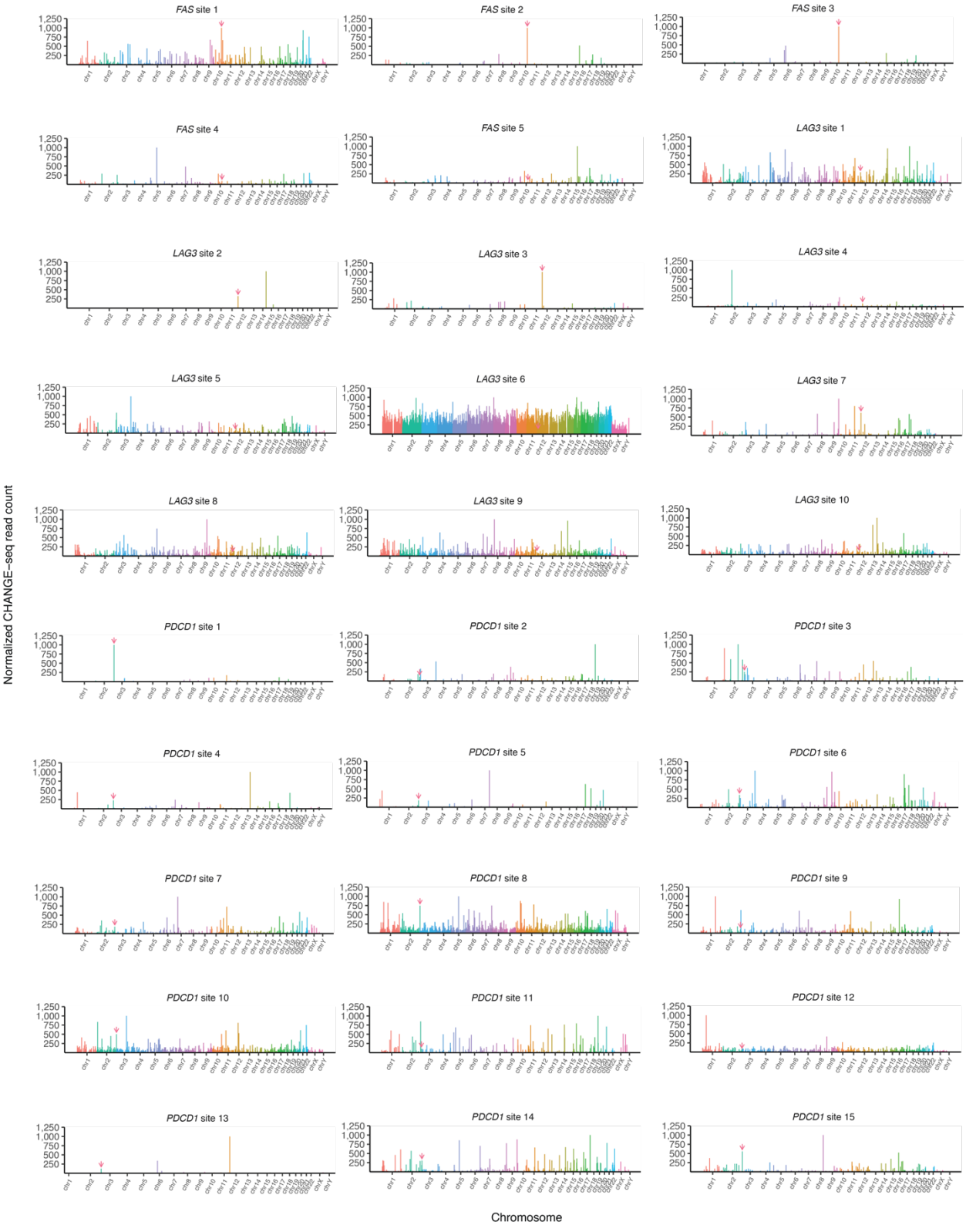
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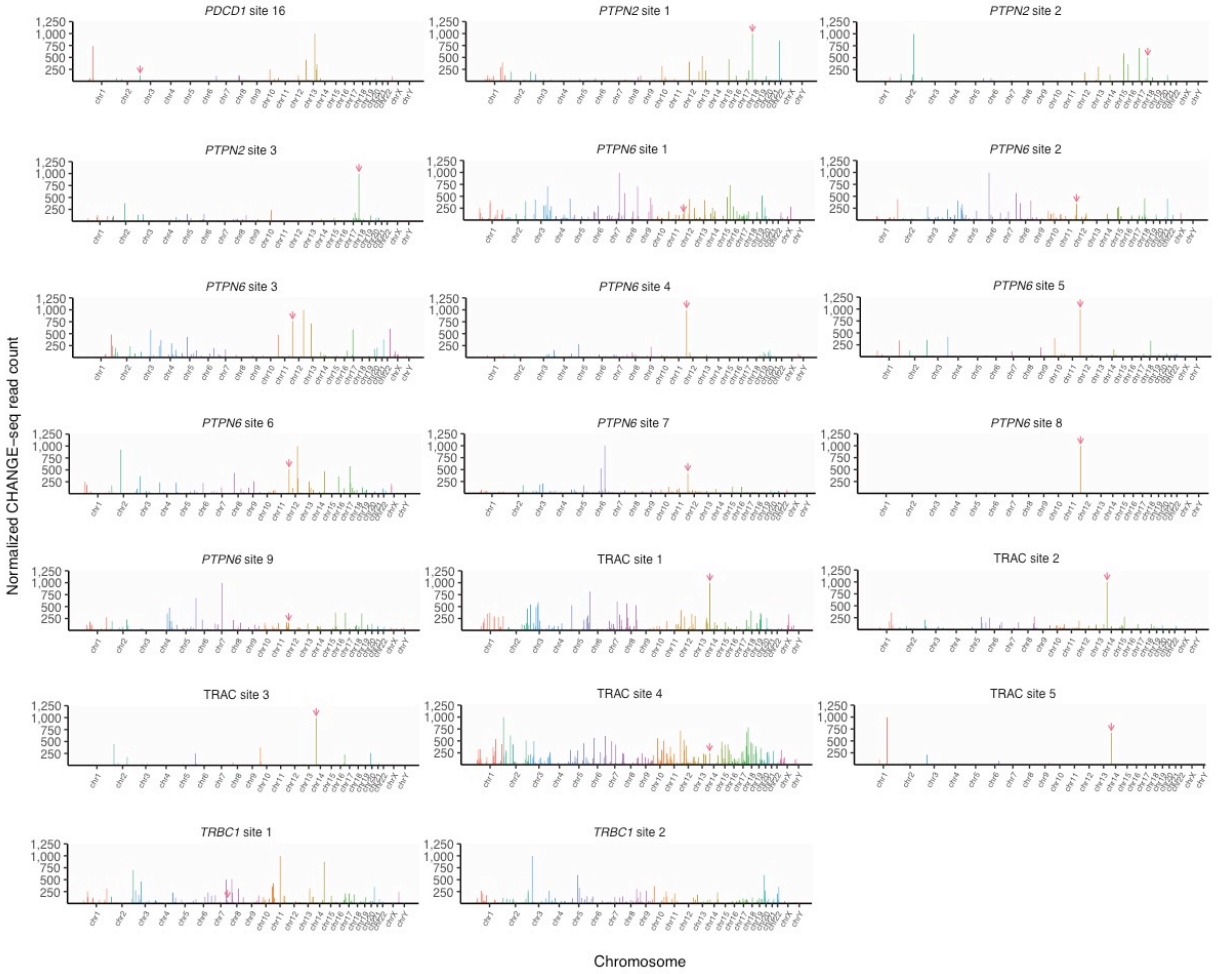
Normalized CHANGE-seq read count
Chromosome



Supplementary Figure 3 | Manhattan plots of CHANGE-seq genome-wide activity profiles for 110 SpCas9 targets. Normalized CHANGE-seq read counts plotted by chromosomal positions. Color of bars is used to distinguish different chromosomes. The height of the bar is proportional to CHANGE-seq read counts and a red arrow indicates the intended on-target sites.