Application of the PURExpress® portfolio for reconstitution of a tRNA-dependent DNA hypermodification pathway

Rebekah M. B. Silva, Ph.D., Y-J Lee, Ph.D., and Peter Weigele, Ph.D.

INTRODUCTION

The PURExpress® *In Vitro* Protein Synthesis system is widely used for cell-free protein synthesis (CFPS). The modular and defined formulation of the product line serves as a powerful platform for reconstitution of enzymatic activity and discovery of new biochemistry. Described here is an expanded application where the PURExpress kit portfolio was used to investigate the biosynthesis of a DNA hypermodification from bacteriophage Mu and to facilitate the identification of the co-substrate used by a phage-encoded enzyme, solving a long-standing complex scientific puzzle.

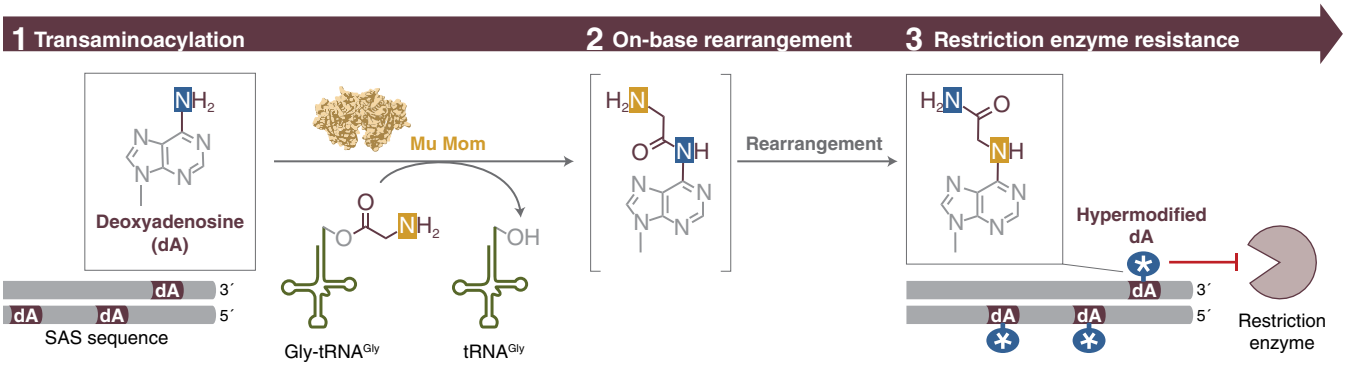
Decades ago, an unusual adenosine modification was found in the gDNA of *E. coli* phage Mu[1]. A Mu gene named *mom* (short for modification of Mu DNA) was associated with the modification; however, the biosynthetic origin of the modification remained unknown for over 40 years[2]. The Mom protein was predicted to belong to the GNAT (Gcn5-related N-Acetyltransferase) superfamily of enzymes, where acyl-CoAs are most often used for acylation of targets. Given that the Mu modification is isomeric with glycine, a tRNA-dependent mechanism for glycine transfer was hypothesized, as tRNA can serve as an amino acid carrier for some GNATs to aminoacylate targets.

By using PURExpress as a source of well-defined, high-quality biochemical components—including amino acids, tRNAs, and aminoacyl-tRNA synthetases—we were able to test the tRNA hypothesis systematically *in vitro*. The modularity of the PURExpress system, including kits lacking amino acids, tRNAs, or ribosomes, allowed for rapid dissection of the minimal requirements for activity. These studies established that glycine is transferred by Mom to dA in a tRNA-dependent process and enabled reconstitution of the complete reaction under simplified, translation-independent conditions (Figures 1 and 2).

