7 U.S. FDA and U.S. NIST Collaboration: Evaluation of Assays and Control Materials for Characterizing Animal Biotechnology Products Generated by Genome Editing

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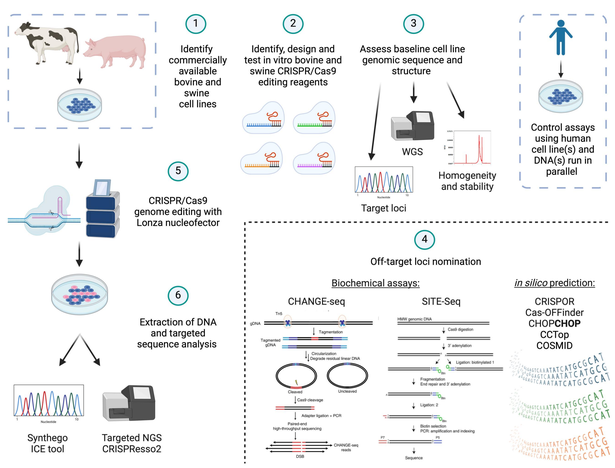
Abstract

Genome editing technology has revolutionized the ability to make targeted changes to an animal's genome (intentional genomic alterations [IGAs]), offering promise for the development of animal biotechnology products that address animal and public health needs. Characterization of these IGAs is an important part of the regulatory process to ensure that the edit to the animal is as intended and to identify any unintended changes. However, there are currently no validated measurements and standards for characterizing unintended edits in animals.

FDA-CVM has established a collaboration with NIST to generate resources for characterizing both intended and unintended alterations in animal biotechnology products resulting from genome editing. These resources will provide developers and FDA regulators with example characterization approaches that they could use as part of the development and regulatory process for IGAs in animals as well as for validating methods, materials, and data.

NIST qualified porcine and bovine cell lines as potential control materials by characterizing their DNA sequences at on-target and potential off-target loci before and after genome editing with multiple guides. The off-target analyses performed include comparisons between off-target loci identified by *in silico* methods and biochemical assays (CHANGE-seq and SITE-Seq), and assessment of editing at nominated loci in edited animal cells. Resulting protocols and datasets will be published and made accessible to developers and the general public. Future work involves generating prototype DNA spike-in control materials used for qualifying DNA sequencing methods that may be used for characterizing animal biotechnology products.

Overview of Resources Generated



Results and Discussion

Characterization of Swine and Bovine Cell Lines and their DNA

NIST selected animal cell lines:
(i) derived from a single animal donor;
(ii) with no restrictions for purchase or research;
(iii) used in multiple publications by the scientific community;
(iv) easily grown in culture.

The cell lines were evaluated for baseline whole genome sequence and genome stability.

No variants were observed at swine or bovine on-target DNA sequences.

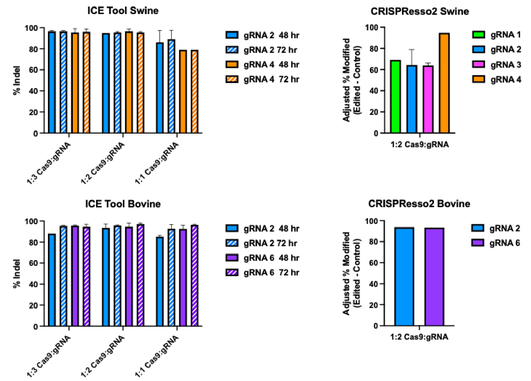
Genomic DNAs from the swine and bovine cell lines as well as a human cell line used as control showed similar stability over time.

Table 1. Genomic DNA stability.

