One-Pot *E. coli* Cell-Free Extract for *in vitro* Expression of Disulfide Bonded Proteins

Author List

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Abstract

In vitro expression of proteins from *E. coli* extract is a useful method for prototyping and production of cytotoxic or unnatural products. However, proteins that have multiple disulfide bonds require custom extract that to date requires careful addition of exogenous, purified isomerase enzymes. Many important proteins such as enzymes, cytokines, and monoclonal antibodies require disulfide bonds for stabilization and/or proper activity. Currently the only solution to expressing these products is to purchase commercial kits (such as PURE*frex*® (GeneFrontier) or PURExpress® (New England BioLabs)) or to grow up the KGK10 strain and supplement with purified enzymes (T7 RNA polymerase and disulfide bond isomerase C). This multistep process can limit the accessibility of such extract to some groups that wish to rapidly prototype proteins with disulfide bonds. In this work, we present a “one-pot” solution that does not require addition of supplemental enzymes. This is done using a commercially available SHuffle® T7 Express *lysY* strain of *E. coli* that can express both the needed T7 polymerase and DsbC isomerase enzymes. We find optimal growth conditions for our 1 L cultures in 2.5 L shake flasks (5.6 and 3.9 hr for harvest and induction respectively) using a luciferase (from *Gaussia princeps*) that contains 5 disulfide bonds as our reporter protein. The method presented here uses a continuous pass homogenizer and pilot scale lyophilizer to produce large volumes of extract; also, optimal reconstitution ratios of the dried extract are found. To show the broad applicability of the extract, three other enzymes containing ≥3 disulfide bonds (hevamine, endochitinase A, and periplasmic AppA) were also expressed from minimal genetic templates that had undergone rolling circle amplification.

Keywords

Disulfide bond, cell-free protein synthesis, luciferase, chitinase

Introduction

Cell free protein synthesis (CFPS) has seen many recent applications in prototyping gene circuits,[1–3] sensors,[4–8] and therapies.[9–11] Cell extract derived from *E. coli* is the most widely used

with efficient extract protocols, multiple supplement recipes, and clear experimental protocols available in literature.[12–18] One major drawback to using *E. coli* extract for protein expression is the inability to perform post-translational modifications. There has been much work to engineer glycosylation[19,20] and disulfide bond-formation[21,22] pathways. However, these improvements still require skilled augmentation of extract, often adding purified chaperones and enzymes that are expressed in other cells. Many extracellular proteins of interest contain multiple disulfide bonds,[23] such as therapeutic proteins like human growth hormone,[24] proinsulin,[25] antibodies,[26] and interleukin-6.[27] Disulfide bonds improve resistance to degradation by proteases and improve thermodynamic stability.[28] This strategy to engineer and improve wild type proteins is employed by industrial enzyme producers and the biopharmaceutical industry.[22,29] In eukaryotes, disulfide bonds are typically formed in the endoplasmic reticulum (ER) while folding in the periplasm of *E. coli*.[30,31] This is not possible for CFPS since the periplasm is compromised during cell lysis.

However, a few *E. coli* strains have been engineered to improve cytoplasmic expression of proteins with disulfide bonds. KGK10 is a strain that has been engineered to improve disulfide bond formation during CFPS. It was derived from the KC6 strain by deleting the glutathione reductase (*gor*) gene and adding a hemagglutinin tag to the thioredoxin reductase (*trxB*) gene.[9,32] The BL21 (DE3) Star strain is broadly used in CFPS and has been directly used to express disulfide bonded proteins, but it is limited to a maximum of two bonds per protein.[33,34] The PURE*frex*® commercial system, made of purified, recombinantly expressed proteins, is another platform capable of reliably producing proteins with disulfide bonds but has higher cost per reaction.[35]

In line with our goal to make CFPS a robust method readily transferable to any research group,[12–14] we desired to identify and optimize a single strain that could be used to grow a single batch (‘one-pot’) of cells to produce extract without need for supplementation with exogenous enzymes. We identified a commercially available *E. coli* strain that has been optimized to support *in vivo* disulfide bonded protein expression in the cytoplasm, T7 SHuffle® by New England BioLabs (NEB). The SHuffle strain is derived from ER2566 and has been modified to overexpress T7 RNA polymerase (T7 RNAP) and disulfide bond isomerase C (DsbC) once chemically induced by the addition of isopropyl β- d-1-thiogalactopyranoside (IPTG) (Fig 1). The SHuffle strain also eliminates unwanted reduction pathways in the cytoplasm due to deletion of the *trxB* and *gor* genes (Fig 1E).[29,36]

To understand the significance of these modifications, it is important to have an understanding of the intracellular redox environment. The indiscriminate oxidation of cysteines is carried out by disulfide bond isomerase A (DsbA) which forms disulfide bonds between successive cysteines (Fig 1A). DsbC allows for thermodynamically driven proof-reading by isomerizing disulfide bonds that have been formed between sub-optimal cysteines, thus allowing for continued folding to the proper state and disulfide linking (Fig 1B). In an unmodified strain, thioredoxin reductase will reduce disulfide bonds or prevent them from forming. Also, in unmodified strains, glutathione reductase will reduce oxidized glutathione (Fig 1D), thus removing its ability to oxidize thiol groups on cysteines.[29,37]

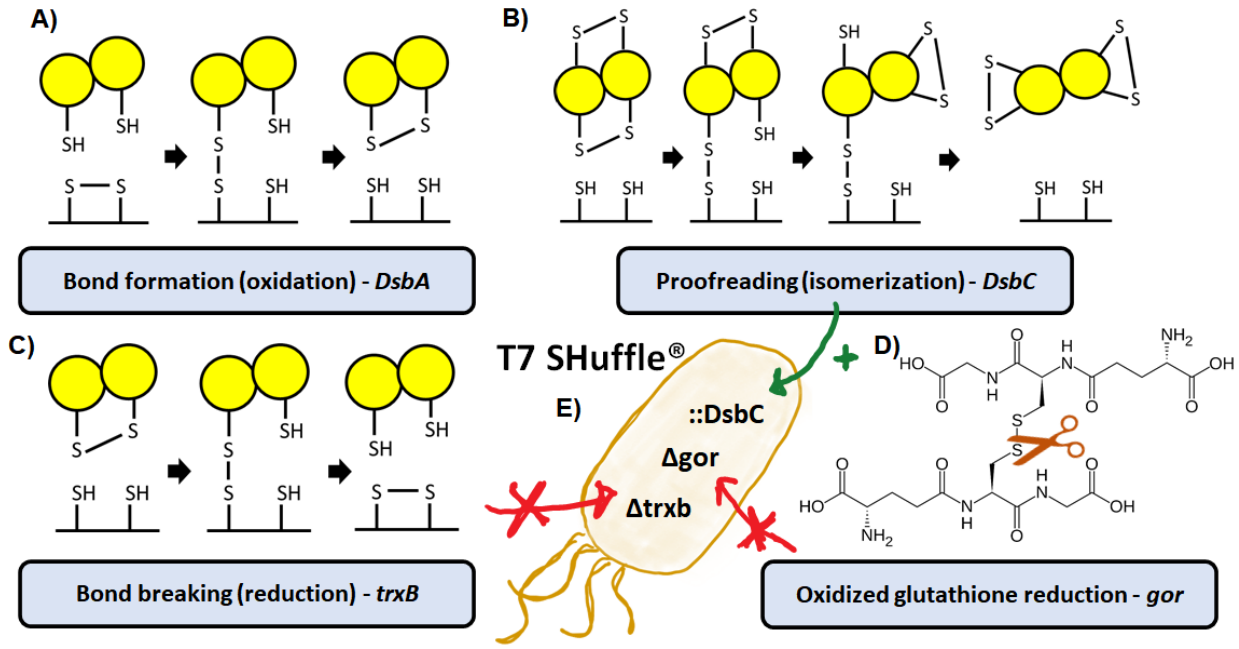

Figure 1 – Schematic of mechanisms that affect disulfide bond formation and implications in the T7 Shuffle strain. (A) Oxidation via DsbA forms bonds between thiol groups on cysteines. (B) DsbC enzymes proofread proteins and isomerize disulfide bonds. (C) Reduction can occur via trxB and (D) gor enzymes that cleave disulfide bonds on the protein. (E) The T7 SHuffle® strain is engineered to support disulfide bond formation by eliminating reducing enzymes and overexpressing the DsbC chaperone. This figure has been modified from a published schematic on disulfide bond formation.[37]