MATERIALS

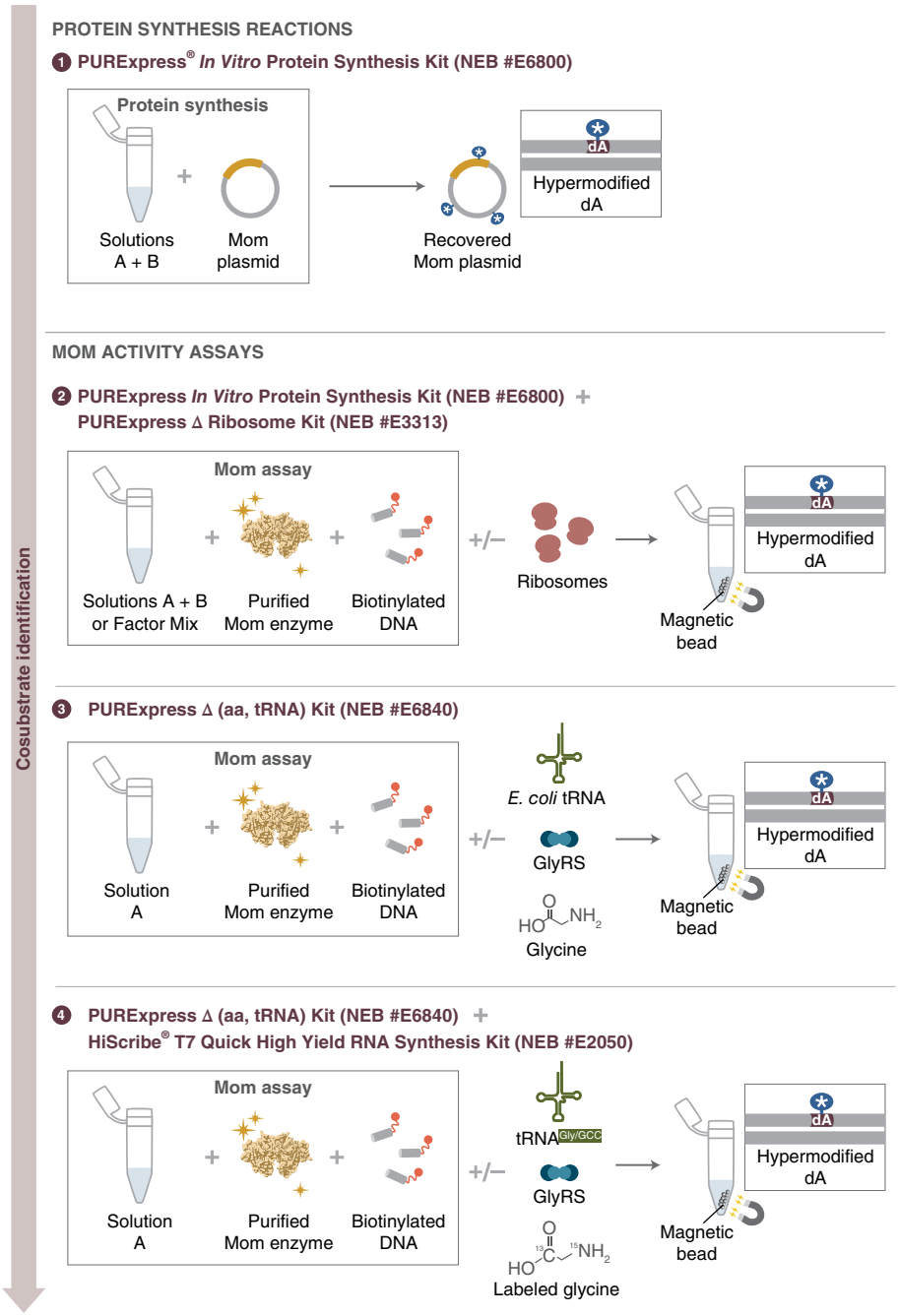
- PURExpress® *In Vitro* Protein Synthesis Kit (NEB #E6800)
- PURExpress® Δ Ribosome Kit (NEB #E3313)
- PURExpress® Δ (aa, tRNA) Kit (NEB #E6840)
- Monarch® Spin PCR & DNA Cleanup Kit (5 µg) (NEB #T1130)
- Streptavidin Magnetic Beads (NEB #S1420)
- 6-Tube (NEB #S1506) or 12-Tube (NEB #S1509) Magnetic Rack
- Nucleoside Digestion Mix (NEB #M0649)
- HiScribe® T7 Quick High Yield RNA Synthesis Kit (NEB #E2050)
- Monarch® Spin RNA Cleanup Kit (500 µg) (NEB #T2050)

