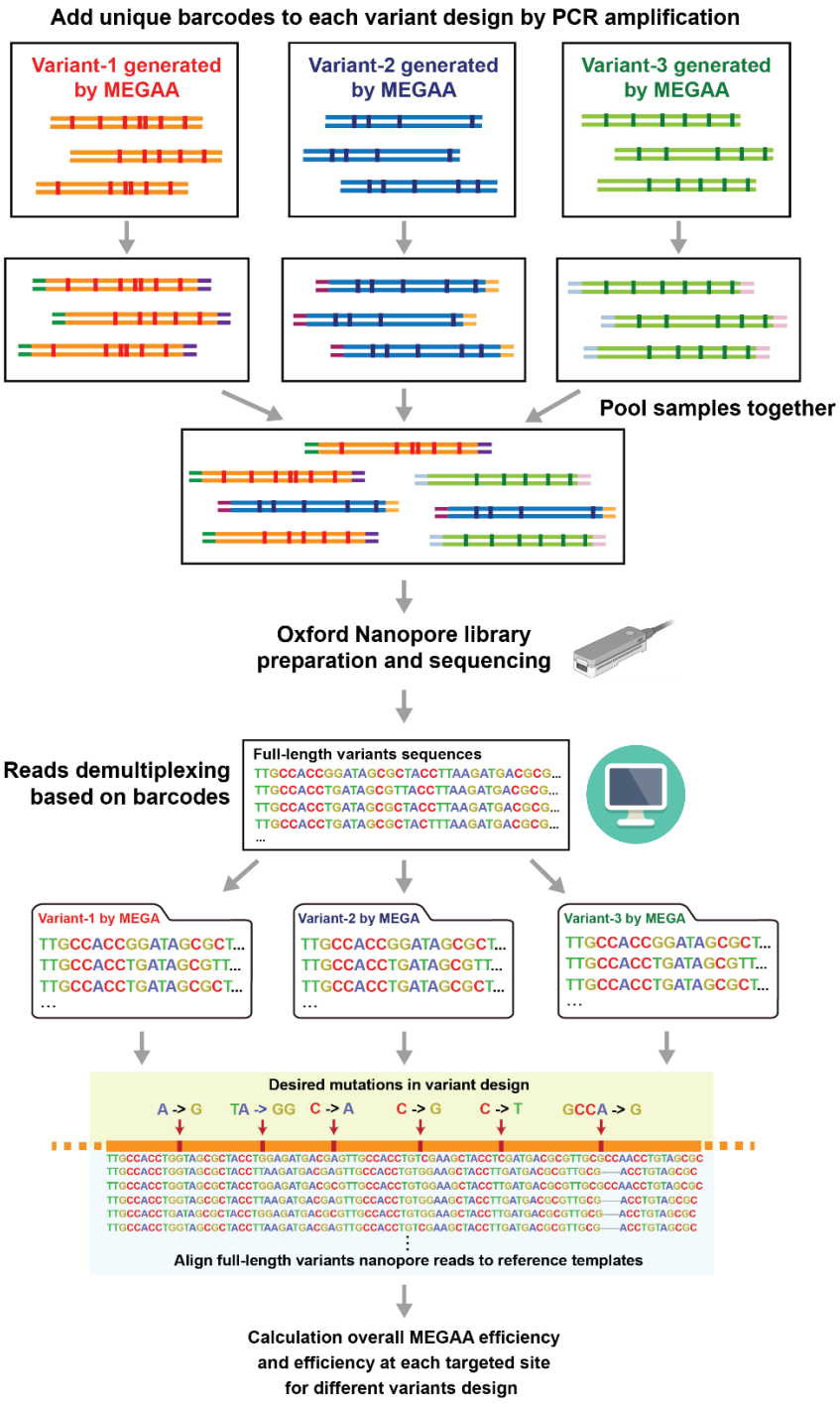
nature methods

Article

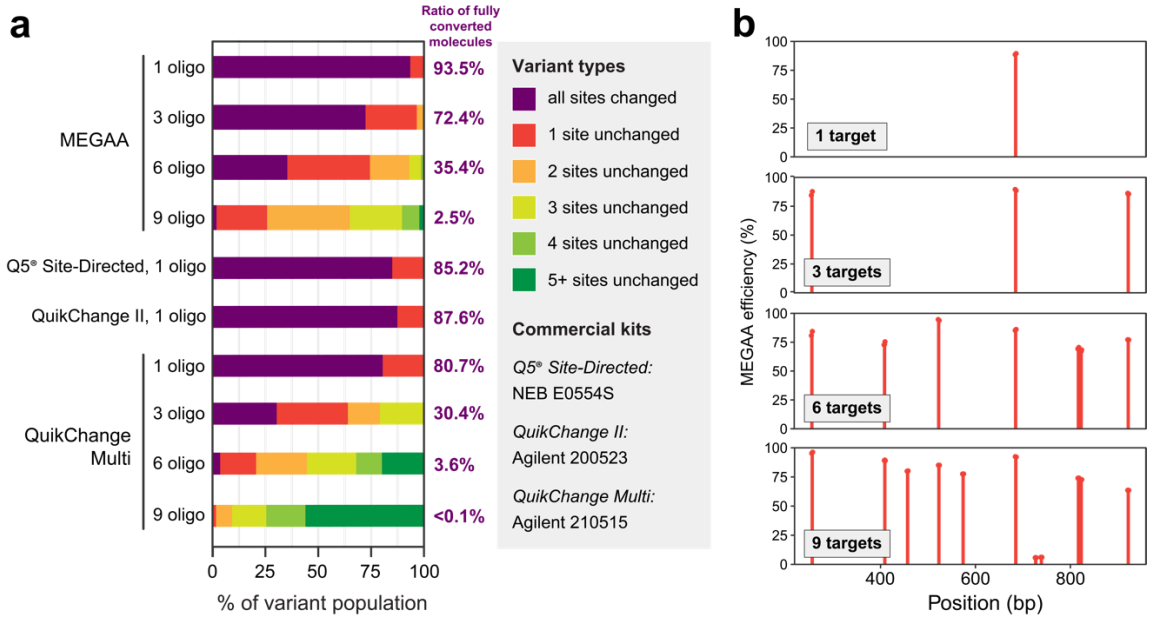
https://doi.org/10.1038/s41592-023-01868-1

