APPLICATION NOTE

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Verifying CRISPR editing with Xdrop® enables the detection of unintended off-target rearrangements

Summary

- Conventional PCR screening for CRISPR–Cas9 editing can miss large deletions and complex rearrangements.
- Here, we show how Xdrop indirect sequence capture facilitates the identification and elucidation of an unintended and otherwise undetected insertion.

Introduction

Current CRISPR–Cas9 editing checkpoints focus on relatively small unintended edits in the immediate vicinity of intended modifications. However, large deletions and complex rearrangements can occur.[1, 2] Conventional PCR screening strategies can easily overlook such changes, particularly if they affect only one of the alleles.

Xdrop supports the verification of CRISPR editing. It enables the confirmation of bioinformatically predicted complex off-target edits and unintended rearrangements (including indels) around the CRISPR cut site. The workflow requires very small amounts of input DNA and the design of only a single standard primer pair. The primer set can be designed kilobases away from the gene editing site, thus avoiding even kilobase-long rearrangements.

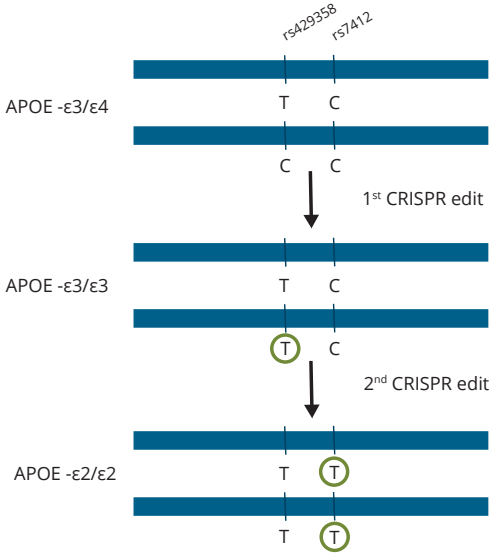

Figure 1. An overview of the edits to Exon 4 of APOE-ε3/ε4 that generated the two homozygous cell lines.

Cell line genome enrichment

Four isogenic cell lines designed as model systems for Alzheimer's disease were created from BIONi010-C, a cell line with the genotype ε3/ε4 for the *APOE* gene on chromosome 19.[3] Two of the novel cell lines were constructed by editing two single-nucleotide positions 139 bp apart in exon 4. First, rs429358 was edited to create a homozygous ε3/ε3 cell line; then rs7412 to create a homozygous ε2/ε2 cell line (Figure 1).

The Xdrop droplet PCR (dPCR) workflow was used to encapsulate DNA from the four cell lines in double-emulsion droplets together with a primer set (the detection sequence) targeting 129 bp on chromosome 19 (position: 44,906,188–44,906,317).

After dPCR, the droplets were stained with a fluorescent dye (Samplix droplet dye) and sorted on a SONY® SH800S cell sorter. Roughly 0.01% of the droplets were positive for the target DNA and were easily gated and sorted.

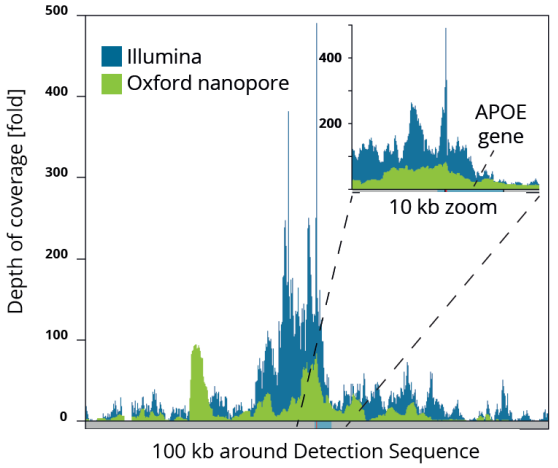

Figure 2. Depth of coverage of Illumina (blue) and Oxford Nanopore (green) sequencing data (sample ε2/ε2). The main graph focuses on the 100kb surrounding the detection sequence designed to capture the APOE gene region. The inset zooms in on the central 10 kb surrounding the detection sequence.

The long target DNA sequences were released into solution and re-encapsulated in single-emulsion droplets for amplification via droplet MDA (dMDA). The amount of enriched DNA subsequently released from these droplets was measured via fluorometry. Fragment size was evaluated on a TapeStation™ (Agilent®).

Library preparation and sequencing

DNA libraries were prepared directly from the enriched DNA samples and sequenced on an Oxford Nanopore® MinION® and on an Illumina® iSeq™.

Enrichment estimation and sequencing coverage

The enrichment with Xdrop and subsequent short- and long-read sequencing enabled the examination of ~100 kb around the edited region with an average enrichment of ~200x. Figure 2 shows the read coverage for both sequencing platforms for a 100 kb and a 10 kb region around the detection sequence.

Detection of unintended edits

Analysis of the Xdrop-enriched fragments detected an unintended insertion of approximately 3.4 kb near the edited region in APO-ε2/ε2 and APOE-ε3/ε3. A fragment from a modified pEasy Flox plasmid containing an insert of the human MAPT gene was in positions 44,908,822–44,908,829 of chromosome 19.[3] This plasmid had been used to co-transfect the cell lines with resistance to neomycin so that the nucleofected cells could be selected. The plasmid should not have been present in the final cell lines nor integrated into their genomes.

Other assessment methods had not detected the insertion because it affected only the C–C haplotype. The original primers used to validate editing amplified a 227 bp region that included the two edited single-nucleotide positions. With the insert, the primer distance expanded to >3.5 kb, preventing amplification with the standard PCR design.

Since the T–C haplotype was still present, its amplification and analysis gave the impression that C-C was correctly modified and that the genotype was T/T–C/C (Figure 3). This insertion occurred in the APOE-ε3/ε3 cell line and was passed on undetected to the constructed APOE-ε2/ε2 line.

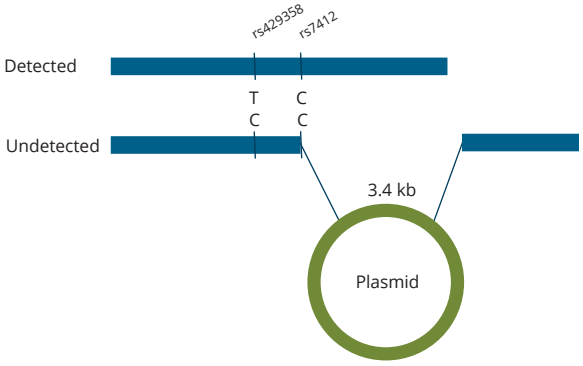

Figure 3. A representation of the plasmid insert affecting the C-C haplotype but not the T-C haplotype.

Conclusion

Indirect sequence capture using Xdrop facilitated the identification and elucidation of an unintended and otherwise undetected insertion that occurred on only one of the alleles. By using easy-to-use microfluidics, Xdrop enriches and amplifies a genomic region of interest (ROI) up to 100 kb in length for downstream long- or short-read library preparation and sequencing.

For more information about Xdrop products and applications, visit samplix.com.

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References

1. Kosicki, M., et al. 2018. Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. Nature Biotech. 36(8): 765–771.

2. Tan, E.P., et al. 2015. Off-target assessment of CRISPR-Cas9 guiding RNAs in human IPS and mouse ES cells. Genesis 53: 225–236.

3. Schmid, B., et al. 2019. Generation of a set of isogenic, gene-edited IPSC lines homozygous for all main APOE variants and an APOE knock-out line. Stem Cell Res. 34:101349.

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