In this work, we investigate the potential of T7 SHuffle® as a platform for CFPS based prototyping of proteins with greater than two disulfide bonds. We optimize the extract preparation based on our scalable techniques previously used for BL21 DE3 Star cells.[13] Luciferase from *Gaussia princeps* is used as a disulfide bonded reporter protein for extract optimization due to its efficient luminescence and previous application in CFPS.[38–40] We then demonstrate the broader applicability of this extract by expressing three additional enzymes with disulfide bonds: hevamine from the rubber tree *Hevea brasiliensis*,[41] endochitinase A (ChitA) from *Zea mays*,[42] and the periplasmic acid phosphatase, phytase (AppA) from *Escherichia coli*[43] which have 3, 7, and 4 disulfide bonds respectively. Some practical applications of the two chitinases include agricultural pathogen control, contribution to asthma, and formation of chitooligosaccharides.[44] Moreover we introduce a custom fusion protein that allows us to measure the ratio between oxidative potential and general productivity of an extract.

Results and Discussion

Designed Experiments

To optimize the growth conditions of the SHuffle strain, we performed two face-centered-cubic (FCC) response surface design of experiments (DoE). This is an established statistical method to optimize an experimental outcome, with minimal experimental runs while accounting for cross-effects of input variables.[45] In this case, the input variables under study are time of induction with ITPG to produce T7 RNAP and DsbC proteins as well as the overall time of harvest. The outcome to optimize is the amount of protein: in the first DoE, we measure the yield of sfGFP to compare to other strains (namely our work with BL21 DE3 Star cells); in the second, we measure the level of correctly folded Gluc to observe the level of disulfide-bonded protein yield. Gluc is a luciferase with five disulfide bonds and a strong luminescent signal making it a convenient reporter for extract optimization.[46] In both cases, we constrained the growth times to encompass a single workday (< 7 hr growth time, as pelleting, washing, and storage must be completed that same day). Each response surface design yielded 13 runs, of which 5 were replicated center points to measure experimental drift. The measured values were then fit to quadratic models and the response surfaces are presented as contour plots (Fig 2 A-B).

The response surface for sfGFP shows that longer growth times would improve the expression (Fig 2A). This indicates that insufficient T7 RNAP is being expressed to maximize overall yield; this is also apparent in the level of sfGFP fluorescence observed which is less than we typically observe with BL21 DE3 Star extract when assayed on the same plate. Since sfGFP contains no disulfide bonds, this response surface is not capable of predicting optimum DsbC production for chaperoning disulfide bond formation. This was resolved using our second reporter protein, Gluc. The response surface for Gluc luminescence output has a clear maximum within our experimental bounds (Fig 2B), indicating the optimum balance of chaperone protein and T7 RNAP to produce disulfide proteins. An optimum growth time of 5.6 hr and an optimum induction point at 3.7 hr are found for these smaller growth vessels (200 mL media in 500 mL Tunair flasks), with greater sensitivity in growth time (Supplementary Figure 1A). These optimum induction and harvest time points were then translated to percentages of the total growth curve (fit by a Verhulst-Pearl logistics function) to allow for application to other growth curves occurring in larger vessels (50.49% and 13.12% respectively), as we have done before.[13] The optimum time points for the 2.5 L shake flasks we use with 1 L culture are then found from these percentages (5.6 and 3.9 hr for harvest and induction respectively). It should be noted that while Gluc is a convenient reporter protein, optimal extract processing conditions may be protein dependent. This is also evident when using Shuffle for *in vivo* expression conditions where temperature, time of induction, and IPTG concentration must be optimized for each new protein to see best results.[29] However, for the prototyping phase of new proteins we have found sufficient level of expression to quantify protein and measure desired properties using a single general extract; the quest for custom, optimal extracts for each protein is better suited in a later stage such as scale up for production of a few desired proteins.

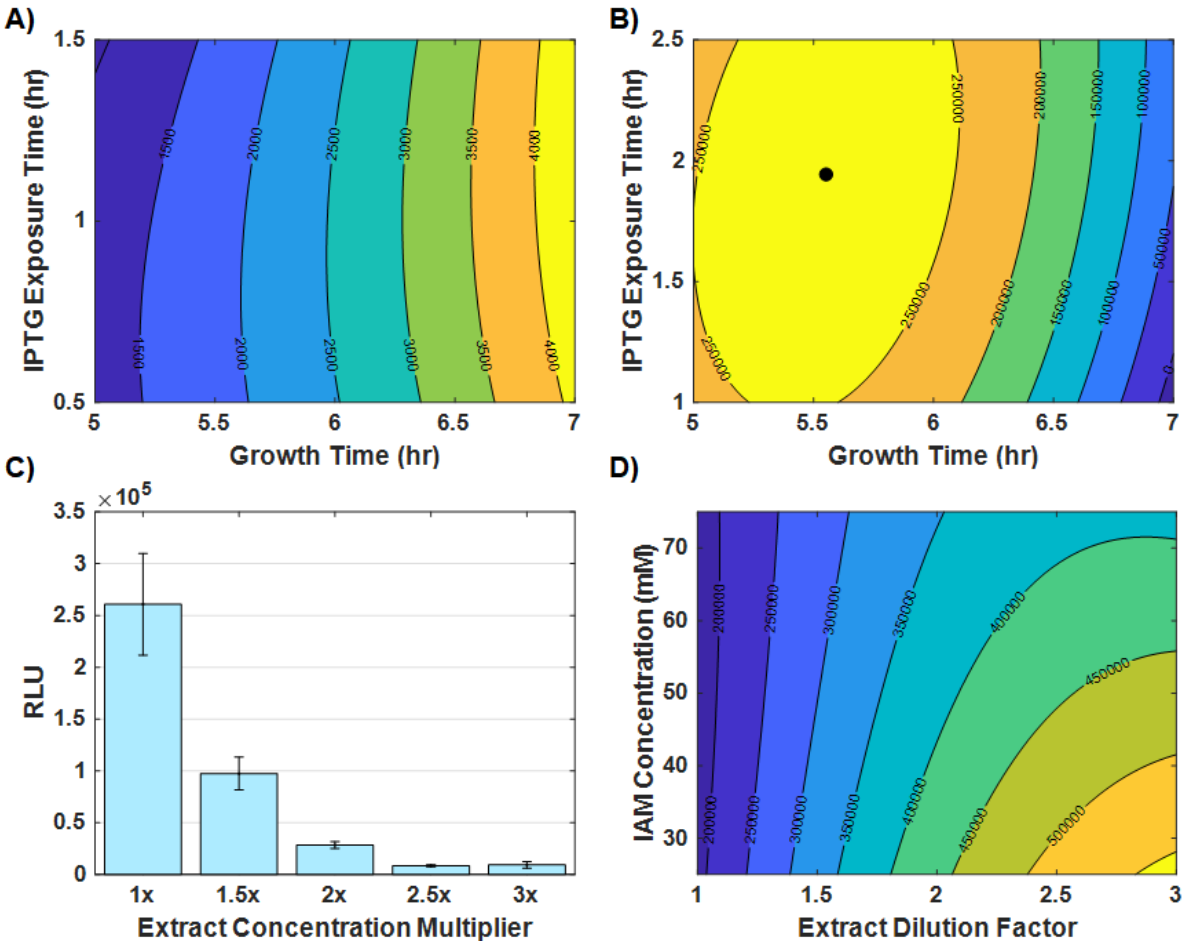

Figure 2 – Experiments to optimize expression from T7 Shuffle extract. (A) Response surface fit to determine optimal growth conditions for sfGFP expression; z axis shows fluorescence (B) Response surface fit to determine optimal growth conditions for Gluc expression; z axis shows luminescence (C) The effect of increasing the extract concentration on the expression of Gluc (n=5). (D) Response surface to observe the effect of diluting extract and iodoacetamide concentration on Gluc luminescent signal (z-axis, RLU).

