GUIDE-seq simplified library preparation protocol (CRISPR/Cas9 off-target cleavage detection)

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ABSTRACT

GUIDE-seq is an experimental method to detect off-target cleavages caused during CRISPR/Cas editing.

See publication https://doi.org/10.1038/nbt.3117 for details about the method, the protocol used, and the analysis performed.

We identified several areas of improvement in the published protocol for GUIDE-seq NGS library preparation and developed a simplified protocol that is easier to perform & less expensive. The new protocol is also load-and-go compatible with standard sequencing runs on all Illumina instruments (using the kit SBS reagents & default instrument settings) compared to the NBT protocol that requires the libraries to be run on a MiSeq with some tweaks to the software configuration.

Notable changes:

1. Moved the UMI (Unique Molecular Identifier) from the Y-adapter to be inline with the insert. UMI is now sequenced as part of the sequencing read (R2 on a paired-end run). This eliminates the need to export index reads from the MiSeq.

2. Removed the sample index from the Y-adapter and changed the PCR scheme. Now, only a single adapter is required for ligation to any number of samples, PCR1 is for enrichment of the dsOligo - genome junction, PCR2 is to add sample-specific indexes to the enriched amplicons.

See oligo designs for further information.

3. Replaced sonication based shearing followed by end-repair with a one-pot, one-step enzymatic fragmentation + end-repair kit from New England Biolabs.

PROTOCOL STATUS

Working

We developed this protocol at the University of Minnesota Genomics Center (UMGC) and it is working. UMGC will prepare GUIDE-seq NGS libraries and sequence them as a service.

GUIDELINES

Time from genomic DNA sample to sequencing-ready library takes 2 days.

Protocol can be performed with 1.5 ml tubes or 96-well plates.

MATERIALS

NAME	CATALOG #	VENDOR
TE buffer		
NEBNext Ultra II Q5 Master Mix	E7649	New England Biolabs
Thermal cycler	View	
NEBNext Ultra II FS DNA Library Prep with Sample Purification Beads - 24 rxns	E6177S	New England Biolabs

MATERIALS TEXT

Oligonucleotides required:

TA_Adaptor_Top - /5Phos/CTCACCGCTCTTGTAGS NNNNNNNN CTGTCTCTTATACACATCTCCGAG*C
TA_Adaptor_Bottom - CTACAAGAGCGGTGAGT

dsODN_Enrich_Plus - TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG NNN *GTTTAATTGAGTTGTCATATGTTAATAACG*G*
dsODN_Enrich_Minus - TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG NNN *CCGTTATTAACATATGACAACTCAATTAAA*C*
Replace the italicized sequences with sequences targeting the dsODN that you used in your experiments.

dsODN_Enrich_Adaptor - GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA*G

Nextera Indexing Oligo design
Nextera_R1 : AATGATACGGCGACCACCGAGATCTACAC [i5] TCGTCGGCAGCGT*C
Nextera_R2 : CAAGCAGAAGACGGCATACGAGAT [i7] GTCTCGTGGGCTCG*G

You will need to replace the [i5] & [i7] with unique index barcodes. You can use the Hamming barcodes from https://doi.org/10.1371/journal.pone.0036852. Order the appropriate number of indexing oligos for your samples based on if you want to do combinatorial indexing (cheaper) or unique dual indexing (better with the patterned flowcells).

Note: When selecting index barcodes, make sure

1) there is no collision with other barcodes that will be run on the same flowcell as your samples
2) don't start with or have consecutive G nucleotides if the samples will be run on instruments that use two-color (NextSeq, NovaSeq) or one-color (iSeq) chemistry.

SAFETY WARNINGS

NOTE:

The NGS library generated by this protocol is NOT plug-and-play compatible with the guideseq analysis package (https://github.com/aryeelab/guideseq).

We have made slight modifications to the guideseq scripts to process these libraries. The output will be the same figure (of off-target sites & their frequencies) as you get with the default package. I will add a comment to this section when the modified package is made available on Github.

BEFORE STARTING

Make sure that the genomic DNA is clean (260/230 & 260/280 > 1.8) and 400 ng of DNA is in a volume less than 26 ul. Measure DNA concentration by fluorometry (Picogreen or Qubit).

Adaptor formation

1

- Use 0.1x TE to prepare 100 µM stock of all the oligos.
- In a 0.2 ml PCR tube, add the following reagents, vortex well, and spin down. Place on the thermal cycler at **95 °C** for **00:03:00** (heated lid).