FIGURE 1: **tRNA-dependent hypermodification of DNA by Mu Mom**



Prior to the work described here, the co-substrate used for hypermodification of phage Mu was unknown. Aided by the PURExpress portfolio of products, a novel mechanism for genome defense was uncovered, in which a viral enzyme from bacteriophage, Mu Mom, co-opts charged $tRNA^{Gly}$ to hypermodify DNA at N6 of adenine through transaminoacylation and isomerization of glycine[2].

FIGURE 2: **Overview of experimental strategies and workflows**



PURExpress kits were used for *in vitro* protein synthesis of Mom and for activity assays with purified Mom and biotinylated DNA substrates. Activity assays with E3313 and E6840 enabled testing of the activity of Mom in the presence and absence of kit components, or with the inclusion of specific components of interest, as in the case for glycine, GlyRS, and $tRNA^{Gly/GCC}$ synthesized by IVT. Plasmids recovered after protein synthesis or biotinylated DNA captured after activity assays were subjected to nucleoside analysis by LC/MS to monitor formation of 6-*N*cmdA.

This work highlights an additional application of the PURExpress portfolio—supporting the study of tRNA-dependent enzymes in aminoacyl-transfer reactions outside of translation. As shown here, PURExpress provides a robust and modular platform for *in vitro* biochemical reconstitution and co-substrate identification, particularly for enzyme systems where cellular context complicates interpretation.

METHODS

Protein synthesis by PURExpress and nucleoside analysis of expression plasmids

Protein synthesis reactions used 1 µg of plasmid encoding Mom under the control of a T7 promoter. Separate control synthesis reactions were assembled using 1 µg of an empty plasmid or 250 ng of the provided DHFR control template. Synthesis reactions using the PURExpress *In Vitro* Protein Synthesis Kit (NEB #E6800) were prepared as recommended and incubated at 25 °C for 24 hours in 30 µl volumes[3,4]. Plasmids were recovered using the Monarch® Spin PCR and DNA Cleanup Kit (NEB #T1130). Plasmids were digested using the Nucleoside Digestion Mix (NEB #M0649), and nucleosides were analyzed by LC/MS as described[5].

Preparation of biotinylated DNA substrates

Biotinylated DNA substrates lacking a T7 promoter were prepared by digesting λ DNA (NEB #N3011) by MseI (NEB #R0525) and backfilling with biotinylated UTP (Jena Biosciences, NU-803-BIO16) as described[2,5]. Alternatively, the pUC19 vector (NEB #N3041) could be used as a DNA substrate for activity assays.

Synthesis and purification of $tRNA^{Gly/GCC}$

The gene for $tRNA^{Gly/GCC}$ from *E. coli* with an upstream T7 promoter was PCR-amplified from a pUC19 vector using Q5® High-Fidelity 2X Master Mix (NEB #M0492) and purified using Monarch PCR & DNA Cleanup Kit (5 µg) (NEB #T1130). $tRNA^{Gly/GCC}$ was synthesized using the HiScribe® T7 Quick High Yield RNA Synthesis Kit (NEB #E2050) using 1 µg of PCR template and GMP (20 mM, Sigma G8377) with a 16-hour incubation at 37 °C in a volume of 20 µl. tRNA was treated with DNase I (NEB #M0303) and purified using the 500 µg Monarch Spin (NEB #T2050).