Extract and Iodoacetamide Concentration

To improve expression, we explored the impact of extract concentration and adding a common reagent used to inactivate thiol containing reductases, iodoacetamide (IAM).[21,32] Previous literature has shown that increasing extract concentration can lead to increased expression rates at the cost of a reduction in total protein produced.[47] In agreement with literature, concentrating the extract led to a sharp decrease in levels of Gluc luminescence as can be seen in Fig 2C. Surprisingly, subsequent dilutions outperformed the standard, non-diluted extract. The small molecule IAM covalently bonds with thiol groups on reductases to reduce their ability to impair disulfide bond formation and is a common supplement to cell free extract reaction

mixes for disulfide bonded proteins. Thus, to observe the effect of both extract and IAM concentration simultaneously, we performed another response surface designed experiment. It makes physical sense that these two variables are interrelated, and thus merit a DoE approach for study; IAM must be optimized to match the reduced number of endogenous reductases in diluted extract and IAM can also interfere with thiol groups in the overexpressed DsbC. For our experiment, the optimum occurred at the edge of our experimental range (Fig 2D) and indicates a 3x dilution with 25 mM IAM. Since water was used for the dilutions, these low extract concentrations also dilute the buffer A concentration (what the cells are lysed in), a lurking variable we did not account for. In follow up work we will include buffer A concentrations as a third parameter and extend the response surface design space in hopes of finding an even more efficient extract. However, for this initial work, it was capable of producing sufficient protein for measurement and we settled on the aforementioned optimal concentrations of IAM and extract (3x dilution with 25 mM IAM) in the following experiments. Statistics for each DoE can be found in Supplementary Table 1.

Exogenous Supplementation

To explore the impact of added enzymes, exogenous DsbC and T7RNAP were added to the SHuffle reactions. We also investigated the effect of removing these supplements from KGK10 reactions. Interestingly, the performance of the SHuffle extract decreased when exogenous proteins were added (Fig 3). With the KGK10 extract, we observed that it can still effectively express Gluc without the addition of DsbC, but that T7RNAP is essential for protein production. The tolerance to no chaperone protein may be isolated to this reporter protein as the majority of Gluc's disulfide bonds occur between consecutive cysteines, thus relying mostly on the native periplasmic isomerase DsbA to form bonds. Previous work with DsbC shows that the bond pattern, or the order in which the cysteines are bonded to one another (consecutive *vs* nonconsecutive), determines how important DsbC is for functional protein production.[29,37,43] While the KGK10 strain is entirely reliant on exogenous T7RNAP and DsbC for expression, the Shuffle extract's best expression occurs without the addition of either enzyme. This correlates well with the DoE showing that diluting the extract increases expression in SHuffle. These data show that SHuffle may have the advantage of being easier to use than KGK10. The only requirement for efficient production using SHuffle extract is the optimized induction time of IPTG and the optimized harvest time. KGK10 extract is more complicated to produce because the strain doesn't produce T7RNAP or DsbC and requires a defined media.[32]

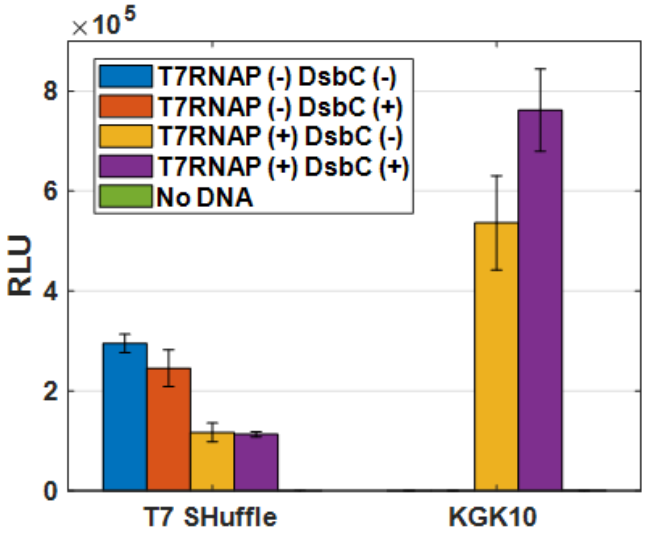

Figure 3 – Exploring the effects of including/excluding exogenous T7 RNAP and DsbC to/from cell-free reactions. Gluc activity was measured with luminescence (RLU) (n=5).

Production Comparison

Relative expression levels of protein are also important information when selecting a strain. To determine this, we expressed sfGFP using BL21 DE3 Star, KGK10, and T7 SHuffle® and found that BL21 DE3 Star has the most productivity with KGK10 closely following (Fig 3A). This corroborates the finding of our designed experiment which identified the optimal extract for disulfide binding to be suboptimal in overall level of protein expression (as measured by sfGFP). These data suggest that the BL21 strain is a better option for proteins with few or no disulfide bonds.

To verify that BL21 DE3 Star can be used for proteins with few, proximal disulfide bonds (due to native DsbA present in the extract), we designed a method to compare the ratio between oxidative potential and general productivity. We did this by designing and expressing a new fusion protein made of a YFP (rxYFP) variant sensitive to the redox environment, a flexible linker, and mCherry to measure overall production. To our knowledge, this is the first fusion protein designed to measure redox/productive potential, especially in cell-free extract. The rxYFP variant has been modified by the addition of 2 non-native cysteine residues. The fluorescent signal from rxYFP is significantly reduced in the presence of an oxidative environment due to disulfide bond formation between these non-native cysteines.[48] The linker selected was previously used to successfully fuse a nanobody to EGFP and still support proper folding.[49] In order to keep consistent molarity of the DNA template between the reactions, the rxYFP-mCherry fusion template was introduced as linear DNA (see methods). To quantify oxidative potential the fluorescence of rxYFP was divided by that of mCherry (the lower the ratio, the better the environment for disulfide bond protein expression). Surprisingly, the Shuffle and KGK10 strains have higher ratios than the BL21 DE3 Star, thus confirming that the Star strain should be used to express protein with few disulfide bonds (Fig 3B). Comparisons in Gluc productivity were also conducted between SHuffle, KGK10, and a commercial, fully recombinant

lysate kit (PURE*frex*®, see Supplementary Fig 3). The kit was 5.19x and 13.4x more productive than KGK10 and Shuffle respectively.

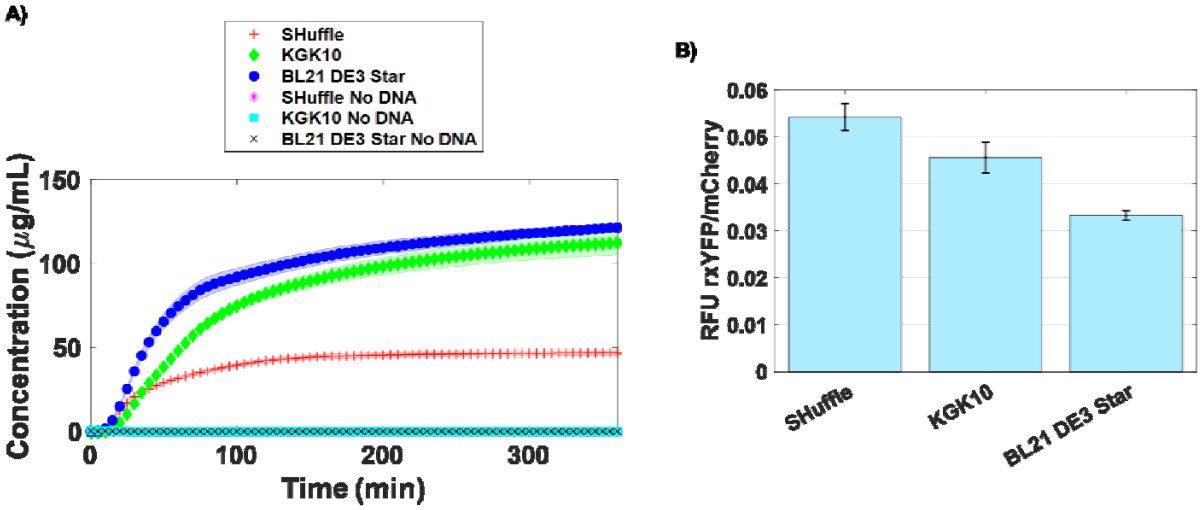

Figure 4 – Experiments to compare the yield of T7 SHuffle®, KGK10, and BL21 DE3 Star. (A) sfGFP to represent expression of proteins without disulfide bonds over 6 hr (n=5). (B) Is a ratio of oxidation potential/productivity using a YFP-mCherry fusion where a S-S bond has been introduced into a YFP variant (n=5).

