



(12) **DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION**

(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2016/09/09
(87) Date publication PCT/PCT Publication Date: 2017/03/16
(85) Entrée phase nationale/National Entry: 2018/04/03
(86) N° demande PCT/PCT Application No.: US 2016/051097
(87) N° publication PCT/PCT Publication No.: 2017/044843
(30) Priorité/Priority: 2015/09/11 (US62/217,690)

(51) Cl.Int./Int.Cl. *C12N 15/10* (2006.01),
C12N 15/113 (2010.01), *C12Q 1/68* (2018.01)
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(54) Titre : INTERROGATION COMPLETE DE DSB NUCLEASIQUES ET SEQUENCAGE (FIND-SEQ)
(54) Title: FULL INTERROGATION OF NUCLEASE DSBs AND SEQUENCING (FIND-SEQ)

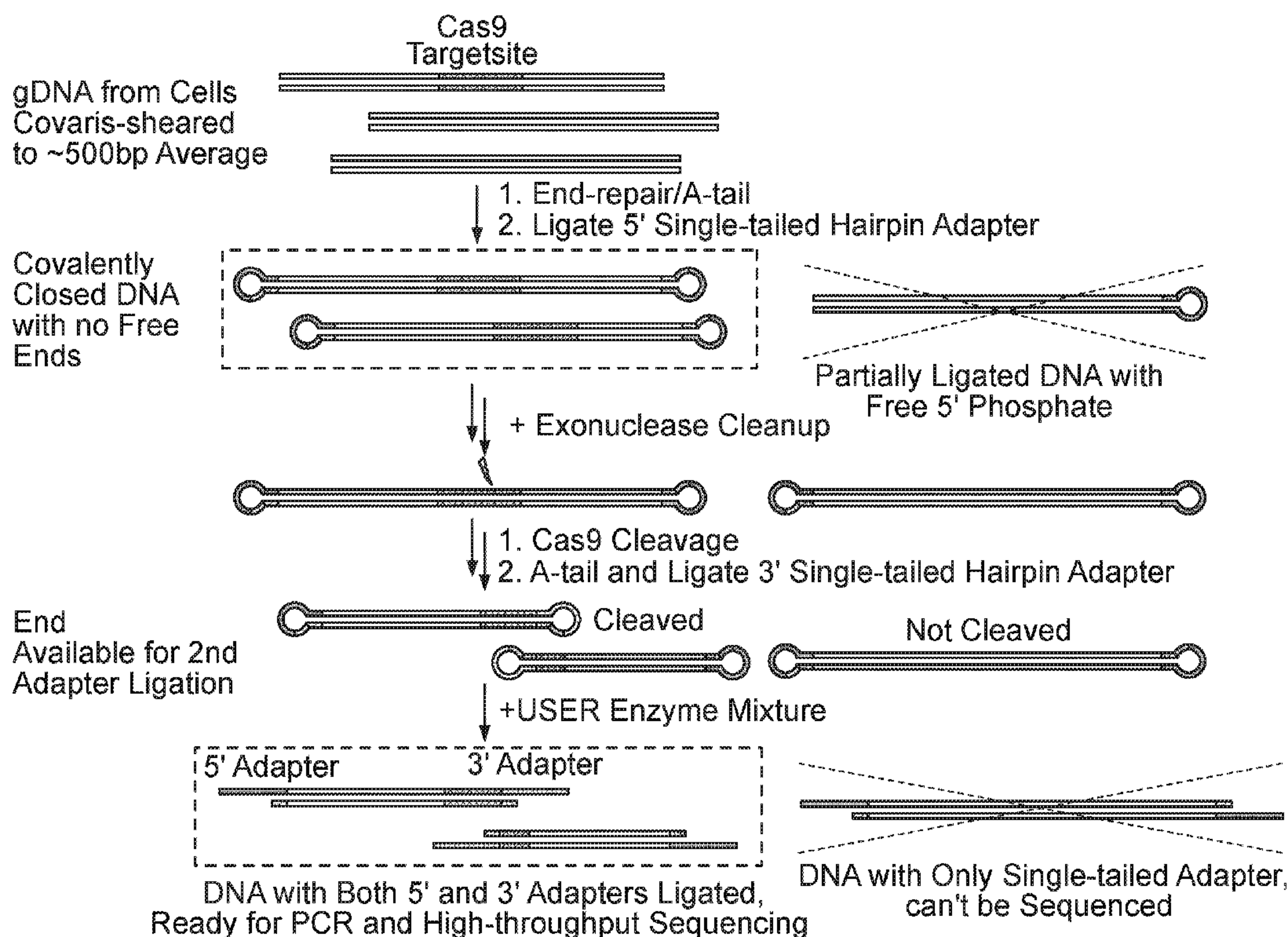


FIG. 1A

(57) **Abrégé/Abstract:**

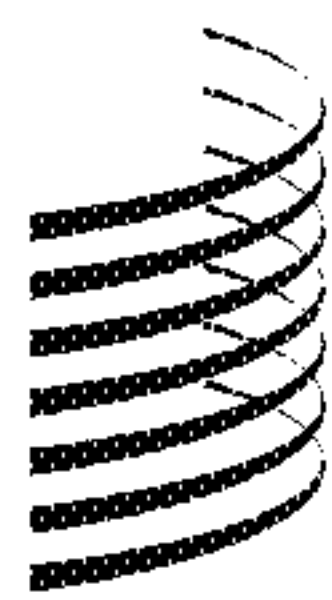
Sensitive, unbiased methods for genome-wide detection of potential CRISPR-Cas9 off-target cleavage sites from cell type-specific genomic DNA samples.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property

Organization

International Bureau



WIPO | PCT



(10) International Publication Number

WO 2017/044843 A1

(43) International Publication Date
16 March 2017 (16.03.2017)

(51) International Patent Classification:

C12N 15/10 (2006.01) C12Q 1/68 (2006.01)
C12N 15/113 (2010.01)

(21) International Application Number:

PCT/US2016/051097

(22) International Filing Date:

9 September 2016 (09.09.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/217,690 11 September 2015 (11.09.2015) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(54) Title: FULL INTERROGATION OF NUCLEASE DSBS AND SEQUENCING (FIND-SEQ)

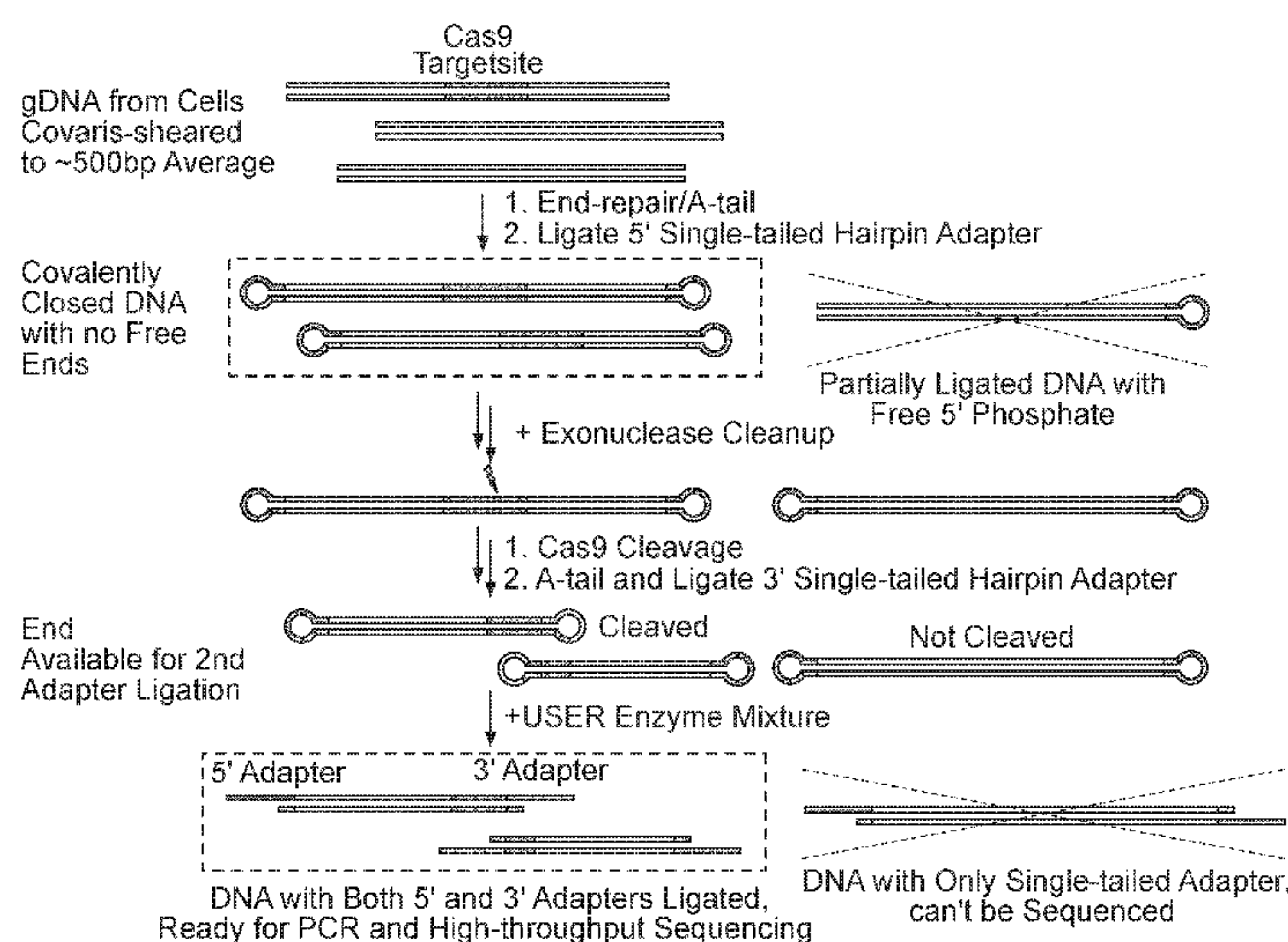


FIG. 1A

(57) Abstract: Sensitive, unbiased methods for genome-wide detection of potential CRISPR-Cas9 off-target cleavage sites from cell type-specific genomic DNA samples.

WO 2017/044843 A1

Full Interrogation of Nuclease DSBs and Sequencing (FIND-seq)

CLAIM OF PRIORITY

This application claims priority under 35 USC §119(e) to U.S. Patent
5 Application Serial No. 62/217,690, filed on September 11, 2015. The entire contents
of the foregoing are hereby incorporated by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under Grant No. DP1
GM105378 awarded by the National Institutes of Health. The Government has
10 certain rights in the invention.

TECHNICAL FIELD

Described herein are *in vitro* methods for defining the genome-wide cleavage
specificities of engineered nucleases such as CRISPR-Cas9 Nucleases.

BACKGROUND

15 Engineered nuclease technology including zinc fingers, TALENs, and
CRISPR-Cas9 nucleases, is revolutionizing biomedical research and providing
important new modalities for therapy of gene-based diseases.

SUMMARY

At least in part, the present invention is based on the development of sensitive,
20 unbiased methods for genome-wide detection of potential engineered nuclease (e.g.,
CRISPR-Cas9) off-target cleavage sites from cell type-specific genomic DNA
samples. The present methods use exonuclease selection of covalently closed DNA
molecules to create a population of genomic DNA molecules with very few free DNA
ends, as a starting population for cleavage-specific enrichment and sequencing.
25 Enrichment of these cleaved fragments, estimated to be >20,000X from human
genomic DNA, enables very sequencing-efficient discovery of *in vitro* cleaved DNA
fragments, in contrast to methods such as Digenome-Seq (Kim et al., Nat Methods.
2015 Mar;12(3):237-43) that rely on whole-genome sequencing. After optimization,
the present *in vitro* assay detected 100% of off-target cleavage sites detected by the in-
30 cell GUIDE-seq assay at the VEGFA site target site used as a test case and described

in Tsai et al., Nat Biotechnol. 2015 Feb;33(2):187-97 (in other words, the present in vitro assay detects a superset of GUIDE-seq detected cleavage sites).

Described herein are methods of enzymatically preparing a library of DNA fragments without DNA double-stranded breaks (DSBs) (the terms “breaks” and “cleavage” are used herein interchangeably) by ligation of stem-loop or hairpin adapters followed by exonuclease selection, and for detecting nuclease-induced cleavage of this enzymatically purified library of covalently closed DNA fragments by sequencing. Together, these two methods comprise a strategy for efficiently mining for nuclease-induced cleavage sites in complex mixtures of DNA. Thus the methods can include creating a population of molecules without ends, treating that population with a nuclease, and finding molecules in this population that have newly created ends as a result of nuclease-induced cleavage.

In a first aspect, the invention provides methods for preparing a library of covalently closed DNA fragments. The methods include providing DNA, e.g., gDNA from a cell type or organism of interest; optionally randomly shearing the DNA to a defined average length, e.g., an average length of about 200-1000 bps, e.g., about 500 bps, to provide a population of DNA fragments; preparing the fragments for end-ligation, e.g., by end-repairing and then A-tailing the sheared DNA; ligating a first hairpin adapter comprising at least a single deoxyuridine (uracil) and a first primer site compatible for use in PCR priming and/or sequencing, e.g., next generation sequencing (NGS) to the ends of the fragments, to prepare a population of ligated fragments; contacting the ligated fragments with a nuclease that induces double stranded breaks; and purifying the ligated fragments using an exonuclease, thereby preparing a library of covalently closed DNA fragments.

