OPTIMIZING A CELL FREE SYSTEM (CFS) FOT *IN-VITRO* TRANSCRIPTION-TRANSLATION (TxTr) OF SYNTHETIC GENE CIRCUITS

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Certificate

This is to certify that this dissertation entitled "Optimizing a Cell Free System (CFS) for *In Vitro* Transcripton-Translation (TxTr) of Synthetic Gene Circuits" submitted towards the partial fulfillment of the BS-MS degree at the Indian Institute of Science Education and Research, Pune (IISER Pune) represents original research carried out by Mr. Ankur Biswas at Indian Institute of Science Education and Research, under my supervision in the Division of Biology, during academic year August 2020 to May 2021.

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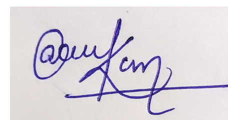
This thesis is dedicated to my father Mr. Ajit Biswas.

Declaration

I hereby declare that the matter embodied in the report entitled "Optimizing a Cell Free System (CFS) for *In Vitro* Transcripton-Translation (TxTr) of Synthetic Gene Circuits" is the result of the work carried out by me at the department of Biology, Indian Institutes of Science Education and Research, Pune (IISER Pune), under the supervision of Dr. Chaitanya A. Athale and the same has not been submitted elsewhere for any other degree.



Ankur Biswas
Date: 15/05/2021

Abstract

Cell free extract (CFE) being an open system, provides a plethora of opportunities like prototyping of genetic circuits, non-canonical amino acid incorporation and high throughput analysis which cannot be performed using intact cells. From the early sixties researchers are utilizing the CFE system for various applications. Targeted high yield protein production being one of the most demanding applications of them all. Expression of toxins can also be achieved in a cell free extract system which is impossible in intact cells. Genetic switches can be designed in such a way that can detect viral RNA when linked to a reporter system. Cell free extract is an excellent platform to express these synthetic gene circuits. Our aim here is to develop and optimise a cell free extract system which would be capable of expressing a synthetic gene circuit. The easiest way to detect expression is to express a fluorescent proteins. Reporter proteins like GFP and variants are prime candidates for such reporter systems. We expressed a monomeric red fluorescent protein (mRFP) and green fluorescent protein (GFP) in *Escherichia coli* DH5α and BL21 (DE3) cells and prepared an extract of them. We wanted to add plasmids that express the genes encoding these proteins in our homemade extract. To this end we needed to establish the stability of expression of these reporter proteins in the extract system. We found out that the iGEM repository supplied mRFP expressing plasmid was constitutive. Both GFP and mRFP were found to be stable in the exact system. We then made our home made cell free extract and optimised conditions for expression. A GFP expression plasmid was added to the homemade extract system and expression levels comparable to cells transformed with the plasmid were observed. We also constructed a LacZ expression plasmid and expressed it in homemade extract system.
We find that our homemade *E. coli* BL21 extract with energy-mix is capable of transcription and translation. In future this would may become part of a larger project to deploy such TxTr systems for viral diagnostics.

Acknowledgements

The accomplishment of this project successfully, many people have bestowed upon me their blessings and heart pledged support, this time I am utilizing it to thank everyone who has been concerned with this project.

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1 Introduction

The pioneering discovery of the system for cell-free protein synthesis was done in Zamecnik laboratory. There they were successfully able to demonstrate that peptide synthesis takes place on the ribosome and requires ATP, GTP, and tRNA (1). During this time, researchers all around the globe started contributing to the field. With combined efforts of Zamecnik (2), Tissie‘res and Hopkins (3), and Nirenberg and Matthaei (4) a cell-free extract was developed. Which when supplied with an appropriate template was capable of polymerizing amino acids into protein. Cell-free system ever since has been a valuable tool for understanding how mRNAs are translated into functional polypeptides. With the emergence of the era of proteomics, the field of Cell Free Extracts (CFE) has experienced a technical renaissance giving rise to a multitude of applications including both structural and functional proteomics (5). As the fascinating discovery started attracting researchers, people started improvising the process of extract preparation. Extracts are prepared by breaking up live cells and followed by harvesting the active cell contents. Considering this fact and with further careful observation, researchers came across the idea that RNA polymerases and ribosomes would be bound and not free which was limiting the protein production efficiency of desired protein. Thereafter, it was discovered that with runoff reaction or incubation increases the overall protein yield and enhances the exact efficiency for protein production. The pre-incubation helps free the RNA-polymerases and ribosomes from their bound state, to make them available freely in the extract for enhanced transcription and translation. The runoff reaction is carried out by incubating the extract without the energy source and DNA or RNA. Thus the incubation helps to make the RNA-polymerases and ribosomes freely available in the extract (6).

