Safety Assessment of Genome Editing in Human Gene Therapy Products Using Next-Generation Sequencing

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Draft Guidance for Industry

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U.S. Department of Health and Human Services
Food and Drug Administration
Center for Biologics Evaluation and Research
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Safety Assessment of Genome Editing in Human Gene Therapy Products Using Next-Generation Sequencing

Draft Guidance for Industry[1]

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I. INTRODUCTION

This guidance provides recommendations for next-generation sequencing (NGS)-based methods used in nonclinical studies that will likely be needed to support initiation of clinical trials of investigational human genome editing (GE) products.[2] These recommendations are in addition to the nonclinical, clinical, and CMC considerations discussed in the "Guidance for Industry: Human Gene Therapy Products Incorporating Human Genome Editing" dated January 2024 (January 2024 GE Guidance) (Ref. 1). Clinical development programs of human GE products should address both the risks associated with the gene therapy product itself as well as the additional risks associated with GE, including off-target editing and unintended changes to the genome for therapies targeting genetic diseases, including individualized therapies. For more information on clinical study design considerations and our science-based approach weighing the benefits and risks for human GE products, please refer to the January 2024 GE Guidance (Ref. 1). The recommendations in this guidance may guide stakeholders on designing nonclinical studies that use NGS methods and bioinformatics to evaluate the potential safety risks associated with off-target editing and loss of genome integrity in human GE products submitted in support of Investigational New Drug (IND) applications and Biologics License Applications (BLAs).

In general, FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the FDA's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in FDA's guidances means that something is suggested or recommended, but not required.

[1] This draft guidance has been prepared by the Office of Therapeutic Products (OTP) in the Center for Biologics Evaluation and Research (CBER) at the Food and Drug Administration.

[2] Human gene therapy products incorporating GE are referred to as human GE products throughout this guidance. For the purposes of this guidance human GE is a process by which DNA sequences are added, deleted, altered or replaced at specified location(s) in the genome of human somatic cells, ex vivo or in vivo, using nuclease-dependent or nuclease-independent GE technologies.

II. BACKGROUND

GE tools or editors have been designed and used to perform editing at a specific (i.e., intended on-target) site(s) in either the genome, the epigenome, or the transcriptome. The distinct sequence recognition process and different mechanisms of action used by various genome editing tools have given rise to many GE modalities. For instance, the clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) system uses a guide RNA (gRNA) to target a specific genomic site harboring the complementary sequence in the presence of a protospacer adjacent motif (PAM) sequence for editing. This system has greatly diversified with the discovery of a wide range of Cas proteins with distinct PAM sequence requirements and nucleotide editing capabilities. Furthermore, fusion of Cas proteins with different types of effector molecules such as deaminases, reverse transcriptase, or DNA methyltransferase has added versatility to an already expanding GE toolbox. In contrast to the CRISPR-Cas, other modalities such as meganuclease, zinc finger nuclease (ZFN), and transcription activator-like effector nucleases (TALENs) use protein domains that recognize specific DNA sequence(s). With continued research efforts, the GE field will likely continue to expand with the discovery of newer, programmable, and specific editing modalities.

Sponsors have used a wide variety of GE modalities to edit cells ex vivo or in vivo. Specifically, GE modalities can be used to edit cells ex vivo using cells obtained from either patients (autologous) or healthy donors (allogeneic). Alternatively, cells can be edited in vivo using messenger RNA (mRNA) encoding the editor or using DNA-based vectors harboring the editor coding sequence that is expressed under a promoter. However, in either case, GE at off-target (i.e., unintended) site(s) and/or chromosomal translocations are a potential safety risk since such edits could be deleterious to normal cell function. Therefore, an adequate assessment to evaluate off-target editing risk and impact on chromosomal integrity of human GE products is crucial to minimize unintended changes in the target cells or tissues. Off-target editing studies use computational and/or NGS-based methods to assess off-target editing activity in a wide range of GE products. This guidance provides FDA's recommendations pertaining to NGS-based studies assessing off-target editing activity, NGS-based chromosomal integrity assessment studies, on-target edit site assessments, sequencing methods, considerations for sample selection, and reporting the findings to FDA.