The methods can also include contacting the library of covalently closed DNA fragments with a nuclease to induce site-specific cleavage; ligating a second hairpin adapter comprising at least a single deoxyuridine and a second primer site compatible for use with the first primer site in PCR priming and/or sequencing; contacting the library with an enzyme, e.g., uracil DNA glycosylase (UDG) and/or endonuclease VIII, a DNA glycosylase-lyase, to nick the DNA at the deoxyuridine; and sequencing those fragments having a first and second hairpin adapter.

Also provided herein are methods for preparing a library of fragments comprising nuclease-induced double stranded breaks in DNA, e.g., genomic DNA

(gDNA). The methods can include providing DNA, e.g., gDNA from a cell type or organism of interest; randomly shearing the DNA to a defined average length, e.g., an average length of about 200-1000 bps, e.g., about 100-500 bps; optionally end-repairing and then A-tailing the sheared DNA; ligating a first single-tailed hairpin adapter, preferably comprising a first region, e.g., of about 10-20, e.g., 12 nucleotides; a second region, e.g., of about 45-65, e.g., 58 nucleotides, that forms one or more hairpin loops and comprises a first primer site compatible for use in PCR priming and/or sequencing, e.g., next generation sequencing (NGS); and a third region, e.g., of about 10-20, e.g., 13 nucleotides (e.g., one longer than the first region) that is complementary to the first region, with a single deoxyuridine nucleotide between the first and second regions; contacting the sample with one or more exonucleases (e.g., bacteriophage lambda exonuclease, E. coli ExoI, PlasmidSafe™ ATP-dependent exonuclease), sufficient to degrade any DNA molecules that lack the first (e.g., 5') single-tailed hairpin adapter ligated to both of their ends; treating the sample with a nuclease to induce site-specific cleavage (e.g., of on- and/or off-target sites, e.g., to induce blunt or staggered/overhanging ends) optionally end-repairing and then A-tailing the resulting ends; ligating a second (e.g., 3') single-tailed hairpin adapter comprising a first region of about 10-20, e.g., 15 nucleotides; a second region of about 40-60, e.g., 48, nucleotides that forms one or more hairpin loops and comprises a second primer compatible for use with the first primer site in PCR priming and/or sequencing, e.g., next generation sequencing (NGS); and a third region of about 10-20, e.g., 16 nucleotides (e.g., one longer than the first region) that is complementary to the first region and that also contains a single deoxyuridine nucleotide between the second and third regions, to create a population wherein the DNA fragments that were cleaved by the nuclease have a first and second single-tailed hairpin adapter ligated to their respective ends; thereby preparing a library of fragments, e.g., wherein one end was created by a nuclease-induced double stranded break in the DNA.

In some embodiments, the methods include contacting the library with uracil DNA glycosylase (UDG) and/or endonuclease VIII, a DNA glycosylase-lyase to nick the DNA at the deoxyuridine; and sequencing those fragments bearing a first and a second hairpin adapter.

Also provided herein are methods for detecting nuclease-induced double stranded breaks (DSBs) in DNA, e.g., in genomic DNA (gDNA) of a cell. The

methods include providing DNA, e.g., gDNA from a cell type or organism of interest;
 randomly shearing the DNA to a defined average length, e.g., an average length of
 about 200-1000 bps, e.g., about 500 bps; optionally end-repairing and then A-tailing
 the sheared DNA; ligating a first single-tailed hairpin adapter, preferably comprising a
 5 first region, e.g., of about 10-20, e.g., 12 nucleotides; a second region, e.g., of about
 45-65, e.g., 58 nucleotides, that forms one or more hairpin loops and comprises a first
 primer site compatible for use in PCR priming and/or sequencing, e.g., next
 generation sequencing (NGS); and a third region, e.g., of about 10-20, e.g., 13
 nucleotides (e.g., one longer than the first region) that is complementary to the first
 10 region, with a single deoxyuridine nucleotide between the first and second regions;
 contacting the sample with one or more exonucleases (e.g., bacteriophage lambda
 exonuclease, *E. coli* ExoI, PlasmidSafe™ ATP-dependent exonuclease), sufficient to
 degrade any DNA molecules that lack the first single-tailed hairpin adapter ligated to
 both of their ends; treating the sample with a nuclease to induce site-specific cleavage
 15 the DNA, e.g., to produce on- and/or off-target cleavage sites; optionally end-
 repairing and then A-tailing the resulting cleaved ends; ligating a second (e.g., 3')
 single-tailed hairpin adapter comprising a first region of about 10-20, e.g., 15
 nucleotides; a second region of about 40-60, e.g., 48, nucleotides that forms one or
 more hairpin loops and comprises a second primer compatible for use with the first
 20 primer site in PCR priming and/or sequencing, e.g., next generation sequencing
 (NGS); and a third region of about 10-20, e.g., 16 nucleotides (e.g., one longer than
 the first region) that is complementary to the first region, and that also contains a
 single deoxyuridine nucleotide between the second and third regions, to create a
 population wherein the DNA fragments that were cleaved by the nuclease have the
 25 first and second hairpin adapters ligated to their respective ends; thereby preparing a
 library of fragments comprising nuclease-induced double stranded breaks in the DNA,
 e.g., wherein one end was created by a nuclease-induced double stranded break in the
 DNA; contacting the library with uracil DNA glycosylase (UDG) and/or
 endonuclease VIII, a DNA glycosylase-lyase to nick the DNA at the deoxyuridine;
 30 and sequencing those fragments bearing a first and a second hairpin adapter; thereby
 detecting DSBs induced by the nuclease.

In some embodiments, the engineered nuclease is selected from the group
 consisting of meganucleases, MegaTALs, zinc-finger nucleases, transcription

activator effector-like nucleases (TALEN), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas RNA-guided nucleases (CRISPR/Cas RGNs).

In some embodiments, treating the sample with a nuclease to induce site-specific cleavage, e.g., at on- and off-target sites, comprises contacting the sample
5 with a Cas9 nuclease complexed with a specific guide RNA (gRNA).

Further, provided herein are methods for determining which of a plurality of guide RNAs is most specific, i.e., induces the fewest off-target DSBs. The methods include, for each of the plurality of guide RNAs: providing gDNA from a cell type or organism of interest; randomly shearing the gDNA to a defined average length, e.g.,
10 an average length of about 200-1000 bps, e.g., about 500 bps; optionally end-repairing and then A-tailing the sheared gDNA; ligating a first single-tailed hairpin adapter, preferably comprising a first region, e.g., of about 10-20, e.g., 12 nucleotides; a second region, e.g., of about 45-65, e.g., 58 nucleotides, that forms one or more hairpin loops and comprises a first primer site compatible for use in PCR priming
15 and/or sequencing, e.g., next generation sequencing (NGS); and a third region, e.g., of about 10-20, e.g., 13 nucleotides (e.g., one longer than the first region) that is complementary to the first region, with a single deoxyuridine nucleotide between the first and second regions; contacting the sample with one or more exonucleases (e.g., bacteriophage lambda exonuclease, E. coli ExoI, PlasmidSafe™ ATP-dependent
20 exonuclease), sufficient to degrade any DNA molecules that lack the first hairpin adapter ligated to both of their ends; treating the sample with a Cas9 nuclease compatible with the guide RNA to induce site-specific cleavage (e.g., of on- and/or off-target sites, e.g., to produce blunt or staggered/overhanging ends); optionally end-repairing and then A-tailing the resulting cleaved ends; ligating a second (e.g., 3')
25 single-tailed hairpin adapter comprising a first region of about 10-20, e.g., 15 nucleotides; a second region of about 40-60, e.g., 48, nucleotides that forms one or more hairpin loops and comprises a second primer compatible for use with the first primer site in PCR priming and/or sequencing, e.g., next generation sequencing (NGS); and a third region of about 10-20, e.g., 16 nucleotides (e.g., one longer than
30 the first region) that is complementary to the first region, and that also contains a single deoxyuridine nucleotide between the second and third regions, to create a population wherein the DNA fragments that have a first and second hairpin adapter ligated to their ends are those that were cleaved by the nuclease; thereby preparing a

library of fragments comprising nuclease-induced double stranded breaks in DNA, e.g., wherein one end was created by a nuclease-induced double stranded break in the DNA; contacting the library with uracil DNA glycosylase (UDG) and/or endonuclease VIII, a DNA glycosylase-lyase to nick the DNA at the deoxyuridine; and sequencing those fragments bearing a first and a second hairpin adapter, thereby detecting DSBs induced by the nuclease in each sample; optionally identifying whether each DSB is on-target or off-target; comparing the DSBs induced by the nuclease in each sample; and determining which of the plurality of guide RNAs induced the fewest off-target DSBs.

In some embodiments, the DNA is isolated from a mammalian, plant, bacterial, or fungal cell (e.g., gDNA).

In some embodiments, the DNA is synthetic.

In some embodiments, the engineered nuclease is a TALEN, zinc finger, meganuclease, megaTAL, or a Cas9 nuclease.

In some embodiments, the engineered nuclease is a Cas9 nuclease, and the method also includes expressing in the cells a guide RNA that directs the Cas9 nuclease to a target sequence in the genome.

In some embodiments, the primer site in the first or second hairpin adapter comprises a next generation sequencing primer site, a randomized DNA barcode or unique molecular identifier (UMI).

The present methods have several advantages. For example, the present methods are *in vitro*; in contrast, GUIDE-seq is cell-based, requiring the introduction of double stranded oligodeoxynucleotides (dsODN) as well as expression or introduction of nuclease or nuclease-encoding components into cells. Not all cells will allow the introduction of dsODNs and/or nuclease or nuclease-encoding components, and these reagents can be toxic in some cases. If a cell type of particular interest is not amenable to introduction of dsODNs, nucleases, or nuclease-encoding components, a surrogate cell type might be used, but cell-specific effects might not be detected. The present methods do not require delivery of the dsODN, nuclease, or nuclease-encoding components into a cell, are not influenced by chromatin state, do not create toxicity issues, and enable interrogation of specific cellular genomes by cleavage of genomic DNA that is obtained from the cell-type of interest.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

Figures 1A-B. Overview of an exemplary in vitro assay for genome-wide identification of CRISPR/Cas9 cleavage sites from complex mixtures of DNA called FIND-seq (Full Interrogation of Nuclease DSBs). (a) Genomic DNA is isolated from human cells and sheared to an average of ~500 bp using a Covaris S220 AFA instrument. Sheared DNA is end-repaired, A-tailed, and ligated with a 5' single-tailed hairpin adapter containing a single deoxyuradine. Successful ligation of the hairpin adapter to both ends of the DNA fragments will result in covalently closed DNA with no free end. Lambda Exonuclease and E. coli ExoI or PlasmidSafe ATP-dependent exonuclease can then be used to dramatically reduce the background of DNA with free ends, leaving a predominantly uniform population of covalently closed DNA molecules. Cas9 cleavage of adapter-ligated gDNA will produce ends with free 5' phosphates for the ligation of a second 3' single-tailed hairpin adapter that also contains a single uracil base. Treatment with USER enzyme mixture and PCR enriches for DNA molecules that have been ligated with both 5' and 3' single-tailed adapters. The use of single-tailed adapters reduces background, as only molecules that contain both 5' and 3' adapters can be amplified and sequenced. (b) Detailed schematic of adapter ligation steps, with sequence and predicted DNA secondary structure of hairpin adapters. Ligation of hairpin adapters at both ends creates covalently closed DNA molecules that can be subsequently reopened at uracil-containing sites.

Figures 2A-B. Reads mapped at on-target and off-target cleavage sites.

(a) Visualization of 'on-target' genomic regions where bidirectionally mapping uniform-end read signatures of Cas9 cleavage can be identified. Reads are indicated by rectangles; coverage is displayed above. The arrow indicates the predicted cut site and data is shown for 3 target sites in the *VEGFA* locus. A sample treated with *Streptococcus pyogenes* Cas9 complexed with EMX1 gRNA is also shown as a negative control. (b) Visualization of example 'off-target' genomic region 20:56175349-56175372 in *VEGFA* site 1. Bidirectionally mapping reads with 'uniform' ends can be observed in this region and are typical of sites detected by this in vitro assay. Reads generally start within 1-bp of the predicted cut-site and originate outward in both directions from this position.

Figures 3A-D. Analysis of FIND-seq in vitro cleavage selection assay results using different exonuclease treatment conditions. (a) Mapped read counts at genome-wide in vitro cleavage assay detected sites for a under different exonuclease treatments. (b) Number of sites detected by genome-wide in vitro cleavage assay with different exonuclease treatments. (c) Average read counts at genome-wide in vitro cleavage assay detected sites under different exonuclease treatments. (d) Percentage of GUIDE-seq detected sites that are detected by genome-wide in vitro cleavage assay. Note there are three conditions where 100% of GUIDE-seq sites are detected using the simple in vitro assay.

Figure 4. Venn diagram of overlap between GUIDE-seq and FIND-seq genomic in vitro cleavage assay performed with 1 hour of lambda exonuclease and *E. coli* exonuclease I, and 1 hour of PlasmidSafe exonuclease treatment at the *VEGFA* target site 1. In this condition, 100% of GUIDE-seq sites are also detected with the in vitro assay.

Figures 5A-B. Analysis of FIND-seq genomic in vitro cleavage assay testing the adapter ligation order with different exonuclease treatment conditions. The condition that detected the highest number of sites was 1 hr PlasmidSafe, 1 hr Lambda exonuclease/*E. Coli* exonuclease I treatment, with the 3' adapter added first. (a) Number of sites by FIND-seq genome-wide in vitro cleavage assay with different exonuclease treatments and adapter ligation order. (b) Read counts at FIND-seq detected off-target sites using different exonuclease treatments and adapter ligation order.

