Directed Evolution using a Deaminase Mutator in T7 Bacterial Systems

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Background

Purpose: Target gene of interest (GOI) in a Directed Evolution system

Directed Evolution[1]:

- Non-rational design using genetic systems for protein modifications.
- Rounds of mutagenesis and selection to accumulate mutations in gene of interest (GOI).

Previous system → mutation plasmid targets all plasmids in host cell, not just GOI.

T7 Bacterial System → mutation plasmid targets GOI between T7 Promoter & T7 Terminator.

How does it work?[2]

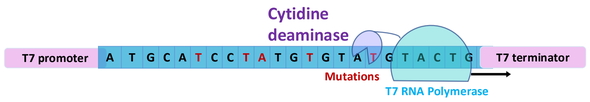


Figure 1. T7 Mutation System. T7 RNA Polymerase recognizes T7 promoter and begins transcription until it reaches the terminator.

- Mutation protein (deaminase) fused to T7RNA Polymerase.
- T7 RNA Pol specifically recognizes T7 promoter and begins transcription.
- Deaminase mutates DNA strand during transcription.
- At T7 terminator, T7 RNA Pol along with deaminase is released.

→ deaminase specifically targets GOI

Phage Assisted Non-Continuous Evolution (PANCE)

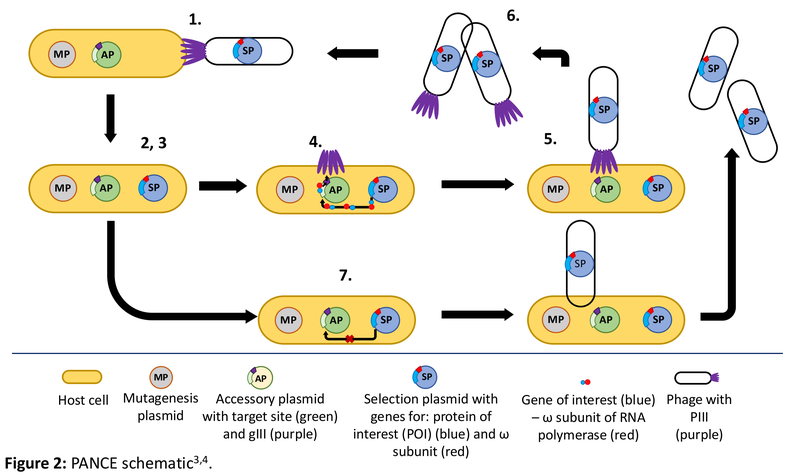


Figure 2: PANCE schematic[3,4].

Selection Phage (SP) = Modified M13 bacteriophage

1. SP infects host cells containing MP & AP via pIII protein.
2. Arabinose induces mutation plasmid (MP) to express mutagenic genes.
3. Mutagenic genes target POI on SP in T7 system.
4. POI expressed by SP → binds to target site on Accessory Plasmid (AP).
5. Binding activates gIII on AP to encode pIII.
6. pIII production forms infectious phages → infect more host cells with SP.
7. No binding → no pIII → phages get washed out.

→ If mutated POI translates protein that does not bind to target site, no gIII is expressed & no phage propagation occurs.

Improved POI = ↑ gIII Expression = ↑ pIII Production = ↑ Phage Propagation
∴ Weak POI = ↓ phage propagation which will be eliminated by natural selection

Mutation Plasmid: eMutaT7[5]

- *pm*CDA1 (cytidine deaminase) fused to T7 RNA Polymerase.
- Mutates DNA during transcription between T7 Promoter + T7 Terminator.

New System: eMutaT7 Phage Assisted Evolution = **eMPAE**

Modifying eMutaT7 For eMPAE

1. UGI Correction

- *pm*CDA1 causes C → T mutations.
- Uracil DNA Glycosylase repairs mutations via Base Excision Repair (BER) Pathway.[6]
- Uracil Glycosylase inhibitor (UGI) inhibits UDG → need UGI expression.

Lack of Shine-Dalgarno (SD) sequence upstream of UGI sequence
∴ no UGI expression results in mutations corrected by BER pathway

→

SDM to introduce native Shine-Dalgarno Sequence in ribosome binding site upstream of UGI
∴ allows UGI expression

Figure 3. DNA segment showing locations of Shine-Dalgarno sequence following SDM.

Kim later released eMutaT7 with an Optimized RBS.

2. Clone in Bacterially Optimized Codons

Kim's *pm*CDA1 not codon-optimized for bacterial systems

- Substituting Kim's *pm*CDA1 with bacterially optimized pmCDA1.[7]

→ New *pm*CDA1 has higher expression in bacterial cells.

