

Use Single-Stranded DNA Donor Templates (ssDNA or ssODNs) for CRISPR Homology Directed Repair (HDR) Mediated Gene Knock-In

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High gene knock-in efficiency and reduced off target integration with single-stranded DNA HDR template

Highlights

By combining the advantages of CAR-T cell therapy with CRISPR/Cas9 technology, it is now possible to insert large genes at specific genetic sites in primary T cells without using viral vectors. This case study demonstrates the benefits of using long single-stranded DNA (ssDNA or ssODN) as non-viral CRISPR HDR templates in T cell engineering, including reduced cell toxicity, high gene knock-in efficiency, and reduced off target integration.

To make ssDNA more readily available for cutting-edge researchers, GenScript has developed proprietary enzymatic approaches for producing ssDNAs with non-detectable levels of dsDNA and minimum DNA base damage. All final deliverables are 100% sequence verified and delivered with flexible quantity options, making CRISPR based gene knock-in easier than ever.

Learn more @ www.genscript.com/new-single-stranded-dna-synthesis-service.html

Background

Over the past decades, cancer remains the second leading cause of death in the US. Recent advances and successes in chimeric antigen receptor T cells (CAR-T) cell therapy have demonstrated its great potential for treating various cancers, especially blood cancers. However, engineering T cells precisely for therapeutic purposes has been challenging. Traditionally, lentiviral and retroviral vectors are used for chimeric antigen receptors (CARs) expression, which are not targeted to specific genetic locus. With the maturation of CRISPR/Cas9 technology, it is now possible to insert large genes at specific genetic sites in human primary T cells for CAR expression without using viral vectors.

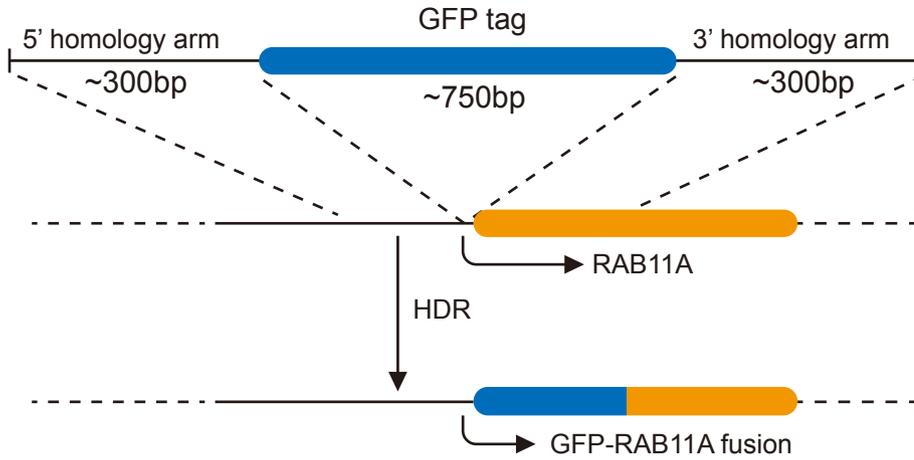
CRISPR/Cas9 technology is commonly used to create precise double stranded breaks (DSBs) at target DNA sites. The guide RNA (gRNA) recognizes the protospacer adjacent motif (PAM) sequence on the target DNA after forming complex with Cas9, then Cas9 exerts its endonuclease function to cause DSBs. This triggers two mechanisms for repair: one is non-homologous end-joining (NHEJ), which introduces mutations in the DSB site. The other mechanism is error-free homology directed repair (HDR) which enables the donor DNA to be inserted at the break site and create gene knock-ins.

Double-stranded DNA (dsDNA) was traditionally used as HDR donor DNA templates, however, recent studies demonstrated that single-stranded DNA (ssDNA or ssODN) is the best HDR templates for CRISPR based gene insertion, replacement, and correction. When compared to double-stranded DNA donors, ssDNAs demonstrated significantly improved editing efficiency and specificity, as well as reduced off-target integration, especially in editing primary cells, stem cells, and developing transgenic animal model.