DETAILED DESCRIPTION

An important consideration for the therapeutic deployment of CRISPR-Cas9 nucleases is having robust, comprehensive, unbiased, and highly sensitive methods for defining their off-target effects. Recently, a number of methods for defining the genome-wide off-target effects of CRISPR-Cas9 and other customizable nucleases have been described. For example, the GUIDE-seq method, which relies on uptake of a short double-stranded oligonucleotide “tag” into nuclease-induced DSBs in living cells, has been shown to define off-target sites on a genome-wide scale, identifying sites that are mutagenized with frequencies as low as 0.1% of the time in a population of cells (Tsai et al., Nat Biotechnol. 2015). Other cell-based methods for defining nuclease-induced off-target breaks include a method that maps translocation fusions to the on-target site and another that relies on uptake of integration-deficient lentivirus (IDLV) genomes into sites of DSBs.

Despite this recent progress, cell-based methods for off-target determination have a number of limitations including: (1) a requirement to be able to introduce both the nuclease components and a tag such as the dsODN or IDLV genome into cells; (2) biological selection pressures that might favor or disfavor the growth of cells harboring certain types of off-target mutations; and/or (3) the potential confounding effects of cell-type-specific parameters such as chromatin, DNA methylation, gene expression, and nuclear architecture on nuclease off-target activities/effects.

In vitro methods using purified genomic DNA provide an attractive alternative because they would sidestep these various limitations of cell-based approaches. However, in vitro methods face the challenge that isolated genomic DNA is by experimental necessity randomly sheared (or broken) into smaller pieces. This poses a challenge because it is not easy to differentially identify DSBs induced by shearing from those induced by treatment of the genomic DNA in vitro by nucleases. The recently described Digenome attempted to use alignment of common ends induced by nucleases in genomic sequence but the signal for this type of event can be challenging to discern relative to the background of random DSBs from shearing of genomic DNA induced during genomic DNA isolation and by deliberate shearing of the DNA to smaller size pieces required for DNA sequencing methods. In addition, and perhaps more importantly, because there is no enrichment for the nuclease-induced DSBs, nearly all of the sequencing data generated with Digenome is just re-sequencing of

random genomic DNA, a factor that further limits the sensitivity of the method since very few reads generated contribute the desired information about nuclease-induced DSBs. Indeed, Digenome failed to identify a number of off-target sites found by GUIDE-seq for a particular gRNA, although the caveat must be added that the two
5 experiments were performed in different cell lines.

Described herein is an *in vitro* method that enables comprehensive determination of DSBs induced by nucleases on any genomic DNA of interest. This method enables enrichment of nuclease-induced DSBs over random DSBs induced by shearing of genomic DNA. An overview of how the method works can be found in
10 **Figures 1A and 1B**. In brief, genomic DNA from a cell type or organism of interest is randomly sheared to a defined average length. In the present experiments, an average length of 500 bps worked well for subsequent next-generation sequencing steps; however, shorter or longer lengths can also be used. The random broken ends of this genomic DNA are end repaired (i.e., the random overhanging ends are filled
15 in/blunted, e.g., using T4 polymerase, Klenow fragment, and T4 Polynucleotide Kinase (PNK)) and then A-tailed (i.e., an A (adenosine) is enzymatically added to the 3' end of a blunt, double-stranded DNA molecule). This then enables the ligation of a hairpin adapter, preferably bearing a 5' end next-generation sequencing primer site, that also contains a single deoxyuridine nucleotide at a specific position (**Figure 1B**)
20 and a 1-nucleotide (thymidine) overhang at the 3' end; hereafter this adapter is referred to as the "5' single-tailed hairpin adapter" or simply "5' hairpin adapter".

Following ligation of this adapter, the sample is then treated with one or more exonucleases (e.g., bacteriophage lambda exonuclease, *E. coli* ExoI, PlasmidSafe™ ATP-dependent exonuclease) that degrade any DNA molecules that have not had the
25 5' single-tailed hairpin adapter ligated to both of their ends. The collection of molecules is then treated to induce blunt-end cuts, e.g., with a Cas9 nuclease complexed with a specific guide RNA (gRNA). The resulting nuclease-induced blunt ends are A-tailed and then a second hairpin adapter bearing a 3' end next-generation sequencing primer site and that also contains a single deoxyuridine nucleotide at a
30 specific position is ligated to these ends; this adapter is also referred to herein as the "3' single-tailed hairpin adapter" or simply "3' hairpin adapter".

As a result of these treatments, the only DNA fragments that should have a 5' and a 3' single-tailed hairpin adapter ligated to their ends are those that were cleaved

by the nuclease. Following treatment (e.g., by the USER enzyme mixture) to nick DNA wherever a deoxyuridine is present, only those fragments bearing the two types of adapters can be sequenced.

The present methods can be used to prepare libraries of fragments for next generation sequencing, e.g., to identify guideRNA/Cas9 combinations that induce the most specific DSBs (e.g., that have the fewest off-target effects), e.g., for therapeutic or research purposes.

Single-Tailed Hairpin Adapters

The present methods include the use of non-naturally occurring 3' and 5' single-tailed hairpin adapters. The hairpin adapters include (from 5' to 3') a first region, e.g., of about 10-20, e.g., 12 or 15, nucleotides; a second region, e.g., of about 45-65, e.g., 58 or 48, nucleotides that forms one or more hairpin loops and includes a sequence compatible for use in PCR priming and/or sequencing, e.g., next generation sequencing (NGS); and a third region, e.g., of about 10-20, e.g., 13 or 16, nucleotides that is complementary to the first region. The lengths of the first, second and third regions can vary depending on the NGS method selected, as they are dependent on the sequences that are necessary for priming for use with the selected NGS platform.

The hairpin adapters include at least one, preferably only one, uracil that allows the adaptor to be opened by Uracil DNA glycosylase (UDG) and Endonuclease VIII, a DNA glycosylase-lyase, e.g., the USER (Uracil-Specific Excision Reagent) Enzyme mixture (New England BioLabs). The UDG catalyzes the excision of uracil bases to form an abasic site but leave the phosphodiester backbone intact (see, e.g., Lindhal et al., J. Biol. Chem., 252:3286-3294 (1977); Lindhal, Annu. Rev. Biochem. 51:61-64 (1982)). The Endonuclease VIII breaks the phosphodiester backbone at the 3' and 5' sides of the abasic site (see, e.g., Melamede et al., Biochemistry 33:1255-1264 (1994); Jiang et al., J. Biol. Chem. 272:32230-32239 (1997)). This combination generates a single nucleotide gap at the location of a uracil. In some embodiments, in the 5' single-tailed hairpin adapters the uracil is placed at or within 1, 2, 3, or 4 nucleotides of the end of the first region and the beginning of the second region, and in the 3' single-tailed hairpin adapters the uracil is placed at or within 1, 2, 3, or 4 nucleotides of the end of the first region and the beginning of the second region.

In the present methods, all parts of the hairpin adapters are preferably orthologous to the genome of the cell (i.e., are not present in or complementary to a

sequence present in, i.e., have no more than 10%, 20%, 30%, 40%, or 50% identity to a sequence present in, the genome of the cell). The hairpin adapters can preferably be between 65 and 95 nts long, e.g., 70-90 nts or 75-85 nts long.

Each of the 5' and 3' single-tailed hairpin adapters should include a primer site that is a randomized DNA barcode (e.g., SHAPE-SEQ, Lucks et al., Proc Natl Acad Sci U S A 108: 11063-11068), unique molecular identifier (UMI) (see, e.g., Kivioja et al., Nature Methods 9, 72-74 (2012); Islam et al., Nature Methods 11, 163-166 (2014); Karlsson et al., Genomics. 2015 Mar;105(3):150-8), or unique PCR priming sequence and/or unique sequence compatible for use in sequencing (e.g., NGS). The sequence compatible for use in sequencing can be selected for use with a desired sequencing method, e.g., a next generation sequencing method, e.g., Illumina, Ion Torrent or library preparation method like Roche/454, Illumina Solexa Genome Analyzer, the Applied Biosystems SOLiD™ system, Ion Torrent™ semiconductor sequence analyzer, PacBio® real-time sequencing and Helicos™ Single Molecule Sequencing (SMS). See, e.g., WO2014020137, Voelkerding et al., Clinical Chemistry 55:4 641-658 (2009) and Metzker, Nature Reviews Genetics 11:31-46 (2010)). A number of kits are commercially available for preparing DNA for NGS, including the ThruPLEX DNA-seq Kit (Rubicon; see U.S. Patents 7,803,550; 8,071,312; 8,399,199; 8,728,737) and NEBNext® (New England BioLabs; see e.g., U.S. patent 8,420,319)). Exemplary sequences are shown in Table A, below.

In some embodiments, the hairpin adapters include a restriction enzyme recognition site, preferably a site that is relatively uncommon in the genome of the cell.

The hairpin adapters are preferably modified; in some embodiments, the 5' ends of the hairpin adapters are phosphorylated. In some embodiments, the hairpin adapters are blunt ended. In some embodiments, the hairpin adapters include a random variety of 1, 2, 3, 4 or more nucleotide overhangs on the 5' or 3' ends, or include a single T at the 5' or 3' end.

The hairpin adapters can also include one or more additional modifications, e.g., as known in the art or described in PCT/US2011/060493. For example, in some embodiments, the hairpin adapters is biotinylated. The biotin can be anywhere internal to the hairpin adapters (e.g., a modified thymidine residue (Biotin-dT) or using biotin azide), but not on the 5' or 3' ends. This provides an alternate method of

recovering fragments that contain the FIND-seq hairpin adapters. Whereas in some embodiments, these sequences are retrieved and identified by PCR, in this approach they are physically pulled down and enriched by using the biotin, e.g., by binding to streptavidin-coated magnetic beads, or using solution hybrid capture; see, e.g., Gnirke et al., Nature Biotechnology 27, 182 - 189 (2009).

Although the present working examples include ligating the 5' adapter and then the 3' adapter, the adapters can be added in either order, i.e., 5' adapter then 3' adapter, or 3' adapter then 5' adapter. The order may be optimized depending on the exonuclease treatment used, e.g., for Lambda exonuclease, which is a highly processive 5' → 3' exonuclease, where adding the 5' adapter second may be advantageous.

Engineered Nucleases

There are presently four main classes of engineered nucleases: 1) meganucleases, 2) zinc-finger nucleases, 3) transcription activator effector-like nucleases (TALEN), and 4) Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas RNA-guided nucleases (RGN). See, e.g., Gaj et al., Trends Biotechnol. 2013 Jul;31(7):397-405. Any of these, or variants thereof, can be used in the present methods. The nuclease can be transiently or stably expressed in the cell, using methods known in the art; typically, to obtain expression, a sequence encoding a protein is subcloned into an expression vector that contains a promoter to direct transcription. Suitable eukaryotic expression systems are well known in the art and described, e.g., in Sambrook et al., *Molecular Cloning, A Laboratory Manual* (4th ed. 2013); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (2006); and *Current Protocols in Molecular Biology* (Ausubel et al., eds., 2010). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (see, e.g., the reference above and Morrison, 1977, J. Bacteriol. 132:349-351; Clark-Curtiss & Curtiss, Methods in Enzymology 101:347-362 (Wu et al., eds, 1983).

Homing Meganucleases

Meganucleases are sequence-specific endonucleases originating from a variety of organisms such as bacteria, yeast, algae and plant organelles. Endogenous meganucleases have recognition sites of 12 to 30 base pairs; customized DNA binding sites with 18bp and 24bp-long meganuclease recognition sites have been described, and either can be used in the present methods and constructs. See, e.g., Silva, G., et

al., *Current Gene Therapy*, 11:11-27, (2011); Arnould et al., *Journal of Molecular Biology*, 355:443-58 (2006); Arnould et al., *Protein Engineering Design & Selection*, 24:27-31 (2011); and Stoddard, Q. *Rev. Biophys.* 38, 49 (2005); Grizot et al., *Nucleic Acids Research*, 38:2006-18 (2010).

5 CRISPR-Cas Nucleases

Recent work has demonstrated that clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems (Wiedenheft et al., *Nature* 482, 331-338 (2012); Horvath et al., *Science* 327, 167-170 (2010); Terns et al., *Curr Opin Microbiol* 14, 321-327 (2011)) can serve as the basis of a simple and
 10 highly efficient method for performing genome editing in bacteria, yeast and human cells, as well as *in vivo* in whole organisms such as fruit flies, zebrafish and mice (Wang et al., *Cell* 153, 910-918 (2013); Shen et al., *Cell Res* (2013); Dicarlo et al., *Nucleic Acids Res* (2013); Jiang et al., *Nat Biotechnol* 31, 233-239 (2013); Jinek et al., *Elife* 2, e00471 (2013); Hwang et al., *Nat Biotechnol* 31, 227-229 (2013); Cong et al., *Science* 339, 819-823 (2013); Mali et al., *Science* 339, 823-826 (2013c); Cho et al., *Nat Biotechnol* 31, 230-232 (2013); Gratz et al., *Genetics* 194(4):1029-35 (2013)).
 15 The Cas9 nuclease from *S. pyogenes* can be guided via simple base pair complementarity between 17-20 nucleotides of an engineered guide RNA (gRNA), e.g., a single guide RNA or crRNA/tracrRNA pair, and the complementary strand of a
 20 target genomic DNA sequence of interest that lies next to a protospacer adjacent motif (PAM), e.g., a PAM matching the sequence NGG or NAG (Shen et al., *Cell Res* (2013); Dicarlo et al., *Nucleic Acids Res* (2013); Jiang et al., *Nat Biotechnol* 31, 233-239 (2013); Jinek et al., *Elife* 2, e00471 (2013); Hwang et al., *Nat Biotechnol* 31, 227-229 (2013); Cong et al., *Science* 339, 819-823 (2013); Mali et al., *Science* 339, 823-
 25 826 (2013c); Cho et al., *Nat Biotechnol* 31, 230-232 (2013); Jinek et al., *Science* 337, 816-821 (2012)).

