A Guide to Selecting the Right Gene Editing Off-Target Assay

Abstract

As CRISPR and other genome editing tools enter the clinic, the ability to accurately and reproducibly measure off-target edits becomes critical. In their 2024 guidance, the FDA recommends using multiple methods to measure off-target editing events, including genome-wide analysis.[1] Until the gene editing field adopts a set of scientific norms concerning off-target analysis, scientists and clinicians have the onerous task of choosing both the general approach (i.e. biased versus unbiased) and the specific assay they use to assess off-target effects. In this assay guide, we review different off-target analysis approaches - including ***in silco***, biochemical, cellular and ***in situ*** methods, and explore the pros and cons of specific assays like GUIDE-seq2[2], CHANGE-seq[3], UDiTaS[4]and DISCOVER-seq[5].

Introduction

Ensuring the safety and efficacy of gene-editing-based therapies depends on rigorous quality control (QC), including both on- and off-target analysis. QC assays are critical for building confidence that engineered cells will perform as intended without introducing unintended risks.

High-quality assays must strike a delicate balance between delivering relevance that reflects true cellular outcomes, assay sensitivity to capture rare editing events, and reproducibility to enable trust across experiments and laboratories. At the same time, the field demands solutions that align with the scale of next-generation sequencing (NGS) projects to support large cohort and therapeutic studies.

Evolving Off-Target Analysis to Align with Clinical Needs

In December of 2023, the FDA approved the first CRISPR-based therapy, exa-cel (exagamglogene autotemcel, CASGEVY™)[6], for the treatment of sickle cell disease. Investigation of exa-cel off-target activity relied on a commonly used approach in which ***in silico***-predicted sites of homology are amplified and sequenced to identify any non-specific cleavage. During the approval of exa-cel, FDA reviewers flagged two potential shortcomings in this approach[7]. First, they questioned whether the database that was used adequately reflected the genetics of people of African descent, a target population of exa-cel. Second was the concern that only 40 patients were tested.

Because of the importance of off-target effects and the limitation of database searches – often referred to as biased assays because they rely on *a priori* knowledge - genome-wide off-target studies, or unbiased assays, may be beneficial, especially during pre-clinical studies rather than waiting until clinical trials. Additional benefits are derived if studies such as these are conducted using cells similar to the target cells to improve physiological relevance.

Approaches to Identify CRISPR Off-Target Effects

A variety of assays have been published[8,9] to examine off-target activity, each having its own strengths and weaknesses, using one of four general approaches: ***in silico* tools** (biased method), or unbiased genome-wide experimental detection which can be conducted using either a **biochemical, cellular**, or ***in situ*** approach. Individual assays within each approach range in their level of sensitivity, throughput, and workflow complexity, with many of the unbiased protocols culminating in NGS.

Different approaches excel within the continuum of off-target evaluation:

- ***In silico*** – sgRNA design/prediction
- **Biochemical** – broad discovery
- **Cellular**– discovery or validation of biological relevance
- ***In situ*** –spatial mapping

Table 1 summarizes their strengths and weaknesses that can help inform decision making. The remainder of this article will review in more depth individual assays within the biochemical and cellular approaches.

Biochemical, NGS-based Off-Target Assays

Biochemical methods rely on *in vitro* assays that use isolated genomic DNA and engineered nucleases to directly map potential cleavage sites without requiring living cells. Assays, such as **Digenome-seq**[10], **CIRCLE-seq**[11], **CHANGE-seq**[3], and **SITE-seq**[12], expose genomic DNA to Cas nucleases under controlled conditions and then enrich and sequence the resulting double-strand breaks. Because they eliminate cellular influences like chromatin structure or repair pathways, biochemical assays are highly sensitive and can reveal a broader spectrum of potential off-target sites than cell-based methods. While they often overestimate editing activity compared to *in vivo* conditions, these