Codon Adaptation Index of 0.66 vs 0.91

→ SDM: introduce new restriction sites into both plasmids
→ Cloned in bacterially optimized *pm*CDA1 into eMutaT7

Figure 4. DNA segment showing Xba1 & Xho1 restriction sites upstream and downstream of *pm*CDA1.

∴ Termed new plasmid **MPT7** = Mutation Plasmid for T7 Systems

Modifying Selection Phage (SP) for eMPAE

1. Add T7 Promoter + T7 Terminator

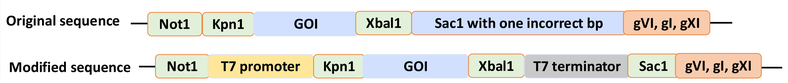


Figure 5. DNA Segments of SP before (top) and after (bottom) modifications made for T7 system.

2. Add Weak Phage Promoter

Low phage titre (activity)
∴ T7 Terminator stopped transcription of essential phage genes

→

Cloned weak promoter to allow transcription of essential phage genes
∴ Genes required for phage maturation

Figure 6. DNA Segment of SP following cloning of weak promoter.

3. Increase Distance between GOI-T7 Terminator

- Short GOI (~300bp).
- T7RNA Pol detaches from DNA before mutations accumulate:

RNA Pol encounters hairpin on terminator faster than deaminase can start mutating GOI.

Steric hinderance between T7 RNA Pol and hairpin on terminator.

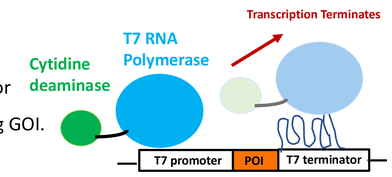
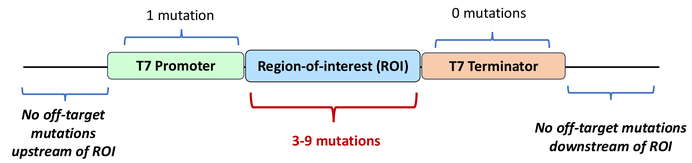


Figure 7: *pm*CDA1-T7 RNA Pol encountering hairpin on terminator and being released.

→ Increase distance between POI – Terminator.
Added ~670 bp linker between POI and Terminator, increasing ROI to ~1000 bp.
→ Allow cytidine deaminase to act on POI before T7RNA Pol encounters hairpin for terminations.

eMPAE Results

- Ran 4x16 hr Passages without selective pressure using MPT7 as mutation vector.
  - Generate a library of protein variants without selection stringency.
- Sequenced ROI for 15 samples.



T7 Promoter: 1 bp mutation in 15/15 samples
ROI: 3-9 bp mutations in 15/15 samples
T7 Terminator: 0 mutations in 5/5 samples
Off-target: 0 mutations in 4/4 samples

Figure 8. Summary of mutations observed after 4 passages.

- Mutation rate calculated based on T7 Promoter + POI sequence.
- 67 total mutations observed in 15 samples.
- 66 C → T mutations; 1 G →A mutation.
- Performed whole plasmid sequencing to determine 4/4 off-target mutations in SP.

→ Mutation rate of **5.6 mutations kbp^{-1} day^{-1}** compared to Kim's 3.5 mutations kbp^{-1} day^{-1}.

∴ 0% Off-Target Mutations in Target Plasmid
∴ High mutation rate compared to previous reported systems

→

∴ Presence of 98% efficient T7 Terminator[8]
∴Codon Optimized base editor (*pm*CDA1) in MPT7

∴ 5 mutations conserved in >70% of sampled population after Passage 10

Mutation Type	Number
Silent	5 (2 conserved)
Missense	10 (3 conserved)
Nonsense	3 (1 conserved)

Table 1. Types of mutations obtained after 10 passages.

Controls:

1. No mutation vector – no mutations observed in ROI
2. MP6 - no mutations observed in ROI
3. eMutaT7 – mutation rate of 1.2 mutations kbp^{-1} day^{-1}

FUTURE DIRECTIONS

- Run selection passages and investigate conserved mutations
- Verify protein evolution through binding assays
- Incorporate other base editors into system (adenine deaminase)
  - Wider mutational spectrum (T→C; A→G)
- eMPAE with additional DNA-binding proteins to generate improved variants

REFERENCES

1. *Nat. Rev. Genetics.* **2015**, 16, 379-394.
2. *Methods Mol Biol*. **2024,** 2793**,** 55-64.
3. *ACS omega.* **2020**, 5(42), 26957-26966.
4. *Nat. Protoc.* **2020**, 15, 4101-4127.
5. *Nucleic Acids Res*. **2021**, 49 (6), e32.
6. *DNA Repair (Amst.).* **2000**, 460 (3–4), 165–181.
7. *Nat. Commun*. **2020**, 11(1), 6436. pmcda2
8. *G3.* **2022,** 12(6).

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