In some embodiments, the present system utilizes a wild type or variant Cas9 protein from *S. pyogenes* or *Staphylococcus aureus*, either as encoded in bacteria or codon-optimized for expression in mammalian cells. The guide RNA is expressed in
 30 the cell together with the Cas9. Either the guide RNA or the nuclease, or both, can be expressed transiently or stably in the cell.

TAL Effector Repeat Arrays

TAL effectors of plant pathogenic bacteria in the genus *Xanthomonas* play important roles in disease, or trigger defense, by binding host DNA and activating effector-specific host genes. Specificity depends on an effector-variable number of imperfect, typically ~33-35 amino acid repeats. Polymorphisms are present primarily at repeat positions 12 and 13, which are referred to herein as the repeat variable-diresidue (RVD). The RVDs of TAL effectors correspond to the nucleotides in their target sites in a direct, linear fashion, one RVD to one nucleotide, with some degeneracy and no apparent context dependence. In some embodiments, the polymorphic region that grants nucleotide specificity may be expressed as a triresidue or triplet.

Each DNA binding repeat can include a RVD that determines recognition of a base pair in the target DNA sequence, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA sequence. In some embodiments, the RVD can comprise one or more of: HA for recognizing C; ND for recognizing C; HI for recognizing C; HN for recognizing G; NA for recognizing G; SN for recognizing G or A; YG for recognizing T; and NK for recognizing G, and one or more of: HD for recognizing C; NG for recognizing T; NI for recognizing A; NN for recognizing G or A; NS for recognizing A or C or G or T; N* for recognizing C or T, wherein * represents a gap in the second position of the RVD; HG for recognizing T; H* for recognizing T, wherein * represents a gap in the second position of the RVD; and IG for recognizing T.

TALE proteins may be useful in research and biotechnology as targeted chimeric nucleases that can facilitate homologous recombination in genome engineering (e.g., to add or enhance traits useful for biofuels or biorenewables in plants). These proteins also may be useful as, for example, transcription factors, and especially for therapeutic applications requiring a very high level of specificity such as therapeutics against pathogens (e.g., viruses) as non-limiting examples.

Methods for generating engineered TALE arrays are known in the art, see, e.g., the fast ligation-based automatable solid-phase high-throughput (FLASH) system described in USSN 61/610,212, and Reyon et al., *Nature Biotechnology* 30,460-465 (2012); as well as the methods described in Bogdanove & Voytas, *Science* 333, 1843-1846 (2011); Bogdanove et al., *Curr Opin Plant Biol* 13, 394-401 (2010); Scholze &

Boch, J. *Curr Opin Microbiol* (2011); Boch et al., *Science* 326, 1509-1512 (2009);
 Moscou & Bogdanove, *Science* 326, 1501 (2009); Miller et al., *Nat Biotechnol* 29,
 143-148 (2011); Morbitzer et al., *T. Proc Natl Acad Sci U S A* 107, 21617-21622
 (2010); Morbitzer et al., *Nucleic Acids Res* 39, 5790-5799 (2011); Zhang et al., *Nat*
 5 *Biotechnol* 29, 149-153 (2011); Geissler et al., *PLoS ONE* 6, e19509 (2011); Weber et
 al., *PLoS ONE* 6, e19722 (2011); Christian et al., *Genetics* 186, 757-761 (2010); Li et
 al., *Nucleic Acids Res* 39, 359-372 (2011); Mahfouz et al., *Proc Natl Acad Sci U S A*
 108, 2623-2628 (2011); Mussolino et al., *Nucleic Acids Res* (2011); Li et al., *Nucleic*
Acids Res 39, 6315-6325 (2011); Cermak et al., *Nucleic Acids Res* 39, e82 (2011);
 10 Wood et al., *Science* 333, 307 (2011); Hockemeyer et al. *Nat Biotechnol* 29, 731-734
 (2011); Tesson et al., *Nat Biotechnol* 29, 695-696 (2011); Sander et al., *Nat*
Biotechnol 29, 697-698 (2011); Huang et al., *Nat Biotechnol* 29, 699-700 (2011); and
 Zhang et al., *Nat Biotechnol* 29, 149-153 (2011); all of which are incorporated herein
 by reference in their entirety.

15 Also suitable for use in the present methods are MegaTALs, which are a
 fusion of a meganuclease with a TAL effector; see, e.g., Boissel et al., *Nucl. Acids*
Res. 42(4):2591-2601 (2014); Boissel and Scharenberg, *Methods Mol Biol.*
 2015;1239:171-96.

Zinc Fingers

20 Zinc finger proteins are DNA-binding proteins that contain one or more zinc
 fingers, independently folded zinc-containing mini-domains, the structure of which is
 well known in the art and defined in, for example, Miller et al., 1985, *EMBO J.*,
 4:1609; Berg, 1988, *Proc. Natl. Acad. Sci. USA*, 85:99; Lee et al., 1989, *Science*.
 245:635; and Klug, 1993, *Gene*, 135:83. Crystal structures of the zinc finger protein
 25 Zif268 and its variants bound to DNA show a semi-conserved pattern of interactions,
 in which typically three amino acids from the alpha-helix of the zinc finger contact
 three adjacent base pairs or a “subsite” in the DNA (Pavletich et al., 1991, *Science*,
 252:809; Elrod-Erickson et al., 1998, *Structure*, 6:451). Thus, the crystal structure of
 Zif268 suggested that zinc finger DNA-binding domains might function in a modular
 30 manner with a one-to-one interaction between a zinc finger and a three-base-pair
 “subsite” in the DNA sequence. In naturally occurring zinc finger transcription
 factors, multiple zinc fingers are typically linked together in a tandem array to achieve

sequence-specific recognition of a contiguous DNA sequence (Klug, 1993, *Gene* 135:83).

Multiple studies have shown that it is possible to artificially engineer the DNA binding characteristics of individual zinc fingers by randomizing the amino acids at the alpha-helical positions involved in DNA binding and using selection methodologies such as phage display to identify desired variants capable of binding to DNA target sites of interest (Rebar et al., 1994, *Science*, 263:671; Choo et al., 1994 *Proc. Natl. Acad. Sci. USA*, 91:11163; Jamieson et al., 1994, *Biochemistry* 33:5689; Wu et al., 1995 *Proc. Natl. Acad. Sci. USA*, 92: 344). Such recombinant zinc finger proteins can be fused to functional domains, such as transcriptional activators, transcriptional repressors, methylation domains, and nucleases to regulate gene expression, alter DNA methylation, and introduce targeted alterations into genomes of model organisms, plants, and human cells (Carroll, 2008, *Gene Ther.*, 15:1463-68; Cathomen, 2008, *Mol. Ther.*, 16:1200-07; Wu et al., 2007, *Cell. Mol. Life Sci.*, 64:2933-44).

One existing method for engineering zinc finger arrays, known as “modular assembly,” advocates the simple joining together of pre-selected zinc finger modules into arrays (Segal et al., 2003, *Biochemistry*, 42:2137-48; Beerli et al., 2002, *Nat. Biotechnol.*, 20:135-141; Mandell et al., 2006, *Nucleic Acids Res.*, 34:W516-523; Carroll et al., 2006, *Nat. Protoc.* 1:1329-41; Liu et al., 2002, *J. Biol. Chem.*, 277:3850-56; Bae et al., 2003, *Nat. Biotechnol.*, 21:275-280; Wright et al., 2006, *Nat. Protoc.*, 1:1637-52). Although straightforward enough to be practiced by any researcher, recent reports have demonstrated a high failure rate for this method, particularly in the context of zinc finger nucleases (Ramirez et al., 2008, *Nat. Methods*, 5:374-375; Kim et al., 2009, *Genome Res.* 19:1279-88), a limitation that typically necessitates the construction and cell-based testing of very large numbers of zinc finger proteins for any given target gene (Kim et al., 2009, *Genome Res.* 19:1279-88).

Combinatorial selection-based methods that identify zinc finger arrays from randomized libraries have been shown to have higher success rates than modular assembly (Maeder et al., 2008, *Mol. Cell*, 31:294-301; Joung et al., 2010, *Nat. Methods*, 7:91-92; Isalan et al., 2001, *Nat. Biotechnol.*, 19:656-660). In preferred embodiments, the zinc finger arrays are described in, or are generated as described in,

WO 2011/017293 and WO 2004/099366. Additional suitable zinc finger DBDs are described in U.S. Pat. Nos. 6,511,808, 6,013,453, 6,007,988, and 6,503,717 and U.S. patent application 2002/0160940.

DNA

5 The methods described herein can be applied to any double stranded DNA, e.g., genomic DNA isolated from any cell, artificially created populations of DNAs, or any other DNA pools, as it is performed *in vitro*.

Sequencing

10 As used herein, “sequencing” includes any method of determining the sequence of a nucleic acid. Any method of sequencing can be used in the present methods, including chain terminator (Sanger) sequencing and dye terminator sequencing. In preferred embodiments, Next Generation Sequencing (NGS), a high-throughput sequencing technology that performs thousands or millions of sequencing reactions in parallel, is used. Although the different NGS platforms use varying assay
15 chemistries, they all generate sequence data from a large number of sequencing reactions run simultaneously on a large number of templates. Typically, the sequence data is collected using a scanner, and then assembled and analyzed bioinformatically. Thus, the sequencing reactions are performed, read, assembled, and analyzed in parallel; see, e.g., US 20140162897, as well as Voelkerding et al., *Clinical Chem.*, 55:
20 641-658, 2009; and MacLean et al., *Nature Rev. Microbiol.*, 7: 287-296 (2009). Some NGS methods require template amplification and some that do not. Amplification-requiring methods include pyrosequencing (see, e.g., U.S. Pat. Nos. 6,210,89 and 6,258,568; commercialized by Roche); the Solexa/Illumina platform (see, e.g., U.S. Pat. Nos. 6,833,246, 7,115,400, and 6,969,488); and the Supported
25 Oligonucleotide Ligation and Detection (SOLiD) platform (Applied Biosystems; see, e.g., U.S. Pat. Nos. 5,912,148 and 6,130,073). Methods that do not require amplification, e.g., single-molecule sequencing methods, include nanopore sequencing, HeliScope (U.S. Pat. Nos. 7,169,560; 7,282,337; 7,482,120; 7,501,245; 6,818,395; 6,911,345; and 7,501,245); real-time sequencing by synthesis (see, e.g.,
30 U.S. Pat. No. 7,329,492); single molecule real time (SMRT) DNA sequencing methods using zero-mode waveguides (ZMWs); and other methods, including those described in U.S. Pat. Nos. 7,170,050; 7,302,146; 7,313,308; and 7,476,503). See,

e.g., US 20130274147; US20140038831; Metzker, Nat Rev Genet 11(1): 31-46 (2010).

Alternatively, hybridization-based sequence methods or other high-throughput methods can also be used, e.g., microarray analysis, NANOSTRING, ILLUMINA, or
5 other sequencing platforms.

Kits

Also provided herein are kits for use in the methods described herein. The kits can include one or more of the following: 5' single-tailed hairpin adapters; 3' single-tailed hairpin adapters; reagents and/or enzymes for end repair and A tailing (e.g., T4
10 polymerase, Klenow fragment, T4 Polynucleotide Kinase (PNK), and/or Taq DNA Polymerase); exonuclease; uracil DNA glycosylase (UDG) and/or endonuclease VIII, a DNA glycosylase-lyase, e.g., the USER (Uracil-Specific Excision Reagent) Enzyme mixture (New England BioLabs); purified cas9 protein; guideRNA (e.g., control gRNA); gDNA template (e.g., control gDNA template); and instructions for use in a
15 method described herein.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Materials

20 The following materials were used in Examples 1-2.

Materials	Vendor	Model Number
HTP Library Preparation Kit	Kapa Biosystems	KK8235
Hifi HotStart ReadyMix, 100 x 25 μ L reactions	Kapa Biosystems	KK2602
Lambda exonuclease (5U/ μ l)	NEB	M0262L
E. Coli Exonuclease I (20U/ μ l)	NEB	M0293S
USER enzyme (1000U/ μ l)	NEB	M5505L
Cas9 enzyme (1000 nM)	NEB	M0386L
Ampure XP 60 ml	Agencourt	A63881

The following Primers were used in Examples 1-2.

