Enzymatically produced piggyBac transposon vectors for efficient non-viral manufacturing of CD19-specific CAR T cells

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The piggyBac transposon system provides a non-viral alternative for cost-efficient and simple chimeric antigen receptor (CAR) T cell production. The generation of clinical-grade CAR T cells requires strict adherence to current good manufacturing practice (cGMP) standards. Unfortunately, the high costs of commonly used lentiviral or retroviral vectors limit the manufacturing of clinical-grade CAR T cells in many non-commercial academic institutions. Here, we present a manufacturing platform for highly efficient generation of CD19-specific CAR T cells (CAR19 T cells) based on co-electroporation of linear DNA transposon and mRNA encoding the piggyBac transposase. The transposon is prepared enzymatically in vitro by PCR and contains the CAR transgene flanked by piggyBac 3’ and 5’ arms. The mRNA is similarly prepared via in vitro transcription. CAR19 T cells are expanded in the combination of cytokines interleukin (IL)-2, IL-7, and IL-21 to prevent terminal differentiation of CAR T cells. The accurate control of vector copy number (VCN) is achieved by decreasing the concentration of the transposon DNA, and the procedure yields up to 1 x 10⁸ CAR19 T cells per one electroporation of 1 x 10⁷ peripheral blood mononuclear cells (PBMCs) after 21 days of in vitro culture. Produced cells contain >60% CAR+ cells with VCN < 3. In summary, the described manufacturing platform enables a straightforward cGMP certification, since the transposon and transposase are produced abiotically in vitro via enzymatic synthesis. It is suitable for the cost-effective production of highly experimental, early-phase CAR T cell products.

INTRODUCTION

Chimeric antigen receptors (CARs) are artificial proteins that can redirect the specificity of T cells to any surface antigen. One of these antigens is the B cell antigen CD19, a well-validated target of already commercially available CD19-specific CAR (CAR19) T cells approved against refractory B cell malignancies. The CAR consists of a single-chain antibody segment (scFv) that defines the specificity, a short linker sequence, a transmembrane domain, and an intracellular signaling domain. Upon recognizing the antigen, the CAR initiates a T cell activation and expansion similarly as an endogenous T cell receptor and subsequent elimination of tumor cells. The development of novel and experimental CAR-T cell therapies requires a cost-effective and rapid production chain. The most complicated step in the manufacture of good manufacturing practice (GMP)-grade CAR T cells is the transfection of T cells, in a majority of cases performed via recombinant lentiviral or retroviral vectors (LV/RV). To meet the current quality standards, these viral vectors must be similarly produced at GMP quality, which is expensive and very slow. Several alternative non-viral approaches were thus developed, including transposons such as piggyBac (PB) or Sleeping Beauty (SB) or genome editing via CRISPR-Cas9. Transposons are usually used as two plasmids; the first one encodes the CAR transgene, and the second one encodes the PB transposase. Both plasmids are electroporated into T cells; the transposase excises the transposon from the first plasmid and randomly integrates it into the genome. Notably, the transposase can be introduced into T cells as mRNA to reduce the risk of random integration of the second plasmid. Several clinical trials demonstrated the efficiency of both PB and SB transposons for generating CAR T cells and validated this approach as safe and feasible. Other non-viral gene engineering techniques such as CRISPR-Cas9-based DNA editing enable transgenesis into a precise DNA locus within the genome. However, this unique feature is impaired by a highly increased risk of chromosomal translocations resulting from the off-target effect of the CRISPR-Cas9 enzyme complex. In contrast, PB transposon integrates its cargo randomly within the genome without producing such severe genomic mutations.