III. SCOPE

This guidance is intended for sponsors developing human gene therapy drug products[3] involving genome editing technologies. The January 2024 GE Guidance describes human GE as a process by which DNA sequences are added, deleted, altered or replaced at specific location(s) in the genome of human somatic cells (Ref. 1). However, the rapid pace of research in the GE field has led to the development of GE technologies that can also modify the epigenome or cleave RNA sequences. For the purpose of this guidance, human GE products also include products editing

[3] For the purposes of this guidance, all references to *drugs* include both human drugs and therapeutic biological products unless otherwise specified.

the epigenome or the transcriptome, and recommendations made for NGS-based evaluation of off-target editing activity would be applicable to all of these products.

Sponsors routinely use NGS-based methods and bioinformatics tools to assess off-target editing risk of their drug product(s). This guidance provides recommendations for nonclinical studies using NGS and bioinformatics to assess off-target editing risk and impact on chromosomal integrity for a broad range of GE modalities such as DNA editors, epigenetic editors, and editors that cleave RNA.

IV. CONSIDERATIONS FOR NEXT GENERATION SEQUENCING (NGS)

The NGS technology and bioinformatics tools generally allow for sensitive and quantitative assessment using targeted methods to assess specific genomic locations or using genome-wide methods to assess the genome. Specifically, these methods may include performing targeted sequencing or using genome-wide sequencing methods to scan for unintentional changes that may be a consequence of an off-target editing activity imparted by a genome editor. In this section, we provide specific recommendations on the use of NGS for off-target edit site assessments and for the evaluation of intended/on-target genomic changes in GE cells.

Sponsors should use an appropriate sequencing strategy that meets the goals of the on-target and off-target edit site analysis method being implemented. For example, for assessing editing at on- and off-target sites that result in changes to a short stretch of DNA (e.g., ≤50-bp), a sequencing strategy utilizing short-read sequencing-based methods may be adequate.[4] However, evaluation of longer stretches of sequence for the presence of large insertions or large deletions may require implementing a sequencing strategy that uses long-read sequencing-based methods that can detect such changes.[5, 6]

Sequencing should be performed using an adequate amount of the input material (genomic DNA, cell number, or RNA, as applicable) at a depth that allows detection of potential off-target editing events occurring at frequencies or edit rates that are usually lower than the on-target edit rate. Sponsors should provide data generated in-house, such as representative data from engineering NGS runs, and/or relevant information from peer-reviewed publications, to support the adequacy and the sensitivity of sequencing depth and to support their strategy to detect low frequency off-target editing events. Where applicable, strategies to minimize PCR amplification bias and/or primer bias are recommended. A detailed report of the NGS and data analysis should include the following:

[4] K. Polonis et al., Innovations in Short-Read Sequencing Technologies and Their Applications to Clinical Genomics; Clin Chem. 2025 Jan 3;71(1):97-108. doi: 10.1093/clinchem/hvae173.

[5] S.L. Amarasinghe et al., Opportunities and challenges in long-read sequencing data analysis; Genome Biol. 2020 Feb 7;21(1):30. doi: 10.1186/s13059-020-1935-5.; T. Xiao et al., The third generation sequencing: the advanced approach to genetic diseases; Transl Pediatr. 2020 Apr;9(2):163-173. doi: 10.21037/tp.2020.03.06.; S.M. Karst et al., High-accuracy long-read amplicon sequences using unique molecular identifiers with Nanopore or PacBio sequencing; Nat Methods. 2021 Feb;18(2):165-169. doi: 10.1038/s41592-020-01041-y.

[6] The recommendations for assessing short (≤50-bp) and long stretch of DNA provided as examples may change with evolving sequencing technologies. Sponsors are encouraged to discuss their strategy with the FDA.

i. A detailed description of cell type, the number of cells used (if applicable), amount of nucleic acid material extracted, nucleic acid extraction process, library preparation, primer information (where applicable), and sequencing method(s).

ii. Bioinformatics tools used for sequence quality/depth assessment, sequence alignment (as applicable), and any additional sequence analysis steps, and command line interface (CLI) information.[7] Include reference(s) to publication(s) with repository information (if applicable).

iii. Reference sequence(s) and database(s) used in the analysis.

iv. A brief description of each step of sequence analysis including information on acceptance criteria applied at each step (as applicable) while processing the sequencing data. The acceptance criteria information should include information on sequencing quality acceptance criteria, sequencing depth acceptance criteria, and alignment metric acceptance criteria.

v. Results from the NGS data analysis and the associated metadata should be submitted in a tabulated format as a text, CSV, or Microsoft Excel (as applicable).