The extract preparation procedures we use these days are mostly similar and resemble the extract preparation procedures developed by Pratt (7) and Zubay (8), which was developed by making few modifications to the Nirenberg procedure. There are quite a few other papers where researchers have tried other ways to make cell-free extract. The most important ingredient for the optimal functioning of cell-free extract is the energy supply. The stability of the energy substrate directly reflects protein production. One of the most interesting work on this was done by Kang and co-workers (9). They used spheroplasts to prepare their extract, that way they were able to reduce the phosphatase activity of the extract by removing periplasmic enzymes. By implementing this together with increased total reaction time they observed a 30% increase in total protein production. This same approach was also used by Kim and Choi (10). Schindler and co-workers (11) took a different approach to make cell-free extract by altering the runoff reaction. However, all of the changes did not make a significant increase in the productivity of the extract. Extract being an open system incapable of producing energy, therefore it is dependent on external energy sources. *E Coli* is widely used as the model organism for extract preparation and one of the primary sources of energy for *E Coli* is glucose. Over the years researchers have used the sugar intermediates of the glycolytic pathway as the main source of energy. The most popular of them was Phosphoenolpyruvate (PEP). PEP is a relatively unstable molecule and gets degraded very fast, creating two big issues. Firstly, degradation leads to huge inorganic phosphate accumulation, inorganic phosphate conjugate with free magnesium ions and result in shrinkage of the production of desired protein. Secondly, due to rapid degradation efficiency of energy production diminishes which leads to decrease in production of desired protein. The efficiency of protein production was compared between PEP and 3-Phosphoglyceric acid (3-PGA) in a study (12). Where it was observed that the 3-PGA is a stable intermediate and decays slowly in the reaction

mixture, which increases the productivity of desired protein and also increases the period of protein production as well (Fig. 1).

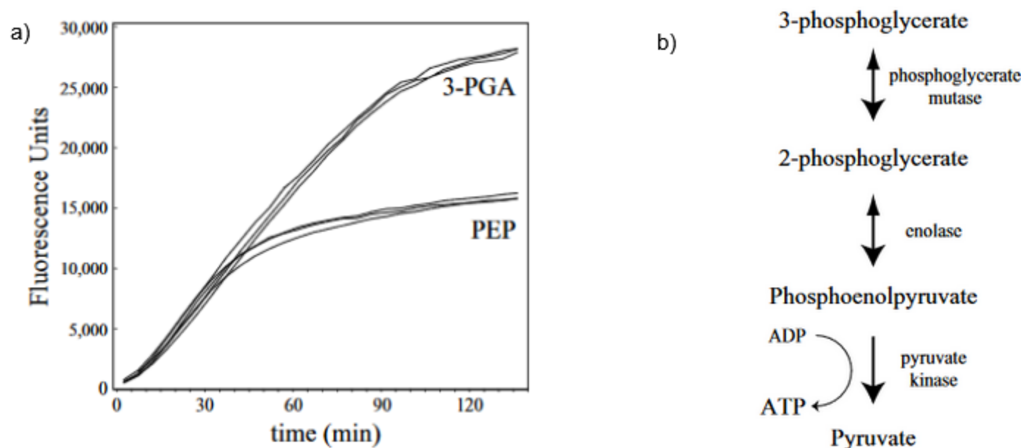

Figure 1: a) The plot represents the difference in expression efficiency of extract with 3-phosphoglycerate (3-PGA) vs the conventional phosphoenolpyruvate (PEP) as the energy source. b) Glycolytic pathway used to generate ATP using 3-phosphoglycerate (3-PGA) as an energy source. Figure taken from Chatarjee et al. Journal of Biotechnology, 10 June 2004, 257-263.