TABLE A		
PrimerName	Sequence	SEQ ID NO:
oSQT1270 5'- Truseq-loop- adapter D501L	/5phos/gatcgggaagagc /ideoxyU/ aatgatacggcgaccaccgagatctacac TATAGCCT acactctttccctacacgacgctcttccgatac*t	1
oSQT1302 5'- Truseq-loop- adapter D502L	/5phos/gatcgggaagagc /ideoxyU/ aatgatacggcgaccaccgagatctacac ATAGAGGC acactctttccctacacgacgctcttccgatac*t	2
oSQT1303 5'- Truseq-loop- adapter D503L	/5phos/gatcgggaagagc /ideoxyU/ aatgatacggcgaccaccgagatctacac CCTATCCT acactctttccctacacgacgctcttccgatac*t	3
oSQT1304 5'- Truseq-loop- adapter D504L	/5phos/gatcgggaagagc /ideoxyU/ aatgatacggcgaccaccgagatctacac GGCTCTGA acactctttccctacacgacgctcttccgatac*t	4
oSQT1305 5'- Truseq-loop- adapter D505L	/5phos/gatcgggaagagc /ideoxyU/ aatgatacggcgaccaccgagatctacac AGGCGAAG acactctttccctacacgacgctcttccgatac*t	5
oSQT1315 5'- Truseq-loop- adapter D506L	/5phos/gatcgggaagagc /ideoxyU/ aatgatacggcgaccaccgagatctacac TAATCTTA acactctttccctacacgacgctcttccgatac*t	6
oSQT1316 5'- Truseq-loop- adapter D507L	/5phos/gatcgggaagagc /ideoxyU/ aatgatacggcgaccaccgagatctacac CAGGACGT acactctttccctacacgacgctcttccgatac*t	7
oSQT1317 5'- Truseq-loop- adapter D508L	/5phos/gatcgggaagagc /ideoxyU/ aatgatacggcgaccaccgagatctacac GACTTGAC acactctttccctacacgacgctcttccgatac*t	8
oSQT1271 3'- Truseq-loop- adapter D701L	/5phos/gatcgggaagagcacacgtctgaactccagtcac ATTACTCG atctcgtatgccgtcttctgcttg /ideoxyU/ gctcttccgatac*t	9
oSQT1306 3'- Truseq-loop- adapter D702L	/5phos/gatcgggaagagcacacgtctgaactccagtcac TCCGGAGA atctcgtatgccgtcttctgcttg /ideoxyU/ gctcttccgatac*t	10
oSQT1307 3'- Truseq-loop- adapter D703L	/5phos/gatcgggaagagcacacgtctgaactccagtcac CGCTCATT atctcgtatgccgtcttctgcttg /ideoxyU/ gctcttccgatac*t	11
oSQT1308 3'- Truseq-loop- adapter D704L	/5phos/gatcgggaagagcacacgtctgaactccagtcac GAGATTCC atctcgtatgccgtcttctgcttg /ideoxyU/ gctcttccgatac*t	12
oSQT1309 3'- Truseq-loop- adapter D705L	/5phos/gatcgggaagagcacacgtctgaactccagtcac ATTCAGAA atctcgtatgccgtcttctgcttg /ideoxyU/ gctcttccgatac*t	13
oSQT1318 3'- Truseq-loop- adapter D706L	/5phos/gatcgggaagagcacacgtctgaactccagtcac GAATTCGT atctcgtatgccgtcttctgcttg /ideoxyU/ gctcttccgatac*t	14
oSQT1319 3'- Truseq-loop- adapter D707L	/5phos/gatcgggaagagcacacgtctgaactccagtcac CTGAAGCT atctcgtatgccgtcttctgcttg /ideoxyU/ gctcttccgatac*t	15
oSQT1320 3'- Truseq-loop- adapter D708L	/5phos/gatcgggaagagcacacgtctgaactccagtcac TAATGCGC atctcgtatgccgtcttctgcttg /ideoxyU/ gctcttccgatac*t	16
oSQT1274 Truseq F1	AATGATACGGCGACCACCGAG	17
oSQT1275 Truseq R1	CAAGCAGAAGACGGCATACGAGAT	18

The sequences in CAPS are dual-index barcodes (for demultiplexing samples) in the Illumina Truseq library preparation system. The * represents a phosphorothioate linkage.

Example 1. Optimization of Exemplary FIND-Seq Methodology

5 Described herein is the development of an *in vitro* method that enables comprehensive determination of DSBs induced by nucleases on any genomic DNA of interest. This method enables enrichment of nuclease-induced DSBs over random DSBs induced by shearing of genomic DNA. An overview of how the method works can be found in **Figures 1A and 1B**. In brief, genomic DNA from a cell type or
10 organism of interest is randomly sheared to a defined average length. In the present experiments, an average length of 500 bps works well for subsequent next-generation sequencing steps. The random broken ends of this genomic DNA are end repaired and then A-tailed. This then enables the ligation of a hairpin adapter bearing a 5' end next-generation sequencing primer site that also contains a single deoxyuridine nucleotide
15 at a specific position (**Figure 1B**); this adapter is also referred to herein as the "5' single-tailed hairpin adapter". Following ligation of this adapter, the sample is then treated with one or more exonucleases (e.g., bacteriophage lambda exonuclease, E. coli ExoI, PlasmidSafe ATP-dependent exonuclease), which degrades any DNA molecules that have not had the 5' single-tailed hairpin adapter ligation to both of
20 their ends. The collection of molecules is then treated with Cas9 nuclease complexed with a specific guide RNA (gRNA). The resulting nuclease-induced blunt ends are A-tailed and then a second hairpin adapter bearing a 3' end next-generation sequencing primer site and that also contains a single deoxyuridine nucleotide at a specific position is ligated to these ends; this adapter is also referred to herein as the "3'
25 single-tailed hairpin adapter". As a result of these treatments, the only DNA fragments that should have a 5' and a 3' single-tailed hairpin adapter ligated to their ends are those that were cleaved by the nuclease. The adapters can be added in any order. Following treatment by the USER enzyme mixture, which nicks DNA wherever a deoxyuridine is present, only those fragments bearing the two types of
30 adapters can be sequenced.

Following paired end next-generation sequencing, the resulting reads can be mapped back to the genome. Sites of nuclease cleavage will have multiple bi-direction reads originating at the site of the nuclease-induced DSB, typically within a nucleotide of the cut site (**Figures 2A and 2B**). As nuclease-induced cleavage

produces 'uniform' ends, in contrast to the staggered ends that results from random physical shearing of DNA, these sites can be simply bioinformatically identified by their signature uniform read alignments.

Using an initial non-optimized version of this approach, off-target sites induced by four different gRNAs and Cas9 nuclease were successfully identified. The target sites are listed in Table 1.

Table 1. List of sgRNA target sites tested with *in vitro* cleavage selection assay.

Target site name	Cells	Target Site (5' → 3')	SEQ ID NO:
VEGFA site1	U2OS	GGGTGGGGGGAGTTTGCTCCNGG	19
VEGFA site2	U2OS	GACCCCTCCACCCCGCCTCNGG	20
VEGFA site3	U2OS	GGTGAGTGAGTGTGTGCGTGNGG	21
EMX1	U2OS	GAGTCCGAGCAGAAGAAGAANGG	22

The results of table 2 were encouraging for this assay, as the majority of GUIDE-seq detected sites were also detected by this method. In the first trial, 58-77% of sites overlapped between methods using 2.5-3M reads. ~20,000X enrichment was estimated for cleaved sites.

Table 2. List of sgRNA target sites tested with *in vitro* cleavage selection assay.

site	cell-based GUIDE-seq total	in vitro total	both	percentage of cell-based GUIDE-seq sites detected
EMX1	16	19	10	63%
VEGFA site 1	22	41	17	77%
VEGFA site 2	152	194	98	64%
VEGFA site 3	60	129	35	58%

A critical parameter for optimization of the present method was the efficiency of degradation of linear DNA fragments following the ligation of the first 5' single-tailed hairpin adapter. Indeed, without wishing to be bound by theory, most of the "background" reads mapped throughout the genome may be due to incomplete digestion of unligated DNA at this step, thereby leaving ends to which the second 3' single tailed hairpin adapter can be ligated.

Therefore, the methods were further optimized by testing various kinds and numbers of exonucleases and lengths of incubation to find an optimal treatment method. As seen in Figures 3A-3D, three treatments consistently yielded the highest

number of reads and sites and also found 100% of the off-target sites identified by matched GUIDE-seq experiments with the same gRNAs: 1 hour PlasmidSafe treatment, two serial 1 hour PlasmidSafe treatments, and 1 hour of Lambda exonuclease treatment followed by 1 hour of PlasmidSafe treatment.

5 Using an optimized exonuclease treatment, the methods were again performed using CRISPR-Cas9 nuclease and a gRNA targeted to VEGFA site 1. This experiment yielded a larger number of off-target sites. Combining the various experiments performed with the VEGFA site 1 gRNA yields a larger number of off-target sites. GUIDE-seq had previously been performed using this same VEGFA site
10 1 gRNA and a range of genome-wide off-target sites identified. Importantly, the present in vitro method performed with the optimized exonuclease treatment found ALL of the off-target sites previously identified by GUIDE-seq for the gRNA tested and a very large number of additional, previously unknown off-target sites as well (Figure 4). This result demonstrates that these new methods are as sensitive as
15 GUIDE-seq but offers the ability to identify sites that are not found by that earlier method. Reasons for this might include greater sensitivity of this in vitro method, negative biological selection against certain off-target mutations when practicing GUIDE-seq in cells, and/or negative effects of chromatin, DNA methylation, and gene expression on the activity of nucleases in cells.

20 **Example 2. Exemplary FIND-Seq protocol**

Selection of Cleaved Amplicons for Next-generation Sequencing (SCANS)
in vitro assay for finding cleavage sites of CRISPR/Cas9 nuclease from complex genomic DNA mixtures

1. End Repair (Red)

Component	1 rxn (ul)	12
Water	8	115
10X Kapa End Repair Buffer	7	101
Kapa End Repair Enzyme Mix	5	72
Total master mix volume	20	288
Input DNA (0.1 - 5 ug)	50	
Total reaction volume	70	

Incubate 30 minutes at 20C.

1.7X SPRI cleanup using 120 ul Ampure XP beads.

2. A-tailing (Blue)

Component	1 rxn (ul)	12
10X Kapa A-Tailing Buffer	5	72
Kapa A-Tailing Enzyme	3	43
Total master mix volume	8	115
TE (0.1 mM EDTA)	42	
End repaired DNA with beads	0	
Total reaction volume	50	

Incubate for 30 min at 30C.

Cleanup by adding 90 ul of SPRI solution.

3. 5' Adapter Ligation (Yellow)

Component	1 rxn (ul)	12
5X Kapa Ligation Buffer	10	144
Kapa T4 DNA Ligase	5	72
Total master mix volume	15	216
A-tailed DNA with beads	0	
TE (0.1 mM EDTA)	30	
5' Truseq Loop Adapter D50X (40 uM)	5	
Total reaction volume	50	

Incubate for 1 hour at 20C.

Cleanup by adding 50 ul of SPRI solution.

Proceed with 1 ug of 5' Adapter-ligated DNA into next step.

4. Exonuclease treatment

Component	1 rxn (ul)	12
10X ExoI buffer	5	72
Lambda exonuclease (5 U/ul)	4	58
E. Coli Exonuclease I (20 U/ul)	1	14
Total master mix volume	10	144
Adapter-ligated DNA with beads	0	
TE (0.1 mM EDTA)	40	
Total reaction volume	50	

Incubate for 1 hour at 37C.

Cleanup by adding 50 ul of 1X Ampure XP beads.

5. Cleavage by Cas9

Component	1 rxn (ul)	5
Water	63	378

10X Cas9 buffer	10	60
Cas9 (1 uM -> 900 nM final)	9	54
sgRNA (300 nM, ~100 ng/ul)	3	18
Total reaction volume	85	510

Incubate at room temperature for 10 minutes.

DNA (~400 bp, 250 ng)	15	
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Incubate for 1 hour at 37C.

Purify with 1X SPRI bead cleanup (100 ul).

6. A-tailing

Component	1 rxn (ul)	5
10X Kapa A-Tailing Buffer	5	30
Kapa A-Tailing Enzyme	3	18
Total master mix volume	8	48
TE (0.1 mM EDTA)	42	
End repaired DNA with beads	0	
Total reaction volume	50	

Incubate for 30 min at 30C.

Cleanup by adding 90 ul of SPRI solution.

7. 3' Adapter Ligation

Component	1 rxn (ul)	5
5X Kapa Ligation Buffer	10	60
Kapa T4 DNA Ligase	5	30
Total master mix volume	15	90
A-tailed DNA with beads	0	
TE (0.1 mM EDTA)	30	
3' Truseq Loop Adapter D70X (40 uM)	5	
Total reaction volume	50	

Incubate for 30 minutes at 20C.

Cleanup by adding 50 ul of SPRI solution.

8. USER enzyme treatment

Add 3 ul of USER enzyme and treat for 30 minutes at 37C.

(Treatment is in TE 10 mM Tris, 0.1 mM EDTA.)

Purify with 1X SPRI solution (50 ul).

9. PCR amplification

Amplify with Kapa Hifi manufacturer's protocol and primers oSQT1274/1275.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of
5 the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of preparing a library of covalently closed DNA fragments, the method comprising:
providing DNA, preferably genomic DNA (gDNA) from a cell type or organism of interest;
optionally randomly shearing the DNA to a defined average length, preferably an average length of about 200-1000 base pairs (bps), to provide a population of DNA fragments;
preparing the fragments for end-ligation, preferably by end-repairing and then A-tailing the sheared DNA;
ligating to the ends of the fragments a first hairpin adapter comprising at least a single deoxyuridine and a first primer site to prepare a population of ligated fragments;
contacting the ligated fragments with a nuclease that induces double stranded breaks; and
purifying the ligated fragments using an exonuclease, thereby preparing a library of covalently closed DNA fragments.
2. The method of claim 1, wherein the first primer site is compatible for use in PCR priming and/or sequencing, preferably next generation sequencing (NGS).
3. The method of claim 1, further comprising contacting the library of covalently closed DNA fragments with a nuclease to induce double stranded breaks, preferably including site-specific cleavage;
ligating a second hairpin adapter comprising at least a single deoxyuridine and a second primer site compatible for use with the first primer site in PCR priming and/or sequencing,
contacting the library with an enzyme to nick the DNA at the deoxyuridine; and
sequencing those fragments having a first and second hairpin adapter.
4. The method of claim 3, wherein the enzyme that nicks the DNA at the deoxyuridine is a uracil DNA glycosylase (UDG) or endonuclease VIII.