Fast and efficient template-mediated synthesis of genetic variants

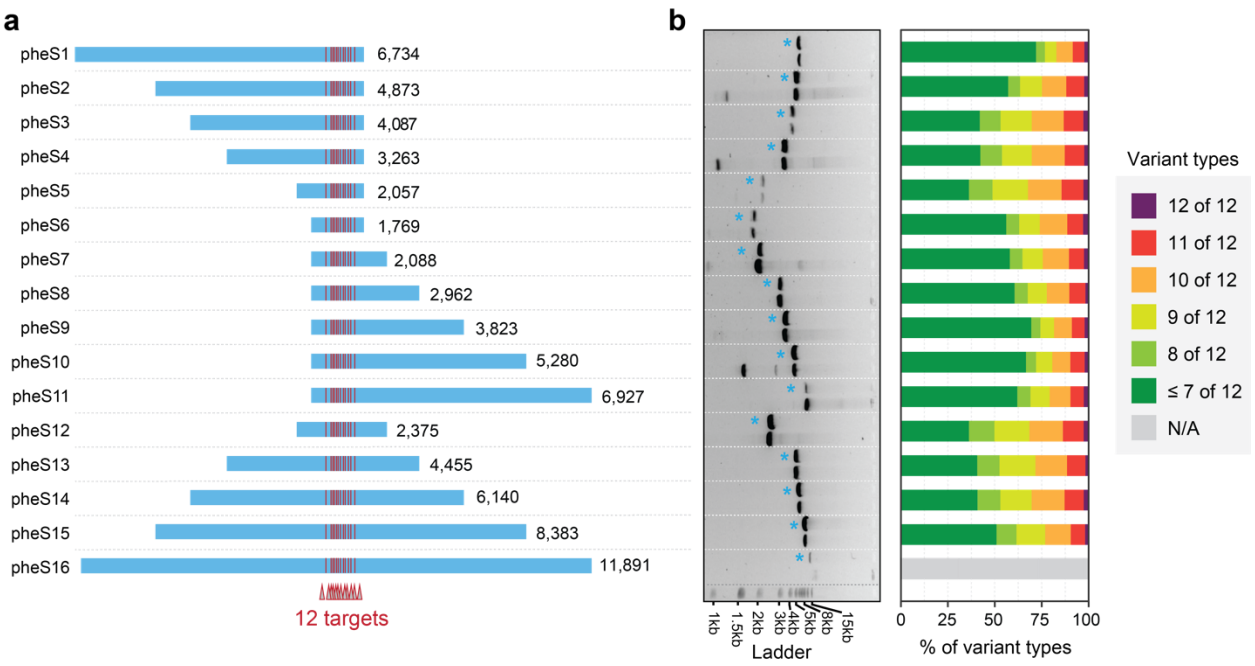
In the format provided by the authors and unedited



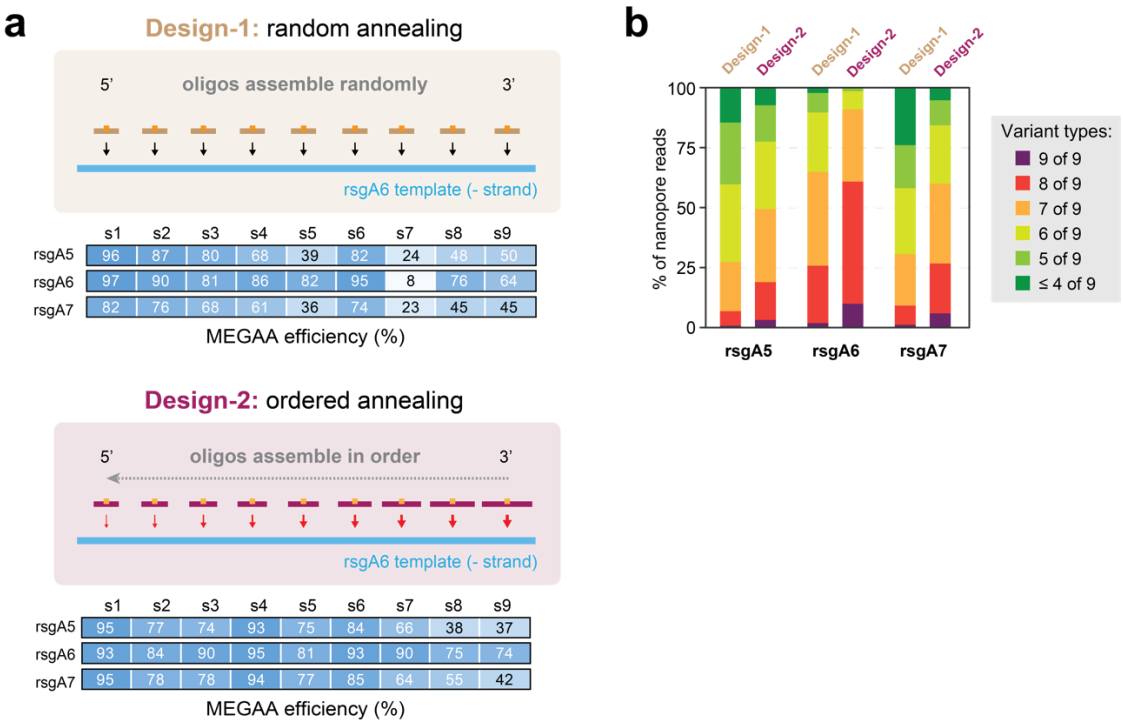
Supplementary Figure 1: Nanopore sequencing and variant calling pipeline for MEGAA product analysis.