It will be of value to develop a universal and straightforward T cell transfection technology to overcome such barriers. Here, we show that abiotogenically (i.e., by in vitro enzymatic synthesis) prepared PB transposon/transposase can be used in this manner to manufacture CD19-specific CAR T cells efficiently. The transposon was prepared by preparative PCR as a linear dsDNA containing the CAR19 transgene and subsequent elimination of tumor cells. The development of novel and experimental CAR-T cell therapies requires a cost-effective and rapid production chain. The most complicated step in the manufacture of good manufacturing practice (GMP)-grade CAR T cells is the transfection of T cells, in a majority of cases performed via recombinant lentiviral or retroviral vectors (LV/RV). To meet the current quality standards, these viral vectors must be similarly produced at GMP quality, which is expensive and very slow. Several alternative non-viral approaches were thus developed, including transposons such as piggyBac (PB) or Sleeping Beauty (SB) or genome editing via CRISPR-Cas9. Transposons are usually used as two plasmids; the first one encodes the CAR transgene, and the second one encodes the PB transposase. Both plasmids are electroporated into T cells; the transposase excises the transposon from the first plasmid and randomly integrates it into the genome. Notably, the transposase can be introduced into T cells as mRNA to reduce the risk of random integration of the second plasmid. Several clinical trials demonstrated the efficiency of both PB and SB transposons for generating CAR T cells and validated this approach as safe and feasible. Other non-viral gene engineering techniques such as CRISPR-Cas9-based DNA editing enable transgenesis into a precise DNA locus within the genome. However, this unique feature is impaired by a highly increased risk of chromosomal translocations resulting from the off-target effect of the CRISPR-Cas9 enzyme complex. In contrast, PB transposon integrates its cargo randomly within the genome without producing such severe genomic mutations.

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transgene flanked with PB 3' and 5' arms. The PB transposase is encoded by mRNA and was prepared by in vitro transcription. According to the already established protocol, the transposon/transposase were co-electroporated into T cells, followed by in vitro expansion. By titrating down the amount of transposon DNA, it was possible to efficiently produce >60% CAR+ T cells containing less than three copies of CAR19 transgene per transduced cell. Generated CAR19 T cells were of early memory immunophenotype and displayed efficient effector functions. This manufacturing platform reduces the complexity of currently used viral-based gene transfection techniques and supports novel immunotherapeutic product development and rapid clinical testing.

RESULTS
CAR19 T cells can be generated with enzymatically in vitro prepared linear DNA transposon and mRNA transposase
A second-generation CAR19 construct previously generated by us was used as a template for the amplification of the transposon by preparative PCR (Figure 1A). The CAR19 construct contains anti-CD19 scFv derived from hybridoma B-D3 and has the following structure: CD8 leader sequence, anti-CD19 scFv, CD8 hinge domain, CD8 transmembrane domain, 4-1BB intracellular domain, TCR zeta intracellular domain; its full sequence is provided in Materials and methods. The manufacturing of the transposon DNA was performed commercially as described in Materials and methods, the purity of DNA was >99%, and there were no mutations within the coding sequence. Next, to manufacture the transposase mRNA, a plasmid pST containing a hyperactive PB transposase (hyPBase) under the T7 promoter was used as a template for the mRNA production by in vitro transcription. As a control reagent for initial experiments, an mRNA encoding GFP was similarly produced. First, we determined the electroporation efficiency of mRNA in T cells under conditions (voltage and pulse length) optimized for the electroporation of DNA by electroporating increasing amounts of GFP mRNA. The results in Figure 1B show that an amount between 10 and 15 µg of mRNA per electroporation should transduce a sufficient number of cells. Based on this estimate, we decided always to use 12 µg of transposase mRNA per one electroporation of 1 × 10^7 peripheral blood mononuclear cells (PBMCs) in a volume of 100 µL, since our goal was to transduce a sufficient number of cells with a lesser amount of transposase to ensure that hyPBase remains active intracellularly only for a short time.

To determine the optimal concentration of the transposon, PBMCs were electroporated with decreasing amounts of DNA (from 9 to 0.1 µg/100 µL). The transfection efficiency was compared to the procedure based on the electroporation of plasmids encoding the transposon/transposase. The data in Figure 2A show that the viability at day 1 and day 4 post electroporation was slightly higher upon electroporation of PCR CAR19 + mRNA hyPBase than the control PBMCs electroporated with plasmids (4 µg transposon plasmid + 2 µg transposase plasmid). As expected, the transfection efficiency depended on the DNA concentration (Figure 2B). The percentage of CAR+ T cells was initially low at day 7 post electroporation, but at later time points (day 14, day 21), we observed an increase in CAR+ cells reaching ~60%–70%. The efficient generation of CAR19 T cells was possible in the range of 3–0.3 µg of DNA per one (100 µL) electroporation. A further decrease below 0.3 µg/
At day 14 the CAR19 T cell yield was significantly lower than the yield of CAR19 T cells compared to electroporation of plasmids. Yields of CAR T cells with acceptable VCN (<10) were produced using PCR DNA/mRNA approach (Figure 3B). Electroporation of DNA in the range of 3–0.3 µg resulted in a substantial reduction of the percentage of transduced cells.