V. CONSIDERATIONS FOR ON-TARGET EDIT SITE ASSESSMENT

An on-target site is a specific sequence in the genome or transcriptome that is intended for editing by the editor. The on-target editing rate can be determined by sequencing the on-target site and evaluating the proportion of reads harboring the intended edit over total reads covering the target site. The sponsor should report the on-target edit rate either as a ratio or percentage of edited reads. Samples used for on- and off-target site analysis should be edited with appropriate parameter(s) so that the resulting on-target editing rates are comparable to the rates proposed in the final drug product. Achieving the intended rate of on-target editing likely ensures the presence of the optimal level of genome editor activity in the samples. The sponsor should report the on-target editing rates from the in vitro studies and may use the data to support the adequacy of assays evaluating off-target editing activity (as feasible). Additionally, the sponsor should use appropriate sequencing-based methods to measure and report the rates of both intended and unintended edits at the on-target genomic edit site(s) in appropriate samples (see section VI.A for recommendations on sample selection). Specific recommendations for sequencing strategy and samples used for on-target edit profiling are provided below.

[7] CLI information is a record of all the commands used by user to run computer program(s) when analyzing sequencing data.

1. Sequencing strategy

Sponsors should use a targeted sequencing strategy that enables the measurement of the rate of intended edits at on-target genomic location(s). Short-read or long-read sequencing-based method(s) are recommended to assess the on-target edit site if the editing modality imparts changes to a single nucleotide (e.g., a genome edit replacing, deleting or inserting a single base pair) or a short stretch of nucleotides (e.g., a genome edit resulting in insertion or deletion of a short stretch of nucleotides). However, on-target edit site analysis using long-read sequencing-based methods should be performed if the editing modality creates double strand breaks and/or has the capacity to cause unintentional changes to a large stretch of DNA (insertions or deletions that are >50bp to several kilobase pair long). The sponsor should provide justification to support the adequacy of their sequencing method used for on-target edit site analysis.

Where applicable, sponsors should use adequate sequencing depth and appropriate strategies to reduce bias (see section IV for information on NGS considerations) and to facilitate the detection of unintended edit outcomes potentially occurring at lower frequency than the intended edit outcome rate. The data from on-target edit site analysis may be provided as a list of all the editing events (substitutions, and/or insertions, and/or deletions) identified and the associated frequencies of edits (edit rates) determined from the sequencing data in a tabulated form.

2. Sample selection for assessing on-target edit sites

An appropriately designed on-target edit site profiling strategy may include using cell samples that are identical to the cells edited in an ex vivo GE product or that are representative of the cell type at the intended site of action for an in vivo genome editing product (see section VI.A for additional recommendation on sample selection).

VI. CONSIDERATIONS FOR STUDIES ASSESSING OFF-TARGET EDITING ACTIVITY

For DNA editors and epigenetic editors, an off-target edit site is any sequence in the genome where editing activity was observed but was not intended. Similarly, for RNA editors, an off-target edit site is any sequence in the transcriptome where an off-target editing event would result in an unintended change to gene expression. These off-target edit sites harbor some degree of homology or similarity when compared to the on-target edit site that is proposed for editing.

This section provides recommendations for approaches used for off-target edit site nomination[8], off-target edit site confirmation[9], and off-target analysis parameters. Specifically, this section provides additional recommendations on sample selection, using modality-specific or generally applicable methods for off-target edit site nomination, specific information that should be included when describing the studies, and reporting of the study results.

To facilitate reproducibility and sensitivity of assays evaluating off-target editing activity, we recommend that the sponsor use appropriate NGS method(s) (see section IV for NGS considerations) and use appropriate samples (see section VI.A for sample considerations) that achieve the intended editing rate at on-target site(s) (see section V for on-target edit site assessment), and use biological replicates.

A. Samples used for off-target editing analysis

Off-target edit site nomination and confirmatory testing studies should be performed in multiple biological replicates. Use of human cells that are either from patients, or from healthy donors is recommended. If obtaining primary human cell samples is not feasible, then a sponsor may consider using representative immortalized human cell lines and provide scientific justification with data (as applicable) to support the adequacy of sample(s) selected for the study. We recommend that sponsors use cells that are representative of the target cell type that they intend to edit either *ex vivo* or *in vivo.* The sample selection is further impacted by factors such as the mode of editing (in vivo or ex vivo), sample availability, and the amenability of cells to in vitro culture conditions.