Activity assays with PURExpress kits

The PURExpress *In Vitro* Protein Synthesis Kit (NEB #E6800), PURExpress Δ (aa, tRNA) Kit (NEB #E6840), and PURExpress Δ Ribosome Kit (NEB #E3313) were utilized for activity assays (without plasmid template), which included PURExpress kit components mixed as recommended, purified WT Mom (2 µM), and 1 µg of biotinylated DNA substrates. Where indicated, reactions were supplemented with $tRNA^{Gly/GCC}$ synthesized through IVT (4 µM), glycine (2 mM, Sigma G7126), glycine-1-^{13}C,^{15}N (2 mM, Sigma 299340), or glycyl-tRNA synthetase (GlyRS, 25-100 µg/ml; NEB internal material) from *E. coli*. Reactions were incubated at 25 °C for 24 hours or 37 °C for ≥ 2 hours, treated with Monarch RNase A (NEB #T3018), and biotinylated substrates were recovered using Streptavidin Magnetic Beads (NEB #S1420) and a 6-Tube (NEB #S1506) or 12-Tube (NEB #S1509) Magnetic Separation Rack[2]. Recovered DNA was subjected to nucleoside analysis as above.

RESULTS

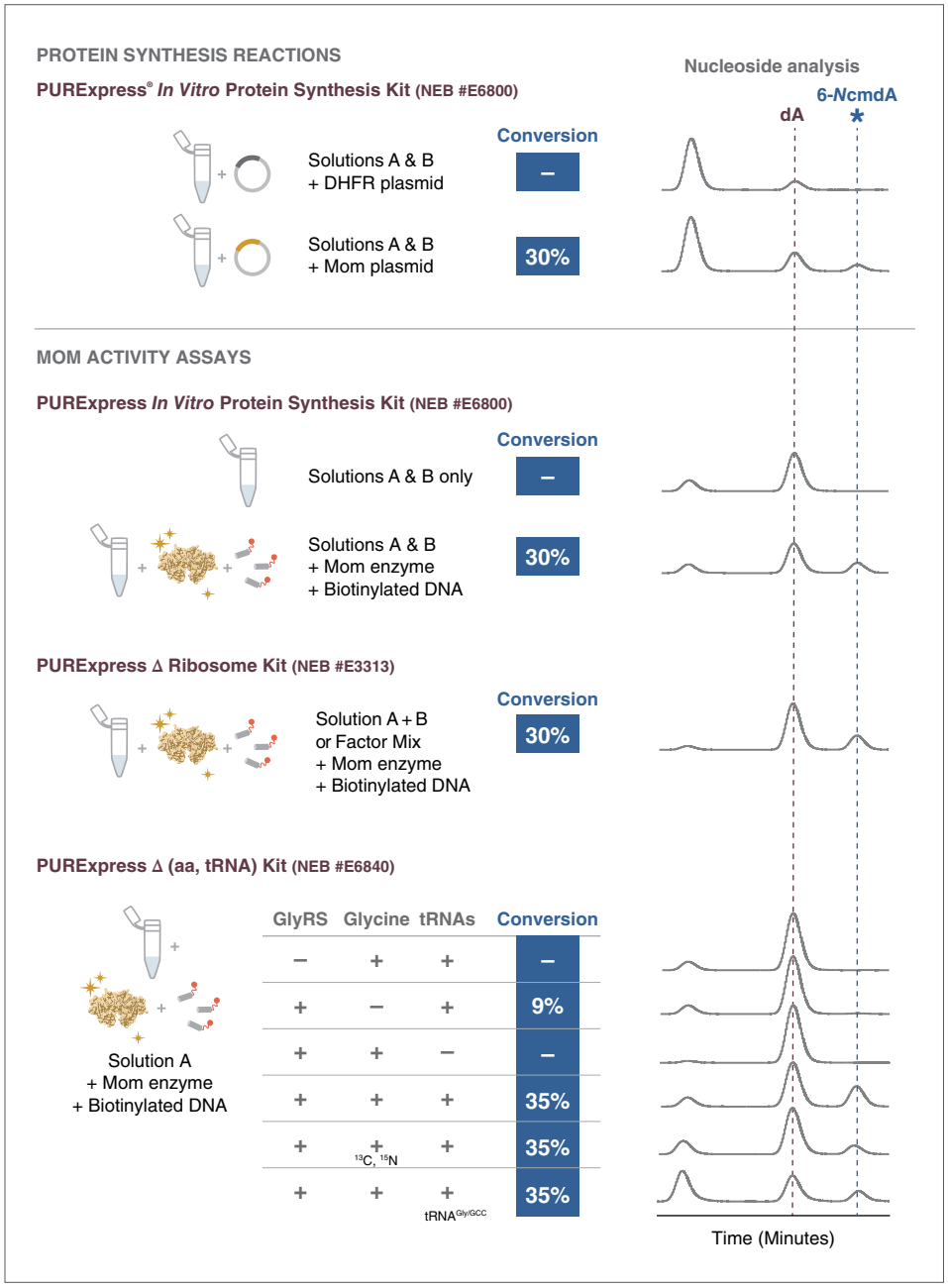
Mu Mom synthesized by *in vitro* transcription and translation with PURExpress is active