Low VCN can be achieved by transfection with minimal amount of transposon DNA using both PCR DNA/mRNA and plasmid/plasmid approach

The vector copy number (VCN) is a critical parameter for the production of clinical-grade CAR T cells. Thus, we quantified the number of integrated CAR19 transposons (i.e., VCN) per cell by digital PCR (ddPCR) and corrected the value to the percentage of CAR+ cells within the sample, which was determined by fluorescence-activated cell sorting (FACS). The data in Figure 3A show that the VCN of CAR19 transposon correlated with the decreasing amount of electroporated PCR CAR19 (the amount of mRNA was constant for all samples). Importantly, the generation of CAR19 T cells with as low as 1–3 copies of transgene was possible by reducing the PCR CAR19 concentration to 0.3 µg/100 µL. In contrast, control electroporation with a high amount of transposon/transposase plasmids (4 µg CAR19 + 2 µg PBASE) produced CAR19 T cells with approximately VCN = 23.

Next, we determined the maximal number of CAR19 T cells generated by this protocol by large-scale cultivations in G-Rex bottles (Figure 3B). Electroporation of DNA in the range 3–0.3 µg, which yields CAR T cells with an acceptable VCN (<10), produced equal numbers of CAR T cells compared to electroporation of plasmids. At day 14 the CAR19 T cell yield was ~1 × 10^7 CAR+ cells per one electroporation, and at day 21 the CAR-T yield was ~1 × 10^8 CAR+ cells per one electroporation. Control PBMCs electroporated with the transposon DNA without transposase mRNA did not expand in vitro and therefore were not further analyzed (data not shown). These data demonstrate that such a transposon dilution technique enables accurate titration of transposon DNA and reliably generates high numbers of CD19-specific CAR T cells with VCN < 5.

Similarly to the manufacturing of CAR19 T cells via PCR-made transposon DNA, we also used this transposon dilution technique to generate CAR19 T cells via electroporation of transposon/transposase plasmids. PBMCs were electroporated with a constant amount of transposase plasmid (1 µg) plus decreasing amounts of CAR19 transposon plasmids. The transfection and CAR19 T cell yield efficiency decreased with the reduced concentration of transposon plasmid, similarly to the PCR DNA/mRNA approach (Figure 4A). The optimal amount of transposon plasmid that enabled an efficient generation of CAR19 T cells with approximately VCN = 2–5 was in the range of 0.125–0.25 µg DNA per electroporation (Figure 4B). Interestingly, the comparison of the plasmid/plasmid approach with the PCR DNA/mRNA approach (Figure 3A versus Figure 4B) showed that the transfection with 1 µg of plasmid transposon produced CAR19 T cells with ~9 copies of transgene per cell and the transfection with PCR DNA/mRNA produced CAR19 T cells with ~3 copies of transgene per cell, while the overall yield of cultivation was similar under both conditions. This finding suggests that the transposition via the plasmid/plasmid approach is more efficient.

Next, to determine any possible changes of VCN during the in vitro expansion, CAR-T cells were generated using 1 µg of transposon/transposase plasmids per electroporation and VCN was measured on day 14 and at the end (day 21) of in vitro expansion. The results presented in (Figure 4C) show that VCN did not significantly change between day 14 and day 21, since ~7 versus 6 copies per cell were detected.