1. *Ex vivo* products

Studies assessing off-target editing activity in ex vivo GE products should be performed using a cell type that is identical to the cell type being edited for drug product generation. Additionally, the samples used to assess off-target risk should have on-target editing rates that are comparable to the rates observed in the final drug product. If the GE strategy is aimed at correcting a mutation in patient cells, then we recommend that sponsors use patient-derived samples or normal cells that are engineered to harbor the mutation. This approach will facilitate reporting of editing rates at the on-target site. The sponsor may use healthy donor samples where appropriate and provide scientific justification to support the adequacy of the approach. If the sponsor chooses a cell type that is different from the cell type they intend to edit, they should provide scientific justification to support the adequacy of their sample selection strategy.

[8] Off-target edit site nomination is synonymous with the term identification of off-target site used in the January 2024 GE Guidance (Ref. 1).

[9] Off-target edit site confirmation is synonymous with the term verification of off-target site used in the January 2024 GE Guidance (Ref. 1).

2. *In vivo* products

For the assessment of off-target editing activity of in vivo products, we recommend that sponsors use appropriate cell types that are representative of the drug product's intended target tissue in vivo and use editing parameters that results in the intended level of editing rates at the on-target site. However, if appropriate cell type(s) are unavailable, difficult to obtain, or if the cells are not amenable to in vitro studies, then sponsors may consider using alternative methods or cell samples to perform studies assessing off-target editing activity and provide scientific justification to support their strategy. For example, the sponsor may use a biochemical assay(s) that requires genomic DNA material for off-target edit site nomination.

If an intended edit is aimed at correcting a disease-causing mutation, then the use of healthy donor samples would not allow for the evaluation of on-target editing rates. In such a case, using patient-derived cells or normal cells engineered to harbor the target mutation is recommended for the assessment of off-target editing activity. If cells harboring the target mutation cannot be used because such cells are unavailable, (due to disease severity or rarity, patient cell availability, etc.,) sponsors may consider using healthy donor samples and provide scientific justification to support the adequacy of the approach. Finally, the data from biodistribution studies, as discussed in the January 2024 GE Guidance (Ref. 1), should inform the need to conduct additional on-target site analysis, off-target edit site nomination, and off-target edit site confirmation analyses in other cell type(s) representative of (non-target) tissues or organs where the product was detected.

When implementing cell-based methods for assessing off-target editing activity, use of a delivery method (e.g., lipid nanoparticle (LNP), adeno associated viral vectors (AAVs), electroporation, etc.) that is identical to the one used in the in vivo drug product is recommended. However, if the in vivo delivery method is not amenable for use in in vitro cell system(s), sponsors may consider alternative methods to deliver the GE components. When performing these studies, the sponsor should evaluate editor expression level, on-target editing rate(s), and submit the data in the study report(s). The sponsor should also provide scientific justification, as applicable, to support the adequacy of their sample selection, delivery method, and editing parameters used in the study.

B. Modality specific off-target edit site nomination methods

The January 2024 GE Guidance (Ref. 1) recommends using multiple approaches such as cell-based assays, biochemical assays, and in silico methods for off-target site nomination. Cell-based assays use genomic DNA from ex vivo edited cultured cells for off-target analysis. Biochemical assays, on the other hand, use purified genomic DNA that are edited in vitro for off-target analysis. Subsequent analysis of the genomic DNA

involves library preparation, NGS, and bioinformatics analysis of sequencing data (see section IV for NGS considerations).

Cell-based and biochemical assays were originally developed for assessment of off-target editing activity of genome editors that create double strand breaks in the genome. However, these assays have been modified, or new assays have been developed, to detect single strand breaks/nicks in the genome. Thus, sponsors should consider the mechanism of action of their proposed editor and choose an appropriate method for off-target edit site nomination. For example, assays designed to detect off-target editing activities resulting in double strand breaks may not be suitable to detect single strand nicks created by base editors. In such a case, sponsors may consider an appropriately designed assay using an endonuclease to create double strand breaks at single strand nick sites to enable detection of edit events post-sequencing.

