# THE PEN CSR, USING EXTERNAL MOLECULAR PROGRAMS TO CONTROL DIRECTED EVOLUTION OF ENZYMES

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Selection-based strategies used in directed evolution methods proved to be very efficient but rely on the possibility of finding a case-specific selection tool that links the enzyme activity to its gene survival. Among all available directed evolution methods, the Compartmentalized Self-Replication (CSR) of Ghadessy and al. (1) and its derivatives allow to perform high-throughput in vitro selection tests thanks to microdroplets compartmentalization. Because engineered enzymes are often intended for use in unnatural environments, this kind of methods are particularly interesting. Yet, they can only be applied to polymerases replicating their own genetic sequence in the droplets.

Here, we used an external DNA-based artificial network to create a feedback loop linking the activity of a nicking enzyme to the replication of its own gene. Molecular networks, such as the one using the PEN DNA toolbox (2), are designed to produce short DNA strands interacting within each other's thanks to a set of enzymes. Taking short oligonucleotides at the input, they can generate short oligonucleotides of arbitrary sequence at the output. Enzyme activity can be assessed by these molecular networks thus allowing to produce a correlated amount of primers at the output. These primers can then be used to run the PCR of the enzyme's gene. Compared to the Compartmentalized Partnered Replication (CPR) (3), our method keeps the ability to perform in vitro assays of the target enzyme and does not necessitate any gene circuitry optimization.

Practically, bacteria carrying and expressing the mutants are co-encapsulated with the molecular program in individual droplets using microfluidics. The isothermal primers amplification (IPA) by the network is next initiated by raising the temperature to 45°C. After running the program for some time, each droplet contains an amount of the enzyme's gene forward and reverse primers corresponding to the activity of the enzyme expressed by the bacterium. A PCR is then launched in the droplets. Its yield in each droplet will then depend on the amount of primers, therefore on the enzyme activity. Up to $10^7$ parallel self-selections can be run simultaneously on individual copy of the gene. After emulsion breakage, we retrieve a library enriched in the best mutant genes that can be used for a new cycle.

Two different selection pressures were applied by playing either on the IPA duration time to select for faster enzymes or by adding a heat shock at 65°C to select for higher thermostability. Next generation sequencing using MinION allowed us to follow the evolution process. This work is the first demonstration of the Programmable External Network based CSR (PEN CSR) method. Programs detecting other types of activity can be envisioned and would allow not only to greatly expand the scope of the CSR but also to implement smart selection functions.

(1) F. J. Ghadessy and al. Proc. Natl. Acad. Sci. U.S.A., vol. 98, Apr. 2001.
(2) K. Montagne and al. Mol. Syst Biol., vol. 7, Feb. 2011.
(3) J.W. Ellefson and al. Nat. Biotechnol., vol. 32, Jan. 2014.