1.1 Application of CFE

Researchers in the early 60s were puzzled trying to decode the codon code associated with amino acids. The cell-free system provided them the right platform to use it for the purpose of investigating the mechanism of protein synthesis in order to decrypt the codon code associated with amino acid. Since the first successful test of protein production with cell-free extract, the system has been constantly utilised for the bulk production of model and therapeutic proteins (13). However, the application didn't stop there, with optimization, the cell-free system evolved so drastically that it became capable of complex and diverse functions. Today the cell-free extract system is used for prototyping cellular metabolism and glycosylation, portable on-demand manufacturing of pharmaceuticals, expressing minimal synthetic cells, virus-like particles, and bacteriophages, incorporation of non canonical amino acids within proteins, prototyping of genetic circuitry, and sensing nucleic acids and small molecules through rapid, low-cost, and field-deployable molecular diagnostics (14). *Escherichia coli* strains are the most successful ones for generation of cell-free extract. Also most progress occurred with *Escherichia coli* strains, mostly because of the fact that *Escherichia coli* genetics and metabolism were well-characterized and fairly simple to work with (15). However, researchers have tried and made progress in generating cell-free extract from eukaryotic and nonmodel organisms, including yeast, Gram-positive bacteria, plants, and mammalian cells (16). Therefore, cell-free extract technology has become a major application in the field of synthetic biology (17). CFEs can be made from various organisms like Leishmania tarentolae, Spodoptera frugiperda, Wheat germ, Tobacco BY-2, *Escherichia*

coli, etc (2). However, we will be focusing here on extract made from *Escherichia coli*.

1.2 Motivation

Cell-free extract opened up a path for in-vitro protein production which can never be achieved with alive and intact cells. An intact cell system carries transcription-translation machinery. Considering an *Escherichia Coli* cell, a protein of interest can be expressed in the cell by utilising the transcription-translation machinery (Fig. 2). The system is well established and very efficient in expression but

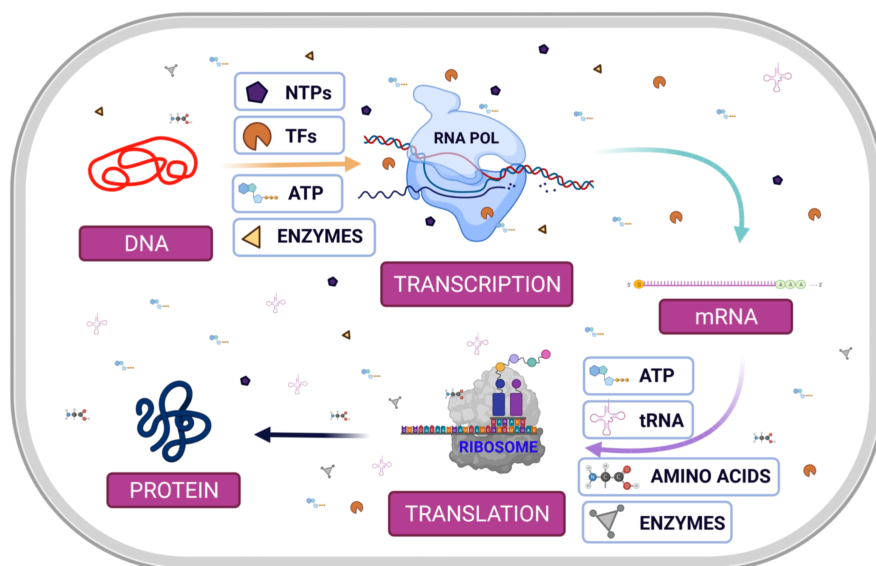

Figure 2: The schematic represents the expression of desired protein in a intact cell utilising the host transcription translation machinery.

