FDA and NIST Collaboration to Evaluate Assays and Control Materials for Characterizing Animal Biotechnology Products Generated by Genome Editing

Patricia Kiesler[1], Natalia Kolmakova[1], Sierra Miller[1], Ayah Shevchenko[1], Stella S. Lee[2], Alexis L. Norris[2], Mayumi F. Miller[3], Adam L. Moyer[2], Samantha Maragh[1]

[1]National Institute of Standards and Technology, Biosystems and Biomaterials Division, Gaithersburg, MD, USA. [2]Office of New Animal Drug Evaluation, US Food and Drug Administration, Center for Veterinary Medicine, Rockville, MD, USA. [3]Office of Applied Science, US Food and Drug Administration, Center for Veterinary Medicine, Laurel, MD, USA

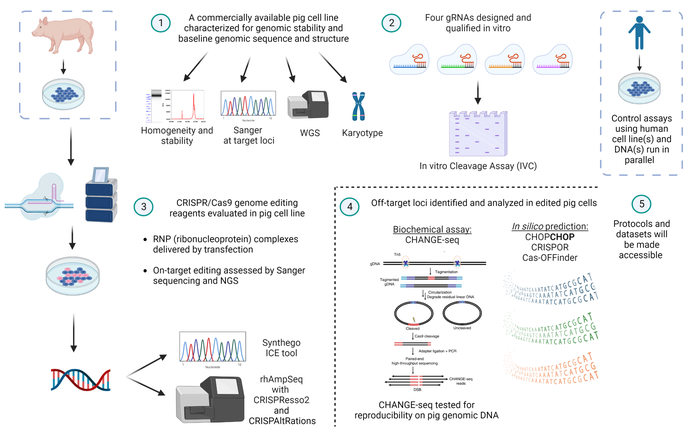
Abstract

Genome editing technology has revolutionized the ability to make targeted changes to an animal's genome (intentional genomic alterations or IGAs), offering exciting 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 intended edit is made to the animal and to identify any unintended changes. However, there are currently no validated measurements and standards for characterizing unintended genomic alterations in animals.

To address these needs, FDA CVM has established a collaboration with the U.S. National Institute of Standards and Technology (NIST) that will generate resources including standardized measurements for characterizing both intended and unintended alterations in animal biotechnology products resulting from genome editing. These resources will provide animal biotechnology product 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/or data. Here, we present preliminary outcomes of this NIST-FDA CVM collaboration.

NIST qualified a commercially available pig cell line and its DNA as potential control materials. The cell line was characterized for genomic stability prior to editing, as well as for sequence before and after genome editing. Four CRISPR/Cas9 editing assays, including two newly developed by NIST, were evaluated using purified pig DNA and the pig cell line. Off-target sites identified from three *in silico* predictors and an existing biochemical assay that detects genomic positions cleaved by genome editing reagents, called CHANGE-seq, were also compared. A subset of the off-target sites identified by *in silico* predictors were also identified by CHANGE-seq and further analyzed for evidence of off-target editing in the edited pig cells. Additionally, the CHANGE-seq assay was evaluated for reproducibility and performed similarly on pig genomic DNA as compared to human genomic DNA. Experimental design, protocols, datasets, and measurements that NIST generated will be published and made accessible to animal biotechnology product developers and the public. Future work will focus on similar qualifications of potential bovine control materials and genome editing assays.

Overview of Resources Generated



Results and Discussion

Characterization of Pig Cell Line and its DNA

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

The pig cell line was evaluated for baseline whole genome sequence as well as genome structure. No chromosomal abnormalities were observed in karyogram analysis.

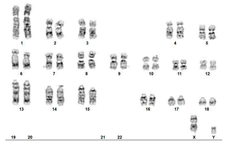


Figure 1. Karyogram analysis.

Genomic DNAs from the pig cell line and a human cell line used as control showed similar stability over time.

Table 1. Genomic DNA stability.