Functional assays of CAR T cells and immunophenotype of CAR19 T cells

To examine the effector functions of CAR19 T cells engineered with PCR DNA/mRNA, their cytotoxic activity and interferon-gamma (IFN-γ) production was measured after antigenic challenge with CD19+ B cell line Ramos and compared to CAR19 T cells generated via DNA plasmids. The amount of transposon used for transfection was the lowest concentration that effectively produced CAR19 T cells with VCN < 5, i.e., 0.3 µg PCR DNA/12 µg mRNA and 0.25 µg transposon plasmid/1 µg transposase plasmid. Both methods produced CAR19 T cells with similar effector functions, as the cytotoxicity (Figure 5A) and the production of IFN-γ (Figure 5B) were not significantly different. Next, we determined the immunophenotype of generated CAR19 T cells via flow cytometry. The representative dot plots in (Figure 5D) demonstrate that...
both methods produced CAR T cells with very similar immuno-
phenotype, characterized by the high level of expression of early
memory antigens CD62L, CD27, and CD28 and absence of
expression of the exhaustion marker PD-1. The graph in (Figure
5C) shows the immunophenotype of all donors analyzed. In sum-
mary, we found no signiﬁcant differences in immunophenotype
or the cytotoxic activity of CAR19 T cells prepared by these two
approaches.

DISCUSSION
The manufacturing of clinical-grade CAR T cells via transfection by
LV/RV is a complicated multi-step process with high costs. Addition-
ally, difficulties in obtaining commercially produced GMP-grade viral
vectors within a reasonable time window create challenging obstacles,
especially for academic centers not equipped with virus production
facilities.12,13

The presented work demonstrates as proof of principle that CAR19
cells can be efficiently manufactured via transfection with abigeni-
cally prepared transposon/transposase vectors. The described
procedure enables accurate control of VCN per cell while providing
sufficient yield of CAR19 T cells for therapeutic purposes. The control
of VCN is achieved by a “transposon dilution technique” that enables
CAR19 T cell generation with VCN below 3, not only after modiﬁ-
cation via PCR DNA/mRNA but also after traditional modiﬁcation with
plasmids encoding the transposon and transposase. The in vitro
expansion of CAR19 T cells is performed in cytokines interleukin
(IL)-4, IL-7, and IL-21, similarly, as we have already described

to preserve an early memory phenotype to
enable cultivation for up to 21 days. The use of
PCR DNA/mRNA has no signiﬁcant adverse ef-
effects on the CAR T cell memory phenotype or
effectort activity compared to the transfection
with plasmids. Notably, the generation of trans-
poson DNA by preparative PCR and the gener-
ation of transposase mRNA are both rapid and
straightforward procedures with no relevant
biohazard issues. The simplicity and the speed
of vector preparation via this method might enable faster clinical
testing of novel types of CAR T cells.

A similar method of T cell transfection was tested by Bishop et al.15
The authors attempted to generate CAR T cells by electroporation
of in vitro-ampliﬁed “doggy bone” DNA (dbDNA) combined with
dbDNA encoding the PB transposase. Our data similarly demonstrate
that enzymatically prepared genetic vectors represent a feasible alter-
native. The transposon copy number can be accurately controlled by a
simple dilution of the PCR DNA without adverse effects on the culti-
vation yield.

Another experimental non-viral technique used to produce CAR
T cells is based on CRISPR-Cas9 genome editing. CRISPR-Cas9 en-
ables the integration of the transgene into a precise locus within the
genome. It has been shown that the insertion of CAR transgene
into the TCR locus disrupts the expression of endogenous TCR,
which leads to improved function of CAR T cells by reducing the
tonic TCR signaling.16 However, CRISPR-Cas9 has signiﬁcant off-
target effects and produces chromosomal translocations.8 Addition-
ally, the procedure for T cell editing via CRISPR-Cas9 is far more
complex than a modiﬁcation via transposons and cannot be as
quickly GMP certiﬁed.17

Lentiviral vectors are currently the gold standard for genetic engineer-
ing of T cells, and their safety was reliably proven in thousands of
patients. This type of vector is especially suited for large-scale phar-
maceutical processes where costs, biohazard, and time are not an
issue. However, their use for small-scale generation of experimental phase 1 products is problematic. The presented technique might fill this gap and provide a rapid methodology for clinical testing of novel types of CAR constructs with unproven medical efficacy. Undeniably, the clinical experience with transposon vectors is insufficient, and thus the likelihood of oncogenesis induced by transposons cannot be reliably determined at this moment.