The sponsor should consider the mechanism of action of their proposed editor when selecting a published modality-specific off-target editing analysis approach and justify their strategy by referencing relevant peer reviewed publication(s) and/or by providing data generated in-house. Any change from the published strategy should be supported by data generated in-house demonstrating the adequacy of the modified strategy. A change in the assay may be any major modification to the assay design that was implemented by the sponsor but was not described in prior publication(s). To support any major modification(s) introduced in the assay evaluating off-target editing activity, the sponsor should provide additional information or studies to support that their proposed major modification(s) did not impact the sensitivity of the assay, impair the data quality, or introduce any bias(es).

When using a novel, unpublished off-target analysis method(s), the sponsor should provide additional information to support the adequacy and sensitivity of their method(s). For CRISPR-Cas-based products, this could be achieved, for example, through reporting off-target edit sites of widely-researched gRNAs identified after implementing the new method and then providing a comparative assessment to support the adequacy of the novel approach. For editing modalities that do not use CRISPR-Cas-based method(s), a similar approach may be used to develop assays evaluating off-target editing activity and may be supported by developing and using positive controls.

The findings from each off-target edit site nomination study should be submitted as a list of nominated off-target sites in a tabulated form (see section VI.F for recommendations on reporting off-target edit sites). Specific recommendations for modality-specific assays are provided in sections VI.B.1 and VI.B.2.

1. Study reports describing the biochemical and cell-based assays should contain the following:

    i. Adequate description of the sample(s). Specifically, indicate if the samples are from healthy donor(s) or patient(s) and discuss if the

samples used for assessing off-target activity harbor sequence(s) that is recognized by the editor to enable measurement of on-target editing rates.

ii. Report the concentrations of the genome editing components.

iii. Report on-target edit rate (if applicable), the total sequencing depth of each sample, read counts at the on-target edit site and at all detected off-target sites.

2. For cell-based off-target edit site nomination assays, the on-target editing rate(s) reported from samples used in this assay should be comparable to the on-target editing rate(s) proposed or measured in the final drug product (see section V for recommendations on on-target edit site assessment). Otherwise, the sponsor should justify the adequacy of the edit rates achieved when implementing the cell-based off-target assays.

C. Generally applicable methods for off-target edit site nomination

In the absence of established empirical methods (biochemical or cell-based assays) to assess the safety of a specific type or class of GE modality, the sponsor may consider implementing other broadly applicable methods to nominate off-target edit sites. These approaches include using computational algorithms or applying established NGS-based methods.

1. In silico methods for off-target edit site nomination

One of the main determinants of the number of potential off-target edit sites and the off-target editing risk is sequence homology of the gRNA or the target sequence to other regions of the human genome. In silico or computational algorithms allow users to scan the reference genome and identify additional regions in the genome that harbor homology to the user-provided gRNA sequence. Sponsors should consider the following points when using in silico off-target edit site nomination tools:

i. For CRISPR-Cas-based products, homology-based off-target edit sites search should include both mismatches and bulge(s) in DNA and gRNA. Additionally, PAM requirements or other modality-specific sequence requirements should be considered when implementing a homology-based off-target edit site search (see section VI.E for recommendations on analysis parameters).

ii. For other editing modalities, a similar in silico sequence homology-based search method that accounts for sequence variations may be

used for off-target edit site nomination. For example, the target sequence that is recognized by the DNA binding domain of an editor may be used to scan the human genome to identify potential off-target edit sites based on homology.

iii. Scientific justification should be provided for the search parameters used, either by citing peer reviewed publication(s) or by providing additional information from data generated in-house to support the approach.

2. Next generation sequencing-based methods for off-target edit site nomination

Current NGS technologies allow for the assessment of changes to genome/transcriptome/epigenome or quantification of mRNA abundance. Depending on the mechanism of action of the editor being used in a drug product, the sponsor may use an NGS-based method that enables evaluation of intended edits in the cells. For example, off-target editing risk of a drug product intended to methylate a cytosine base at a specific genomic site using an epigenome editor should be assessed with an NGS-based genome-wide DNA methylation analysis method. Using appropriate NGS-based method(s) would facilitate off-target editing analysis of a wide range of genome editing modalities, irrespective of whether an editor targets the genome, transcriptome, or epigenome.

