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Improved HTGTS for CRISPR/Cas9 Off-target Detection

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[Abstract] Precise genome editing is essential for scientific research and clinical application. At present, linear amplification-mediated high-throughput genome-wide translocation sequencing (LAM-HTGTS) is one of most effective methods to evaluate the off-target activity of CRISPR-Cas9, which is based on chromosomal translocation and employs a “bait” DNA double-stranded break (DSB) to capture genome-wide “prey” DNA DSBs. Here, we described an improved HTGTS (iHTGTS) method, in which size-selection beads were used to enhance reaction efficiency and a new primer system was designed to be compatible with Illumina Hiseq sequencing. Compared with LAM-HTGTS, iHTGTS is lower cost and has much higher sensitivity for off-target detection in HEK293T, K562, U2OS and HCT116 cell lines. So we believe that iHTGTS is a powerful method for comprehensively assessing Cas9 off-target effect.
Keywords: CRISPR-Cas9, Off-target activity, Chromosomal translocation, LAM-HTGTS, iHTGTS

[Background] The CRIPSR-Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins) has been widely used as a powerful genome editing tool (Cong *et al.*, 2013; Jinek *et al.*, 2013; Mali *et al.*, 2013). However its off-target activity causes DNA DSBs at imperfectly matched loci. During the past few years, several methods based on next-generation sequencing have been published to detect off-target sites. LAM-HTGTS (Frock *et al.*, 2015; Hu *et al.*, 2016), which is based on chromosomal translocation, makes use of a “bait” DSB to capture “prey” DSBs to sensitively identify off-target hotspots; GUIDE-seq inserts a specific DNA oligo into break site and applies PCR to enrich DSBs (Tsai *et al.*, 2015). *In vitro* methods such as Digenome-seq (Kim *et al.*, 2015) and CIRCLE-seq (Tsai *et al.*, 2017) are more sensitive but often need to be verified *in vivo*. Though LAM-HTGTS is very sensitive, there is still room for improvement.

In the improved HTGTS (iHTGTS), we applied size-selection beads to deplete surplus biotinylated primer for the bridge adapter ligation efficiency enhancement. iHTGTS showed 4 times greater sensitivity than LAM-HTGTS. Also a new primer system was employed which can accommodate 150 bp x 2 Hiseq sequencing instead of 250 bp x 2 Miseq, so that the sequencing cost is much saved. Taken together, iHTGTS is a cost-effective and high efficient method. We believe iHTGTS can give researchers deeper insights into the off-target activity of CRIPSR/Cas9.

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Materials and Reagents

1. Pipette tips (Quality Scientific Plastics)
2. 1.5 ml tube (Axygen, catalog number: MCT-150-C)
3. 0.22 μm syringe filter (MILLEX, catalog number: PR03683)
4. 200 μl PCR tubes (Axygen, catalog number: 14-222-261)
5. Agarose (Thermo Fisher, catalog number: R0492)
6. 1 kb DNA plus ladder (Transgen Biotech, catalog number: BM211)
7. Streptavidin C1 beads (Thermo Fisher, catalog number: 65001)
8. AMPure XP beads (Axygen)
9. Protease K (Sigma, catalog number: P8044)
10. FastPfu (Transgen Biotech, catalog number: AP221-02)
11. dNTPs (Transgen Biotech, catalog number: AD101-11)
12. AxyPrep MAG PCR Clean-Up (Axygen, catalog number: MAG-PCR-CL)
13. NaCl (VWR Life Sciences, catalog number: 97061-266)
14. EDTA (Amresco, catalog number: BDH9232)
15. Tris (VWR Life Sciences, catalog number: 97062-420)
16. PEG 8000 (Sigma, catalog number: 89510-250G-F)
17. T4 ligase (Thermo Fisher, catalog number: EL0011)
18. EasyTaq (Transgen Biotech, catalog number: AP111-01)
19. Gel extraction kit (Thermo Fisher, catalog number: K0691)
20. 75% Ethanol (Beijing Chemical Works)
21. Isopropyl (Beijing Chemical Works)
22. EDTA-$Na_2 \cdot 2H_2O$ (Amresco, catalog number: BDH9232)
23. NaOH (Sigma, catalog number: 221465)
24. HCl (Sigma, catalog number: 7647-01-0)
25. SDS (Sigma, catalog number: 72455)
26. TAE (see Recipes)
27. Proteinase K (see Recipes)
28. 5 M NaCl (see Recipes)
29. 0.5 M EDTA (pH 8.0) (see Recipes)
30. 1 M Tris-HCl (pH 7.4) (see Recipes)
31. Cell lysis buffer (see Recipes)
32. TE buffer (see Recipes)
33. 50% (wt/vol) PEG 8000 (see Recipes)
34. 2x B&W buffer (see Recipes)
35. Annealing buffer (see Recipes)
36. 50 mM bridge adapter (see Recipes)