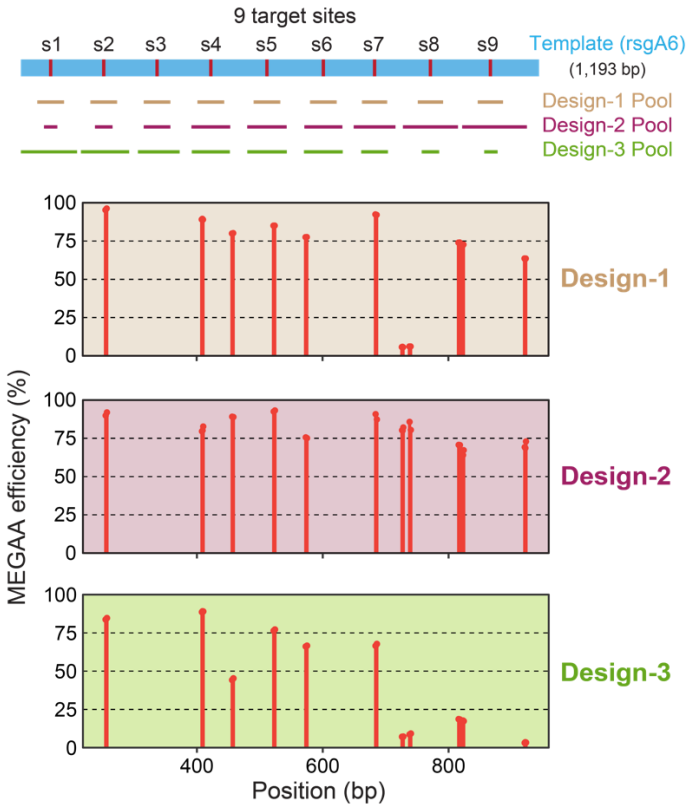
Supplementary Figure 2: MEGAA efficiency using different numbers of oligoes in each pool against the *rsgA*6 template and comparison with commercial kits. **(a)** Variant population generated with increasing number of oligos for MEGAA and 3 commercial kits. **(b)** Per target efficiency with different numbers of oligos per pool for MEGAA.



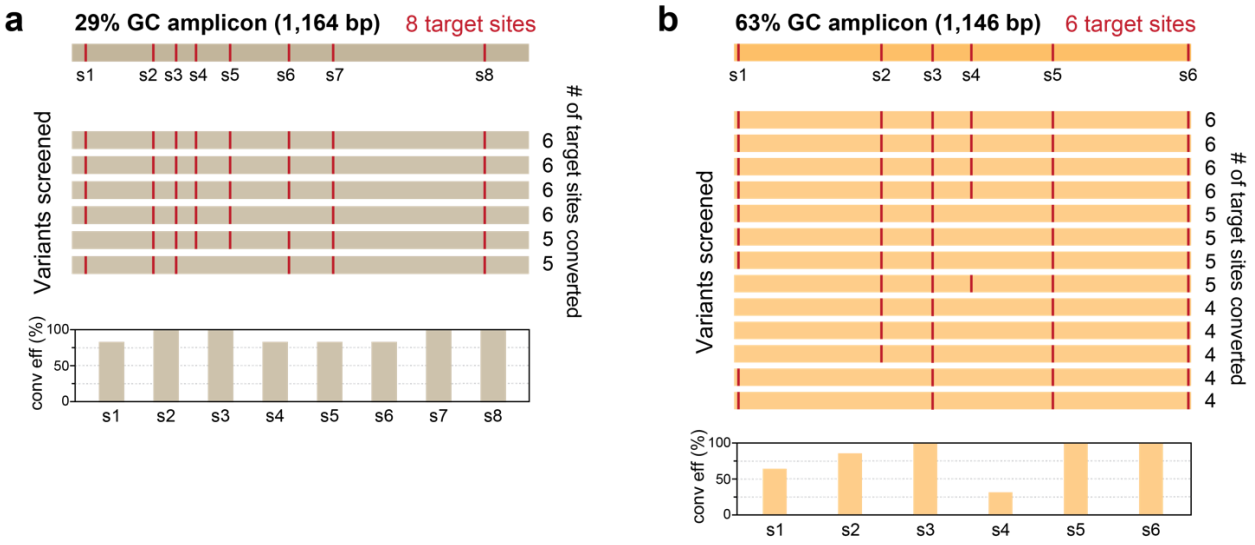
Supplementary Figure 3: MEGAA reaction using U-templates (*pheS*1-*pheS*16) generated near the *pheS* gene in *E. coli* K12 genome. **(a)** A 12-pool oligo was used to target 12 sites spanning a ~1kb region across *pheS*1-*pheS*16 templates. The numbers on the right side of the bar show the sizes of the fragments, and the span of the bar is the starting and ending positions of the fragments **(b)** Left panel shows gel electrophoresis of the initial U-templates and their corresponding MEGAA products (asterisks indicate U-containing templates). Right panel shows the distribution of variants generated as determined by nanopore sequencing of the MEGAA products.



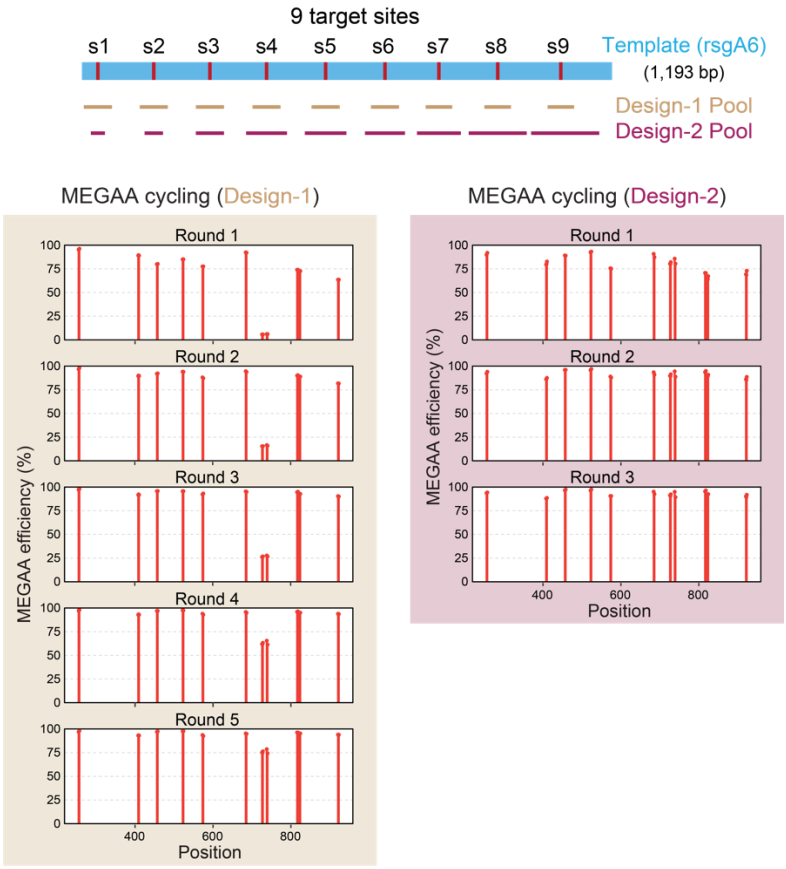
Supplementary Figure 4: Increasing MEGAA efficiency using improved oligo design. **(a)** Design-1 does not consider order of oligo assembly to the template, while Design-2 provides an ordered annealing process for oligo assembly favoring 3' regions first. Corresponding MEGAA efficiencies across 9 target sites are shown in the bottom panels for templates *rsgA*5, *rsgA*6 and *rsgA*7, indicating improved oligo incorporation efficiency using Design-2. **(b)** Overall population of variants generated using Design-1 and Design-2 for different templates.