Prototyping Proteins - Enzymes

To investigate the SHuffle strain's ability to quickly prototype proteins, we chose to express three enzymes (hevamine, endochitinase A (ChitA), and periplasmic AppA which have 3, 7, and 4 disulfide bonds) using our previously established minimal, rapid-order genetic template method.[12] Hevamine is a class IIIa chitinase from the rubber tree *Hevea brasiliensis* and exhibits exochitinase activity.[50] ChitA is a class IV chitinase found primarily in developing maize kernels of *Zea mays* that exhibits endochitinase activity.[51] Both chitinases are responsible for protecting the plants against fungal pathogens.[41,42,51,52] AppA is a periplasmic protein native to *E. coli* that acts as a phytase.[43] Phytases are an interesting class of enzymes because they break down phytate which is considered an antinutrient and reduces nutrient absorption from seeds (grains, beans, and nuts) in monogastric animals.[53,54] Undigested phytates from monogastric livestock are also a large source of environmental phosphorous pollution in agriculture.[54,55] To our knowledge, these enzymes have not been express in an *E. coli*-based cell-free system.

The resulting products can be screened directly in the lysate without purification. Hevamine samples demonstrated 1.3x greater activity than no DNA controls over the length of the entire reaction (Fig 5A). The substrate chose for this reaction was 4-Methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside which is a fluorogenic used to characterize endochitinases. Hevamine's kinetics and preferred substrate have been published in previous literature.[52] In contrast, ChitA samples performed 2.4x better than the no DNA controls when comparing initial reaction rate (change in fluorescence over time during the first 60 sec, Fig 5B). The signals eventually converged suggesting that the substrate had been quickly consumed (supplement). The

substrate used for ChitA was 4-methylumbelliferyl N-acetyl-β-D-glucosaminide which is fluorogenic substrate used to characterize exochitinases When screening the expressed AppA we found the sample with enzyme reaches a kinetic rate of 0.041±.005 abs/min over the span of 5 minutes while the control (no DNA) only reaches 0.031±.005 abs/min (Fig 5C). The substrate used was p-nitrophenyl phosphate which is used to characterize alkaline and acid phosphatase activity.

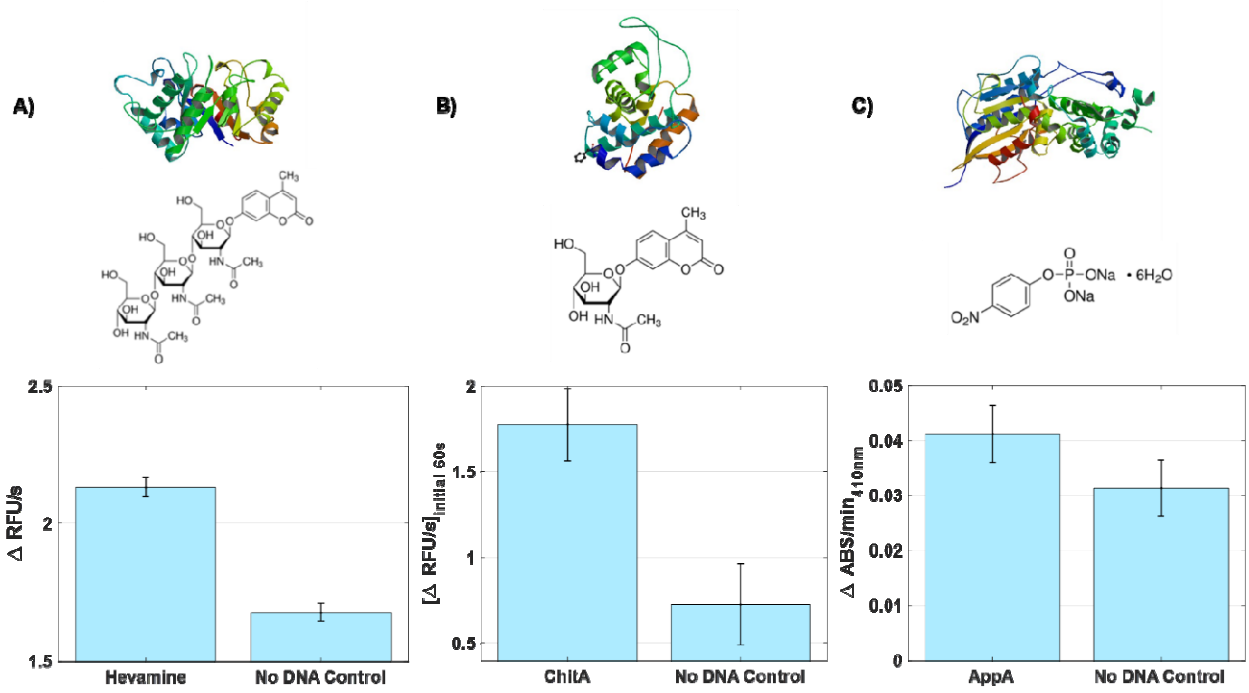

Figure 5 – The crystal structure, corresponding substrate, and activity of enzymes used in this work. (A) Total hevamine (RCSB PDB # 2HVM) activity on 4-Methylumbelliferyl β-D-N,N′,N′′-triacetylchitotrioside (p-value = 1.10×10^{-5}) (n=3). (B) Initial rate (60 sec) of ChitA (RCSB PDB # 4MCK) activity on 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (p-value = 5.92×10^{-4}) (n=4). (C) The activity of AppA (RCSB PDB # 1DKL) on p-nitrophenyl phosphate, disodium salt hexahydrate (p-value = 0.034) (n=4).

Conclusion

From the experiments performed, we conclude that the commercial cell line T7 SHuffle® can be used to create extract for cell free expression of proteins with many disulfide bonds. Conveniently in processing, this extract is lyophilized to drive off extra water used for the continuous homogenizer which increases the flexibility of extract storage and distribution. This extract can be used for rapid prototyping of enzymes using minimal genetic templates that are amplified with isothermal rolling circle amplification. This extract is also more economical due to the scalable production method, simple steps and lower required running concentration (3x

dilution). However, the convenience of this 'one-pot' cell extract preparation comes at the expense of lower yield (13.4x less than recombinant commercial systems and 2.58x less than KGK10 exogenously supplemented when tested with sfGFP). In the case of prototyping, where enough protein is produced to measure activity or desired properties, we do not see this as a concern. In cases where increased yield is needed, there are some general approaches to further improve yield that we have yet to apply to this strain, such as: increasing the surface area to volume ratio,[56–58] implementing machine learning to improve reaction composition,[59] and conducting metabolomic studies to identify and optimize key metabolites.[60] However, even with these improvements, the SHuffle strain may continue to express less protein than other strains that are exogenously supplemented with T7RNAP and DsbC simply due to exhausted machinery. Yet, the facile, 'one-pot' approach presented in this work can still make a significant impact at the prototyping phase where a large amount of extract is needed to test many sequence candidates. Sufficient protein is produced to be tested, as we have found with our candidate enzymes. Once desired enzymes, therapies, or protein-based materials are discovered, they can be scaled up in more efficient extract or using traditional cell-based methods.