Approach	Assays/Tool	Input Material	Detection in…	Strengths	Limitations
In silico	Cas-OFFinder, CRISPOR, CCTop, MIT CRISPR tool	Genome sequence + computational models	Predicted sites (based on sequence similarity, PAM rules, models)	Fast, inexpensive, no lab work; useful for guide DNA design	Predictions only; no chromatin, repair, or nuclease activity captured
Biochemical	CIRCLE-seq, CHANGE-seq, SITE-seq, DIGENOME-seq	Purified genomic DNA	Naked DNA (no chromatin)	Ultra-sensitive; comprehensive; standardized	May overestimate cleavage; lacks biological context
Cellular	GUIDE-seq, DISCOVER-seq, UDiTaS	Living cells (edited)	Native chromatin + repair	Reflects true cellular activity; identifies biologically relevant edits	Requires efficient delivery; less sensitive; may miss rare sites
In situ	BLISS, BLESS, END-seq, GUIDE-tag	Fixed/permeabilized cells or nuclei	Chromatinized DNA in native location	Preserves genome architecture; captures breaks *in situ*	Technically complex; lower throughput; variable sensitivity

Table 1: Summary of off-target analysis approaches.

	DIGENOME-seq	CIRCLE-seq	CHANGE-seq	SITE-seq
General description	Treats purified genomic DNA with nuclease, then detects cleavage sites by whole-genome sequencing	Uses circularized genomic DNA and exonuclease digestion to enrich nuclease-induced breaks	Improved version of CIRCLE-seq with tagmentation-based library prep for higher sensitivity and reduced bias	Uses biotinylated Cas9 RNP to capture cleavage sites on genomic DNA, followed by sequencing
Sensitivity	Moderate; requires deep sequencing to detect off-targets	High sensitivity; lower sequencing depth needed compared to DIGENOME-seq	Very high sensitivity; can detect rare off-targets with reduced false negatives	High sensitivity; strong enrichment of true cleavage sites
Input DNA	Micrograms of purified genomic DNA	Nanogram amounts of purified genomic DNA	Nanogram amounts of purified genomic DNA	Microgram amounts of purified genomic DNA
Enrichment step	None (direct WGS of digested DNA)	Circularization of DNA → exonuclease removes linear DNA, enriching cleavage products	DNA circularization + tagmentation → efficient capture of nuclease cuts	Biotinylated Cas9 binds and pulls down cleaved DNA fragments
Reference	Kim *et al.*, Nat Methods 2015	Tsai *et al.*, Nat Methods 2017	Lazzarotto *et al.*, Nat Biotechnol 2020	Cameronet *al.*, Nat Methods 2017

Table 2: Summary of biochemical off-target assays. DIGENOME-seq - DIGested GENOME Sequencing; CIRCLE-seq - Circularization for In vitro Reporting of Cleavage Effects by Sequencing; CHANGE-seq - Circularization for High-throughput Analysis of Nuclease Genome-wide Effects by Sequencing; SITE-seq - Selective enrichment and Identification of Tagged genomic DNA Ends by Sequencing

techniques provide valuable first-line data for identifying off-target risks, prioritizing candidate sites for further validation, and improving nuclease design.

Cellular NGS-based Off-Target Assays

Cellular methods assess nuclease activity directly in living or fixed cells, capturing the influence of chromatin structure, DNA repair pathways, and cellular context on editing outcomes. Techniques such as **HTGTS**[13], **DISCOVER-seq**[5], **BLESS**[14], **UDiTaS**[4] and **GUIDE-seq**[15], rely on introducing specialized tags, sequencing adapters, or monitoring endogenous repair proteins to map double-strand breaks as they occur in cells. These assays provide biologically relevant insights by identifying which off-target sites are edited under physiological conditions. While they may have lower sensitivity than biochemical assays and often require efficient delivery of both nuclease and detection reagents, cellular methods are essential for validating the clinical relevance of off-target effects and are particularly valuable in therapeutic development.