Supplementary Figure 5: MEGAA conversion efficiency of 9 target sites for the *rsgA*6 variant using oligo Design-1 pool, Design-2 pool or Design-3 pool. For each site, the % of converted sites (the MEGAA efficiency) quantified across all nanopore sequencing reads are shown for Design-1 (same T_m), Design-2 (increasing T_m from 5' to 3'), and Design-3 (decreasing T_m from 5' to 3').

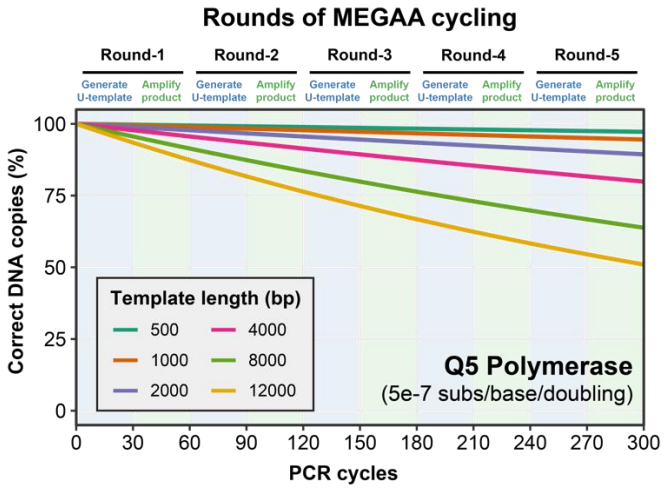


Supplementary Figure 6: MEGAA reactions using templates of different GC content. **(a)** Template with 29% GC content generated from the *sdpB* gene of *Bacillus subtilis* 168 and the associated MEGAA efficiency based on 6 clones analyzed by Sanger sequencing. Mean conversion efficiency per site (s1-s8) shown on bottom panel. **(b)** Template with 63% GC content generated from the *pheS* gene of *Pseudomonas aeruginosa* PAO1 and the associated MEGAA efficiency based on 13 colonies analyzed by Sanger sequencing. Mean conversion efficiency per site (s1-s6) shown on bottom panel.

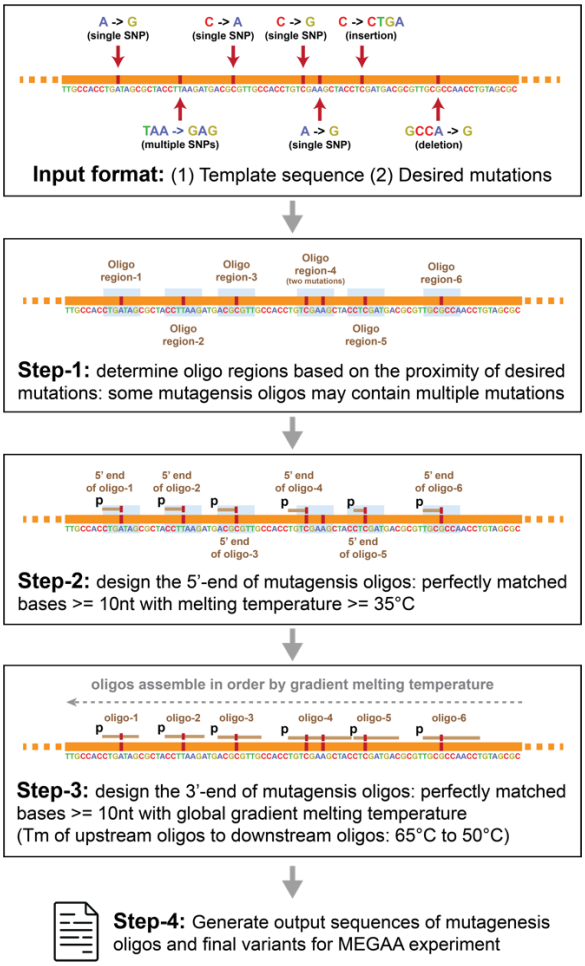


Supplementary Figure 7: MEGAA conversion efficiency of 9 target sites for the *rsgA*6 variant using oligo Design-1 pool or Design-2 pool through iterative rounds of MEGAA cycling. Five rounds are performed with Design-1 pool and three rounds are performed with Design-2 pool. For each site, the % of converted sites (the MEGAA efficiency) quantified across all nanopore sequencing reads are shown for increasing rounds of MEGAA cycling.

Supplementary Figure 8: Sanger sequencing traces on picked colonies illustrate the efficiency of variants generation by single-round MEGAA with Design-2 oligos. One of the randomly selected 5 colonies was fully converted.



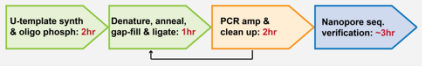
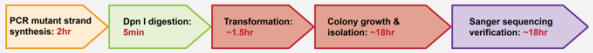
Supplementary Figure 9: Estimation of correct DNA copies after 5 rounds of MEGAA reactions. Error rate of Q5 Hot Start High-Fidelity DNA Polymerase was previously estimated as 5E-07 substitution/base/doubling (PMID: 28060945) and the proportion of correct DNA copies, i.e., DNA without any accumulated amplification error, was calculated using the same model of NEB PCR Fidelity Estimator (https://pcrfidelityestimator.neb.com).