Moreover, animal studies can hardly answer such complex questions. On the other hand, mature T cells are inherently resistant to malignant hematopoietic transformation, compared to, for example, undifferentiated hematopoietic precursors, or B cells. In our experience, pharmaceutical regulatory agencies require additional quality control (QC) tests such as mapping of integration sites and monitoring of T cell clonality for approval of clinical trials with transposon-engineered T cells.

In summary, the manufacture of clinical-grade CAR T cells via lentiviral or retroviral transfection is a complicated and expensive task primarily resulting from GMP rules regulating the preparation of viral vectors. Currently, the major bottleneck is the difficulty in obtaining the GMP-grade viral vectors within a reasonable time. Enzymatically prepared transposon vectors are surprisingly highly efficient for small-scale manufacturing of this type of therapeutic product. In our opinion, the development of an easily certifiable GMP-grade method of T cell transfection is critical for the rapid development of T cell-based therapies in the future.

MATERIALS AND METHODS
Preparation of DNA and mRNA amplicons and plasmid cloning
The mRNA encoding the hyperactive PB transposase (hyPbase) was prepared by in vitro transcription with the HiScriber T7 High Yield RNA Synthesis Kit (New England Biolabs). To increase the stability of mRNA, we used 3'-O-Me-m7G (5') PPMPS (5') G RNA cap structure analog at a concentration of 8 mM (New England Biolabs). Before an in vitro transcription, the pSTI plasmid template encoding the hyPbase, or GFP, was linearized via BspQI (New England Biolabs) and gel-purified. The reaction was carried out for 3 h at 37°C using 1 μg of linearized plasmid template per 50-μL reaction. Prepared mRNA was purified with the MEGAclear Transcription Clean-Up Kit (Thermo Fisher Scientific) and analyzed with the Experion Automated Electrophoresis System (Bio-Rad Laboratories). The yield was ~100 μg per one 50-μL reaction.

The transposon containing the CAR19 transgene was prepared commercially by preparative PCR (LinearX, USA) using M13 fwd and M13 rev primers and a Taq DNA polymerase, followed by a purification step on a chromatographic column. The declared purity of the PCR product was >99%. Furthermore, no mutations in the coding sequence were detected by Sanger sequencing. Plasmids were amplified in E. coli and purified by QIAGEN plasmid kits.

T cell cultivation and electroporation
PBMCs were separated from buffy coats obtained from healthy donors with Ficoll density gradient centrifugation (Ficoll-Paque, GE Healthcare). All donors gave written informed consent in accordance with Helsinki ethical guidelines. Cells were cultivated at 37°C in 5% CO2 in CellGenix GMP DC medium (CellGenix) supplemented with heat-inactivated 10% fetal bovine serum (Thermo Fisher Scientific), penicillin/streptomycin 100 U/mL (Thermo Fisher Scientific), and cytokines IL-4 (20 ng/mL), IL-7 (10 ng/mL), and IL-21 (40 ng/mL), all from Miltenyi Biotec. For large-scale expansion, we used G-Rex10 flasks (Wilson Wolf). For electroporations, 1 × 107 PBMCs were resuspended in 100 μL of buffer T containing plasmid DNA or linear transposon+ mRNA. Cells were then electroporated with the Neon Electroporation System (Thermo Fisher Scientific, USA) by a single 20-ms/2,300-V pulse and then were transferred to complete cell media. Next day, cells were polyclonally stimulated with TransAct reagent (anti-CD3/CD28, Miltenyi Biotec, Germany) and expanded for 21 days in the presence of IL-4, IL-7, and IL-21, identically as already described.13

Functional assays and flow cytometry
The functionality of CAR19 T cells was tested by FACS-based cytotoxic assay against Ramos B cells. Briefly, Ramos cells were labeled with 0.5 μM CFSE (Thermo Fisher Scientific) in PBS for 10 min at room temperature, washed in CellGenix medium, and mixed with target cells at a ratio of 3:1 and 1:1. After 24-h incubation, cells were harvested, labeled with DAPI, and analyzed by FACS to determine the percentage of dead Ramos cells.