The adequacy of the experimental strategy and analysis parameters used when implementing these methods should be supported with references to peer-reviewed publication(s) or any analyses performed in-house. In addition to the recommendations made on the use of NGS in section IV and on-target edit site assessment in section V, we have the following additional recommendations when implementing NGS for an off-target edit site assessment:

i. Sequencing quality and depth acceptance criteria should be provided with scientific justifications to support the sensitivity, adequacy of genome or exome coverage (if applicable), and reproducibility of the assay.

ii. A detailed description of filtering step(s) used to either subset reads or variants based on base quality[10] or mapping quality[11], should be supported with scientific justification.

[10] Base quality score is a measure of probability of identifying an incorrect nucleotide during sequencing.
[11] Mapping quality for short sequencing reads is a measure of probability of an incorrectly mapped read. An appropriate mapping quality score that is adapted for long sequencing reads may be used, where applicable.

iii. Any other factor(s) used in determining off-target site edit(s) should be presented and supported with rationale, where applicable.

D. Confirmatory testing methods

The sponsor should use appropriate cell type(s) to verify the nominated off-target edit sites and to measure the editing frequency at the nominated sites. Confirmatory testing methods may include probe-based sequence enrichment, targeted sequence amplification, and/or other equivalent methods. While performing confirmatory testing at all the nominated off-target edit sites is recommended, the sponsor may select a subset of nominated off-target sites for confirmatory testing and provide scientific justification to support their strategy. The rationale for selecting a subset of nominated off-target edit sites may include applying statistical cut-offs, editing rate cut-off, detection of off-target sites in multiple samples, or other factors. However, use of stringent filtering criteria should be avoided to enable evaluation of editing rate at the nominated off-target edit sites.

When using a confirmatory testing method, the sponsor should select a sequencing strategy that minimizes bias and implement it at predetermined sequencing depth and quality that enables evaluation of low frequency editing events (see section IV for sequencing considerations). The sponsor may report the sensitivity of the confirmatory testing method and any other information as a rationale to justify their testing strategy. Confirmatory testing should be performed in appropriate cell type(s) (see section VI.A). Additionally, use of unedited control and edited sample pairs to determine edit rates at the on-target and all the off-target edit sites is recommended. The editing rates at the on-target edit site(s) reported from this study should be comparable to the intended on-target edit rates proposed by the sponsor in their final drug product. Please refer to section VI.F for recommendations on reporting the findings of the confirmatory testing study.

E. Analysis parameters

Analysis parameters used for off-target edit site nomination and confirmation studies are specific to the experimental design and NGS method(s). For CRISPR-Cas-based editors that have been shown to recognize different versions of PAM sequence for editing, off-target edit site identification strategy should include different PAM versions. For example, NGG is a canonical PAM sequence for spCas9, but spCas9 has been shown to recognize non-canonical PAM sequences as well. The sponsor should reference peer-reviewed publication(s) or data generated in-house, as applicable, to support their PAM selection or exclusion strategy. For NGS data generated using either unbiased whole genome/exome/transcriptome/epigenome sequencing methods or targeted sequencing methods, details of the filtering parameters used for data analysis should be reported and supported with scientific justification. Any additional analysis parameters that are sequencing-method specific should also be reported and supported with justification.

Please refer to our recommendations from section IV and section VI.C.2 when describing the analysis steps and parameters.

F. Reporting off-target edit sites

When reporting the nominated and confirmed off-target edit sites, the sponsor should report the read counts and/or editing frequencies measured from the respective off-target analysis study (as applicable). Additionally, we recommend that the sponsor describe the tool(s) that they used to annotate the genomic coordinates of off-target edit sites. An annotated list of all the off-target edit sites reported from nomination and confirmatory testing studies should also include the following:

i. Genomic coordinate information.

ii. Number of mismatches and bulges compared to the gRNA or target sequence.

iii. PAM information for CRISPR-Cas-based editors.

iv. Whether the off-target edit site is intergenic, exonic, or intronic and include the following information (as applicable):

   a. For intergenic off-target edit site(s), information on the distance to the nearest gene should be provided, and the impact on promoter or regulatory regions should be discussed.
   b. For exonic off-target edit site(s), the name and function of the associated gene should be reported, and the impact of genome editing on the amino acid sequence, gene expression, and splicing should be discussed.
   c. For intronic off-target edit site(s), the distance to the nearest exon should be reported and the potential impact on splicing should be discussed.

