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DEVELOPMENT OF MG-SCALE CELL-FREE PRODUCTION METHOD FOR GENE-SIZE SSDNA FRAGMENTS

Background. Compared to dsDNA, single-stranded DNA (ssDNA) offers greater structural flexibility, lower immunogenicity, and is utilized in CRISPR-Cas9 homology-directed repair, recombinase-mediated insertions, molecular probes, and expression vectors. We developed a protocol based on the selective 5'-3' degradation of 5'-phosphorylated dsDNA, followed by magnetic carboxylated bead capture, to generate and purify μ g-scale, transfection-grade ssDNA for immunocompetent mammalian cells. **Methods.** Input dsDNA was generated via preparative PCR (Q5 DNA polymerase, 5'-phosphorylated primer), purified with AmpliClean™ magnetic beads, and digested with Lambda exo (NEB). ssDNA was purified using Quick-DNA/RNA™ MagBeads (Zymo Research) and verified by agarose gel electrophoresis. Residual dsDNA was quantified via Qubit™ 1X dsDNA HS Assay post-S1 nuclease digestion. Endotoxins were measured using the PyroSmart NextGen® kinetic protocol. **Results.** To upscale the standard Lambda exo digestion (100 ng/ μ L, 1 U/ μ g DNA, 30-min incubation) to a 100 μ L reaction with 20 μ g of dsDNA, the enzyme-substrate ratio and incubation time were titrated. Complete digestion was achieved at an enzyme-substrate ratio of 1.25 U/ μ g without degrading the non-phosphorylated strand. Optimal incubation times scaled with fragment length: 2 h (>3500 bp), 1.5 h (>1500 bp), and 1 h (<500 bp). Because Lambda exo digests retained a stable fraction of residual dsDNA, even in an over-digestion regime, enzymatic removal using dsDNA-selective nucleases (ExoIII (NEB) and ezDNase™ (ThermoFisher)) was tested. In all tested conditions, neither nuclease exhibited the required selectivity, causing major ssDNA degradation, likely due to residual secondary structures. We then evaluated TRI Reagent® (Sigma) extraction and Quick-DNA/RNA™ MagBeads to selectively capture ssDNA. While the TRI Reagent® extraction resulted in recovering both ssDNA and dsDNA in an organic phase, Quick-DNA/RNA™ MagBeads successfully isolated pure ssDNA in the unbound fraction (recapitulating RNA behavior). The protocol was tested at various dsDNA loads, confirming robust performance up to 20 μ g of initial mass load. Processed ssDNA maintained homogeneity during all processing stages, yielding <2% residual dsDNA carryover and endotoxins <0.08 EU/ μ g in a final prep at end-to-end yields of 33–52% (for the longest and the shortest fragments, respectively). **Conclusions.** Our optimized Lambda exo protocol effectively scales ssDNA production across diverse fragment sizes. Combined with Quick-DNA/RNA™ MagBeads purification, it reliably yields homogeneous, low-dsDNA and endotoxin-free ssDNA, suitable for mammalian cell transfection.

Keywords: ssDNA, Lambda exonuclease, Quick-DNA/RNA™ MagBeads.

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