Methods

Extract Preparation

T7 SHuffle®

Extract from the SHuffle® T7 Express *lysY* strain (gift from the Mansell lab) was prepared according to previously established protocols.[13] Cells were grown and harvested in 200 mL 2x YTPG media using a 500 mL shake flask at 37°C and 230 RMP. Cultures were harvested at different growth/induction times according to the DoE design (Supplementary Table 2). The cells were centrifuged for 15 minutes at 5,000 xg and 4°C. The cell pellets were collected and washed in 10 mL of cold buffer A and centrifuged for 15 minutes at 4,255 xg and 4°C.[14] The supernatant was discarded, and the wet cell mass was measured before freezing the cells at -80°C. Cells were thawed and resuspended in 1 mL of buffer A per gram of wet cell mass. Suspended cells were tip sonicated (QSonica) in 1 mL aliquots at -4°C using a 10 sec on/off pulse and 50% amplitude until the energy input reached 532 J.[17] Sonicated cell suspensions were then centrifuged for 10 minutes at 4°C and 12,000 xg to create S12 extract. The supernatant (S12 extract) was collected and stored at -80°C in 45 uL aliquots. Once the optimum growth/induction times were determined for 200 mL cultures, Matlab was used to fit a response surface to 200 mL and 1 L culture growth curves. The regression used was the Verhulst-Pearl equation (VPE) used in other similar studies.[61] These curve fits were used to correlate the optimum at 200 mL with a predicted optimum at 1 L. Optimized 1 L cultures were then grown in 2.5 L Tunair flasks and harvested according to the aforementioned protocol but were lysed using a French press homogenizer (Avestin EmulsiFlex C3) according to previous protocols.[13] Frozen cells were resuspended in 1 mL buffer A + 1 mL ddH_2O per 1 g of wet cell mass. This double dilution reduces the viscosity and maintains buffer composition upon

reconstitution. The suspension was then fed through the French press and lysed at 20,000 - 30,000 psig. The crude lysate was then centrifuged for 10 min at 12,000 xg and 4°C. The supernatant was then collected, frozen at -80°C, and lyophilized in a VirTis pilot lyophilizer (SP Scientific) overnight. The resulting powder was collected, weighed, and stored at -80°C. Concentrated extract was obtained by re-lyophilizing extract and adding less water. Dilute extract was obtained by adding more water.

KGK10

The KGK10 strain (gift from Swartz lab) was grown in a 500 mL shake flask and monitored every 15 min to obtain a growth curve. This growth curve was used to find the point at which the rate of growth was the highest. KGK10 was then grown and harvested at 240 min (Supplementary Fig 4). The cells were centrifuged for 15 minutes at 5,000 xg and 4°C. The cell pellets were collected and washed in 10 mL of buffer A and centrifuged for 15 minutes at 4,255 xg and 4°C. The supernatant was discarded, and the wet cell mass was measured before freezing the cells at -80°C. Cells were thawed and resuspended in 1 mL of buffer A per gram of wet cell mass. Suspended cells were tip sonicated (QSonica) in 1 mL aliquots at -4°C using a 10 sec on/off pulse and 50% amplitude. Since there is no data on KGK10 sonication methods, the 1 mL samples were sonicated to an energy input of 705 J as previous literature states is best for a K12 strain.[17]

DNA Amplification

All Gluc and sfGFP reporter protein expression experiments were carried out using plasmids. The sfGFP plasmid used was pJL1-sfGFP and the Gluc plasmid was pET24a-Gluc-6H (gift from the Swartz lab).

The production of minimal genetic templates for prototyping new enzymes has been described in previous work.[12] In brief, the gene of interest was codon optimized using IDT's codon optimization tool and purchased in the form of a gene fragment. The gene fragment was then amplified using OneTaq (NEB), digested to form sticky ends with HindIII (NEB), ligated with T4 ligase (NEB), and isothermally amplified with TempliPhi (GE Healthcare). This was all done in under 24 hr using a standard C1000 Touch Thermal Cycler (BioRad). The sequences for all linear and minimal genetic templates used are in the accompanying supplementary information.

Cell-Free Reaction

T7 SHuffle®

The supplement recipe used for the designed experiments and initial tests is a modified version of the PANOx-SP system that is improved to form disulfide bonds.[62] Since the SHuffle strain can be induced to produce T7 RNAP and DsbC, these proteins were omitted from the reaction mix. The cell-free reaction included: 1.2 mM ATP, 0.85 mM each of GMP, UMP, and CMP, 30 mM phosphoenolpyruvate (Roche), 130 mM potassium glutamate, 10 mM ammonium glutamate, 12

mM magnesium glutamate, 1.5 mM spermidine, 1 mM putrescine, 34 µg/mL folinic acid, 171 µg/mL *E. coli* tRNA mixture (Roche), 2 mM oxidized glutathione (GSSG), 2 mM each of 20 unlabeled amino acids, 0.33 mM NAD, 0.27 mM Coenzyme A (CoA), 4 mM potassium oxalate, 57 mM HEPES-KOH buffer (pH 7.5), 15 ng/uL plasmid, 0.24 volumes of E. coli S12 extract. Reactions were carried out in a 384 black-walled, flat-bottom well plate and shaken at 300 rpm using a ThermoMixer® (Eppendorf). Because proteins with disulfide bonds are typically expressed at 30°C *in vivo* to reduce the rate of translation,[62,63] all proteins were expressed at 30°C. Gluc, hevamine, rChiA, and AppA reactions were run for 16 hrs. The other proteins (sfGFP and rxYFP-mCherry) were expressed for 6 hrs. Reactions for the fluorescent proteins were carried out in a Synergy Neo2 HTS Multi-Mode Microplate Reader (BioTek) at 237 cpm.

KGK10

The cell-free reaction mixture for the KGK10 strain is identical to that used for the T7 SHuffle® with the addition of 100 µg/mL DsbC (GeneFrontier) and 3.33 Units/uL T7 RNAP (Roche). Previously prepared KGK10 extract (gift from the Swartz lab) was used instead of the cells grown in-house. As an aside, the in-house KGK10 extract we made showed little activity. We suspect KGK10 is sensitive to growth conditions and media.[64]

PURE*frex*®

The components for the PURE*frex* reactions were scaled down to suit 15 uL reactions. The components from the PURE*frex*® 2.1 (GeneFrontier) were supplemented with DsbC and GSSG from the DS Supplement kit (GeneFrontier) according to manufacturer's protocols.

Assays

Bioluminescence

The Gluc assay is based on previous work in the Swartz lab.[40] 5 µL of CFPS reaction was diluted in 100 µL nickel affinity EB buffer containing 1% w/v bovine serum albumin (BSA). The activity was measured by adding 1 µL of diluted sample to 100 µL assay buffer with 1 µL of 0.5 µg/ µL coelenterazine (in ethanol) and immediately mixing before reading the luminosity. The sample buffer consisted of PBS pH 7.4 with 0.01% v/v Tween 20. Readings taken in a U-bottom 96 well plate. Luminescence was measured every 10 sec for 60 sec using a Synergy Neo2 HTS Multi-Mode Microplate Reader (BioTek). The plate reader settings used a 1536 filter, top optics position, and a gain of 150.

Fluorescence

Fluorescence measurements were also taken using a Synergy Neo2 HTS Multi-Mode Microplate Reader (BioTek). Readings were taken every 5 min. The fluorescence of sfGFP was measured at an excitation of 485 nm and emission of 528 nm using a ±20 bandpass window and 61 gain setting. The reaction was also stirred in orbital motion at 237 cpm. For rxYFP,

readings were taken at an excitation of 512 and emission of 523 using a ±5 bandpass window. Measurements for mCherry were taken at an excitation of 587 ±11 and an emission of 610 ±10.