the efficiency differs with cell lines. Various commercial industries use the intact cell culture system to produce protein that we consume in our day to day life, starting from protein supplements to the very essential drug insulin. However, the intact cell system is not that suitable if we want to prototype a genetic circuit which expresses a protein that is toxic to the host. Similarly, the system is not appropriate if we want to express thousands of gene circuits at a time, as there are multiple steps associated in the process like transformation, selection, expression etc. Instead if we take the cell and break open the cell membrane to get all the cellular components out along with the cytoplasm, that will still contain all the constituents of the transcription-translation machinery and theoretically that should still be capable of performing transcription and translation. If we get rid of the genomic DNA from that what we would be left with is the cell free extract. Due to the fact that the cell free extract is devoid of cellular compartmentalization and regulations therefore cannot produce energy and other substrates that is used in the process of transcription and translation. Hence, additional supplementation of energy substrate such as PEP, glucose-6-phosphate, 3-PGA which are intermediates of glycolytic pathway is required, along with NTPs, amino acids, tRNAs which are key component of

transcription-translation machinery. Also to stabilize the energy production reaction in extract Mg, K ions are required. PEG is used as a crowding agent. PEG is a polyner of poly ethylene glycol, which forms a mesh-work of branched polymer and increases the viscosity of the system and reduces the intermediate space between molecules which in turn increases the inter-molecular interaction. These all together makes the cell free system. If we add all these to a tube and a template DNA, theoretically it should express the protein of interest (Fig. 3). Due to the openness of the system the system is very sensitive, which makes the in-vitro protein production challenging. Also the system is very hard to make functional due to the complexity associated with the system. The motivation behind this project was to accept the challenge and prepare a homemade cell-free extract which would be efficient enough in expression of the protein of our interest. Here, I proceed to describe the work where we produced homemade *E. coli* cell free extract. Then we proceeded to test the expression level of our home made extract with different expression plasmids. We also constructed lacZ expression plasmids and expressed them in our homemade extract.

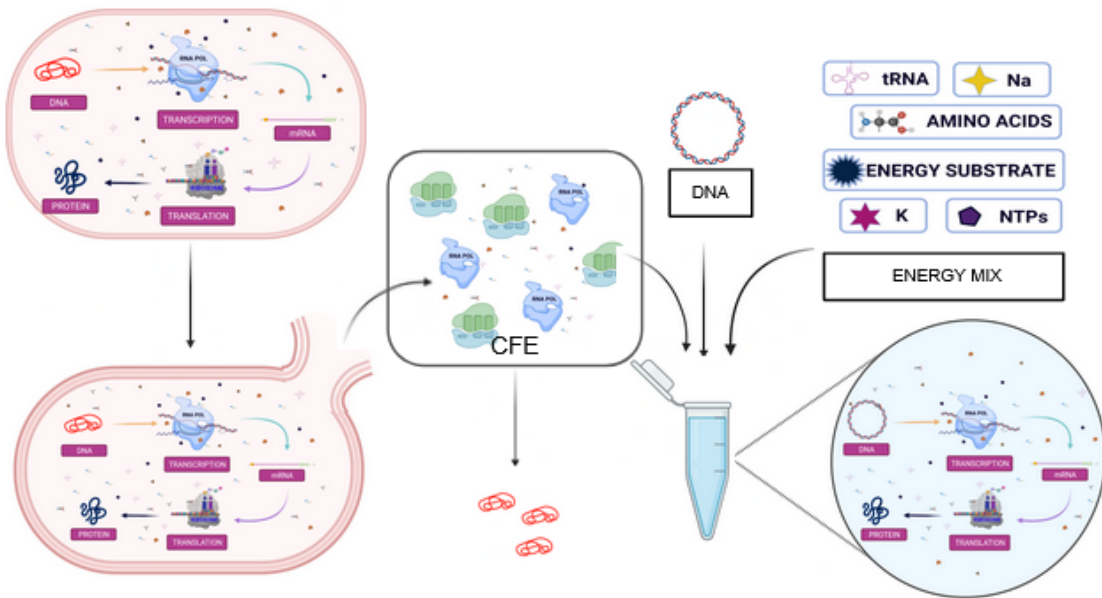

Figure 3: The schematic represents the *in-vitro* transcription translation system.