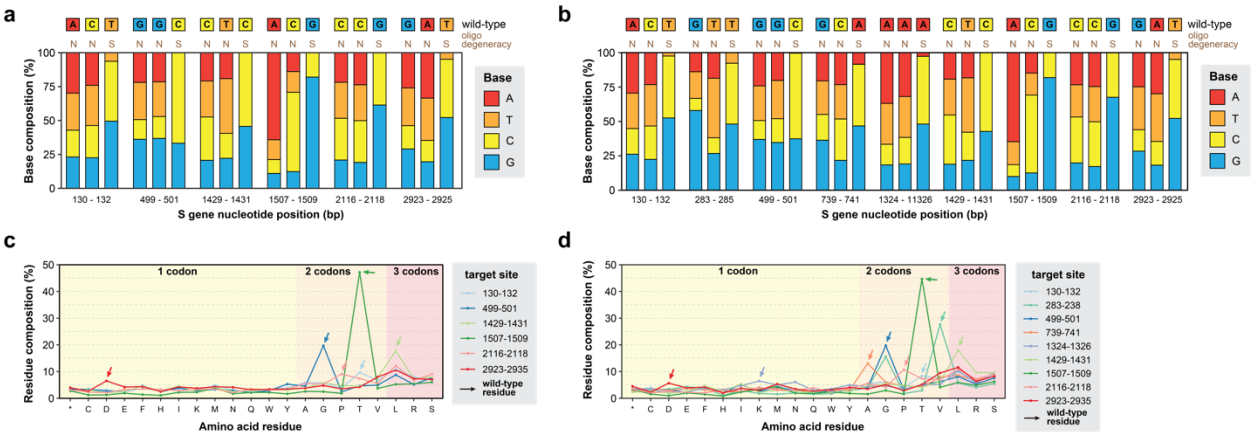
Supplementary Figure 10: MEGAA design tool (MEGAA-dt) flow chart for oligo design.

		MEGAA genes		Commercial genes	
Gene length	# of mutations	Cost/gene	Production	Cost/gene	Production
1,800 bp	>30	$23-$73	<2 days	$187	6-9 days
3,200 bp	>60	$23-$73	<2 days	$409	11-17 days
5,000 bp	>90	$23-$73	<2 days	$775	11-17 days
5,000 bp	degeneracy	$23-$73	<2 days	not available	not available
7,000 bp	>90	$23-$73	<2 days	not available	not available

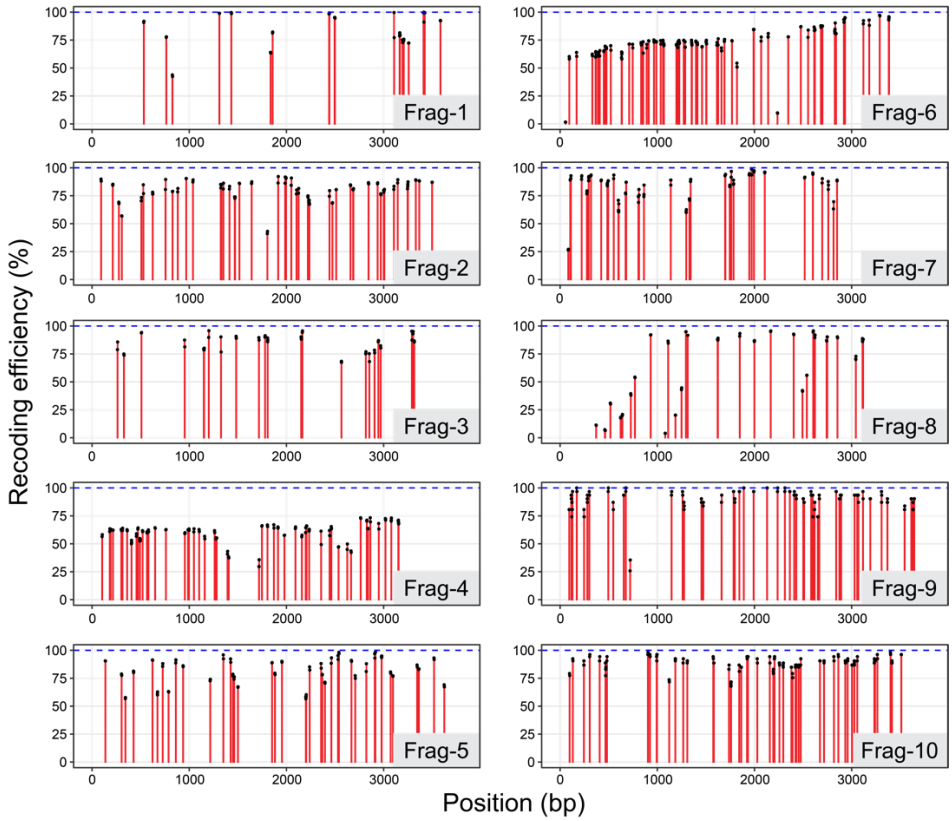
Supplementary Figure 11: Cost and speed comparisons of MEGAA variant synthesis with commercial gene synthesis. Comparison is made with standard cost and delivery times for commercial vendor Twist Biosciences. MEGAA costs are estimated with reagent and raw material costs only.

Methods	Total time	Reaction steps & times
MEGAA (this work)	~8 hours (+3 hr/cycle)	
Q5® Site-Directed Mutagenesis Kit	~40 hours	
QuikChange II Site-Directed Mutagenesis Kit	~41 hours	
QuikChange Lightning Multi Site-Directed Mutagenesis Kit	~40 hours	
Darwin Assembly (target region <2kb, modified oligos)	~48 hours	
Darwin Assembly (target region >2kb, long oligos)	~46 hours	