Genomic DNA	Number of Days Stored at -20℃	Avarage Smear Size (bp)	GQN (Genomic Quality Number) Set at 10 kb
Pig	89	39,685	9.7
Pig	39	39,685	9.5
Pig	0	32,580	9.2
Human	153	36,740	9.4
Human	89	31,038	9.4

CRISPR/Cas9 Genome Editing in Pig Cells

Guide RNA (gRNA) sequences targeting four pig genomic loci were obtained from published studies (previously shown to edit relevant targets in Porcine Fetal Fibroblasts [PFFs], gRNA 3 and gRNA 4) or newly designed to target regions with 100% sequence identity across pig 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 1:9 Cas9:gRNA (40nM:360nM in *in vitro* cleavage [IVC] or 1μM:9μM in nucleofection), 1:2 Cas9:gRNA (40nM:80nM in IVC or 1.5μM:3μM in nucleofection) or 1.95:2 Cas9:gRNA (2.9μM:3μM in nucleofection) ratio and used to cleave relevant DNA substrate (4nM) generated by PCR amplification of the on-target DNA region or introduced into 350,000 cells by nucleofection.

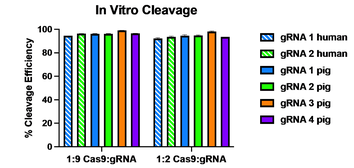


Figure 2. Guide RNAs cleave on-target DNA with high efficiency.

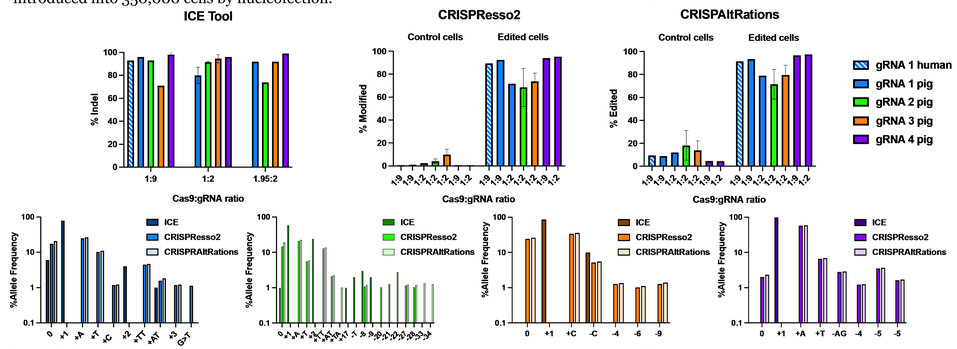


Figure 3. Guide RNAs successfully edit on-targets. On-target regions were sequenced by Sanger sequencing or targeted next-generation sequencing (NGS). The ICE (Inference of CRISPR Edits) tool (1) was used to compare Sanger sequence traces of on-target amplicons generated from control cells, those nucleofected without RNP, and with gRNAs. CRISPResso2 (2) and CRISPAltRations (3) were used to analyze NGS data. Notably, the edits detected at ≥1% frequency by the ICE tool and the NGS analysis tools were highly concordant.

CHANGE-seq Nomination of Off-target Sites

CHANGE-seq was successful on pig DNA. Cross-species gRNA 2 had similar reproducibility within pig replicates and within human replicates. The discordance observed between replicates occurred at coordinates that had the lowest read counts, reproducing what NIST has previously observed with human CHANGE-seq assays.

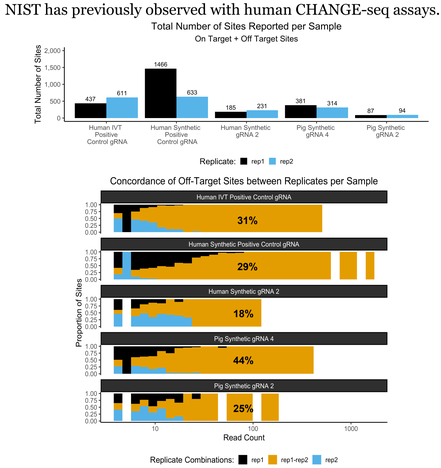
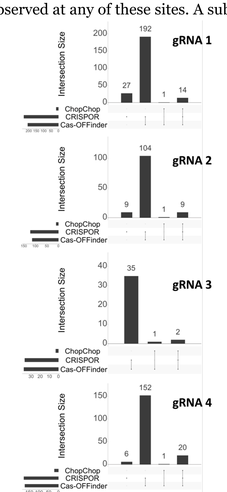


Figure 4. CHANGE-seq analysis. CHANGE-seq (4) performed on pig gRNA 4 and cross-species gRNA 2 identified potential off-targets. Human genomic DNA with IVT (in vitro transcribed) and synthetic control gRNAs served as positive assay controls.