Enzyme Activity

Hevamine activity was determined by adding 10 µL CFPS reaction to 90 µL McIlvaine buffer pH 6.0 with 10 µL of 1 mM 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside (4-MUF-TriNAG). rChiA activity was determined by adding 10 µL CFPS reaction to McIlvaine buffer pH 4.0 with 10 µL 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MUF-NAG). Fluorescence was measured at an excitation of 360 and emission of 445 with a bandpass window of ±20. Measurements were taken every 60 sec for 2 hrs in a black walled, flat bottom 384 well plate. AppA activity was determined by adding 10 µL CFPS reaction to 100 µL glycine buffer (250 mM glycine + 25 mM p-nitrophenyl phosphate) titrated to pH 3.0 with HCl. Absorbance readings were taken at 410 nm every 20 sec for 5 min in a 96 well plate at 37°C.

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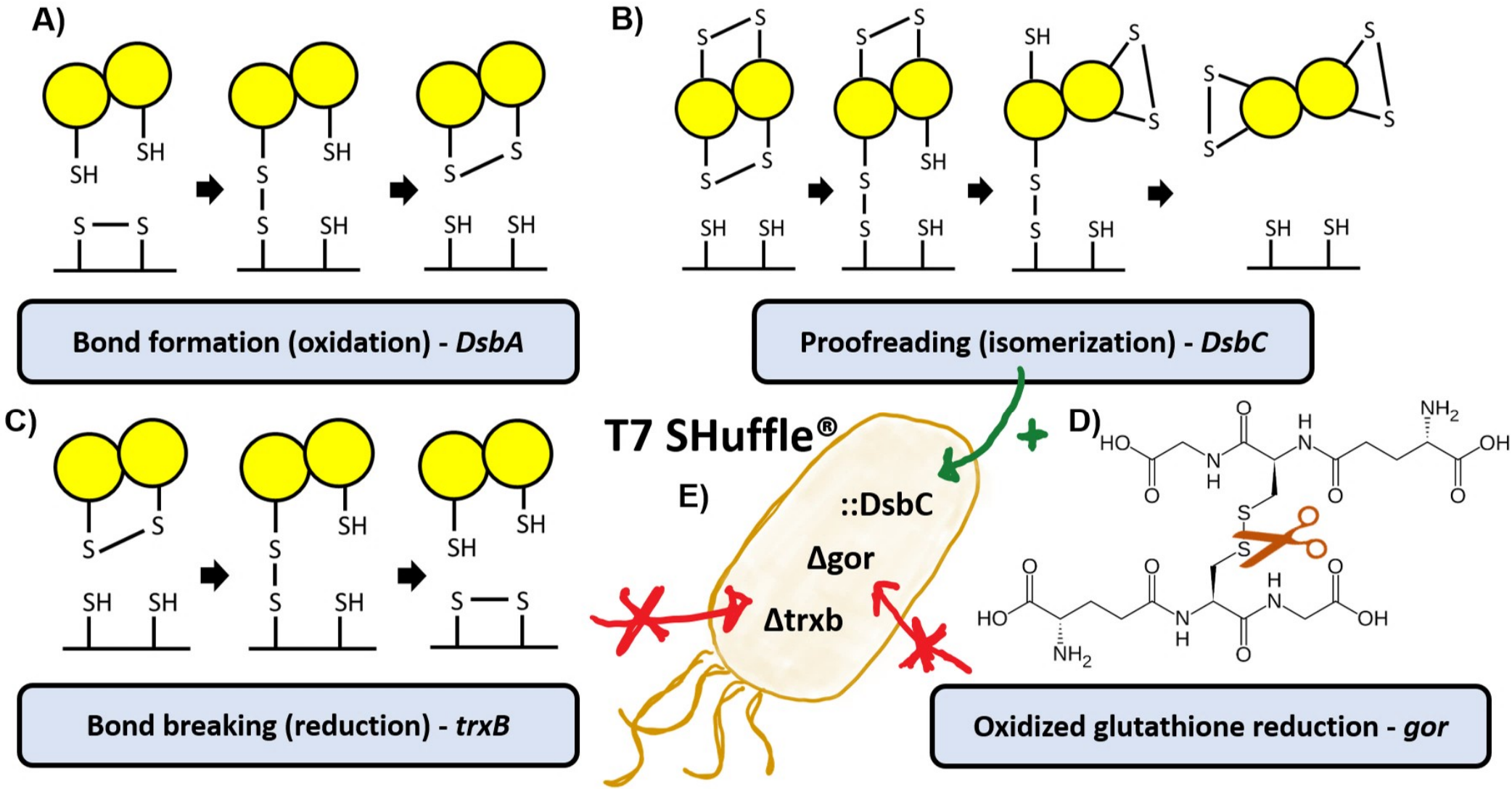
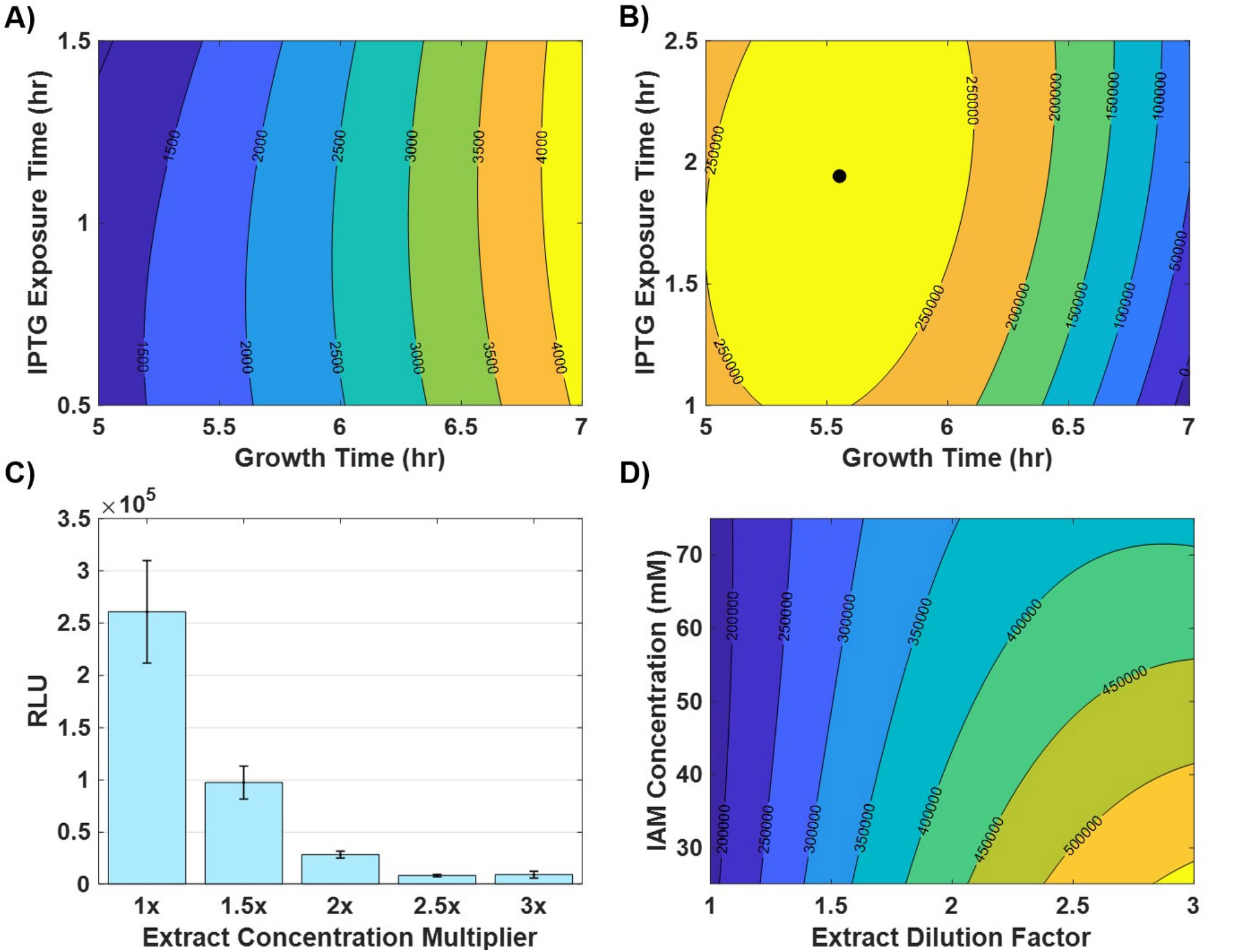