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Equipment

1. Foam floating tube rack
2. 8-well magnet stand
3. Pipettes (GILSON)
4. Thermomixer C (Eppendorf)
5. PCR Thermal Cyclers (Applied Biosystems)
6. Covaris (M220 Focused-ultrasonicator)
7. Centrifuge (Eppendorf, model: 5418R)
8. NanoDrop (DeNOVIX, DS11)
9. Incubator (Thermo Fisher)
10. Autoclave

Procedure

A. Design the primers used for iHTGTS

Choose Cas9-generated on-target site DSB as the “bait” to capture other genome-wide “prey” DSBs. To be compatible with 2 x 150 bp Hiseq sequencing, biotinylated primer for LAM-PCR was designed to bind 150 bp upstream of Cas9 binding site; nested primer was designed annealling to the downstream of the biotinylated primer about 90 bp away from the cut site. (Figure 1, Table 1)

Note: Avoid designing primers at DNA repetitive region.

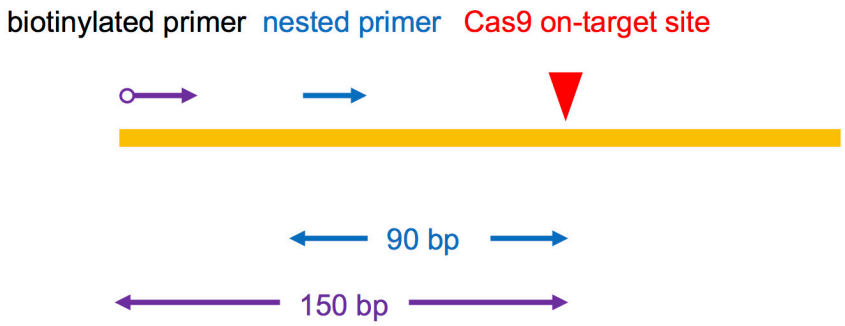

Figure 1. Primer design for iHTGTS. The red arrow indicates the Cas9 on-target cutting site. The blue arrow indicates the nested primer and purple indicates the biotinylated primer. The distance to the cutting site is shown below.

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Table 1. The sequences of primers and bridge adapters. The Phor indicates the phosphorylation. MID indicates the index base for data demultiplex. REDPRIMER indicates nested primer, which varies between different loci.

Adapter-upper-6N	TGTAGAGCACGCGTGGNNNNNN-NH2
Adapter-lower-NH2	/5Phor/CCACGCGTGCTCTACAAGATCGGAAGAGCACACGTCTGAACTCCAGT-NH2
P5-I5	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGC
P7-I7	CAAGCAGAAGACGGCATACGAGAT
I5-Red	ACTCTTTCCCTACACGACGCTCTTCCGATCTMIDREDPRIMER
I7-Blue	CAGAAGACGGCATACGAGATMIDGTGACTGGAGTTCAGACGTGTGC

B. Extract Genomic DNA (gDNA)

1. Forty-eight hours after transfecting HEK293T cells with the Cas9 plasmids, collect 10^7 transfected HEK293T cells (we have also tried K562, HCT116 and U2OS, all work well) in a 1.5 ml tube and add 500 µl cell lysis buffer. Incubate the tube in Thermomixer at 56 °C, 500 *x g* for 10-18 h.

   Note: Protease K solution should be added into lysis buffer just before use (10 ng/ml final concentration).
2. Add 500 µl isopropyl and mix thoroughly till you can see a white flocculent DNA pellet.
3. Using a pipet to transfer the pellet into another 1.5 ml tube with 1 ml 70% ethanol. Centrifuge at 13,000 *x g* for 5 min.
4. Discard the supernatant. Centrifuge again and deplete residual 70% ethanol. Add 500 µl TE and incubate the tube in Thermomixer at 56 °C, 500 *x g* for at least 2 h.

   Note: Adjust the volume of added TE to make sure the DNA concentration not less than 200 ng/µl.
5. Quantify the DNA using NanoDrop. The A_{260}/A_{280} should be higher than 1.8.
6. Recommend 20 µg gDNA for iHTGTS library construction.