In this case study, the knock-in efficiency and off-target rates of using ssDNA or dsDNA as HDR templates were evaluated.

a

HDR template design: GFP insertion in RAB11A



b

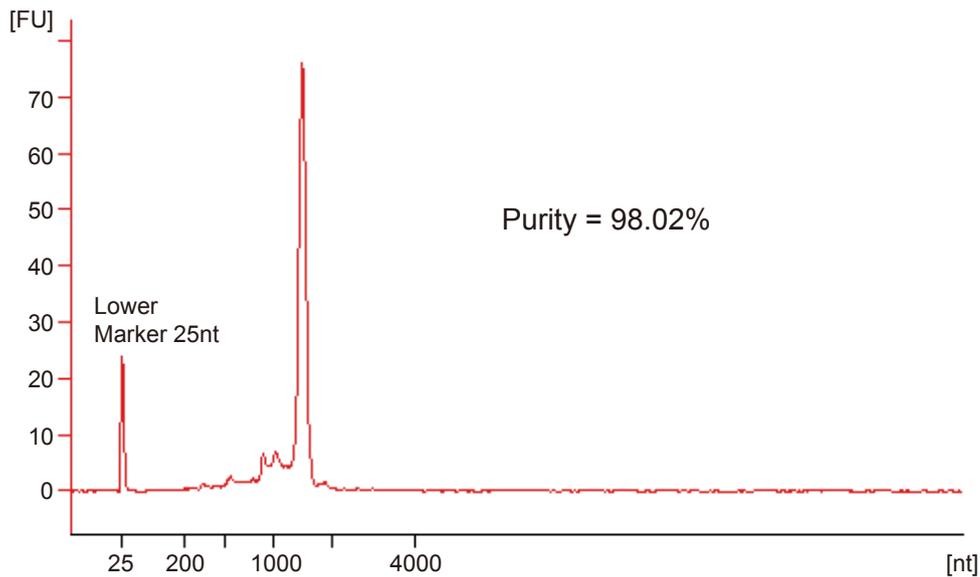


Figure 1. CRISPR homology directed repair (HDR) template design. **a**, Both dsDNA and ssDNA HDR template were designed to integrate an N-terminal green fluorescent protein (GFP) fusion tag to a house-keeping gene, RAB11A. HDR dsDNA templates were amplified from sequence verified plasmids containing the GFP insert and homology arms. ssDNA templates were provided [by GenScript GenCRISPR ssDNA synthesis service](#). Figure 1a was modified from Roth, et al., Nature 559 (2018) 405–409. **b**, GenScript ssDNA product purity was detected using Agilent 2100 Bioanalyzer and purity was calculated by dividing the % of peak area under the curve over total corrected area. Results showed that the final ssDNA product purity was greater than 98%.