A)
B)
Bond formation (oxidation) - DsbA
Proofreading (isomerization) - DsbC
C)
T7 SHuffle®
E)
::DsbC
Δgor
Δtrxb
D)
Bond breaking (reduction) - trxB
Oxidized glutathione reduction - gor


A)
IPTG Exposure Time (hr)
Growth Time (hr)
1.5
1
0.5
5
5.5
6
6.5
7
1500
2000
2500
3000
3500
4000
B)
IPTG Exposure Time (hr)
Growth Time (hr)
2.5
2
1.5
1
5
5.5
6
6.5
7
250000
200000
150000
100000
50000
C)
×10^5
RLU
3.5
3
2.5
2
1.5
1
0.5
0
1x
1.5x
2x
2.5x
3x
Extract Concentration Multiplier
D)
IAM Concentration (mM)
Extract Dilution Factor
70
60
50
40
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2.5
3
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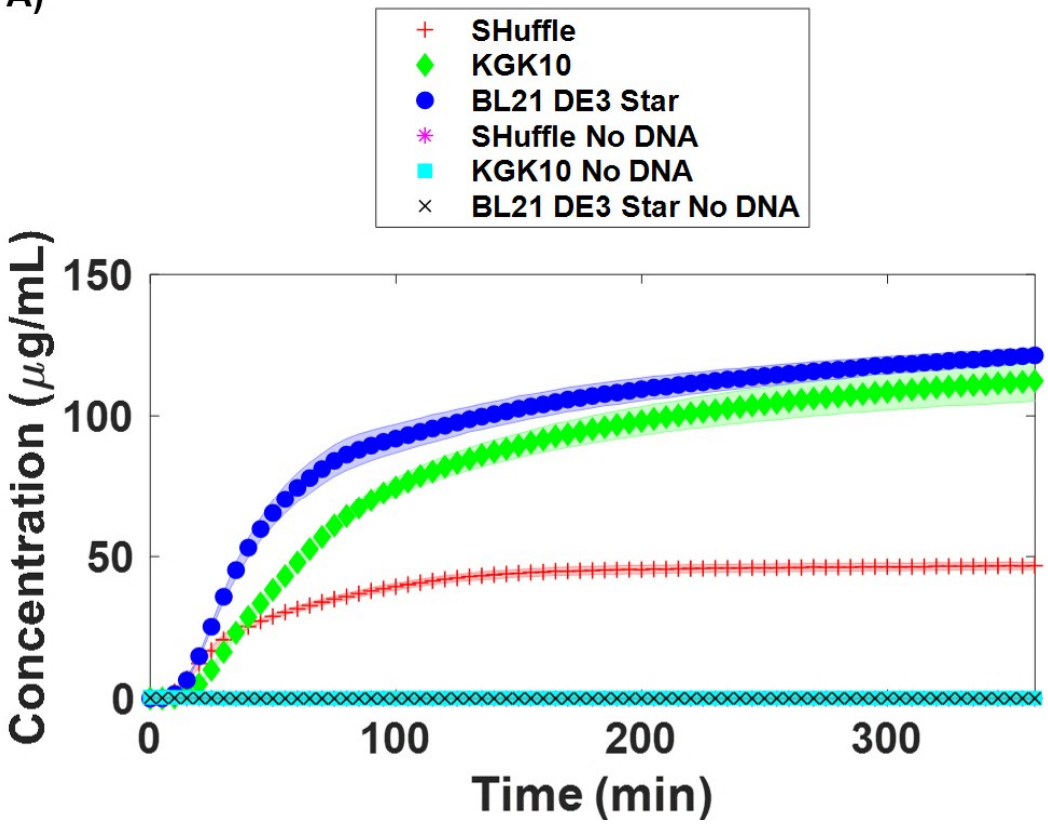
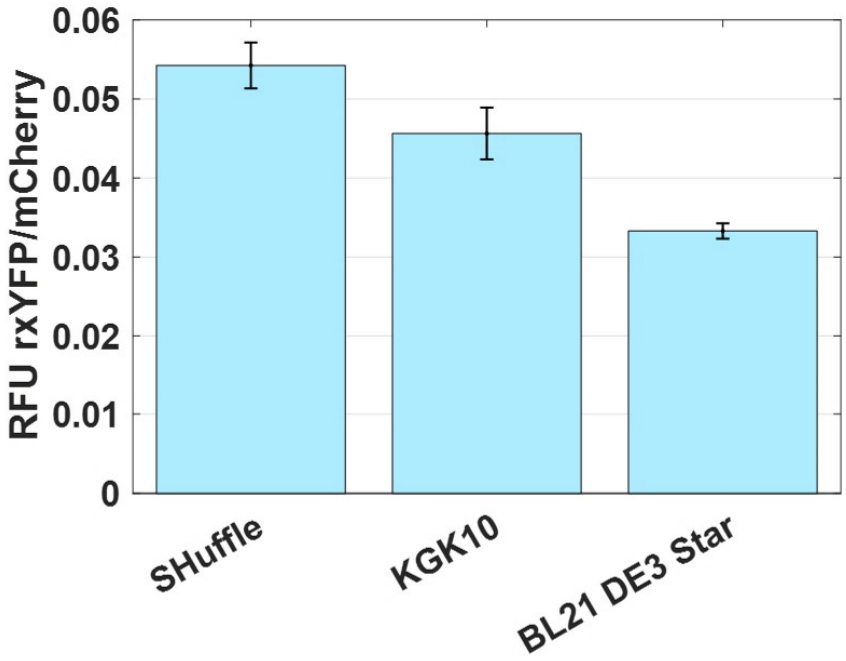
250000
300000
350000
400000
450000
500000

A)


SHuffle
KGK10
BL21 DE3 Star
SHuffle No DNA
KGK10 No DNA
BL21 DE3 Star No DNA
Concentration (μg/mL)
150
100
50
0
0
100
200
300
Time (min)