C. Fragment gDNA by sonication

1. Add 20 µg gDNA into a PCR tube. Set Covaris with the following parameters: PIP = 50 watts, DF = 30%, CPB = 200, Time = 60 s.
2. After sonication, take 200 ng DNA for 1% agarose page. The range of the DNA smear should be at 0.2-2 kb with a peak at 0.75 kb. (Figure 2)

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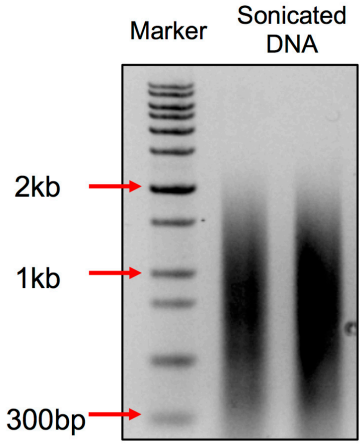

Figure 2. DNA smear pattern after sonication. The smear ranges from 300 bp to 2 kb with the peak at about 750 bp.

D. LAM-PCR

1. Set up the reaction in 4 x 50 µl PCR tubes as following (DNA template for each PCR reaction can be 1-10 µg):

	1x (µl)	4x (µl)
5x FastPfu buffer	10	40
dNTPs (2.5 mM)	1.5	6
Bio-primer (1 µM)	0.5	2 (1 µM)
FastPfu	0.5	2
Sonicated DNA	25	100
H_2O	12.5	50

2. Set up the PCR program:
   95 °C for 2 min
   [95 °C for 30 s, 58 °C for 30 s, 72 °C for 1.5 min] (80 cycles)
   72 °C for 2 min
   10 °C forever
3. Deplete surplus biotin primer using AMPure XP beads
   a. Add 50 µl AMPure XP beads into each PCR tube, mix gently and incubate the tubes at RT for 5 min.
      Note: This step aims to remove fragments less than 300 bp; the volume of the beads can be upscaled according to different batches.

   Note: All the following Steps (D3b-D3f) are operated at RT.
   b. Put the tubes on an 8-well magnet stand for 5 min.
   c. Remove the supernatant, add 200 µl 70% ethanol. After standing for 30 s, remove the supernatant.

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d. Repeat the Step D3c.
e. Add 50 µl dH_2O, mix the beads completely using a pipette and incubate at RT for 2 min.
f. Put the tubes on the magnet for 2 min and pool the supernatants (about 200 µl) into a new 1.5 ml tube.

E. Streptavidin beads binding

1. Add 50 µl 5 M NaCl, 2.5 µl 0.5 M EDTA into the PCR product from the last step. Add 30 µl streptavidin beads and rotate for 4 h at RT. (The streptavidin beads should be washed twice with 1x B&W buffer before use)
2. Put the beads against the 1.5 ml tube magnet stand and remove the supernatant. Wash the beads three times each using 400 µl 1x B&W buffer.
3. Wash the beads with 400 µl dH_2O and then resuspend the beads in 42.4 µl dH_2O.

F. On-beads ligation for bridge adapter

	1x (µl)
10x T4 DNA ligase buffer	8
Bridge adapter (50 µM)	1.6
T4 DNA ligage (5 U/µl)	4
50% (wt/vol) PEG8000	24
DNA-beads complex	42.4
Total	80

Set the reaction in a 1.5 ml tube in a rotator and ligate overnight at RT.

G. Nested PCR

1. Add 80 µl 2x B&W buffer and 160 µl 1x B&W buffer. Put the beads against the 1.5 ml tube magnet stand and remove the supernatant. Wash the beads using 400 µl 1x B&W buffer three times and 400 µl dH_2O once. Resuspend the beads in 80 µl dH_2O.
2. Set up the PCR reaction in 2x PCR tubes as following:

	1x (µl)	2x (µl)
10x EasyTaq PCR buffer	5	10
dNTPs (2.5 mM)	4	8
I5-Red (10 µM)	2	4
I7-Blue (10 µM)	2	4
EasyTaq polymerase	0.5	1
DNA-beads complex	40	80

3. Set up the PCR program:
   95 °C for 5 min
   [95 °C for 1 min, 58 °C for 30 s, 72 °C for 1 min] (15 cycles)

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72 °C for 10 min
10 °C forever

4. Recycle the PCR products using AMpure XP beads as described in Step D3. Elute the PCR products with 35 µl dH_2O for each PCR tube. Gather the PCR products together and measure the concentration.
   Note: This step aims to remove DNA fragments less than 500 bp, upscale the volume of the beads according to different batches.

Enzyme Blocking (Optional)

1. Add 8 µl 10x enzyme buffer, 10 U blocking enzyme, incubate at 37 °C for 1 h or longer.
2. Purify the DNA with GeneJET column, elute with 70 µl dH_2O, and check the concentration.

