DNASCRIPT

APPLICATION HIGHLIGHT

Guide RNA Production

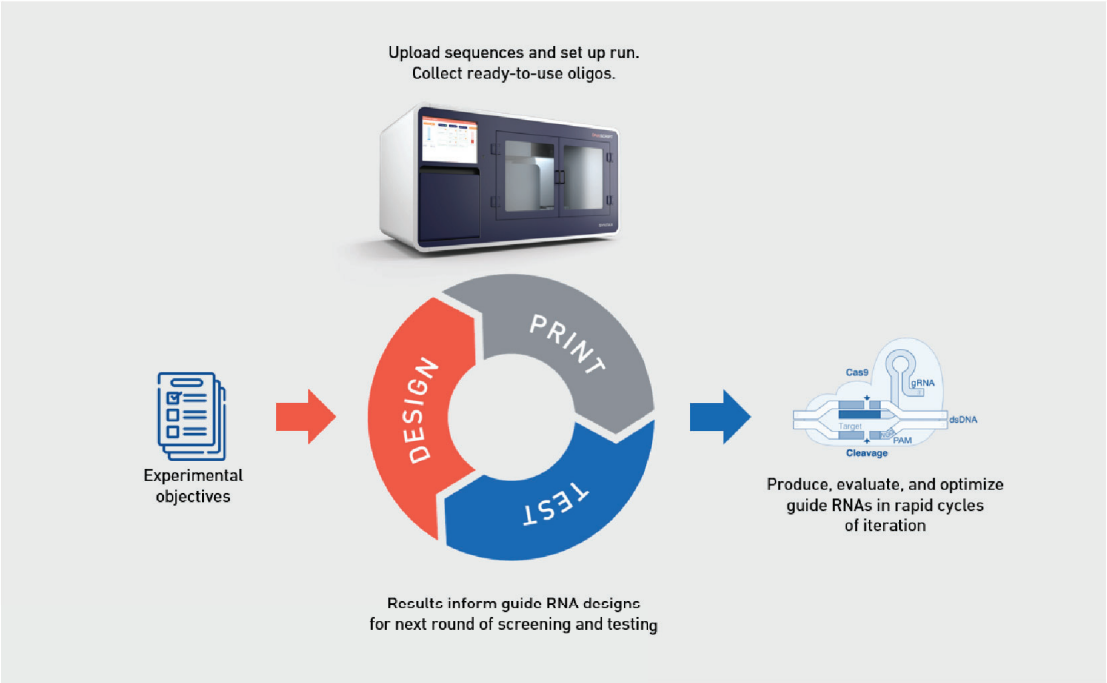
DNA Script has developed novel Enzymatic DNA Synthesis (EDS) technology[1] that enables in-house synthesis of DNA oligos with an easy-to-use benchtop instrument. Guide RNAs prepared with the SYNTAX System have been demonstrated to support CRISPR/Cas9-based gene editing. EDS enables rapid production and screening of guide RNAs, with full control over sequence information.

INTRODUCTION

The past decade has seen rapid and widespread adoption of CRISPR/Cas9-based methods for genome editing,[2] in biological systems ranging from cultured cells to entire animals. The targeting specificity of CRISPR/Cas9 is determined by 20 nt of sequence at the 5'-end of a so-called "guide RNA." In vitro methods for the production of different components of the CRISPR/Cas9 system are gaining popularity as they allow for high-throughput production and screening of functional Cas9-ribonucleoprotein (RNP) complexes.[3]

DNA Script's EDS-powered SYNTAX System is ideally suited for the synthesis of single guide RNAs (sgRNAs) for in vitro Cas9-RNP preparation and screening. Custom synthesis plates with the T7 promoter sequence in the initiator DNA (iDNA)[1,4] enables the production of 96 desalted, normalized, ready-to-use CRISPR RNA (crRNA) oligos in a standard workday. This capability accelerates the production, screening, and optimization of sgRNAs in rapid cycles of iteration, each only requiring a few days.

In a simple experimental model, crRNAs produced by EDS performed comparably to those ordered from a commercial supplier.



DISCUSSION

The CRISPR/Cas9 system requires two components: a Cas9 nuclease that cleaves the target sequence and a single guide RNA (sgRNA) that directs the nuclease to the targeted cleavage site. The sgRNA is composed of two parts: a target-specific CRISPR RNA (crRNA) sequence and a trans-activating crRNA (tracrRNA) scaffold sequence that is common to all sgRNAs.

