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Synthesis and purification of site-specifically modified T7 RNA polymerase for bioorthogonal conjugation applications

D.O. Semeniuk, N.O. Hrubiian, S.O. Novosolov, M.O. Trokoz,
S.D. Panashchuk, O.M. Hubar

Yuria-Pharm LLC
19, Sviatoslava Khorobroho Str., Kyiv, Ukraine, 03161
dmytro.semeniuk@uf.ua

Background. Flow reactor synthesis of RNA using immobilized T7 RNA polymerase is a promising technological approach for large-scale therapeutic RNA production. Immobilization of T7 RNAP for flow reactor systems requires protocol for site-specific immobilization that allows controling the immobilization position to maximize activity and half-life of the enzyme. **Aim.** To develop protocol for site-specific introduction of bioorthogonal reaction groups into T7 RNAP while preserving activity of the enzyme. **Methods.** N-terminal 8xHisTag, FGE motif and G3S linker were introduced into T7RNAP CDS by long-overhang PCR and modified CDS was cloned into pGEX-2T backbone under lac-promoter. TAG codon was introduced into 8xHisTag-FGEmotif-GS3-T7RNAP in place of Cys codon of FGE motif by SDM. T5pLacO-driven cassette for over-expression of C-terminal fragment of L11 ribosome protein was cloned downstream of T7RNAP cassette by Golden Gate assembly. CDSs for mtbFGE, pAzFRS.2.t1 and CTA-tRNA were synthesized from oligo pools (Yuria-Pharm, Ukraine) by OE-PCR. Helper plasmids carrying expression cassette for mtbFGE and for pAzFRS.t1/CTA-tRNA were assembled by Golden Gate cloning according to reference constructs (AddGene ID102615, AddGene ID73546). In both systems, the expression and helper vectors were co-transformed into *E.coli* BL21 strain, ON cultures were inoculated into fresh media (in presence of arabinose and 1mM of pAzF for pAzF system) and induced at OD 0.6 by addition of IPTG/arabinose (and 100uM of $CuSO_4$ for FGly system). The cultures were incubated for 16h at RT, harvested and purified by Ni-NTA spin columns. The label incorporations were quantified by RP-LC-MS of an intact protein or modification-containing peptide, cleaved by TEV protease. **Results.** FGly system. Investigation of initial unsuccessful attempts to obtain modified protein identified *in cellulo* aggregation of mtbFGE as a root cause. Induction and cultivation of producer cells at reduced temperatures (18 °C) allowed to obtain protein with >94% modification rate at final titers of 6 mg/L. Quantitative *in vitro* modification of FGE motif by purified mtbFGE has also been demonstrated for T7RNAP and can be used as well. pAzF system. Initial attempts of co-translational pAzF installation failed to produce detectable level of T7 RNAP. Investigation confirmed issues with helper plasmid toxicity, possibly due to sequestration of ribosomes by uncharged CTA-tRNA. Addition of pAzF and arabinose into culture media during cultivation (at 18 °C) allowed to rescue production of fully modified T7RNAP achieving final titers of 3mg/L. Mass-spectrometry of purified products showed presence of ~35% of reduced azide form, which was lowered by removal of DTT from purification buffers. **Conclusion.** For both systems, the optimized protocols were developed, which allow successful incorporation of the bioorthogonal groups with near-quantitative modification efficacy and practical yields. Due to a greater flexibility of pAzF system for tether group positioning it will be used for immobilization studies.

Keywords: T7 RNA polymerase, immobilization, FGE, pAzF, flow reactor synthesis, IVT, therapeutic RNA.