To determine the production of IFN-γ, we similarly co-cultivated CAR19 T cells with Ramos B cells at a 1:1 ratio overnight. The production of IFN-γ was determined in supernatants with the ELISA MAX Deluxe Set Kit (BioLegend). The absorbance at 450 nm was measured on an ELISA reader (Tecan Infinite 200 Microplate Reader). The IFN-γ concentration was calculated according to the diluted IFN-γ standard calibration curve in the GraphPad Prism software.

To determine the immunophenotype of CAR19 T cells, the cells were washed in 1× PBS, stained with fluorochrome-conjugated monoclonal antibodies (mAbs) in FACS buffer (1% BSA, 0.1% sodium azide, 1× PBS) for 30 min on ice, washed with PBS, and resuspended...
in FACS buffer containing DAPI 100 ng/mL (Merck). In the case of indirect labeling, the primary antibody was washed out with PBS, and cells were then stained with fluorochrome-conjugated secondary antibody for 30 min on ice. The expression of CAR19 transgene was determined with Alexa Fluor 647-conjugated F(ab')2 fragment of Goat Anti-Mouse immunoglobulin G (IgG) (Jackson ImmunoResearch). This polyclonal antibody reacts with mouse anti-CD19 scFv. The immunophenotype of CAR T cell was determined similarly as already described. First, cells were stained either with Alexa Fluor 647- or Alexa Fluor 488-labeled F(ab')2 fragment of Goat Anti-Mouse IgG (Jackson ImmunoResearch, UK) to detect the CAR transgene. Cells were then washed twice and blocked with 10% mouse serum, followed by staining with fluorescently labeled mouse mAbs specific to human antigens: CD45RA-CA480 (clone HI100), CD62L-BV650 (clone DREG-56), and CD3-BV786 (clone UCHT1) purchased from BD Biosciences (USA). CD8-AF700 (clone MEM-31) and CD27-PE-Dy590 (clone S3.5) and CD28-PE-Cy7 (clone CD28.2) were from BioLegend (USA). Live cells were identified with the Fixable Blue Dead Cell Stain Kit (Thermo Fischer Scientific, USA). Staining specificity was extensively tested to rule out possible binding of mouse antibodies to cells via goat anti-mouse Ab used to detect CAR. Cells were analyzed with BD LSRFortessa (BD Biosciences), and the data were processed with FlowJo software.

### DNA sequence of the CAR19 transposon

(M13) GTAAACAGCGGCCGCAAGTACGACGCGCGTAATACGACA
CAGCTCGAGTACGACGGTGACGCACACCTCTCGAGTCATGAGCTAC
CACTAGAGCTTATTGATTTGTATACTTATAC

| Table 1. The sequences of primers and probes |
|--------------------------------------------|
| **CAR19**                                |
| forward                                    |
| 5'-atggtatctggtgagac-3'                   |
| reverse                                    |
| 5'-gactagctggcaagaggtg-3'                 |
| probe                                     |
| 5'-FAM-ctctctgcctggctcaggtga-BHQ1-3'      |
| **PBae**                                  |
| forward                                    |
| 5'-CCCTGAACATCGGAGA-3'                    |
| reverse                                    |
| 5'-ATCCGGCTGCGGAGAAGAC-3'                 |
| probe                                     |
| 5'-FAM-CCCGACCAAGATGTCAGGACATCT-BHQ1-3'   |
| **Albumin**                               |
| forward                                    |
| 5'-TAGAATCATCGTTTCAAAAGAGTTT-3'           |
| reverse                                    |
| 5'-CTCTCTCCTCAGAAAAGTGTCATAT-3'           |
| probe                                     |
| 5'-HEX-TGGTGAACAC-3'                      |

10 min at 95°C, 45 cycles of a two-step of 30 s at 94°C and 60 s at 54°C, ending with a final hold of 10 min at 98°C for droplet stabilization and cooling to 4°C. Droplets were counted on/divided by a QX200 droplet reader based on their fluorescence amplitude into positive or negative. Data were analyzed with QuantaSoft Analysis Pro version 1.0.596 software (Bio-Rad), including automatic Poisson distribution. The VCN was determined as the ratio of CAR copies/albumin copies × 2 and divided by the fraction of CAR+ T cells in the sample.
Amino acid sequence of CAR19 construct