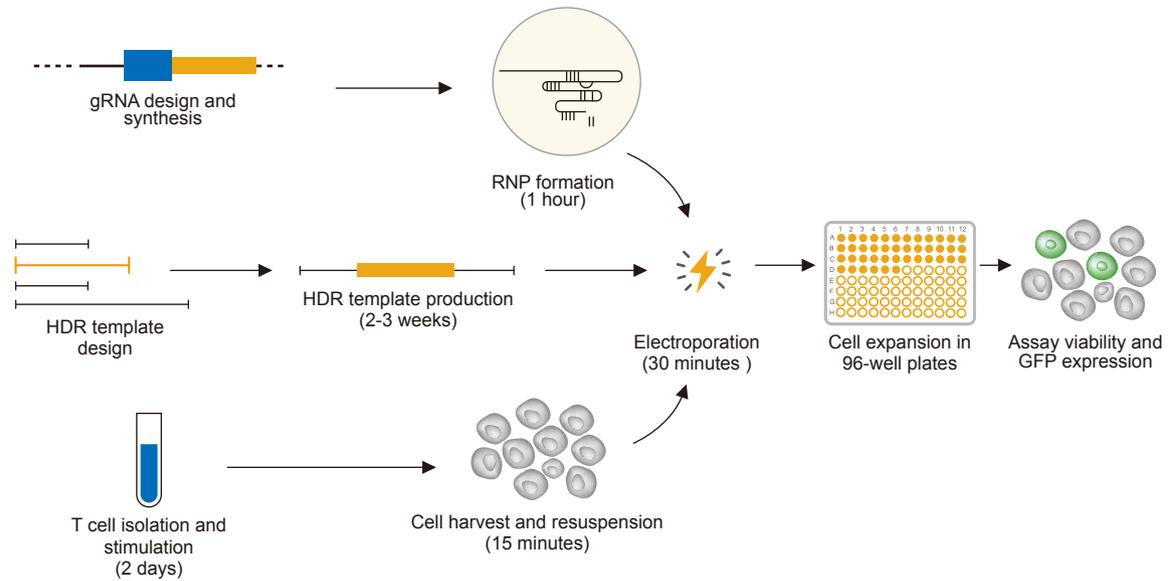


Figure 2. Workflow for electroporation. Primary human T cells were isolated from blood and stimulated two days before electroporation. HDR templates were first added to 96-well plates, then the ribonucleoprotein (RNP) complex, consisting of sgRNA and Cas9 protein, were added to the HDR templates. After 30 seconds incubation at room temperature, cells suspended in electroporation buffer were added and mixed with the HDR templates and RNP by pipetting. After electroporation, engineered T cells can be readily expanded for downstream measurement. Figure modified from Roth, et al., Nature 559 (2018) 405–409.

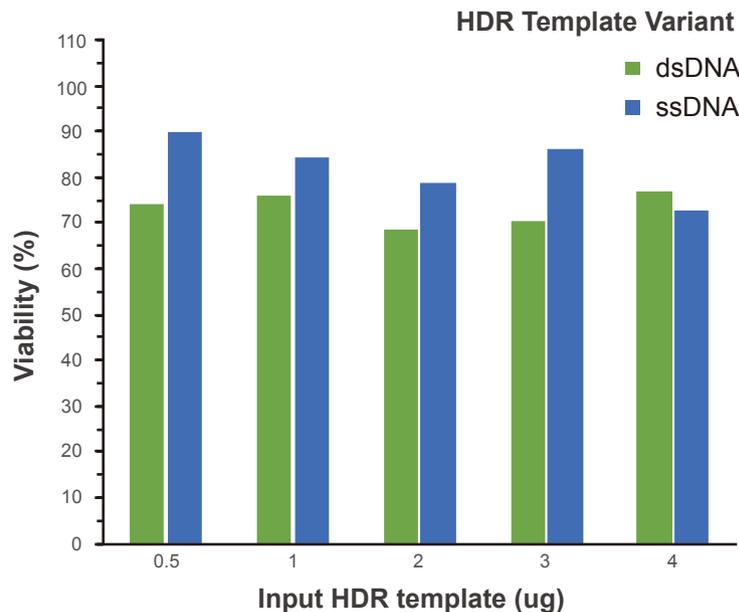


Figure 3. ssDNA template demonstrated less cytotoxicity compared to dsDNA templates. Cell viability was measured 2 days after electroporation with either ssDNA or dsDNA HDR templates. Overall, cells preserved high viability after electroporation with either templates. With 0.5ug to 3 ug HDR templates, ssDNA groups showed higher viable cell numbers compared to dsDNA groups. With 4ug HDR template, cell viability was similar between dsDNA and ssDNA groups.

