APPLICATION NOTE

Samplix

Localizing lentiviral transduction-inserted CAR cassettes in T cells using Xdrop®

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Summary

- Starting from just 10 ng of DNA, Xdrop reveals gene cassettes inserted using lentivirus and other transduction systems.
- Here, Xdrop discloses 1,000 unique insertion sites of the CAR cassette all over the genome.

Introduction

Safety and efficacy are key considerations in engineered cell therapies. Any technology that modifies the genome has the potential to cause genotoxicity.[1,2] The accuracy of editing and the potential occurrence of unintended rearrangements should be assessed to understand the associated risks, such as cassette insertion in the vicinity of an oncogene or a tumor suppressor. However, conventional PCR screening for editing outcomes can overlook such unintended insertions.

The Xdrop-based workflow is proven to overcome this limitation to identify the precise localization of the CAR insertion. What's more, it requires just 10 ng of DNA as starting material. This low requirement for the scarce biological material makes Xdrop relevant during engineered cell development, after cell expansion, for post-transfusion monitoring, and in unexpected clinical event investigation.

Here we demonstrate how Xdrop was applied to identify ~1000 CAR cassette insertion sites in a lentiviral CAR T cell sample using long read sequencing to yield high quality candidate integration sites, which was validated by Sanger sequencing of PCR amplified CAR cassette border regions.

Experimental setup

The detection sequence is defined by a single primer pair and was designed using the sequence of the CAR cassette. DNA extracted from CAR T cells (CD19scFv-CD28-4-1BB-CD3ζ, PM-CAR1003-1M, Amsbio Ltd.) was used for Xdrop enrichments. The DNA was sequenced using Oxford Nanopore® technology.

The sequencing results were analyzed following Samplix recommendations for insert data analysis.

Genome wide CAR insertions

We performed six Xdrop enrichments, each from 10 ng DNA, and sequenced the enriched DNA in pools of two to produce 11.5 Gb of data. The analysis revealed ~1,000 CAR cassette insertion sites (Figure 1). The insertion sites are on all chromosomes, with very few in regions with low gene density.

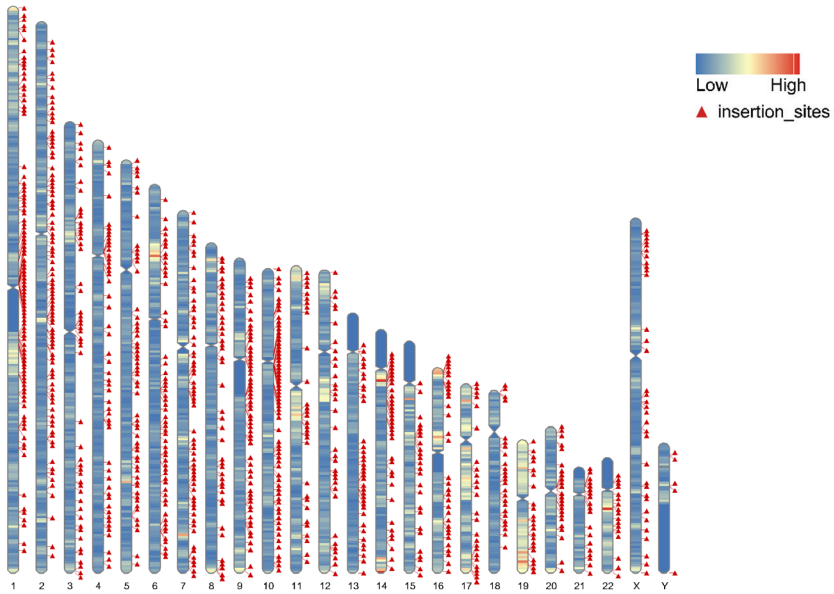

Figure 1. Identified CAR cassette insertion sites (red triangles). Many of the sites are found in gene dense regions (low gene density: blue, high gene density: red). Fewer insertions are found in regions that have low gene density and are thus less transcriptionally active, such as the short arms of Chr 13, Chr 14, and Chr 15.

CAR insertions near genes

Insertional mutagenesis is the main concern when using viral vectors with semi-random integration. Of the ~1000 integration events identified here, 64% were found close to genes including promoters (Figure 2). Disruptive integration within oncogenes and tumor suppressors is obviously very problematic, but altered expression of other genes induced by insertions into promoters and other regulatory elements may also have critical impact.