Successful in vivo gene editing depends on delivery of the Cas9/sgRNA complex to the target genome. This is achieved by either expressing the Cas9 and sgRNA in target cells or by pre-assembling the Cas9-ribonucleoprotein (RNP) complex in vitro and delivering this to the target cells.

A second strategy (outlined in Figure 1) is gaining popularity, as it does not require cloning and allows for high-throughput production of fully functional Cas9-RNP complexes that are active upon delivery and are quickly degraded, thereby reducing the potential for off-target effects.[3]

In an initial proof-of-concept experiment, designed to determine whether oligos produced by EDS are of sufficient quality to support in vitro sgRNA production, crRNA sequences were designed to delete the kanamycin resistance (Kan^R) and lacI promoter cassettes from a commercial plasmid. For each of the cut sites, multiple crRNA oligos were designed with the Geneious Prime software.

The unique ability of the SYNTAX System to extend existing ssDNA[2] was utilized for crRNA synthesis by including the 20-nt T7 promoter sequence in the initiator DNA (iDNA). De novo synthesis commenced immediately after the iDNA, starting with a G for all oligos. This was followed by (i) 19 or 20 nucleotides unique to the target site, and (ii) the 14-nt complement of the 5'-portion of the tracrRNA, to yield full-length crRNA oligos of 54 – 55 nt.

For comparison, full-length crRNA oligos were ordered from a commercial supplier.

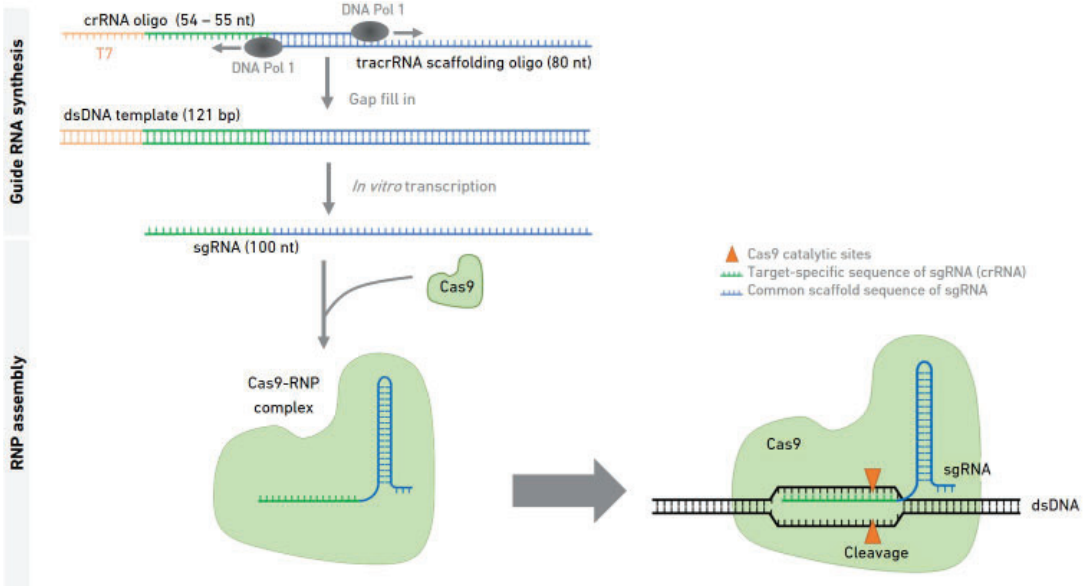


FIGURE 1. SCHEMATIC REPRESENTATION OF IN VITRO CAS9-RNP PRODUCTION. Partially overlapping crRNA (orange/green) and tracrRNA (blue) oligos are extended using DNA polymerase I, creating a 121-bp dsDNA template with a T7 promoter sequence (orange) for in vitro transcription (IVT). The IVT reaction yields the single-stranded sgRNA, which is purified and incubated with Cas9 protein to form the active Cas9-RNP complex that is delivered to target cells. The target-specific sequence of the crRNA must always start on a G to ensure efficient transcription with T7 RNA polymerase and is included prior to synthesis if not present in the oligo design.

RESULTS AND CONCLUSION

The EnGen® sgRNA Synthesis Kit, S. pyogenes (New England Biolabs) was used for in vitro transcription of sgRNAs, which were purified using the Monarch® RNA Cleanup Kit (New England Biolabs). All crRNA oligos were used in combination with the tracrRNA oligo supplied in the EnGen kit, and yielded single-stranded sgRNAs with the expected length of 100 nt (Figure 2).

Cas9-RNP complexes were assembled and editing of the pET-28(+) plasmid (Novagen) was performed according to standard protocols included with the EnGen kit. Results are shown in Figure 3. No difference in the efficiency of sgRNA production or the outcome of nuclease assays was observed for crRNA oligos produced by EDS vs. those obtained from a commercial supplier (CS).