After incubation of Mom plasmid (1 µg) for 24 hours at 25 °C, nucleoside analysis of recovered plasmids (Figure 3, Trace 2) showed that 30% of dA was converted to a product with the same mass and retention time as the native modification from phage Mu (not shown). We assign the novel peak in Trace 2 to 6-*N*cmdA. No modification was observed in the control synthesis reaction with DHFR (Figure 3, Trace 1). In a single set of straightforward experiments, we determined that Mom could be synthesized in an active form by *in vitro* transcription and translation using PURExpress and that components of transcription/translation support 6-*N*cmdA formation.

Purified Mom is active on dsDNA substrates in the PURExpress reaction mixture

We next tested if Mom (2 µM), which had been heterologously expressed and purified, was active on biotinylated dsDNA substrates (1 µg) in the PURExpress reaction mixture in the absence of active transcription and translation by omitting the typical DNA template encoding for T7 promoter-driven protein. After overnight incubation at 25 °C, biotinylated substrates were recovered, digested to nucleosides, and analyzed.

FIGURE 3: **The PURExpress portfolio facilitates reconstitution of 6-*N*cmdA *in vitro* and systematic identification of the co-substrate used by Mu Mom to hypermodify dA.**



It was first established that components of transcription/translation support 6-*N*cmdA formation in protein synthesis reactions expressing Mom (1 µg of plasmid) or (Traces 1–2) in activity assays where purified Mom (2 µM) is incubated with DNA substrates (1 µg) and protein synthesis reaction components in the absence of expression template (Traces 3–4). Activity assay conditions were then varied systematically using a combination of kits and reagents (Traces 5–10) and GlyRS, glycine, and tRNA were found to be necessary to observe full modification (Traces 6–9). Using glycine labeled with ^{13}C and ^{15}N, it was further demonstrated that a glycyl group is used by Mom to hypermodify dA (Trace 10, 6-*N*cmdA +2 in mass relative to unlabeled, not shown). Finally, 6-*N*cmdA could be reconstituted in a reaction with fully purified components – $tRNA^{Gly/GCC}$, GlyRS, ATP, glycine, Mom, and dsDNA (Trace 11). The modification is not detected (ND) in controls where a Mom expression plasmid or purified Mom was not included. Displayed traces were aligned at dA.

LC/MS of digested nucleosides confirmed that Mom was purified in an active state and could install 6-*N*cmdA using the substrates and cofactors already present in the PURExpress system in the absence of active protein synthesis driven by a T7 promoter (Figure 3, Traces 3 and 4).

The PURExpress Δ Ribosome Kit and the PURExpress Δ (aa, tRNA) Kit allow for systematic identification of hypermodification components

Reconstituting 6-*N*cmdA *in vitro* enabled the determination of the specific reaction component(s) used by Mom to hypermodify dA. Using the PURExpress Δ Ribosome Kit for an activity assay, we determined that ribosomes were not necessary for Mom to install 6-*N*cmdA (Figure 3, Trace 5). The PURExpress Δ (aa, tRNA) Kit enabled direct testing of our hypothesis that Mom may hypermodify dA through aminoacylation. In a series of activity assays, we omitted either GlyRS, glycine, or tRNAs (Figure 3, Traces 6–8). A small amount of modification was detected without the addition of glycine (Trace 7). We observed full modification only when GlyRS (25 µg/ml), glycine (2 mM), and tRNAs (2.5 µl) were included in an activity assay with Mom and dsDNA (Figure 3, Trace 9).