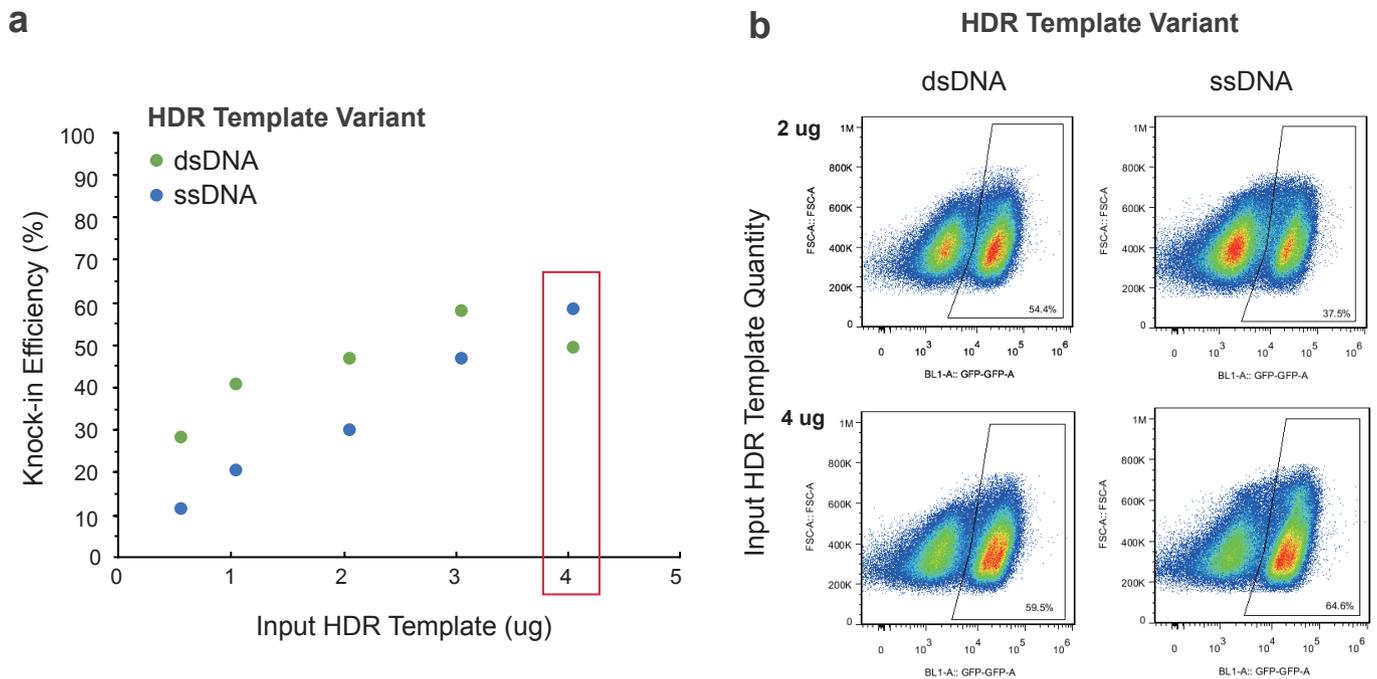


Figure 4. ssDNA templates demonstrated similar knock-in efficiency as dsDNA templates. To test the knock-in efficiency of different HDR templates, GFP expression was measured 4 days after electroporation. Results showed that with increasing amount of HDR template, either dsDNA or ssDNA, the knock-in efficiency increases. Despite higher knock-in efficiency was observed with 3 ug of DNA template or less for dsDNA, ssDNA template exhibits higher knock-in efficiency at 4 ug level.

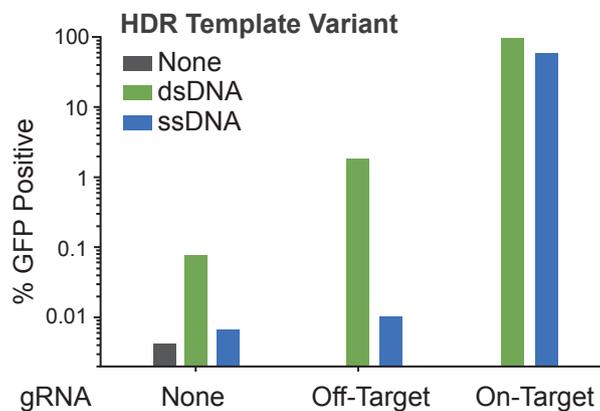


Figure 5. ssDNA templates reduced off target integrations compared to dsDNA. Off target GFP integration analysis demonstrated that dsDNA template induced significantly higher off target integration compared to ssDNA template. In fact, the off target integration rate of ssDNA template was reduced to the limit of detection, which is similar to the level of the control group with no gRNA and Cas9 protein electroporated. Figure from Roth, et al., Nature 559 (2018) 405–409.

Conclusion

This case study demonstrated the advantages of using long ssDNA as non-viral HDR templates in T cell engineering, including reduced cell toxicity, high gene knock-in efficiency, and reduced off target integration compared to traditional dsDNA templates.



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