Transforming Off-Target Assays to the Population-Scale

The biochemical and cellular assays reviewed thus far culminate with NGS read outs. Pre-clinical and translational studies typically examine large sample sets. NGS throughput can become burdensome.   Scalability of sequencing pipelines, particularly during NGS library preparation, is an area of concern, often causing a bottleneck. Simple library

	HTGTS	DISCOVER-seq	BLESS	UDiTaS	GUIDE-seq
General description	Captures translocations from programmed DSBs to map nuclease activity	Recruitment of DNA repair protein MRE11 to cleavage sites by ChIP-seq	Labels DSB ends in situ with biotin linkers	Amplicon-based NGS assay to quantify indels, translocations, and vector integration at target loci	Incorporates a double-stranded oligonucleotide at DSBs, followed by sequencing
Input DNA	Cellular DNA after nuclease expression	Cellular DNA; ChIP-seq of MRE11 binding	Fixed/permeabilized cells; in situ DNA labeling	Genomic DNA from edited cells (amplicon sequencing)	Cellular DNA from edited, tagged cells
Sensitivity	Moderate; dependent on translocation frequency	High; captures real nuclease activity genome-wide	Moderate; detects DSBs but limited by labeling efficiency	High for indels and rearrangements at targeted loci	High sensitivity for off-target DSB detection
Detects Translocations	Yes	No	No	Yes	No
Detects Indels	No	No	No	Yes	Yes
Reference	Frock *et al.*, Nat Biotechnol 2015	Wienert *et al.*, Science 2019	Crosetto *et al.*, Nat Methods 2013	Giannoukos *et al.*, BMC Genomics 2018	Tsai *et al.*, Nat Biotechnol 2015

Table 3: Summary of cellular off-target assays. HTGTS – High Throughput Genome-wide Translocation Sequencing; BLESS – Breaks Labeling, Enrichment on Streptavidin and Sequencing; UDiTaS – Uni-Directional Targeted Sequencing; GUIDE-seq - Genome-wide, Unbiased

prep workflows, such as seqWell **tagmentation**, can significantly streamline library prep, improve lab productivity, and allow cost reductions without sacrificing data quality.

Case Study: Evolution of CIRCLE-seq to CHANGE-seq

CIRCLE-seq maps CRISPR off-target activity by circularizing genomic DNA, exposing it to Cas nucleases, and enriching the cleavage products via exonuclease digestion prior to sequencing. This genome-wide assay was first published by Tsai *et al.* in 2017 in Nature Methods11 and has the benefits of low DNA input requirements and a high level of sensitivity. This assay, however, uses traditional NGS library preparation based on enzymatic fragmentation and adapter ligation that is not easily scaled to accommodate the required throughput. To overcome this and other assay limitations, CHANGE-seq was developed in 2020 by Lazzarotto *et al.* and reported in Nat Biotechnol3. Both CIRCLE-seq and CHANGE-seq assays rely on circular DNA enrichment of nuclease cleavage events; however, CHANGE-seq's adoption of tagmentation-based library prep greatly improves the ease of automation and overall throughput, while maintaining the high sensitivity and low DNA input aspects of CIRCLE-seq. CHANGE-seq has now become a well-known assay commonly used for biochemical off-target analysis.

Taking Tagmentation to the Cellular Level: The GUIDE-seq Challenge

Similar to the evolution of CIRCLE-seq to CHANGE-seq, **GUIDE-seq** (Genome-wide, Unbiased Identification of Double-Stranded Breaks Enabled by Sequencing) – a broadly adopted, genome-wide cellular off-target assay – was updated in 2025 to **GUIDE-seq2**[2] by incorporating tagmentation to address assay limitations.