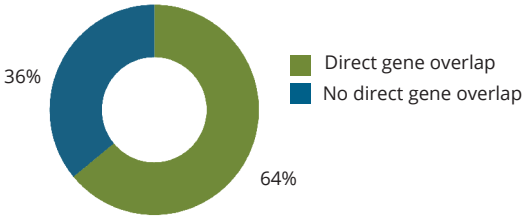

Figure 2. Insertion sites are in many cases close to genes. This figure is based on the overlap of an Oxford Nanopore read with an annotated gene (NCBI).

High heterogenicity among CAR T cells

The data was analyzed both per pool and combined to investigate the heterogenicity of the CAR T cells. The limited overlap between the pools shows high heterogenicity among the CAR T cells (Figure 3). This reflects the need to analyze many cells to understand viral insertion patterns.

Validation of borders by PCR and Sanger sequencing

We also analyzed the number of reads per identified border. This shows that most borders are supported with 1 to 10 reads. We therefore decided to validate several borders using PCR and Sanger sequencing. Primers were designed to match the cassette and the flanking genomic region. PCR was performed on the heterogenic sample, aiming at one specific band, which could be Sanger sequenced (Figure 4). Specificity of the PCR was difficult to obtain probably due to the high number of CAR cassettes and the low amount of clonality meaning the border was potentially no longer present. We validated 18 out of the 39 borders. Borders with down to one long Oxford Nanopore read were validated like this (Figure 4).

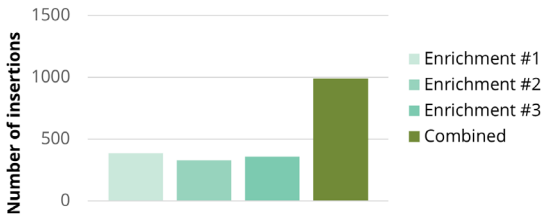

Figure 3. Each enrichment (1 to 3) revealed unique insertion sites. Thus, the combined data set is close to equal to the sum of enrichment 1 to 3. This shows that a high variability of insertions exists between cells.

Conclusion

Xdrop can reveal transgene insertion sites in both homogenic and heterogenic samples, starting from just a few nanograms of DNA. The technology can readily be applied to increase the understanding of edits introduced via viral integrations, but also those occurring using more targeted systems as CRISPR and TALEN.

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

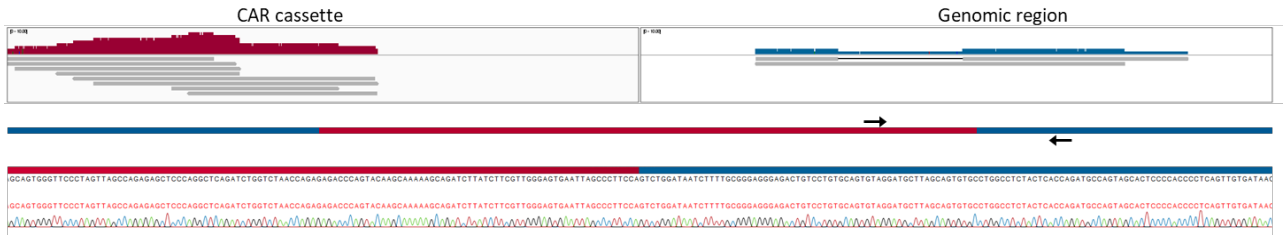

Figure 4. Insertion sites were validated using PCR and Sanger sequencing. Top: Reads were mapped to the CAR cassette and genome. One read (grey) was found to map primary and supplementary to the start of CAR cassette and a genomic region on Chr 9. This indicated a reverse insertion of the CAR cassette in Chr 9. Primers were designed to generate a PCR product over the border (middle). The resulting PCR product was sequenced using Sanger sequencing and a match read across the border was generated (bottom) validating the insertion in the genomic region.

References

1. Schubert, M.-L., et al. 2021. Side-effect management of chimeric antigen receptor (CAR) T-cell therapy. Annals of Oncology. 32(1): 34–48.

2. Nobles, C.L., et al. 2020. CD19-targeting CAR T cell immunotherapy outcomes correlate with genomic modification by vector integration. J. Clin. Invest. 130(2): 673–685.

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