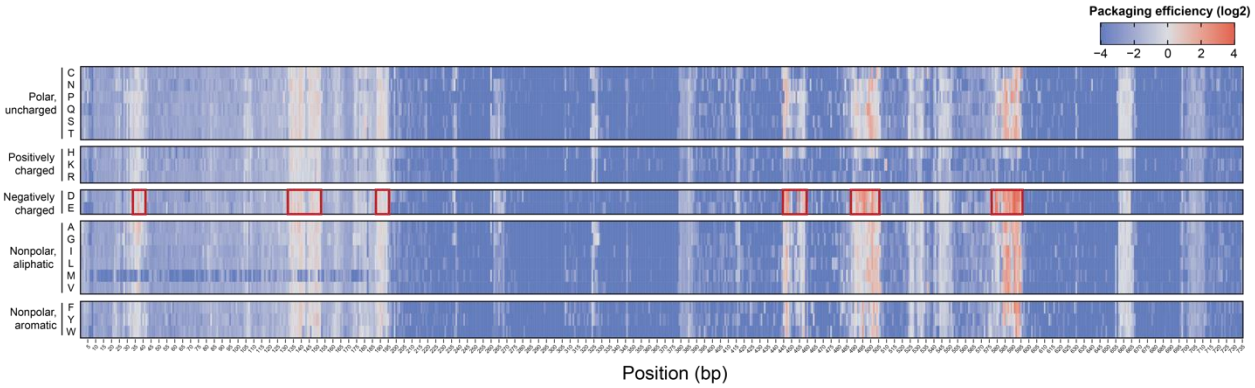
Supplementary Figure 12: Comparison of workflows for MEGAA, Darwin Assembly (PMID: 29409059), and other commercial kits.



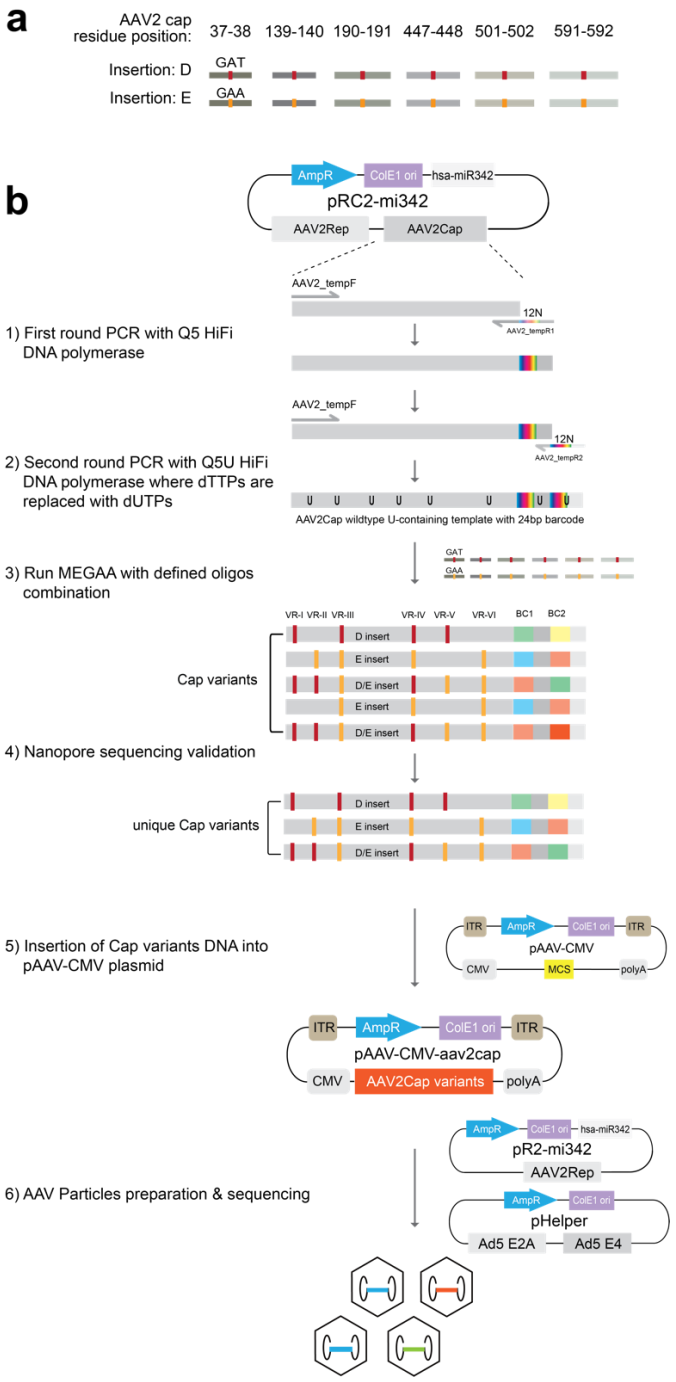
Supplementary Figure 13: Illumina NGS data of MEGAA reactions on SARS-CoV2 *S* gene using oligos containing degenerate bases (NNS) to produce Spike variant populations containing combinatorial mutations across multiple regions. **(a, b)** Per-base nucleotide frequency of targeted sites for *S* genes variants with 6 combinatorial mutation regions **(a)** and 9 combinatorial mutation regions **(b)**. Oligo degeneracy design and wild-type base at each site are shown above the frequency bar. **(c, d)** Amino acid residues frequency of targeted sites for S genes variants with 6 combinatorial mutation regions **(c)** and 9 combinatorial mutation regions **(d)**. Amino acid residues frequency were calculated based on trinucleotide frequency and the wild-type amino acid residue of targeted sites are indicated by arrow. Amino acid residues were classified into three groups based on number of codons they are mapped to in oligo degeneracy designs: 32 potential codons of NNS cover 13 single-codon residues, 5 double-codon residues and 3 triple-codon residues.



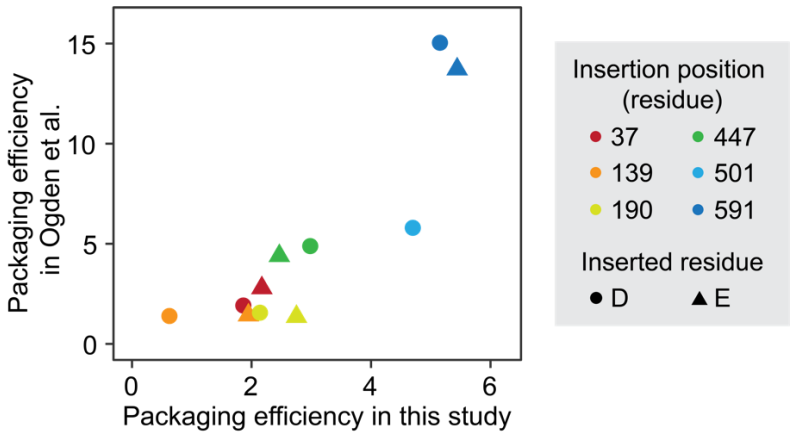
Supplementary Figure 14: Nanopore sequencing traces showing mutation efficiency per site across the 10 genomic recoding fragments generated by MEGAA.