15 µl Oligo TA_Adaptor_Top (100 uM)
15 µl Oligo TA_Adaptor_Bottom (100 uM)
70 µl dH2O
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100 µl Total

- After incubation, terminate the incubation and let the heat block cool down to room temperature. Remove the tube and place it on the bench for 1 hr.

- The oligos will have annealed and formed the adaptor.

DNA fragmentation, end-repair, ligation

2

This section of the protocol is from the manual for NEB Ultra II FS kit - Catalog # E7805.

(Refer to https://www.neb.com/-/media/catalog/datacards-or-manuals/manuale7805.pdf : Section for Input > 100 ng for detailed protocol)

- Aliquot **400 ng** of genomic DNA into a well of a 96-well plate and use 1x TE to bring the volume to **26 µl** .
- Add the NEBNext Ultra II FS buffer + enzyme mix to the DNA (follow protocol from the linked manual)
- Set up the following program on the thermal cycler **75 °C heated lid**

37 °C for **00:10:00**
65 °C for **00:30:00**
4 °C hold.

NOTE
This should fragment the library to have a distribution between 300 - 700 bp.

- Add the NEBNext Ultra II ligation mix + enhancer to the well. Add **2.5 µl** of the adaptor to the mixture. Mix well and incubate at **20 °C** for **00:15:00 heated lid off** . Post incubation, add **28.5 µl** of 0.1x TE and bring volume to **100 µl** .
- Perform bead clean-up of the ligation mix using the sample purification beads that came with the kit.

Use **20 µl** of beads for 1st bead addition
10 µl of beads for 2nd bead addition (to select for an insert size distribution of 350 - 600 bp).

- Elute in **17 µl** of dH_2O.

Enrichment PCR

3

Make 10 uM dilutions of the following primers.

dsODN_Enrich_Plus
dsODN_Enrich_Minus
dsODN_Enrich_Adaptor

For each sample set up the following PCRs in a 96-well plate.

PCR 1.1 - Plus_Strand_Enrichment

7.5 µl Adaptor ligated DNA
12.5 µl Ultra II Q5 master mix

2.5 µl dsODN_Enrich_Adaptor

2.5 µl dsODN_Enrich_Plus

PCR 1.2 - Minus_Strand_Enrichment

7.5 µl Adaptor ligated DNA

12.5 µl Ultra II Q5 master mix

2.5 µl dsODN_Enrich_Adaptor

2.5 µl dsODN_Enrich_Minus

PCR program:

98 °C | 00:00:30 Initial denaturation

98 °C | 00:00:10 Denaturation

70 °C Gradient -1 ºC / cycle | 00:00:30 Annealing

72 °C | 00:01:00 Extension

7 cycles

98 °C | 00:00:10 Denaturation

63 °C | 00:00:30 Annealing

72 °C | 00:01:00 Extension

13 cycles

4 °C Hold

After end of PCR, use 1 µl of the PCR end-product for Picogreen / Qubit.

Use 2 µl to run the sample on a Tapestation (D5000 high-sensitivity) to check for library size.

Each sample library should be a unimodal distribution between 400 - 1300 bp. All libraries should have an average size distribution within 15% across all samples.

Indexing PCR

4

Make 10 uM dilutions of the **Nextera_R1** & **Nextera_R2** primers.

For each sample, mix

5 ng of ***PCR 1.1 +***

5 ng of ***PCR 1.2***

Set up the following PCR:

10 µl Ultra II Q5 master mix

1 µl Nextera_R1

1 µl Nextera_R2

10 ng Template

0 µl dH2O As required

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20 µl Total

PCR Program:

98 °C 00:00:30 Initial Denaturation

98 °C 00:00:10 Denaturation

65 °C 00:00:30 Annealing

72 °C 00:01:00 Extension

10 cycles

4 °C Hold

After end of PCR, use 1 µl of the PCR end-product for Picogreen / Qubit.

Library pooling, QC, Sequencing

5

- Pool samples by equal mass (assuming the library size distribution for the samples are similar).
- Do a 1.2x SPRI bead clean-up. Elute in 30 µl 0.1x TE
- Check concentration of the pool by Picogreen/Qubit and quantify library using Kapa Illumina Library quantification kit. Dilute the pool to appropriate molarity required for the sequencing instrument.
- Load the pool on the sequencer and run with 15% PhiX spike-in for base diversity (works on MiSeq, should work with other Illumina 4-color instruments. Might need more PhiX for NextSeq.)