For all the confirmed off-target edit sites, the sponsor should submit a summary of the risk assessment performed using prior knowledge such as information from peer-reviewed publication(s) about the associated genomic location, gene expression information (if applicable), gene disease association studies (if available), and/or any other relevant information as applicable.

G. Additional considerations

Off-target editing activity of a drug product is further impacted by additional factors such as amino acid modification(s) made to the editor and the mode of delivery of the editor (see section VI.A.2 for recommendations on use of delivery methods for studies assessing

off-target activity). If an editor is altered by changing amino acid residue(s) to modify editing activity and/or specificity, then a detailed description of such modification(s) should be provided. The sponsor should also provide additional data generated in-house or reference peer-reviewed publication(s) to support the modified editing activity or specificity of their editor. Studies assessing off-target editing activity should be performed using the genome editor proposed in the final drug product. The durability of editor expression is also crucial for safety assessment. For example, the activity of a genome editor delivered as an mRNA molecule using a LNP will likely be short term compared to the same editor delivered using an AAV and this information should be reported as a part of the safety assessment. Additional long-term off-target editing risk assessments should be provided if the editing modality is likely to have sustained long-term expression in cells (e.g., AAV delivered editors, in vivo delivered editors).

VII. CONSIDERATIONS FOR OFF-TARGET EDIT SITE ANALYSIS ACCOUNTING FOR HUMAN GENETIC VARIATION

Individual human genomes harbor several million nucleotide variations. For CRISPR-Cas-based editors, some of these nucleotide variation(s) may either reduce mismatch(es) between a genomic site and a gRNA or may create a PAM sequence resulting in a new genomic site that is potentially amenable to editing. Similarly, for other editing modalities, a variant may reduce mismatch between the target sequence and a genomic off-target edit site making it potentially more amenable to recognition by sequence recognition domain of an editor. In either case, the off-target editing risk will be present in individuals harboring that specific variant. An adequate in silico off-target analysis method accounting for human genetic variation should enable reporting of potential off-target edit sites contributed by variant(s). Specific recommendations and additional considerations for this analysis are provided below.

A. Database selection

Sponsors should choose one or more human genetic variation databases with variant information from healthy and/or patient populations. The variant information should be derived from published publicly available genomic databases or equivalent sources. Adequate scientific justification for using one or more databases should be provided in the context of disease prevalence in the intended population, knowledge of common nucleotide variations, and disease-causing variations.

B. Population stratification

If the GE drug product is used to treat a disease with well-characterized prevalence, then sponsors may consider an in silico off-target edit site nomination strategy that accounts for genetic variability by selecting variants based on allele frequency in a population stratified based on disease prevalence in a specific genetic ancestry. The sponsor should provide scientific justification to support their strategy for using population stratification in their analysis.

C. Variant allele frequency

Inclusion of both common and rare variants present in the genome for the in silico analysis is recommended. The sponsor may use all the variants reported in a database(s) or provide scientific justification for selecting variants that are present above a certain frequency for this analysis. Inclusion of all genic and intergenic variants in this analysis is recommended. The sponsor should provide justification to support their strategy for using variant allele frequency threshold in their analysis.

D. In silico search parameters to identify variant contributed off-target edit sites

The sponsor should use criteria that enables identification of sites where the variant(s) increased homology between the gRNA (or target sequence) and the genomic site either by reducing mismatches and/or by lowering gaps. Additional criteria for CRISPR-Cas-based products should include identifying variants that increased off-target editing potential by creating a PAM sequence.

E. Assessing editing potential at variant contributed off-target edit sites

The sponsor may perform additional analyses to evaluate editing potential at variant contributed off-target edit sites. These analyses may include experimental methods to measure editing rates in samples or genomic samples harboring variant(s) of interest or computational methods that report scores/metrics as a readout for editing potential.

F. Reporting variant contributed off-target edit sites

A detailed report of the off-target edit site analysis accounting for human genetic variation should include the following:

i. A detailed description of the database used, variant frequency thresholds applied, and any population stratification applied with scientific justification.

ii. Specific criteria used to identify variant contributed off-target edit sites.

iii. The results of this analysis should be submitted as an annotated list of variant-contributed off-target edit sites in a tabulated format as a text file, Microsoft Excel file, or CSV as feasible. Please refer to section VI.F for annotation information.