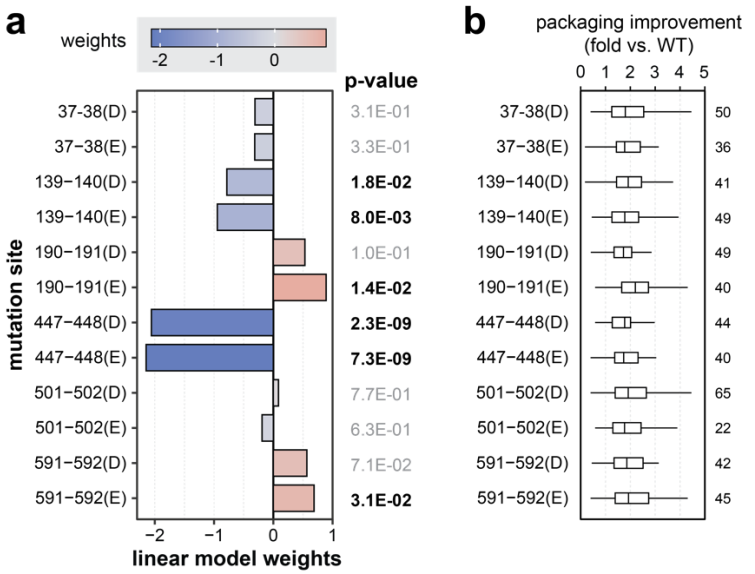
Supplementary Figure 15: Packaging efficiency of an AAV saturation insertion mutagenesis library, with data from Ogden et al (PMID: 31780559). Regions of interest selected in the current study are boxed in red.



Supplementary Figure 16: AAV2 *cap* gene variants generation and particle preparation **(a)** MEGAA oligos design with GAT or GAA insertion mutations. **(b)** Overview of AAV variants generation using MEGAA and subsequent cloning and packaging.



Supplementary Figure 17: Comparison of packaging efficiency improvement relative to wild type of single insertion mutants measured in Ogden et al (PMID: 31780559) and measured in our study showing high concordance.



Supplementary Figure 18: Linear model of AAV variants in packaging efficiency improvement. **(a)** Weights of individual mutation sites in the linear model with p-value shown on the right. The p-values were calculated by two-sided t-test on the linear regression to test if coefficient of the variable (presence or absence of mutations site) equals to zero in the model and no adjustment was performed on p-values. **(b)** Boxplot of packaging efficiency of all variants with the mutation site. Numbers on the right represents numbers of variants with the mutation site. Definition of box-plot elements: center line: median; box limits: upper and lower 25th quartiles; whiskers: 1.5x interquartile range.

Supplementary Methods

MEGAA mutagenesis protocol

The MEGAA protocol was performed first by using a DNA seed (e.g., *E. coli* genomic DNA, pcDNA3.1 SARS-CoV-2 S D614G DNA, pRC2-mi342 Vector containing AAV2 *cap* gene) to generate an uracil-containing template, then by performing rounds of denaturing, ligation, and extension steps, followed by a final amplification step. Details steps are as follows.

MEGAA reaction mixes and buffers

10× MEGAA reaction enzyme mix	2× MEGAA reaction master mix buffer
• 50 mM Potassium Acetate (Sigma-Aldrich #P1190) • 20 mM Tris-acetate (Thermo Scientific™ # 434500250) • 10 mM Magnesium Acetate (Thermo Scientific™ #J60041.AD) • 200 µg/mL BSA (Thermo Scientific™ #B14) • 1 mM DTT (Thermo Scientific™ # D1532) • 50% Glycerol (Invitrogen™ # 15514011) • 10 U/µL Taq DNA Ligase (NEB # M0208L) • 0.1 U/µL Q5U® Hot Start High-Fidelity DNA Polymerase (NEB #M0515SVIAL) • pH 7.4 @ 25°C	• 1.7× Taq DNA Ligase Reaction Buffer (NEB # B0208SVIAL) • 1.7× Q5U® Reaction Buffer (NEB #B9037SVIAL) • 1.7× CutSmart Buffer (NEB # B6004S) • 0.17 mM dNTPs (NEB # N0447L) • 0.33 mM NAD (NEB # B9007S) • 6.67 mM DTT (Thermo Scientific™ # D1532) • 16.7% DMSO (Sigma-Aldrich #D8418) • pH 7.2 @ 25°C

MEGAA reaction conditions

Step 1: PCR generation of uracil-containing template and clean up

PCR setup to amplify uracil-containing template:

a) 1 µL of DNA template (e.g., 1 ng/µL *E. coli* genomic DNA) or diluted MEGAA product from last round of reaction.
b) 1 µL of forward primer 20 µM
c) 1 µL of reverse primer 20 µM
d) 1 µL of 10 mM dNTPs (dATP, dCTP, dUTP and dGTP at 2.5 mM each, NEB #N0446S, #N0459S)
e) 10 µL of 5× Q5U® reaction buffer
f) 1 µL of Q5U® hot-start high-fidelity DNA polymerase
g) 35 µL of nuclease-free water (Invitrogen #AM9937)

PCR protocol:

h) 98°C for 30 seconds [initial denaturation]
i) 30 cycles of the following:
   - 98°C for 10 seconds
   - *55–72°C for 10 seconds
   - 72°C for X minutes (20-30 seconds/kb)
   - 72 °C for 5 min [final elongation]

*Use of the NEB T_m Calculator is highly recommended.

SPRI beads purification of amplified uracil-containing template:

j) Resuspend the SPRI beads by vortexing.
k) Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.
l) Add the 1× ratio of suspended SPRI beads to the uracil-containing template DNA and mix by flicking the tube.
m) Incubate for 5 minutes at room temperature.
n) Prepare 1mL of fresh 80% ethanol in nuclease-free water.
o) Spin down the sample and pellet on a magnet until eluate is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant.
p) Keep the tube on the magnet and wash the beads with 500 µL of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
q) Repeat the previous step.
r) Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet o the point of cracking.
s) Remove the tube from the magnetic rack and resuspend the pellet in 20-30 µL nuclease-free water. Incubate for 2 minutes at room temperature.
t) Pellet the beads on a magnet until the eluate is clear and colourless.
u) Remove and retain 20-30 µl of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube or LoBind PCR tube.