GCGTAATCATGGTCATAGCTGTTTCCTG (CCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGAGGGTTAATCTAGTATACGCGTATGCGGCCGCTTAATTAATATGATTATCTTTAACGTACGTCACAATATGATTATCTTTCTCGATATACAGACCGATAAAACACATGCGTCAATTTTACGCAAATTGTTTGTTGAATTTATTATTAGTATGTAAGTGTAAATT TGAGTTTTTGTTTTTTTTTAATAAATAAATAAACATAAATGACATACTAGTTAAAAGTTTTGTTACTTTATAGAAGAAATT AATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGGTCGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAC TGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGATGA TGAGAATTCCGACTGTGCCTTCTAGTTGCCAGCCATCTCAGGGCCTGAGCACC GCCACCAAGCCACCGCTCGTGGTCAATTTTACGCAAATTGTTTGTTGAATTTATTATTAGTATGTAAGTGTAAATT TGAGTTTTTGTTTTTTTTTAATAAATAAATAAACATAAATGACATACTAGTTAAAAGTTTTGTTACTTTATAGAAGAAATT AATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGGTCGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAC TGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGATGA TGAGAATTCCGACTGTGCCTTCTAGTTGCCAGCCATCTCAGGGCCTGAGCACC GCCACCAAGCCACCGCTCGTGGTCAATTTTACGCAAATTGTTTGTTGAATTTATTATTAGTATGTAAGTGTAAATT TGAGTTTTTGTTTTTTTTTAATAAATAAATAAACATAAATGACATACTAGTTAAAAGTTTTGTTACTTTATAGAAGAAATT AATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGGTCGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAC TGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGATGA TGAGAATTCCGACTGTGCCTTCTAGTTGCCAGCCATCTCAGGGCCTGAGCACC GCCACCAAGCCACCGCTCGTGGTCAATTTTACGCAAATTGTTTGTTGAATTTATTATTAGTATGTAAGTGTAAATT TGAGTTTTTGTTTTTTTTTAATAAATAAATAAACATAAATGACATACTAGTTAAAAGTTTTGTTACTTTATAGAAGAAATT AATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGGTCGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAC TGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGATGA TGAGAATTCCGACTGTGCCTTCTAGTTGCCAGCCATCTCAGGGCCTGAGCACC GCCACCAAGCCACCGCTCGTGGTCAATTTTACGCAAATTGTTTGTTGAATTTATTATTAGTATGTAAGTGTAAATT TGAGTTTTTGTTTTTTTTTAATAAATAAATAAACATAAATGACATACTAGTTAAAAGTTTTGTTACTTTATAGAAGAAATT AATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGGTCGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAC TGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGATGA TGAGAATTCCGACTGTGCCTTCTAGTTGCCAGCCATCTCAGGGCCTGAGCACC GCCACCAAGCCACCGCTCGTGGTCAATTTTACGCAAATTGTTTGTTGAATTTATTATTAGTATGTAAGTGTAAATT TGAGTTTTTGTTTTTTTTTAATAAATAAATAAACATAAATGACATACTAGTTAAAAGTTTTGTTACTTTATAGAAGAAATT AATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGGTCGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAC TGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGATGA TGAGAATTCCGACTGTGCCTTCTAGTTGCCAGCCATCTCAGGGCCTGAGCACC GCCACCAAGCCACCGCTCGTGGTCAATTTTACGCAAATTGTTTGTTGAATTTATTATTAGTATGTAAGTGTAAATT TGAGTTTTTGTTTTTTTTTAATAAATAAATAAACATAAATGACATACTAGTTAAAAGTTTTGTTACTTTATAGAAGAAATT AATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGGTCGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAC TGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGATGA TGAGAATTCCGACTGTGCCTTCTAGTTGCCAGCCATCT

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AUTHOR CONTRIBUTIONS

I.K., P.P., and M.S. designed and performed experiments; H.Z., V.S., and K.S. analyzed data; P.O. conceptualized the study and wrote the manuscript.

The authors declare no competing interests.

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