H. Tagged PCR

1. Set up the PCR reaction in 2 x PCR tubes as following:

	1x (µl)	2x (µl)
5x FastPfu PCR buffer	10	20
dNTPs (2.5 mM)	4	8
P5-I5 (10 µM)	2	4
P7-I7 (10 µM)	2	4
FastPfu polymerase	0.5	1
DNA	35	70

2. Set up the PCR program:
   95 °C for 3 min
   [95 °C for 20 s, 60 °C for 30 s, 72 °C for 1 min] (10-15 cycles)
   72 °C for 5 min
   10 °C forever
   Note: The PCR cycle number is dependent on the DNA concentration from the last step.

DNA concentration (ng/µl) (no enzyme blocking)	DNA concentration (ng/µl) (enzyme blocking)	Cycle number
> 15	> 10	11
10-15	7-10	12-13
< 10	< 7	14-16

I. Purified PCR products

Pool the DNA together, run all the DNA on 1% agarose gel in TAE buffer, cut products between 500-900 bp (Figure 3), purify through a Gel extraction column, elute with 30 µl dH_2O twice. Now the PCR product is ready for Hiseq sequencing.

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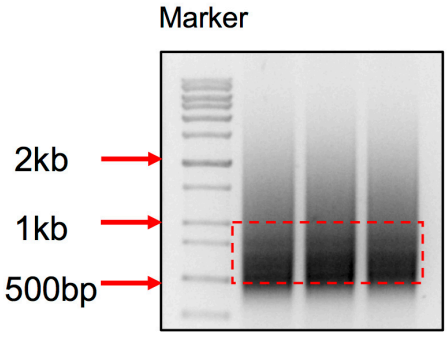

Figure 3. DNA smear pattern for iHTGTS library. Cut the DNA within the range from 500 bp to 1 kb.

Data analysis

The detailed operation and mechanism for data analysis is the same with LAM-HTGTS, which can be found in the step 48 to step 51 for Hu *et al.* (2016).

Recipes

Note: The reagents used in this protocol are almost the same with those in Hu et al. (2016). The following recipes are adapted from it.

1. TAE
   40 mM Tris-HCl
   20 mM Acetic Acid
   1 mM EDTA
2. Proteinase K
   Dissolve 0.1 g of proteinase K powder in 5 ml of H_2O to make a 20 mg/ml stock
   Divide the solution into 0.5 ml aliquots and store them at -20 °C for up to 3 months
3. 5 M NaCl
   Dissolve 292.5 g of NaCl in H_2O, and adjust the total volume to 1 L
   Autoclave the solution and store it at room temperature (RT; 20-25 °C) for up to 1 year
4. 0.5 M EDTA (pH 8.0)
   Dissolve 186.12 g of EDTA-Na_2·$2H_2O$ in H_2O, adjust the pH to 8.0 using 2.5 N NaOH and then adjust the total volume to 1 L
   Autoclave the solution and store it at RT for up to 1 year
5. 1 M Tris-HCl (pH 7.4)
   Dissolve 121.14 g of Tris base in H_2O, adjust the pH to 7.4 using HCl, and then bring the total volume to 1 L
   Autoclave the solution and store it at RT for up to 1 year
6. Cell lysis buffer

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200 mM NaCl
10 mM Tris-HCl (pH 7.4)
2 mM EDTA (pH 8.0)
0.2% (wt/vol) SDS
Store it at RT for up to 6 months

7. TE buffer
   10 mM Tris-HCl (pH 7.4)
   0.5 mM EDTA (pH 8.0)
   Store it at RT for up to 6 months
8. 50% (wt/vol) PEG 8000
   Dissolve 5 g of PEG 8000 in H_2O at 56 °C, and then adjust the total volume to 10 ml
   Filter the solution through a 0.22 μm syringe filter, prepare 1 ml aliquots and store them at -20 °C for up to 1 year
9. 2x B&W buffer
   2 M NaCl
   10 mM Tris-HCl (pH 7.4)
   1 mM EDTA (pH 8.0)
   Dilute it with H_2O to make 1x B&W buffer. Store it at RT for up to 1 year
10. Annealing buffer
    25 mM NaCl
    10 mM Tris-HCl (pH 7.4)
    0.5 mM EDTA (pH 8.0)
    Store it at RT for up to 1 year
11. 50 mM bridge adapter
    a. Dissolve the two DNA oligos (Table 1) in annealing buffer to a final concentration of 400 μM
    b. Mix equal volumes of the two dissolved oligos in a new 1.5 ml microtube, put the tube in 1 L of boiling water with a foam floating tube rack, boil it for 5 min and then cool it down slowly in water to ~30 °C on the bench (adapter concentration is 200 μM). Alternatively, the oligos can be annealed on a PCR thermoblock
    c. Dilute fourfold (concentration is 50 μM) with H_2O, prepare 100 μl aliquots and store them at -20 °C for up to 2 months
    d. Thaw the adapter on ice before use

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Competing interests

The authors declare they have no conflict of interest or competing interests.

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