GUIDE-seq assays have been a cornerstone assay for cellular measurement of CRISPR off-target activity since the method was first published in 2015 by Tsai, *et.al.* in Nature Biotechnology[15]. By leveraging double-stranded oligodeoxynucleotide (dsODN) integration at double-stranded breaks, GUIDE-seq provides powerful insights into real-world off-target events within cellular contexts. However, the method suffers from limitations:

- **Complex library preparation**: Traditional workflows require multiple enzymatic steps, ligations, and nested PCR, increasing time and variability. Library preparation takes an 8-hour day to complete (Figure 1).
- **Lower throughput**: The hands-on nature limits scalability, constraining adoption for large-scale studies.
- **Sensitivity trade-offs**: The complex workflow can reduce sensitivity and reproducibility, particularly in challenging samples.

These limitations leave a gap between GUIDE-seq's powerful biology and the streamlined, NGS-compatible workflows demanded in today's gene editing landscape.

Enter GUIDE-seq2: Power Meets Efficiency

The introduction of **GUIDE-seq2** marks a major milestone in addressing these challenges. By integrating **tagmentation into the library preparation workflow**, GUIDE-seq2 delivers

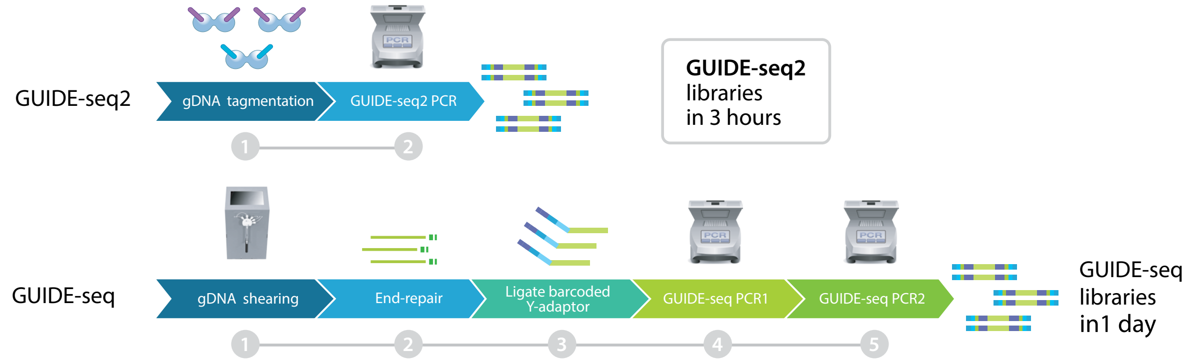

Figure 1. Original GUIDE-seq protocol[3] compared to GUIDE-seq2[4]. Tagmentation saves several steps significantly contributing to the reduction in library preparation time.

a more sensitive, reproducible and scalable solution while retaining the assay's proven biology. Second-generation GUIDE-seq2 has been validated across five therapeutic loci in human primary T cells, demonstrating strong correlation with the original GUIDE-seq method, establishing the ability to evolve the assay without compromising assay results[2].

Key Change from the Original GUIDE-seq

Fragmentation and Adapter Addition in One Step via Tagmentation

During library preparation, instead of mechanical shearing followed by end-repair, A-tailing, ligation and nested PCR, GUIDE-seq2 uses ***Tn5 transposase*** to simultaneously fragment and directly tag gDNA with UMIs and sequencing adapters

The original GUIDE-seq library preparation method requires fragmentation of gDNA (often using mechanical or enzymatic shearing) followed by end-repair/A-tailing of resulting fragments, ligation and nested PCR of library molecules. GUIDE-seq2 removes physical DNA shearing along with multiple enzymatic steps and required cleanups, using tagmentation, while also eliminating the need for nested PCR. Tn5 transposase is loaded with a unique molecular identifier (UMI) and i5 adaptor (commercially available as Tagify™ from seqWell) which is directly tagged onto DNA during enzyme fragmentation. Tagmented gDNA undergoes a single round of PCR amplification using a tag-specific primer with an i7 barcode primer (Figure 1).