Modularity of the PURExpress Δ (aa, tRNA) kit enables reaction monitoring through stable isotope labeling experiments with glycine-1-^{13}C,^{15}N

The formulation of PURExpress Δ (aa, tRNA) allowed us to supplement an activity assay for Mom with a stable isotope of glycine labeled with ^{13}C and ^{15}N (Figure 3, Trace 10).

The mass of 6-*N*cmdA formed in the presence of glycine-1-^{13}C,^{15}N was heavier by 2 Daltons (310 Da, not shown) relative to unlabeled reactions (308 Da), demonstrating that glycine is the source of the aminoacyl group transferred by Mom to dA.

Hypermodification of dA by Mu Mom with glycine is dependent on tRNA$^{Gly/GCC}$

In the final set of experiments with the PURExpress Δ (aa, tRNA) Kit, we substituted the mixture of tRNAs from *E. coli* for tRNA$^{Gly/GCC}$ synthesized by IVT from a template encoding *glyV* from *E. coli* using the HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB #E2050) and purified using the Monarch Spin RNA Cleanup Kit (500 µg) (NEB #T2050). As can be seen in Figure 3, Trace 11, the formation of 6-*N*cmdA by Mom is supported when tRNA$^{Gly/GCC}$ synthesized by IVT is included. In total, these data indicate that Mom transfers a glycyl group, likely from Gly-tRNA^{Gly}, to hypermodify dA.

CONCLUSION

The PURExpress portfolio was used here in an efficient, systematic workflow for deciphering the co-substrate needed by Mu Mom to hypermodify dA. This work also highlights an additional application of PURExpress kits, which accelerated the discovery of biochemistry utilizing amino acids and tRNA to hypermodify DNA[6,7]. The approach used here may be valuable for exploring aminoacyl transfer by other GNATs and tRNA-utilizing enzymes part of natural product biosynthesis pathways[8–10]. In these cases, PURExpress kits can be used in one-pot assays for *in vitro* reconstitution of activity.

References

1. Weigele, P. & Raleigh, E. A. Biosynthesis and Function of Modified Bases in Bacteria and Their Viruses. Chem Rev 116, 12655–12687 (2016).
2. Silva, R. M. B. et al. A single viral enzyme drives tRNA-dependent hypermodification of DNA at adenine. Nat. Commun. (2026) doi:10.1038/s41467-026-70671-1.
3. Shimizu, Y. & Ueda, T. Cell-Free Protein Production, Methods and Protocols. Methods Mol Biology 607, 11–21 (2009).
4. Tuckey, C., Asahara, H., Zhou, Y. & Chong, S. Protein Synthesis Using a Reconstituted Cell-Free System. Curr Protoc Mol Biology 108, 16.31.1-16.31.22 (2014).
5. Silva, R. M. B. et al. Modification of DNA by a viral enzyme and charged tRNA. bioRxiv 2023.03.24.534169 (2023) doi:10.1101/2023.03.24.534169.
6. Ulrich, E. C. & Donk, W. A. van der. Cameo appearances of aminoacyl-tRNA in natural product biosynthesis. Curr Opin Chem Biol 35, 29–36 (2016).
7. Francklyn, C. S. & Minajigi, A. tRNA as an active chemical scaffold for diverse chemical transformations. Febs Lett 584, 366–375 (2010).
8. Scott, T. A. & Piel, J. The hidden enzymology of bacterial natural product biosynthesis. Nat. Rev. Chem. 3, 404–425 (2019).
9. Maruyama, C. & Hamano, Y. tRNA-dependent amide bond–forming enzymes in peptide natural product biosynthesis. Curr. Opin. Chem. Biol. 59, 164–171 (2020).
10. Burckhardt, R. M. & Escalante-Semerena, J. C. Small-Molecule Acetylation by GCN5-Related N -Acetyltransferases in Bacteria. Microbiol Mol Biol R 84, 10.1128/mmbr.00090-19 (2020).