B)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0.05
0.04
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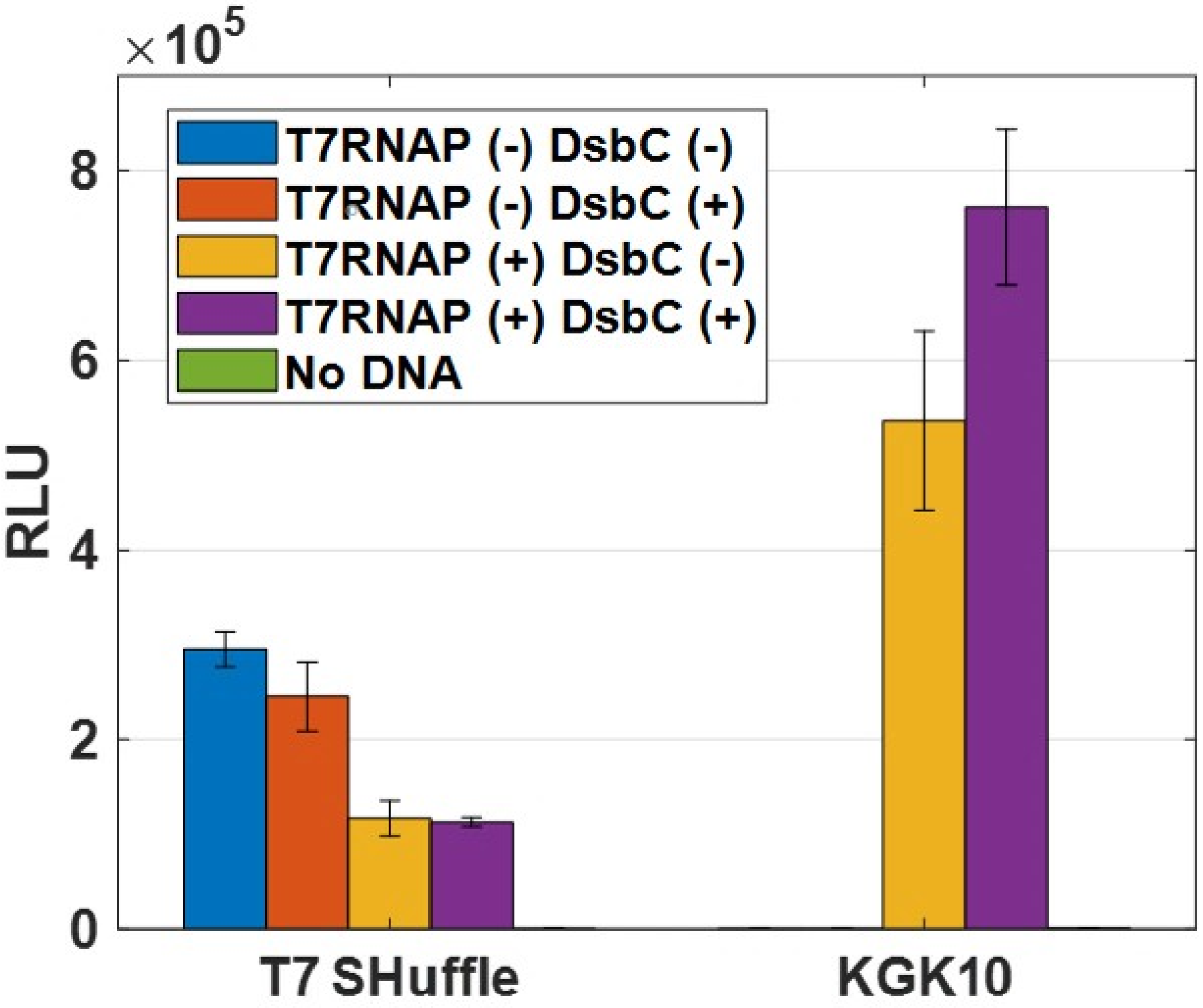
0
SHuffle
KGK10
BL21 DE3 Star


×10^5
8
6
4
2
0
RLU
T7RNAP (-) DsbC (-)
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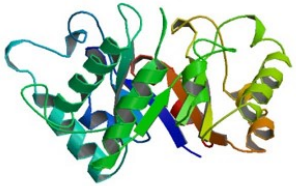
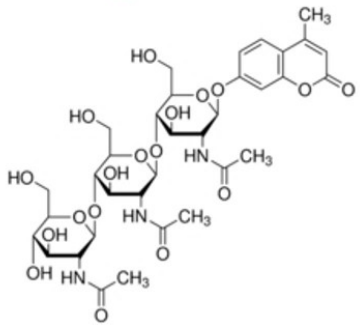
T7RNAP (+) DsbC (-)
T7RNAP (+) DsbC (+)
No DNA
T7 SHuffle
KGK10

A)

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HO
OH
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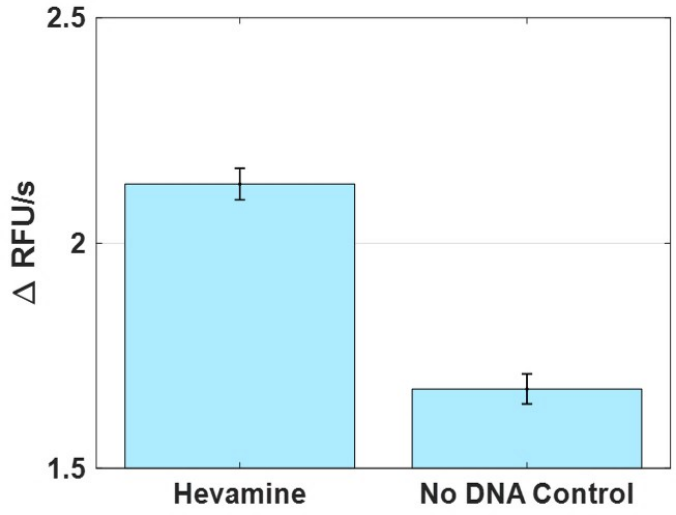
CH3
O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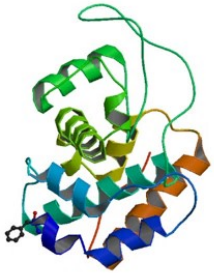
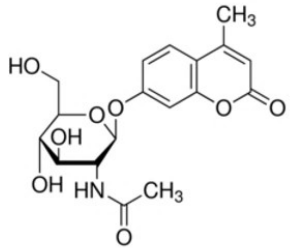
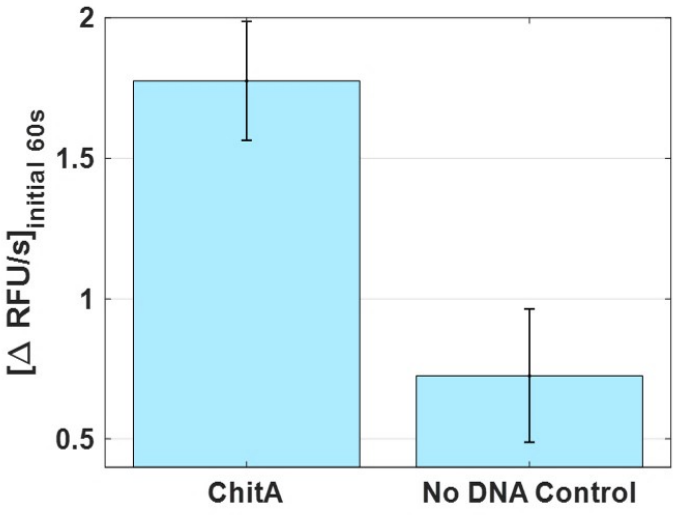
2
1.5
Δ RFU/s
Hevamine
No DNA Control

B)




CH3
HO
OH
HN
CH3
O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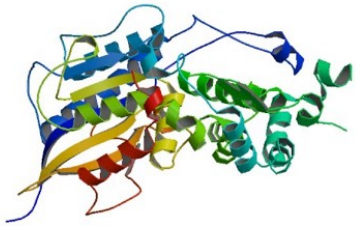
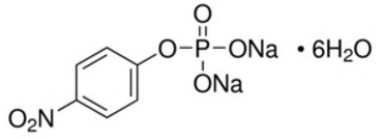
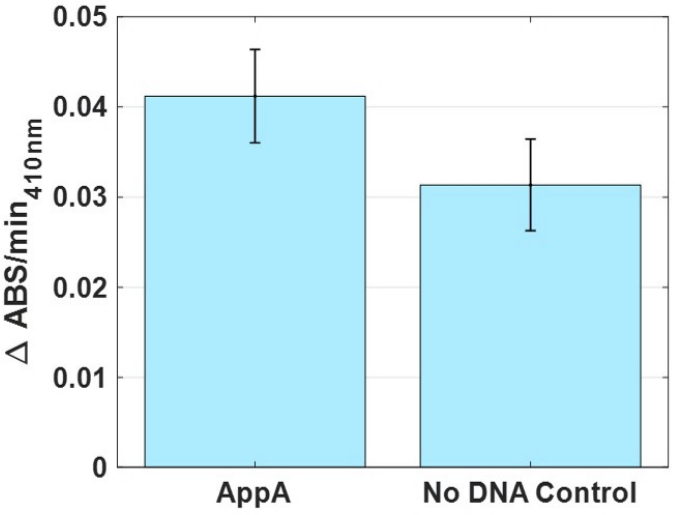
1.5
1
0.5
[Δ RFU/s]initial 60s
ChitA
No DNA Control

C)




O
O–P–ONa • 6H2O
ONa
O2N


0.05
0.04
0.03
0.02
0.01
0
Δ ABS/min410nm
AppA
No DNA Control