5. A method of preparing a library of fragments comprising nuclease-induced double stranded breaks in DNA, the method comprising:
providing DNA;
randomly shearing the DNA to a defined average length, preferably an average length of about 200-1000 base pairs (bps);
optionally end-repairing and then A-tailing the sheared DNA;
ligating to the DNA a first hairpin adapter, preferably comprising a first region, of about 10-20, nucleotides; a second region of about 45-65 nucleotides that forms one or more hairpin loops and comprises a first primer site compatible for use in PCR priming and/or sequencing; and a third region of about 10-20 nucleotides that is complementary to the first region, with a single deoxyuridine nucleotide between the first and second regions;
contacting the sample with one or more exonucleases, sufficient to degrade any DNA molecules that lack the first hairpin adapter ligated to both of their ends;
treating the sample with a nuclease to induce site-specific cleavage;
optionally end-repairing and then A-tailing the resulting ends;
ligating a second hairpin adapter comprising a first region of about 10-20 nucleotides; a second region of about 40-60 nucleotides that forms one or more hairpin loops and comprises a second primer compatible for use with the first primer site in PCR priming and/or sequencing; and a third region of about 10-20 nucleotides that is complementary to the first region and that also contains a single deoxyuridine nucleotide between the second and third regions, to create a population wherein the DNA fragments that were cleaved by the nuclease have a first and second hairpin adapter ligated to their respective ends; thereby preparing a library of fragments wherein one end was created by a nuclease-induced double stranded break in the DNA.
6. The method of claim 5, further comprising:
contacting the library with uracil DNA glycosylase (UDG) and/or endonuclease VIII to nick the DNA at the deoxyuridine; and
sequencing those fragments bearing a first and a second hairpin adapter.

7. A method for detecting nuclease-induced double stranded breaks (DSBs) in DNA, the method comprising:
 - providing DNA;
 - randomly shearing the DNA to a defined average length;
 - optionally end-repairing and then A-tailing the sheared DNA;
 - ligating a first hairpin adapter, preferably comprising a first region of about 10-20 nucleotides; a second region of about 45-65 nucleotides that forms one or more hairpin loops and comprises a first primer site compatible for use in PCR priming and/or sequencing; and a third region of about 10-20 nucleotides that is complementary to the first region, with a single deoxyuridine nucleotide between the first and second regions;
 - contacting the sample with one or more exonucleases, sufficient to degrade any DNA molecules that lack the first hairpin adapter ligated to both of their ends;
 - treating the sample with a nuclease to induce double stranded breaks, preferably including site-specific breaks, of the DNA;
 - optionally end-repairing and then A-tailing the resulting cleaved ends;
 - ligating a second hairpin adapter comprising a first region of about 10-20 nucleotides; a second region of about 40-60 nucleotides that forms one or more hairpin loops and comprises a second primer compatible for use with the first primer site in PCR priming and/or sequencing; and a third region of about 10-20 nucleotides that is complementary to the first region, and that also contains a single deoxyuridine nucleotide between the second and third regions, to create a population wherein the DNA fragments that were cleaved by the nuclease have the first and second hairpin adapters ligated to their respective ends; thereby preparing a library of fragments comprising nuclease-induced double stranded breaks in the DNA, wherein one end was created by a nuclease-induced double stranded break in the DNA;
 - contacting the library with uracil DNA glycosylase (UDG) and/or endonuclease VIII, a DNA glycosylase-lyase to nick the DNA at the deoxyuridine; and
 - sequencing those fragments bearing a first and a second hairpin adapter; thereby detecting DSBs induced by the nuclease.
8. A method of determining which of a plurality of guide RNAs is most specific, the method comprising, for each of the plurality of guide RNAs:

providing DNA;
randomly shearing the DNA to a defined average length;
end-repairing and then A-tailing the sheared DNA;
ligating a first hairpin adapter, preferably comprising a first region of about 10-20 nucleotides; a second region of about 45-65 nucleotides, that forms one or more hairpin loops and comprises a first primer site compatible for use in PCR priming and/or sequencing; and a third region of about 10-20 nucleotides that is complementary to the first region, with a single deoxyuridine nucleotide between the first and second regions;
contacting the sample with one or more exonucleases, sufficient to degrade any DNA molecules that lack the first adapter ligated to both of their ends;
treating the sample with a Cas9 nuclease compatible with the guide RNA to induce double stranded breaks, preferably including site-specific double stranded breaks, of the DNA;
optionally end-repairing and then A-tailing the resulting cleaved ends;
ligating a second hairpin adapter comprising a first region of about 10-20, nucleotides; a second region of about 40-60 nucleotides that forms one or more hairpin loops and comprises a second primer compatible for use with the first primer site in PCR priming and/or sequencing; and a third region of about 10-20 nucleotides that is complementary to the first region, and that also contains a single deoxyuridine nucleotide between the second and third regions, to create a population wherein the DNA fragments that have a first and second hairpin adapter ligated to their ends are those that were cleaved by the nuclease; thereby preparing a library of fragments comprising nuclease-induced double stranded breaks in DNA wherein one end was created by a nuclease-induced double stranded break in the DNA;
contacting the library with uracil DNA glycosylase (UDG) and/or endonuclease VIII to nick the DNA at the deoxyuridine; and
sequencing those fragments bearing a first and a second hairpin adapter, thereby detecting DSBs induced by the nuclease in each sample;
optionally identifying whether each DSB is on-target or off-target;
comparing the DSBs induced by the nuclease in each sample; and

determining which of the plurality of guide RNAs induced the fewest off-target DSBs.

9. The method of any of claims 1-8, wherein treating the sample with a nuclease to induce site-specific cleavage comprises contacting the sample with a Cas9 nuclease complexed with a specific guide RNA (gRNA).
10. The method of any of the preceding claims, wherein the DNA is genomic DNA isolated from a mammalian, plant, bacterial, or fungal cell.
11. The method of any of the preceding claims, wherein the DNA is synthetic.
12. The method of any of the preceding claims, wherein the nuclease that induces double stranded breaks is selected from the group consisting of meganucleases, MegaTALs, zinc-finger nucleases, transcription activator effector-like nucleases (TALEN), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas RNA-guided nucleases (CRISPR/Cas RGNs).
13. The method of claim 12, wherein the nuclease is a Cas9 nuclease, and the method also includes expressing in the cells a guide RNA that directs the Cas9 nuclease to a target sequence in the genome.
14. The method of claims 1-8, wherein the primer site in the first or second hairpin adapter comprises a next generation sequencing primer site, a randomized DNA barcode or unique molecular identifier (UMI).

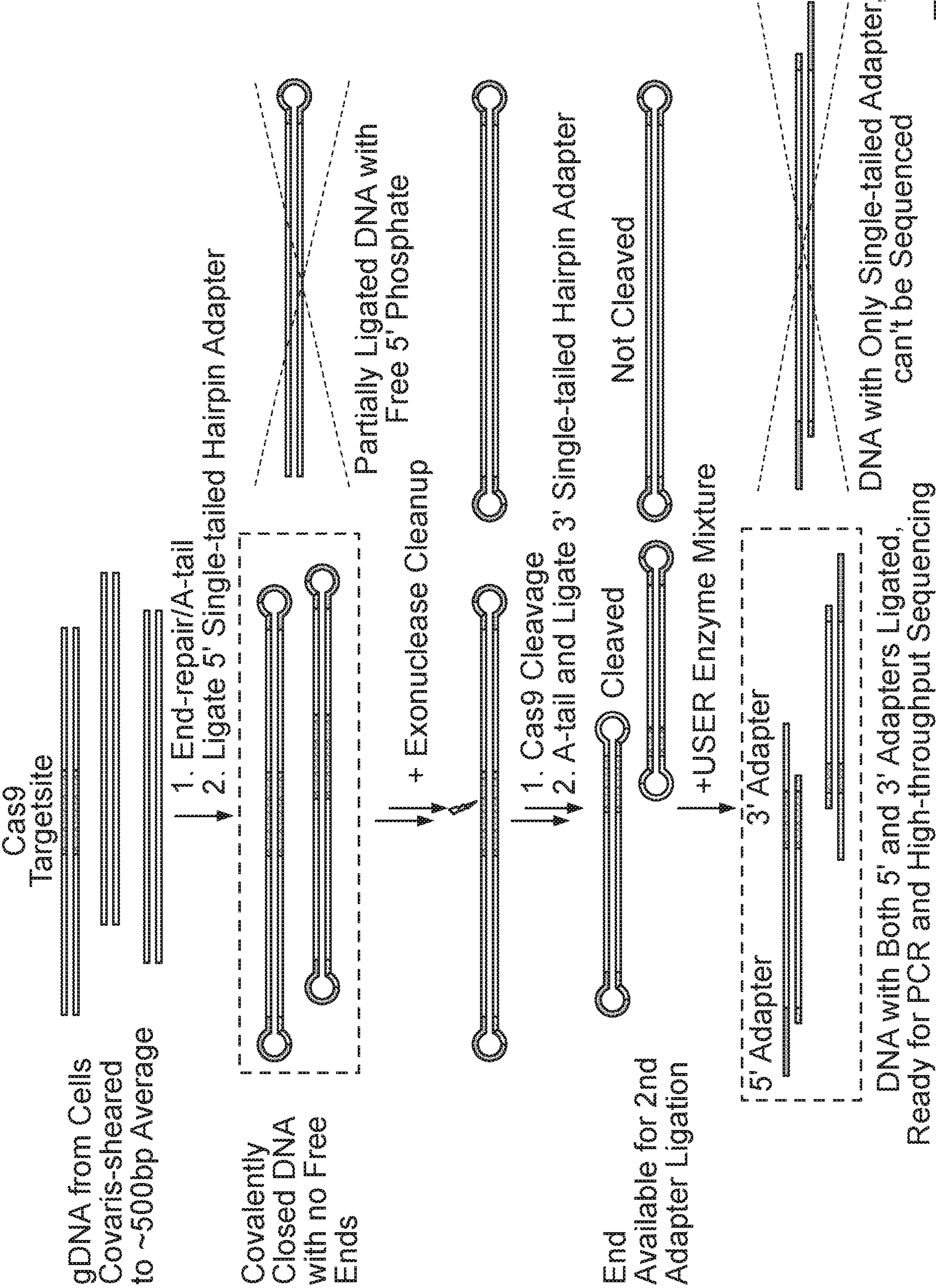
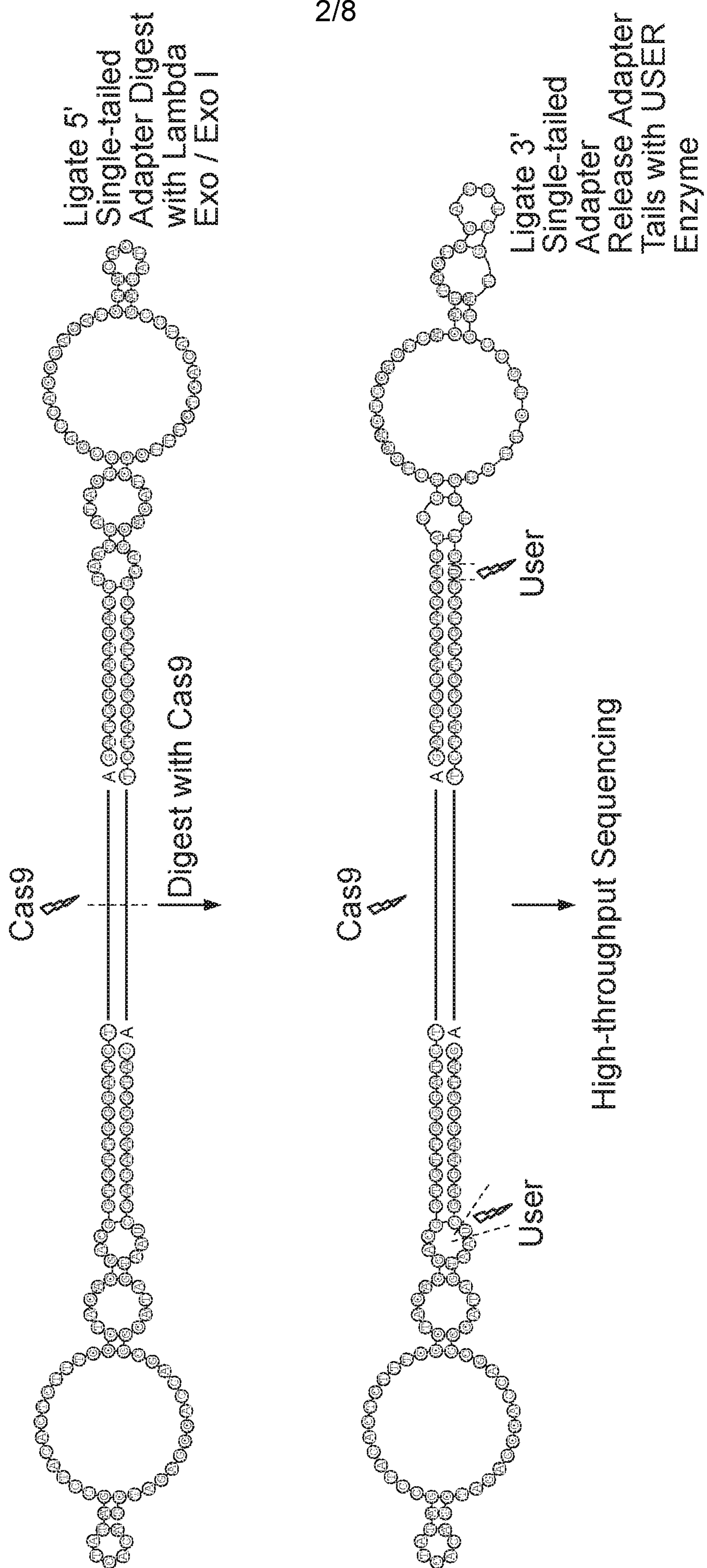