GUIDE-seq2 advantages include:

- **Streamlined workflow**: Tagmentation eliminates multiple library prep steps, reducing assay complexity and hands-on time. Total library preparation can now be completed in 3 hours.
- **Scalability**: GUIDE-seq2 can be readily scaled for large panels or therapeutic pipelines, meeting the needs of high-throughput labs.
- **Improved reproducibility**: Simplified workflows reduce sources of technical variability, making results more consistent across experiments.
- **Compatibility with modern sequencing**: GUIDE-seq2 aligns with the latest NGS platforms and best practices, ensuring smoother integration into existing workflows.

Population-Scale Off-Target Effects

As genome editing technologies such as CRISPR-Cas9 move closer to population and clinical applications, it has become clear that genome-based off-target analysis using reference genomes alone is not enough. Even small genetic variants—such as single nucleotide polymorphisms (SNPs) or structural variations—can create or abolish CRISPR off-target sites, substantially altering editing specificity. This means that assays limited to a single genome background risk overlooking clinically relevant off-targets in diverse populations. Incorporating population-scale off-target analysis ensures that editing outcomes are evaluated across the spectrum of human genetic diversity, providing greater confidence in safety, sensitivity, and therapeutic applicability. The evolution to GUIDE-seq2 now offers an off-target assay that fulfills the throughput requirement and commercial availability of critical assay reagents to power large-scale studies.

GUIDE-seq2 Publication SNIP-IT: Population-scale cellular GUIDE-seq2 and biochemical CHANGE-seq-R profiles reveal human genetic variation frequently affects Cas9 off-target activity.

Lazzarotto *et.al.* developed GUIDE-seq2 to better understand how genetic variation influences Cas9 off-target activity in human cells. Using GUIDE-seq2, they analyzed 665 libraries from six gRNA targets in lymphoblastoid cells across 95 individuals representing four ethnic groups:

- African ancestry (Southwest U.S.) - ASW
- European ancestry (Utah) - CEU
- Han Chinese (Beijing) - CHB
- Mexican ancestry (Los Angeles) - MXL

The results of their studies revealed that GUIDE-seq2 detected off-target events frequently overlapped with human genetic variants, the frequency of which depended on ethnicity. The highest frequency of overlap with one or more genetic variations was seen in the African ancestry group (16.6%) and the lowest in the Han Chinese group (7.8%).

These findings indicate that individual genetic variants may frequently have unintended off-target activity and emphasize the importance of population-scale CRISPR analyses.

> “The simplified GUIDE-seq2 workflow substantially streamlines the process and enables high-throughput experiments, while also decreasing the requirement of input genomic DNA for library preparation by approximately 4-fold.”
>
> *–Developers of GUIDE-seq2*[2]

Looking Forward

The evolution from GUIDE-seq to GUIDE-seq2 mirrors the successful transition seen in CIRCLE-seq to CHANGE-seq. Tagmentation is redefining how off-target assays keep pace with the scale and precision of modern sequencing. For researchers and developers, GUIDE-seq2 represents not just an incremental update, but a transformational one—making in-cell off-target detection more accessible, reliable, and ready for population-scale studies.

In a landscape where regulatory scrutiny and clinical demands are rising, having assays that combine biological relevance with operational efficiency is critical. GUIDE-seq2 demonstrates how thoughtful technical innovation can unlock the next phase of genome editing research and development.

Enhancing Gene Editing QC with Tagify Adapter-Loaded Transposases

The GUIDE-seq2 protocol incorporates Tn5 transposase loaded directly with P5/i5 sequence adapters and UMIs (Tagify™ i5 UMI from seqWell), demonstrating how loaded transposases can streamline workflows, increase efficiency, and reduce manual steps. While laboratories can express, purify and load Tn5 in-house, the correlation to the commercially available counterpart from seqWell provides researchers easy access to a highly consistent and fully-QC’d source of this critical assay reagent.