Step 2: Mutagenesis using MEGAA

Phosphorylation of oligos (optional step for MEGAA cycling):

a) Prepare oligo phosphorylation reaction as follows:
   - 2.5 µL T4 DNA ligase buffer (10×)
   - 5 µL T4 kinase (PNK) (NEB #M0201L)
   - oligos (100 pmolar)
   - 2.5 µL PEG 8000 (50%) (Thermo Scientific™ #50-488-949)
   - Add nuclease-free water to 25 µL
b) Incubate at 37°C for 60-90 minutes
c) Incubate at 65°C for 20 minutes to heat inactivate

One-pot MEGAA mutagenesis:

d) Add the following into each tube
   - 0.5 pmol extension primer (non-phosphorylated)
   - 0.5 fmol uracil-containing template
   - 0.5 pmol individual mutagenic oligo or pool oligos (0.5 pmol each)
   - 2 µL 10× MEGAA reaction enzyme mix
   - 10 µL 2× MEGAA reaction master mix buffer
   - Nuclease-free water to 20 µL final volume

e) MEGAA Program on PCR thermocycler:
   - 95°C for 90 seconds denaturation
   - 4°C for 60 seconds annealing
   - 55-65°C for 3 minutes extension/ligation
   - 65°C for 60-90 minutes final ligation

Step 3: Amplification of MEGAA product

Reaction setup to PCR amplify MEGAA product

a) 2 µL of MEGAA product
b) 1 µL of forward primer 20 µM
c) 1 µL of reverse primer 20 µM
d) 25 µL of 2 × Q5® Hot Start High-Fidelity Master Mix (NEB # M0494L)
e) 21 µL of nuclease-free water (Invitrogen)

PCR protocol:

f) 98°C for 30 seconds [initial denaturation]
g) 30 cycles of the following:
   - 98°C for 10 seconds
   - *55–72°C for 10 seconds
   - 72°C for X minutes (20-30 seconds/kb)
   - 72 °C for 5 minutes [final elongation]

*Use of the NEB T_m Calculator is highly recommended.

The MEGAA product is now ready for downstream application and MEGAA cycling can be performed if the efficiency is not ideal.

Step 4: MEGAA cycling

a) Repeat MEGAA Steps 1-3 with 1:40000 diluted product as input to minimize the contamination in uracil-containing template.

MEGAAtron automation system components and operation

Premade MEGAAtron master mix

MEGAAtron template generation master mix

- 49.5 µL in total
- 1 µL of forward primer 5 µM (5 pmol)
- 1 µL of reverse primer 5 µM (5 pmol)
- 1 µL of 10 mM dNTPs (dATP, dCTP, dUTP and dGTP at 2.5 mM each)
- 10 µL of 5× Q5U® reaction buffer
- 1 µL of Q5U® hot-start high-fidelity DNA polymerase
- 35.5 µL of nuclease-free water

MEGAAtron reaction master mix

- 2.8 µL in total
- 0.2 µL of extension primer 0.5 µM (non-phosphorylated)
- 0.4 µL 10× MEGAA reaction enzyme mix
- 2 µL 2× MEGAA reaction master mix buffer
- 0.2 µL of nuclease-free water

MEGAAtron amplification master mix

- 46 µL in total
- 2 µL of forward primer 20 µM (40 pmol)
- 2 µL of reverse primer 20 µM (40 pmol)
- 25 µL of 2 × Q5® Hot Start High-Fidelity Master Mix
- 17 µL of nuclease-free water

Protocol details

Step 1: PCR generation of uracil-containing template and clean up

PCR setup to amplify uracil-containing template:

a) 0.5 µL of DNA template (e.g., 1 ng/µL *E. coli* genomic DNA) or diluted MEGAA product from last round of reaction
b) 49.5 µL premade MEGAAtron template generation master mix

PCR protocol:

c) 98°C for 30 seconds [initial denaturation]
d) 30 cycles of the following:
    - 98°C for 10 seconds
    - *55–72°C for 10 seconds
    - 72°C for X minutes (20-30 seconds/kb)
    - 72 °C for 5 min [final elongation]

*Use of the NEB T_m Calculator is highly recommended.

SPRI beads purification of amplified uracil-containing template:

e) Transfer 0.4 µL of generated template and mix with 19.6 µL nuclease-free water. Add the 1× ratio of suspended SPRI beads (20 µL) and mix by pipetting.
f) Perform SPRI beads cleanup according to the same protocol above with volume scaled down for PCR plates.
g) Templates are eluted into 80 µL nuclease-free water, yielding 0.0005 µM uracil-containing template.

Step 2: Mutagenesis using MEGAA

One-pot mutagenesis:

a) Transfer 2.8 µL premade MEGAAtron reaction master mix to each well of the plate on Thermocycler module.
b) Transfer 0.2 µL 0.0005 µM uracil-containing template to the plate respectively.
c) Transfer 1 µL 0.1 µM corresponding oligos pool to the plate respectively and mix by pipetting.

MEGAA Program on thermocycler module:

d) 95°C for 90 seconds denaturation
e) 4°C for 60 seconds annealing
f) 55-65°C for 3 minutes extension/ligation
g) 65°C for 60-90 minutes final ligation

Step 3: Amplification of MEGAA product

Reaction setup to PCR amplify MEGAA product

a) Directly transfer 46 µL premade MEGAAtron amplification master mix to the plate and mix by pipetting.

PCR protocol:

b) 98°C for 30 seconds [initial denaturation]
c) 30 cycles of the following:
    - 98°C for 10 seconds
    - *55–72°C for 10 seconds
    - 72°C for X minutes (20-30 seconds/kb)
    - 72 °C for 5 minutes [final elongation]
d) *Use of the NEB T_m Calculator is highly recommended.

The MEGAA product is now ready for downstream applications or MEGAA cycling can be performed if the efficiency is insufficient.

Step 4: MEGAA cycling

a) Dilute the product 200-fold by mixing 0.4 µL amplification product with 79.6 µL nuclease-free water.
b) Perform the second dilution to yield 40000-fold diluted product.
c) Repeat MEGAA Steps 1-3 with 1:40000 diluted product as input to minimize the contamination in uracil-containing template.