Genomic DNA	Number of Days Stored at -20ºC	Avarage Smear Size (bp)	GQN (Genomic Quality Number) Set at 10 kb
Swine	89	39,685	9.7
Swine	39	39,685	9.5
Swine	0	32,580	9.2
Bovine	265	28,103	9.1
Bovine	61	29,446	9.3
Bovine	37	48,152	9.1
Bovine	0	32,393	9.1
Human	153	36,740	9.4
Human	89	31,038	9.4

CRISPR/Cas9 Genome Editing in Swine and Bovine Cells

Guide RNA (gRNA) sequences targeting swine and bovine genomic loci were obtained from published studies (previously shown to edit relevant targets in swine, gRNA 3 and gRNA 4, or bovine cells, gRNA 5 and gRNA 6) or newly designed to target regions with 100% sequence identity across swine, bovine and human (gRNA 1 and gRNA 2). This cross-species gRNA design enabled their use in control human assays.
Each gRNA was complexed with Cas9 at 1:2 Cas9:gRNA (40 nM:80 nM) ratio and used to cleave relevant DNA substrate (4 nM) generated by PCR amplification of the on-target DNA region in *in vitro* cleavage (IVC) assays or complexed with Cas9 at 1:2, 1:1.2 and 1:3 Cas9:gRNA (3 µM:6 µM, 3 µM:3.6 µM and 3 µM:9 µM, respectively) ratios and introduced into 350,000 cells by nucleofection. Editing efficiencies of Cas9:gRNA complexes were measured by sequencing human, swine and bovine target regions.



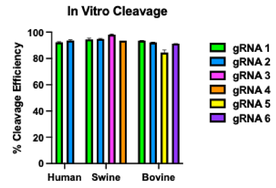


Figure 1. Guide RNAs cleave on-target DNA efficiently. Cleavage efficiency for each gRNA was calculated as the fraction of DNA substrate that was cleaved by each CRISPR/Cas9 ribonucleoprotein (RNP) complex after a 60-minute incubation period with relevant DNA substrates.

Figure 2. Guide RNAs successfully edit on-targets. Genomic DNA was extracted from nucleofected swine and bovine cell lines at the indicated time points and on-target regions were sequenced by Sanger sequencing or targeted next-generation sequencing (NGS). The ICE (Inference of CRISPR Edits) tool (1) was used to compare Sanger sequence traces of on-target amplicons generated from control cells, those nucleofected without RNP, and cells nucleofected with gRNAs. CRISPResso2 (2) was used to analyze NGS data. Editing efficiency of each RNP is expressed as indel percentage in the ICE tool analysis or as adjusted percent modified corresponding to the proportion of modified reads minus the proportion of unmodified or control reads in the CRISPResso2 analysis.

Comparison between Off-target Site Nomination Approaches

Replicated off-target sites nominated by CHANGE-seq and SITE-Seq for cross-species gRNA 2, swine gRNA 4 and bovine gRNA 6 were compared. Similar results were found with human and animal DNA. Concordant off-target sites between assays had normalized read counts closer to the on-target read count while discordant off-target sites between assays had lower normalized read counts.

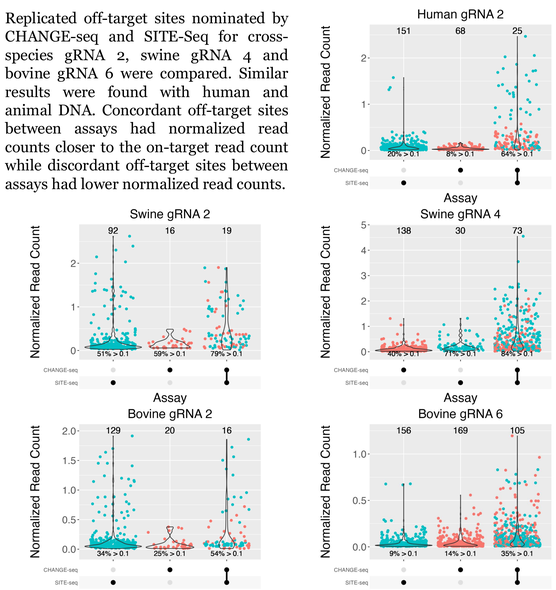


Figure 5. Comparison of off-target nominations. Off-target nominations for gRNAs 2, 4 and 6 from biochemical assays were compared. Violin plots were constructed as indicated for each assay.