FIG. 1A



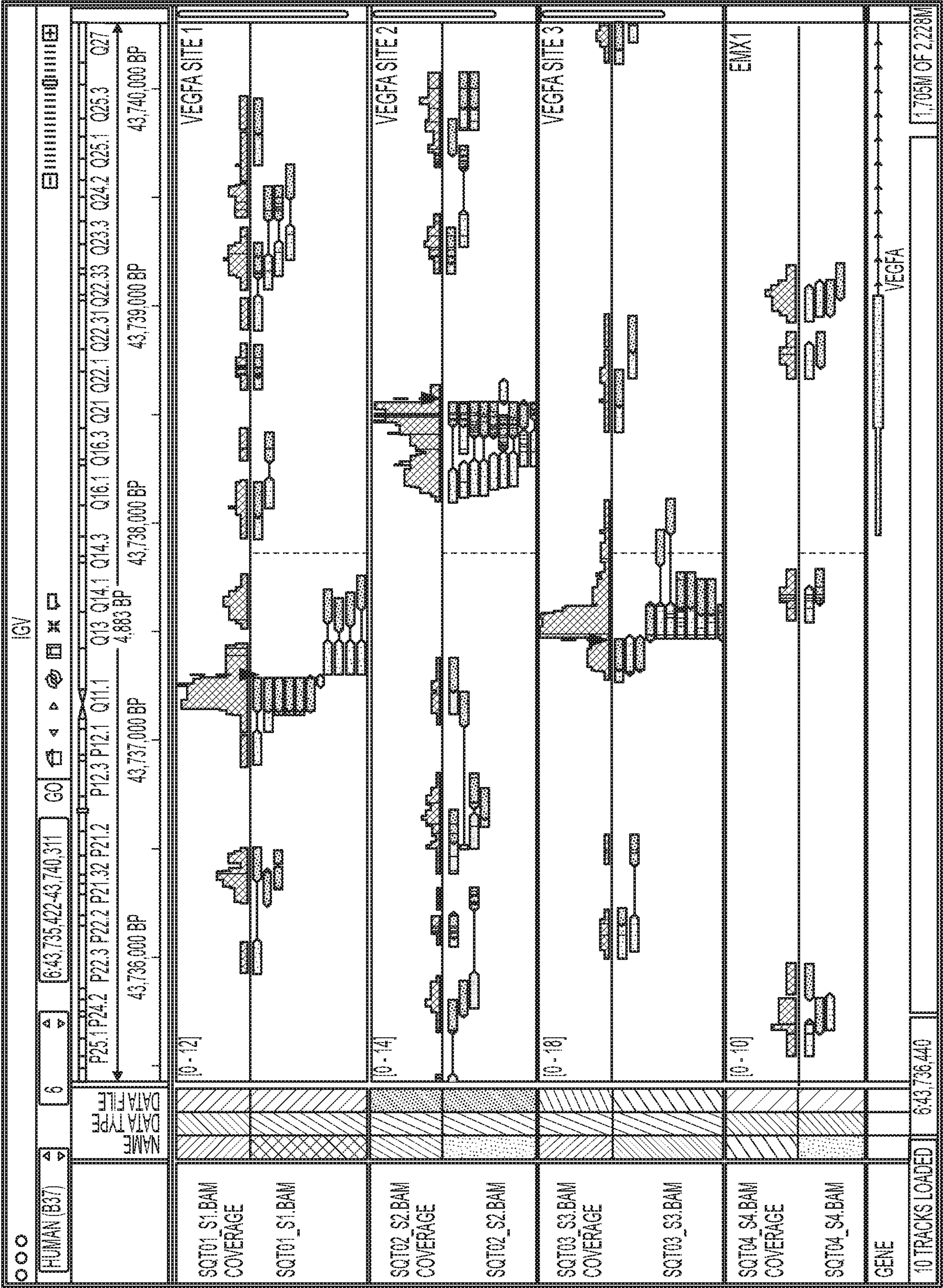


FIG. 2A

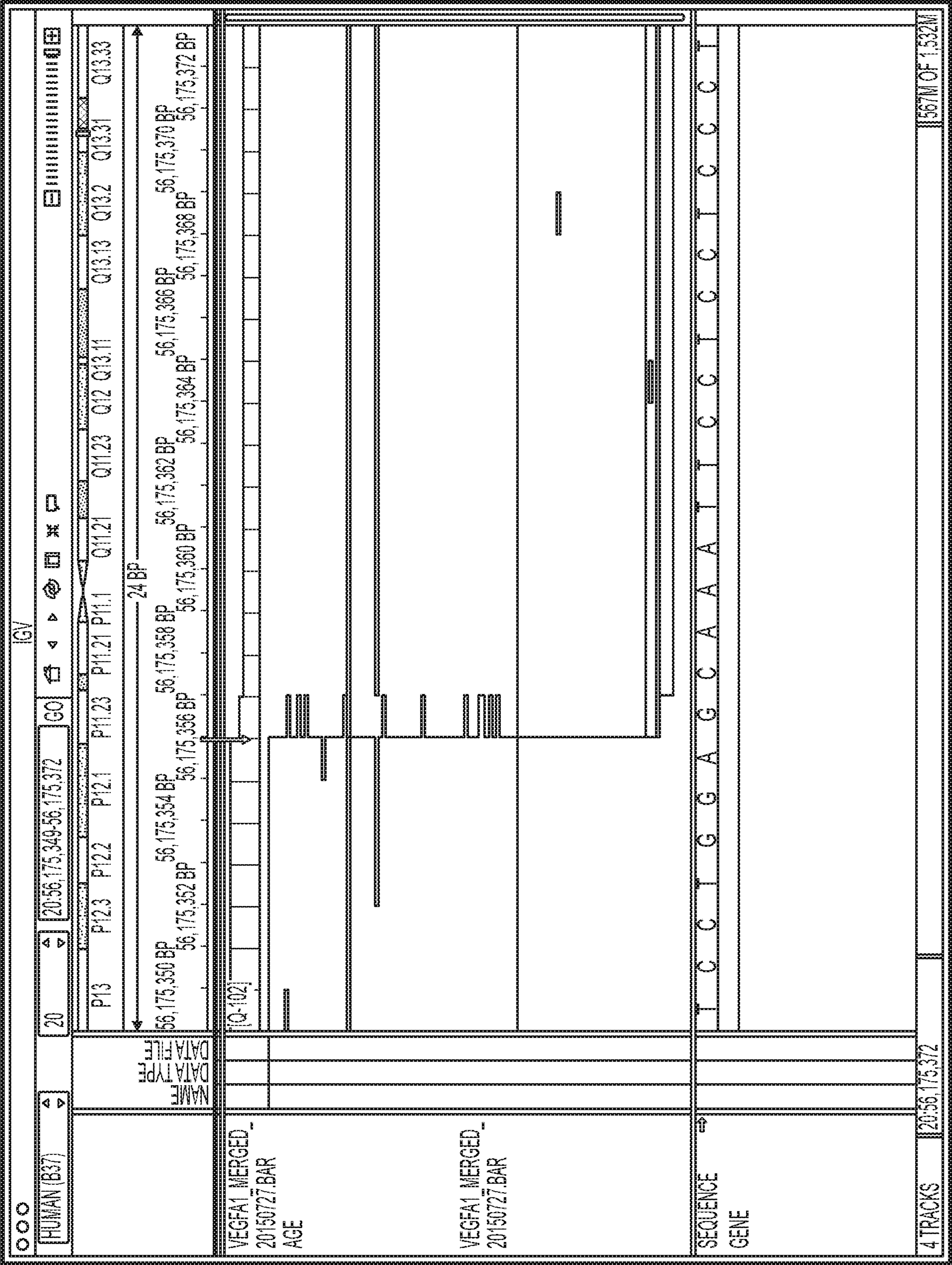


FIG. 2B

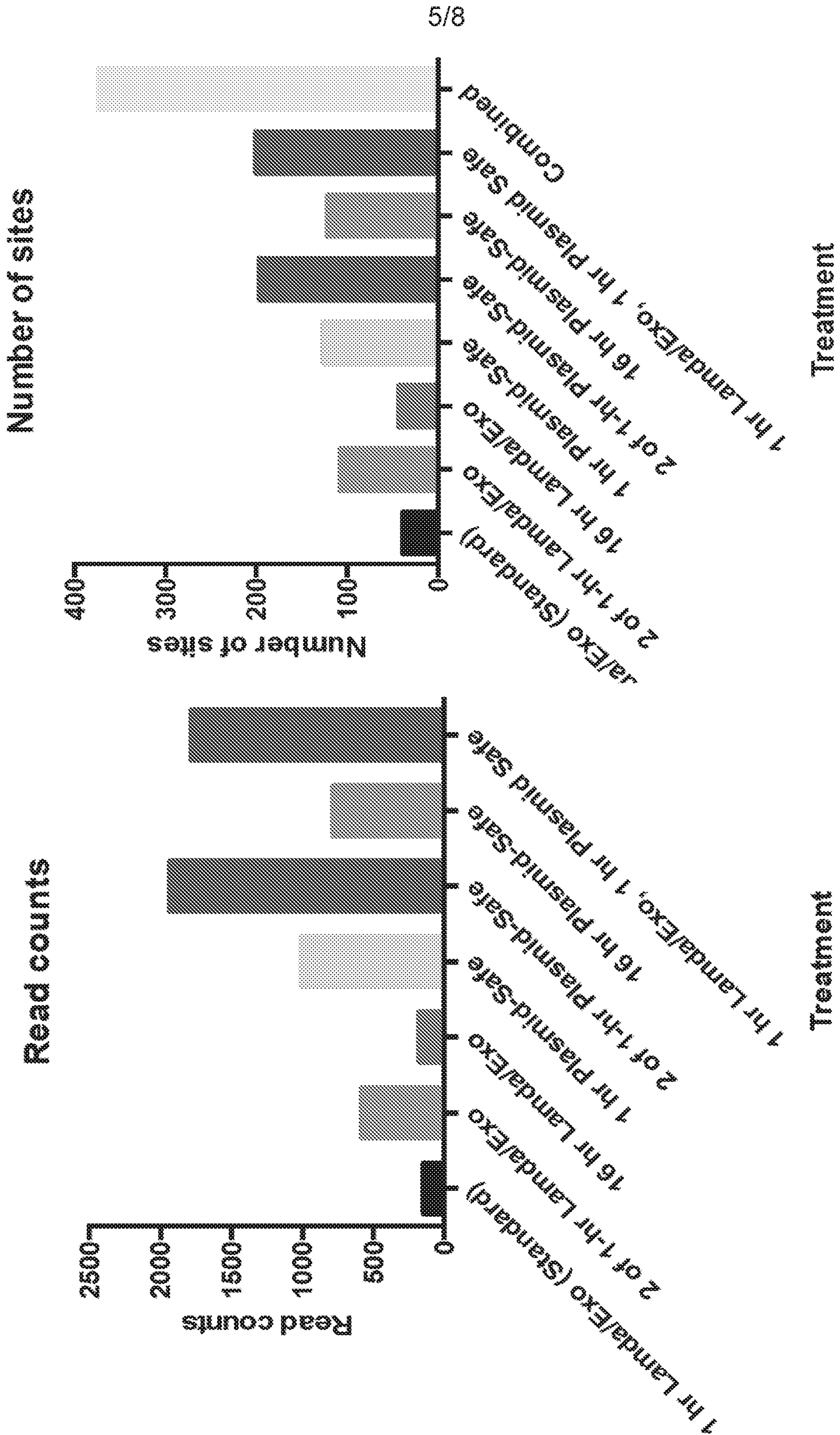


FIG. 3B

FIG. 3A

FIG. 3D

7/8

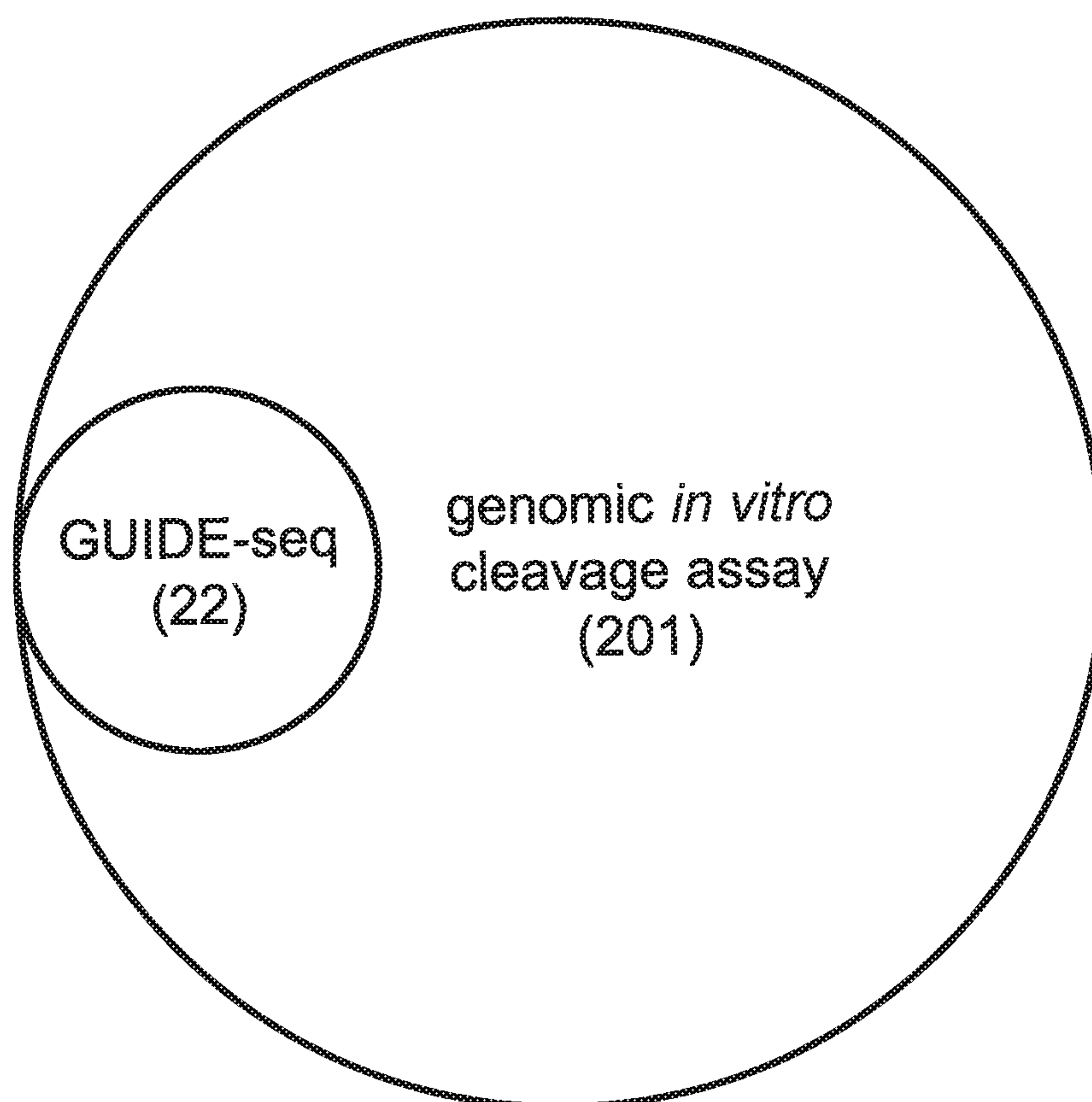
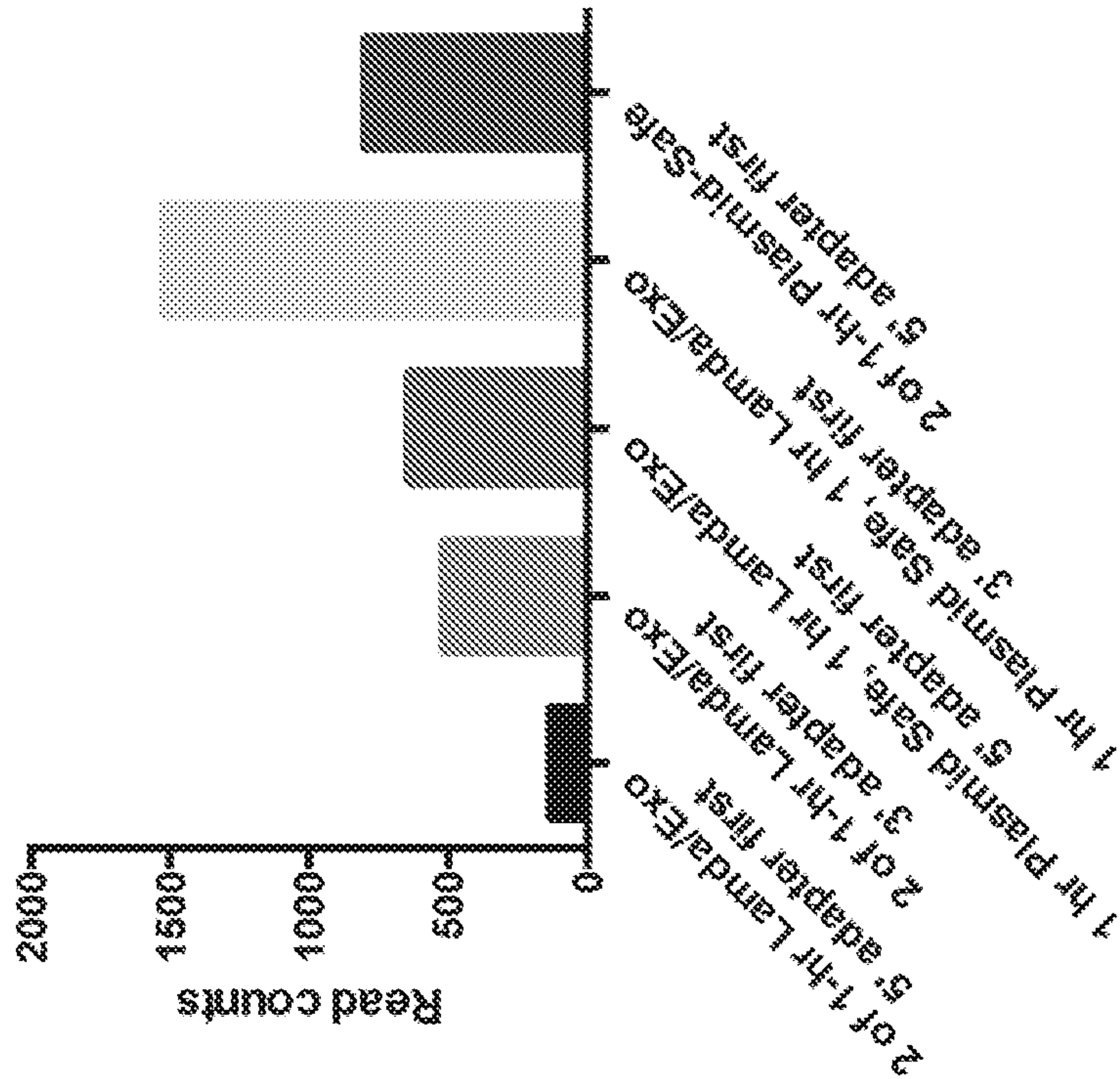