In silico and CHANGE-seq Off-targets

For all guide RNAs, CHOPCHOP off-target sites (with up to 3 mismatches) were also found by Cas-OFFinder and CRISPOR while Cas-OFFinder off-target sites (with up to 4 mismatches) were also found by CRISPOR. The off-target sites that were predicted by the three tools were analyzed via targeted NGS for evidence of off-target editing in edited pig cells. No editing was observed at any of these sites. A subset of the *in silico*-predicted off-targets for gRNA 2 and gRNA 4 were also nominated by both CHANGE-seq replicates for these gRNAs.



In Silico Tool/Assay	Off-Targets with 0-6 Mismatches 0	1	2	3	4	5	6
gRNA 1							
ChopChop	1	0	0	14	-	-	-
CRISPOR	0	0	3	30	200	-	-
Cas-OFFinder	1	0	0	14	192	1530	9773
gRNA 2							
ChopChop	1	0	2	7	-	-	-
CRISPOR	0	0	3	12	107	-	-
Cas-OFFinder	1	0	2	7	104	924	8029
CHANGE-seq rep1	1	0	2	6	12	18	38
CHANGE-seq rep2	1	0	2	3	15	22	37
CHANGE-seq rep1-rep2	1	0	2	3	9	5	8
gRNA 3							
ChopChop	1	0	0	2	-	-	-
CRISPOR	0	0	0	2	35	-	-
Cas-OFFinder	1	0	0	2	35	372	3376
gRNA 4							
ChopChop	1	0	2	18	-	-	-
CRISPOR	0	0	3	22	153	-	-
Cas-OFFinder	1	0	2	18	152	1596	11613
CHANGE-seq rep1	1	0	1	13	47	113	133
CHANGE-seq rep2	1	0	1	12	44	85	114
CHANGE-seq rep1-rep2	1	0	1	12	35	61	56

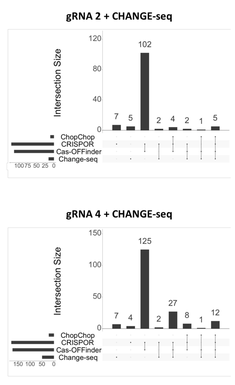


Figure 5. Comparison of off-target predictions. Off-target predictions for gRNAs 1-4 from three *in silico* tools CHOPCHOP (5), CRISPOR (6) and Cas-OFFinder (7) were compared to each other. Off-target predictions for gRNAs 2 and 4 were also compared to CHANGE-seq nominations for these gRNAs.

Conclusions & Future Directions

1. The commercially available pig cell line characterized in this study was successfully edited with CRISPR/Cas9 RNP.
2. The CHANGE-seq off-target assay can be used on pig DNA with similar assay performance to human DNA, while off-targets nominated by *in silico* tools vs. CHANGE-seq did not completely overlap and will be evaluated further.
3. Protocols and datasets will be made public at the completion of this study.
4. Future work will focus on similar qualifications of potential bovine control materials and genome editing assays.

References

1. Conant D. et al., CRISPR J. 2022, 5(1): 123-1301
2. Clement K. et al., Nat Biotechnol. 2019, 37(3): 224-226
3. Kurgan G. et al., Mol Ther Methods Clin Dev. 2021, 21: 478-491
4. Lazzarotto C. et al., Nat Biotechnol. 2020, 38(11): 1317-1327
5. Labun K. et al., Nucleic Acids Res. 2019, 47(W1): W171-W174
6. Concordet JP. et al., Nucleic Acids Res. 2018, 46(W1): W242-W245
7. Bae S. et al., Bioinformatics. 2014, 30(10): 1473-1475