VIII. CONSIDERATIONS FOR NGS METHOD-BASED CHROMOSOMAL INTEGRITY ANALYSIS

For genome editing modalities known to create DNA double-strand breaks and/or medium to large insertions or deletions (or indels), erroneous DNA repair processes at double strand break sites may result in chromosomal translocation event(s) that can impact genomic integrity of cells post-genome editing. In these instances, sensitive and quantitative NGS-based assessment of genomic integrity in edited cells is recommended. The sponsor may consider choosing targeted sequencing and/or primer-based sequencing or other genome-wide NGS-based methods to detect low frequency translocation events and report chromosomal translocation rate(s) between on-target genomic site(s) where double strand break events occurred during GE. We recommend that the sponsor use a sequencing strategy that is sensitive and that minimizes bias when performing this type of analysis (see section IV for NGS considerations). If confirmed off-target edit site(s) were reported from safety analyses of the drug product, then an additional assessment to evaluate potential translocation events between on-target and off-target edit sites should be performed. Please refer to section VI.A for sample selection, IV for sequencing considerations, and VI.F for reporting an annotated list of sites identified from this analysis.

IX. SUBMISSION OF STUDY REPORTS DESCRIBING OFF-TARGET EDITING AND CHROMOSOMAL TRANSLOCATION ANALYSES TO FDA

All the recommended nonclinical off-target editing and chromosomal translocation studies should be performed based on the recommendations made in this guidance and completed prior to the submission of an original IND application. A detailed study report should include all the information recommended in this guidance and should be submitted with the original IND application. The results of the off-target editing and chromosomal translocation studies should also be submitted in recommended format(s) with the original IND application. Studies assessing off-target editing activity by accounting for human genetics variations may not be necessary with an original IND submission in some cases such as when the proposed indication is an ultra-rare disease or when it is intended for the treatment of a single patient. Sponsors are encouraged to discuss their study plan with the FDA prior to the submission of their IND application.

Specifically, sponsors are encouraged to explore meeting options to discuss their strategies assessing off-target editing activity in an INTERACT or pre-IND setting (Refs. 2, 3). The sponsor may provide a brief description of their off-target analysis plan in these submissions. The type of information that may be included in the pre-submission files is summarized in the table below.

Minimum recommended information to be submitted in the indicated submission type.

Submission type	Minimum Information

INTERACT	• Editor used (any modification made to the editor)
	• Mechanism of action (what is known)
	• Nonclinical study strategy to assess off-target editing activity (brief description)
	  • Method(s) for off-target edit site nomination
	  • Method(s) for off-target edit site confirmation
	  • Method(s) for chromosomal translocation analysis

Pre-IND	• Editor used (any modification made to the editor) • Mechanism of action (what is known) • Nonclinical study strategy to assess off-target editing activity (brief descriptions of methods, analysis parameters, criteria used for off-target site nomination/off-target site confirmation/evaluation of chromosomal translocation, or editor specific analyses) • Reports of completed studies assessing off-target editing risk (if available) • Sequencing strategies with specifics about data quality, depth, alignment • Samples used in each analysis

Additional off-target editing analysis may be needed during product development if major changes to the manufacturing process(es) impacts either the editor activity and/or editing rate(s) at on-target site(s) that may potentially impact the off-target edit site profile of the drug product. Furthermore, additional off-target editing analysis may be needed if safety issues are identified. Sponsors are encouraged to explore formal meeting options to discuss strategies to assess the impact of changes in manufacturing process(es) on off-target editing risk of their drug product with the FDA (Ref. 3).

During the clinical studies conducted under an IND or in the BLA submission, the sponsor may need to submit the raw sequencing data and the associated software script(s) used to analyze the sequencing data. The sponsor should discuss this with the FDA during product development and/or prior to the submission of their BLA.

X. REFERENCES

1. Human Gene Therapy Products Incorporating Human Genome Editing; Guidance for Industry, January 2024, https://www.fda.gov/media/156894/download.
2. OTP Pre-IND Meetings, last updated Aug. 2024, https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/otp-pre-ind-meetings.
3. Formal Meetings Between the FDA and Sponsors or Applicants of PDUFA Products; Draft Guidance for Industry*, Sept. 2023, https://www.fda.gov/media/172311/download.

* When finalized, this guidance will represent FDA's current thinking on this topic.