FIG. 4

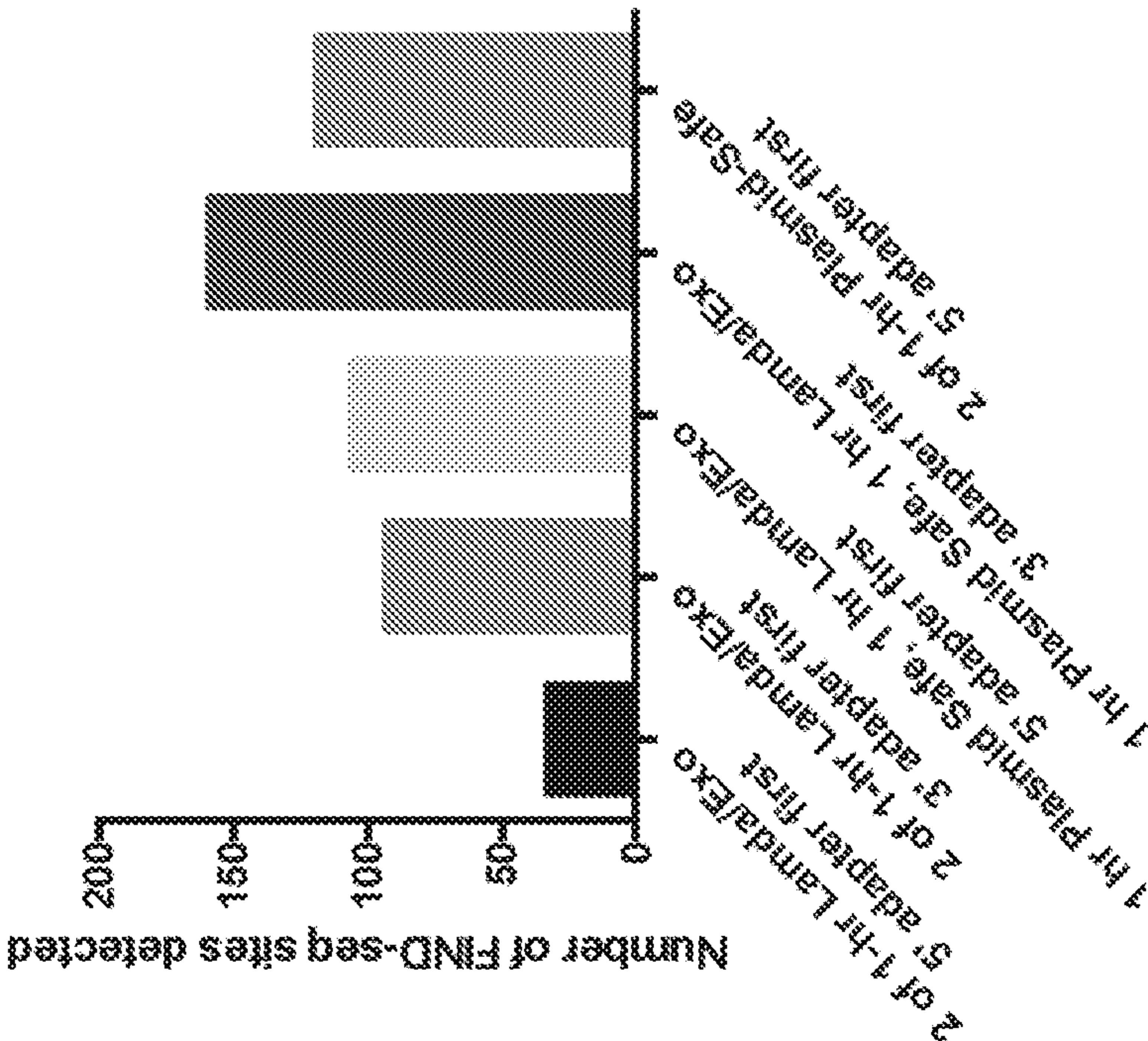
Test of adapter-ligation order at VEGFA site 1



Treatments

FIG. 5B

Test of adapter-ligation order at VEGFA site 1



Treatments

FIG. 5A

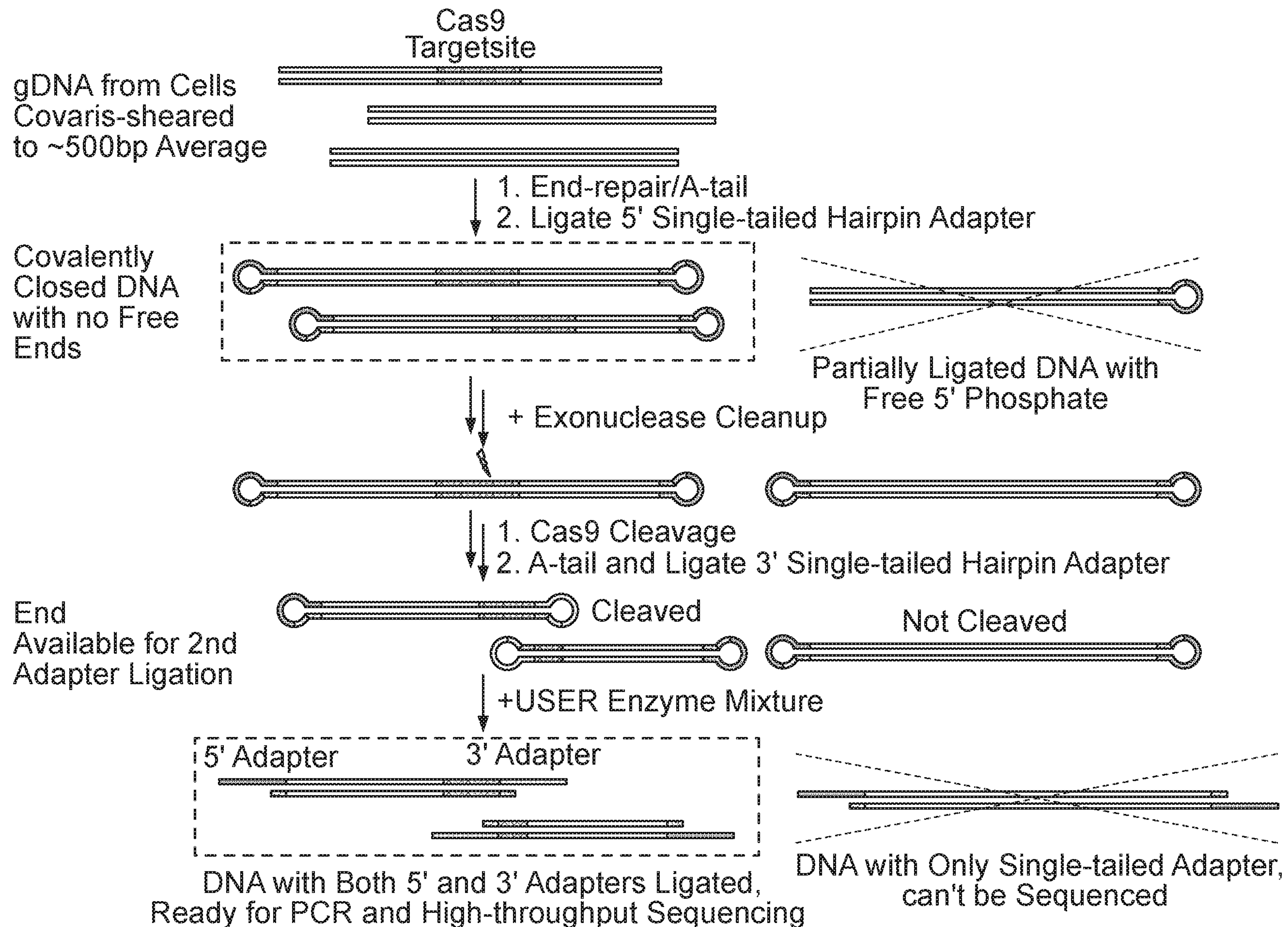


FIG. 1A