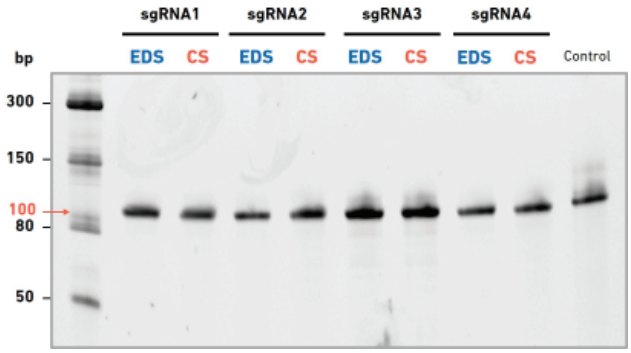


FIGURE 2. ELECTROPHORETIC ASSESSMENT OF SELECT SGRNAS PRODUCED BY IVT USING EDS OR COMMERCIAL (CS) CRRNA OLIGOS. Guide RNAs (100 nt) were produced as described above. sgRNA1 and sgDNA2 target the 3'- and 5'-ends of the Kan^R cassette of the pET-28(+) plasmid, respectively; whereas sgRNA3 and sgRNA4 target the 3'- and 5'-ends of the lacI cassette. The Control lane contains an sgRNA produced with the control crRNA oligo provided in the EnGen kit. Electrophoresis was performed using denaturing polyacrylamide gels (10% TBE-Urea; Novex) and RNA was visualized with GelRed® stain (Biotium).

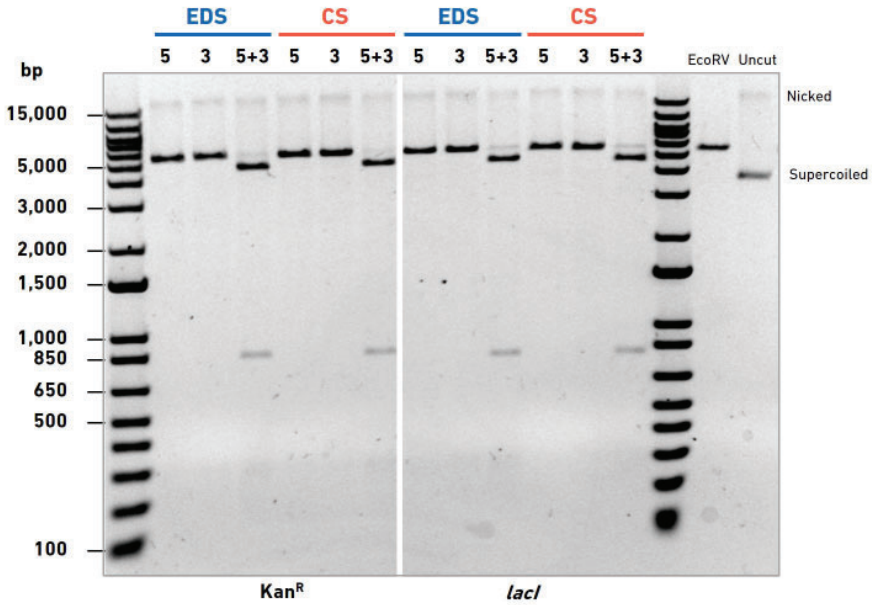


FIGURE 3. EDITING OF THE PET28A(+) PLASMID WITH IVT-PRODUCED SGRNAS. Lanes labeled 5 or 3 designate assays performed with only one of the sgRNAs targeting a specific cassette (the 5'- or 3'-end only) and were expected to yield a linear 5.4-kb product, the same size as obtained after digestion of the plasmid with restriction enzyme EcoRV (second lane from the right).

Lanes labeled 5+3 indicate that both sgRNAs were included in the reaction, to excise the 0.8-kb Kan^R cassette (left half of gel), or 1.0-kb lacI cassette (right half).

Faint bands corresponding to uncut, nicked plasmid (last lane) were observed in all reaction products, suggesting the nicked plasmid DNA may be resistant to cleavage with the Cas9-RNP complex.

All products from reactions performed with both sgRNAs displayed a small amount of linearized plasmid, irrespective of whether crRNAs were produced by EDS or conventional phosphoramidite chemistry.

REFERENCES

1. DNA Script Tech Note (TN900100). https://www.dnascript.com/wp-content/uploads/2021/04/DNAScript_EDS_3Pillars_TN900100.pdf.
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