

Use of xGen® Lockdown® Probes for NGS Capture Enrichment to Assess CRISPR Gene Editing Events

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Introduction

Characterization of on- and off-target CRISPR/Cas9 gene editing events requires further investigation even as the *in vivo* application of this technology has become commonplace. Here, we investigate CRISPR gene editing using both Sanger and next-generation sequencing (NGS) methods to evaluate on-target and off-target effects (OTEs). The scope of OTEs needs to be easily queried by the investigator, and methods to minimize their occurrence implemented. A custom enrichment panel for NGS was designed using xGen® Lockdown® Probes (IDT) to capture on-target as well as potential sgRNA off-target sites.

Methods

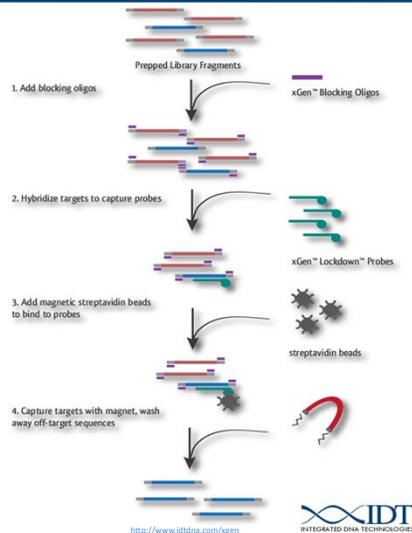
HEK293 cells stably expressing *S. pyogenes* Cas9 were transfected (48 hrs) with either gBlocks® Gene Fragments (dsDNA) containing a U6 promoter-driven sgRNA targeting *HPRT1* or a ssRNA *in vitro* transcription (IVT) product of the chimeric *HPRT1* sgRNA. Genomic DNA was purified and used for NGS library prep.

CLONING/SANGER SEQUENCING—PCR products from genomic DNA amplification were blunt cloned into One Shot TOP10 Chemically Competent Cells (Invitrogen). Primers flanking the insert were used to sequence clones from the *HPRT1* target region to interrogate on-target editing events using Sanger BigDye Terminator sequencing (ABI).

NEXT-GENERATION SEQUENCING—Libraries were prepared from transfected HEK293 cells (as well as various negative controls), enriched by hybridization/capture using the HPRT-OTE xGen® Lockdown® panel. Sequencing was performed as an 8-plex on an Illumina miSEQ instrument.

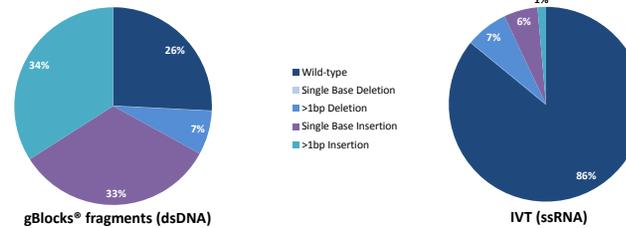
PANEL DESIGN—Probes were made for all sites (460) in the human genome having at least a 13/20 match to the guide RNA targeting site in *HPRT1*. The 120 nt Lockdown® probes targeted all of these theoretical off-target sites. An additional 250 xGen® Lockdown® Probes were included in the panel to capture the entire *HPRT1* locus.

xGen® Lockdown® Probes



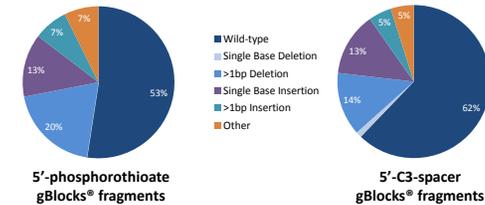
On-Target CRISPR Activity

Sanger sequencing of *HPRT1* showed a high frequency of gene editing (26% remaining wild-type) at the target site in DNA isolated from cells transfected with the *HPRT1*-targeting gBlocks®. IVT *HPRT1* sgRNA transfections were far less effective (86% remaining wild-type), which we suspect was largely due to the observed ssRNA innate-immune-induced toxicity and cell death.



Primer modifications: reducing NHEJ insertions

The CRISPR gBlocks® fragment expression cassettes (U6-driven sgRNA) are PCR amplified to increase mass prior to transfection. In order to reduce enzymatic processing and/or recombination events near the ends of the amplicons (hypothesized to contribute to insertion of transfected DNA into the breakpoint via NHEJ), the 5'-ends of the primers were protected by either phosphorothioate linkage (PS) modification or were end-blocked using a 5'-propanediol (C3 spacer). Modified gBlocks®-derived PCR products were transfected and Sanger sequencing performed as before. Compared to the unmodified gBlocks® fragments, the incidence of large insertions was reduced.



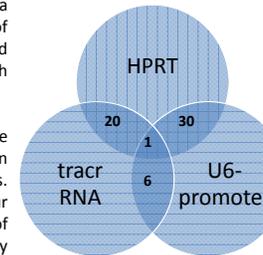
Percentage of insertions that contain fragments of the transfected sgRNA expression cassette

DNA gBlocks fragments	45%
5'-PS gBlocks fragments	24%
5'-C3 gBlocks fragments	7%

NGS: On-Target CRISPR Activity

NGS results at the *HPRT1* target site agreed with the Sanger data in the top panel. The large insertions created by repair of CRISPR/Cas9 cleavage included fragments of the transfected dsDNA sgRNA expression cassette, as confirmed by both sequencing methods.

Initially, NGS data analysis was complicated by the nature of the CRISPR gene editing products, which comprise insertion/deletion events ranging from a single nucleotide to several hundred bases. The Sanger results directed our analysis of the NGS data, and our bioinformatics pipeline was modified to detect these kinds of gene editing events without implementing a computationally heavy split-read aligner.



Of 2875 reads that contained partial match sequence to the reference genome at the expected target site in *HPRT1*, many of the larger insertions can have what appears to be part of the transfected DNA expression cassette at the expected site of double-stranded DNA cleavage mediated by Cas9, presumably due to insertion by NHEJ.

Future Directions

The data analysis tools to detect on-target gene editing events are valuable as we move forward with an off-target panel of xGen® Lockdown® Probes. In particular, the xGen® panel will enrich for potential CRISPR off-target sites, and the improved bioinformatics pipeline can detect reads that contain partial match sequence to the reference genome as well as the characteristic large insertion/deletion events. This approach comprises a rapid method for using NGS to survey off-target CRISPR gene editing events throughout the genome.

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