In the GUIDE-seq2 publication from *Lazzarotto, et. al.*, the authors compared seqWell’s off-the-shelf Tagify™ i5 UMI reagent with lab-generated loaded transposase. They concluded that:

- Tagify i5 UMI provides a reliable, commercial source of loaded transposase that produces equivalent assay performance
- 0.99 - 1.00 correlation between seqWell’s Tagify and in-house produced Tn5 for all four of the targets examined

Evolve Your Assay Using Tagify™ Custom-loaded Transposases

In addition to Tagify i5 UMI, seqWell offers Tagify™ Custom-loaded Transposases: Tn5 or TnX - our next-generation

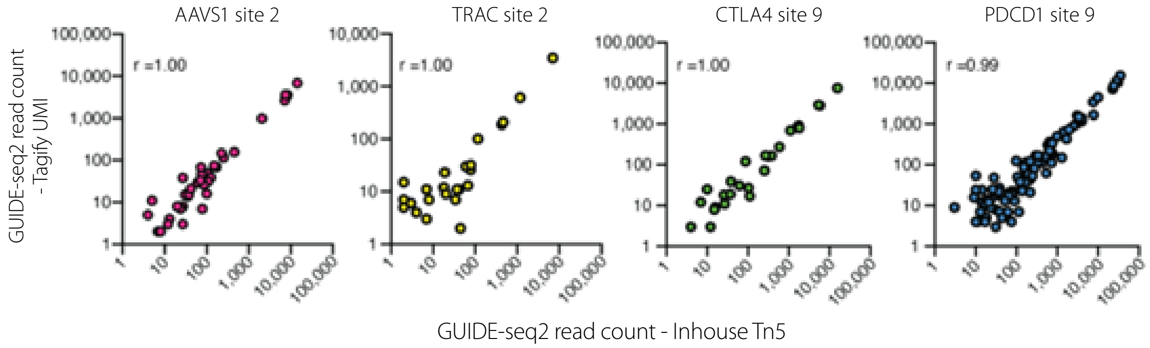

Figure source: Lazzarotto, C, *et.al.,* bioRxiv, 2025-02. Supplementary Figure 1. GUIDE-seq2 profile correlates well between in-house Tn5 and commercially available Tn5. Scatterplots of GUIDE-seq2 read counts (log10) from experiments performed on primary human T cells for 4 target sites. Genomic DNA were tagmented using inhouse Tn5 (x-axis) and seqWell Tagify-UMI (y-axis) before GUIDE-seq2 PCR. GUIDE-seq2 library prepared with each method was sequencd individually with NextSeq2000. r = Pearson’s correlation coefficient.

transposase that provides enhanced sequencing performance - loaded with custom payloads to help drive other transposase-based assays, such as UDiTaS™, ATAC-seq, SHARE-seq, GUIDE-tag, CHANGE-seq, TTIS-seq, or RGen-seq.

Interested in converting your assay library preparation to a highly scalable tagmentation-based method using seqWell Tagify™ reagents? Contact Us today!

Excellent Reviews of Assay Methods:

- Guo, C., Ma, X., Gao, F., & Guo, Y. (2023). Off-target effects in CRISPR/Cas9 gene editing. *Front Bioeng Biotechnol, 11:1143157*.
- Atkins, A., Chung, C. H., Allen, A. G., Dampier, W., Gurrola, T. E., Sariyer, I. K., Nonnemacher, M.R., & Wigdahl, B. (2021). Off-target analysis in gene editing and applications for clinical translation of CRISPR/Cas9 in HIV-1 therapy. *Front Genome Ed, 3, 673022*.

References:

1. FDA Guidance https://www.fda.gov/media/156894/download
2. Lazzarotto, CR, Li, Y, Flory, AR, Chyr J, Yang, M, Katta, V, Urbina, E, Lee, G, Wood, R, Matsubara, A, Rashkin, SR, Ma, J, Cheng, Y, & Tsai, SQ. (2025). Population-scale cellular GUIDE-seq2 and biochemical CHANGE-seq-R profiles reveal human genetic variation frequently affects Cas9 off-target activity. ***bioRxiv*** 02.10.637517; doi: https://doi.org/10.1101/2025.02.10.637517
3. Lazzarotto, CR, Malinin, NL, Li, Y, Zhang, R, Yang, Y, Lee, G, Cowley, E, He, Y, Lan, X, Jividen, K, Katta, V, Kolmakova, NG, Petersen, CT, Qi, Q, Strelcov, E, Maragh, S, Krenciute, G, Ma, J, Cheng, Y, & Tsai, S. Q. (2020). CHANGE-seq reveals genetic and epigenetic effects on CRISPR-Cas9 genome-wide activity. *Nat Biotechnol, 38(11), 1317-1327*.
4. Giannoukos, G, Ciulla, DM, Marco, E., Abdulkerim, HS, BarreraLA, Bothmer, A, Dhanapal, V, Gloskowski, SW, Jayaram, H, Maeder, ML, Skor, MN, Wang, T, Myer, VE & Wilson, CJ. (2018). UDiTaS™, a genome editing detection method for indels and genome rearrangements. *BMC Genomics, 19(1), 212*.
5. Wienert, B., Wyman, S. K., Richardson, C. D., Yeh, C. D., Akcakaya, P., Porritt, M. J., Morlock, M., Vu, J.T., Kazane, K.R., Watry, H.L., Judge, L.M., Conklin, B.R., Maresca, M., & Corn, J. E. (2019). Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq. *Science, 364(6437), 286–289*.
6. Philippidis, A. (2024). CASGEVY Makes History as FDA Approves First CRISPR/Cas9 Genome Edited Therapy. *Hum Gene Ther. 35(1-2):1-4*.
7. From Ledford, H. (2023). Is CRISPR safe? Genome editing gets its first FDA scrutiny. *Nature, 623(7986), 234-235*.
8. Guo, C., Ma, X., Gao, F., & Guo, Y. (2023). Off-target effects in CRISPR/Cas9 gene editing. *Front Bioeng Biotechnol, 11:1143157*.
9. Atkins, A., Chung, C. H., Allen, A. G., Dampier, W., Gurrola, T. E., Sariyer, I. K., Nonnemacher, M.R., & Wigdahl, B. (2021). Off-target analysis in gene editing and applications for clinical translation of CRISPR/Cas9 in HIV-1 therapy. *Front Genome Ed, 3, 673022*.
10. Kim, D., Park, J, Bae, S, Kim, E., Kim, S, Yu, HR, Hwang, J, Kim, JI, & Kim, J. S. (2015). Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods, 12 (3), 237–243*.
11. Tsai, S. Q., Nguyen, N. T., Malagon-Lopez, J., Topkar, V. V., Aryee, M. J., & Joung, J. K. (2017). CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. *Nat Methods, 14(6), 607–614*.
12. Cameron, P, Fuller, CK, Donohoue, PD, Jones, BN, Thompson, MS, Carter, MM, Gradia, S, Vidal, B, Garner, E, Slorach, EM, Lau, E, Banh, LM, Lied, AM, Edwards, LS, Settle, AH, Capurso, D, Llaca, V, Deschamps, S, Cigan, M, Young, JK, & May, A.P. (2017). Mapping the genomic landscape of CRISPR-Cas9 cleavage. *Nat Methods, 14(6), 600–606*.
13. Frock, R. L., Hu, J., Meyers, R. M., Ho, Y. J., Kii, E., & Alt, F. W. (2015). Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat Biotechnol, 33(2), 179–186*.
14. Crosetto, N., Mitra, A., Silva, M. J., Bienko, M., Dojer, N., Wang, Q., Karaca, E, Chiarle, R, Skrzypczak, M, Ginalski, K, Pasero, P, Rowicka, M, & Dikic, I. (2013). Nucleotide-resolution DNA double-strand break mapping by next-generation sequencing. *Nat Methods, 10(4), 361–365*.
15. Tsai, S. Q., Zheng, Z., Nguyen, N. T., Liebers, M., Topkar, V. V., Thapar, V., Wyvekens, N., Khayter, C., Le, L.P., Aryee, M. & Joung, J. K. (2015). GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol, 33(2), 187–197*.