CHANGE-seq Nomination of Off-target Sites

CHANGE-seq (3) was successful on swine and bovine DNAs. Cross-species gRNA 2 had similar reproducibility within animal replicates and within human replicates. The discordance observed between replicates occurred at off-target sites that had the lowest normalized read counts while a greater proportion of off-target sites that replicated had normalized read counts closer to the on-target read count, reproducing what NIST has previously observed with human CHANGE-seq assays.

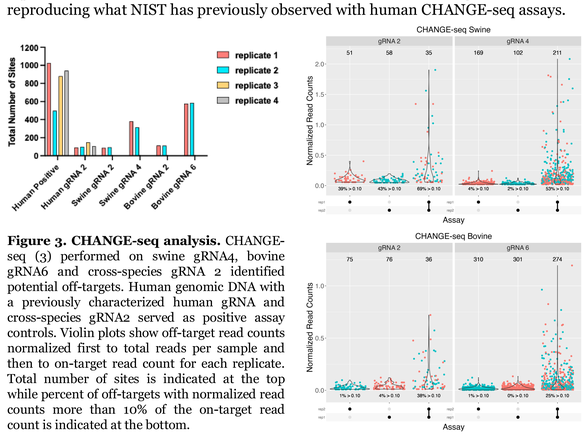


Figure 3. CHANGE-seq analysis. CHANGE-seq (3) performed on swine gRNA4, bovine gRNA6 and cross-species gRNA 2 identified potential off-targets. Human genomic DNA with a previously characterized human gRNA and cross-species gRNA2 served as positive assay controls. Violin plots show off-target read counts normalized first to total reads per sample and then to on-target read count for each replicate. Total number of sites is indicated at the top while percent of off-targets with normalized read counts more than 10% of the on-target read count is indicated at the bottom.

SITE-seq Nomination of Off-target Sites

SITE-Seq (4) was on-boarded using human genomic DNA and cross-species gRNA 2 and then carried out with animal DNA to identify potential off-targets for gRNAs 4, 6 and cross-species gRNA 2. The latter produced similar results on human and animal DNA. Reported off-targets sites were present in all 3 replicates except for one bovine gRNA 2 off-target site which was present in 2 replicates. The proportion of sites with normalized read counts close to the on-target read count varied across gRNAs.

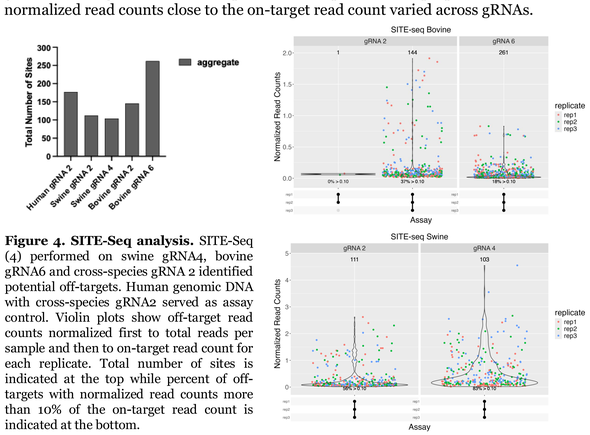


Figure 4. SITE-Seq analysis. SITE-Seq (4) performed on swine gRNA4, bovine gRNA6 and cross-species gRNA 2 identified potential off-targets. Human genomic DNA with cross-species gRNA2 served as assay control. Violin plots show off-target read counts normalized first to total reads per sample and then to on-target read count for each replicate. Total number of sites is indicated at the top while percent of off-targets with normalized read counts more than 10% of the on-target read count is indicated at the bottom.

Conclusions & Future Directions

1. The commercially available animal cell lines characterized in this study were successfully edited with CRISPR/Cas9 RNP.
2. The CHANGE-seq and SITE-Seq off-target assays can be used on swine and bovine DNA with similar assay performance to human DNA, while off-targets nominated by these biochemical assays did not completely overlap and will be evaluated further. Preliminary comparisons indicate that a subset of these off-targets overlap with *in silico* tool predictions.
3. Protocols and datasets will be made public at the completion of this study.
4. Future work will focus on generating spike-in control materials that may be used to qualify DNA sequencing methods relevant to characterizing animal biotechnology products.

References

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