1.3 Comparison of extract preparation protocol

The system was developed in the 60's and ever since people are persistently trying to modify it to simplify the overall process of extract preparation. Hence there are multiple protocols available for preparation of extract. Therefore the first step in this project was to select the most simplified extract preparation protocol. We therefore compared between various protocols (Fig. 4). The protocol originally developed was very complex, lengthy and comprised 30,000 x g for separating lysate from other components of the cell. Over time the protocol is made simple and more robust. Here for this project we selected the protocol reported by Krinsky et al. as this was the one of the simplest and

robust ones. Here they used 12,000 x g for separating the lysate. As centrifugation with 12,000 x g very minimal cellular components can be sedimented therefore it is also called crude extract. So we followed this protocol to make our cell free extract.

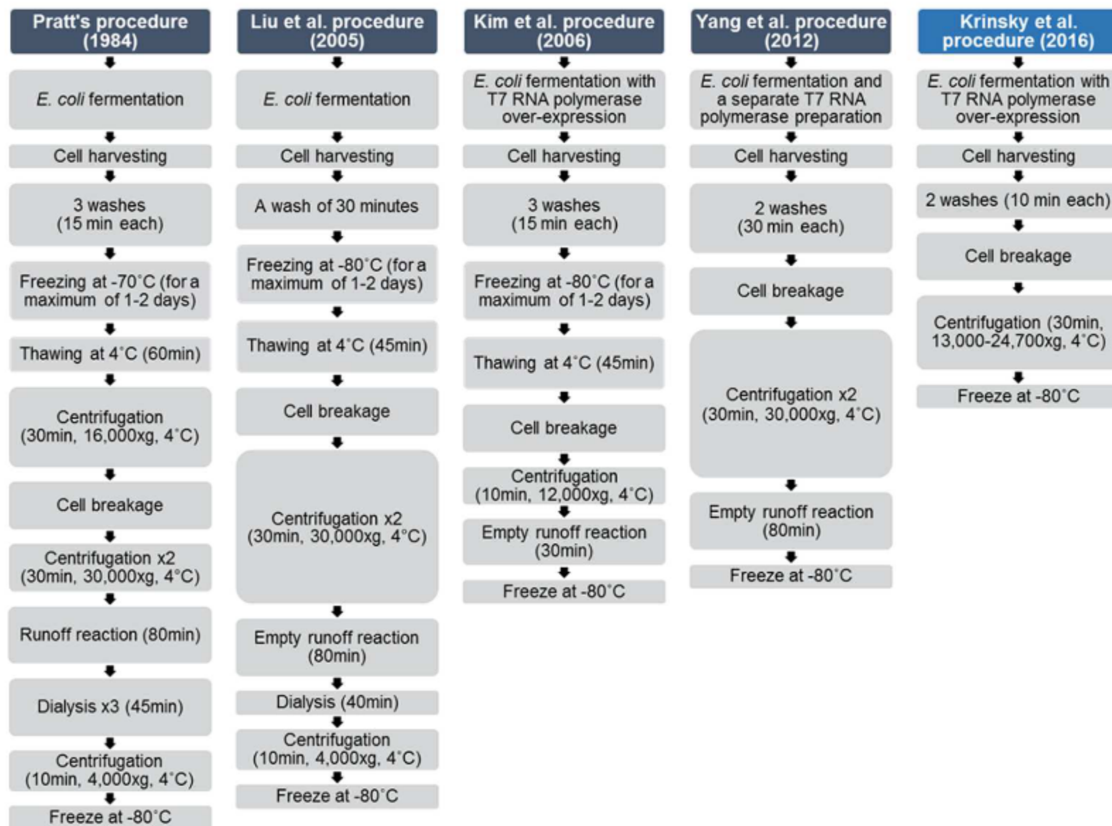

Figure 4: Comparison of various extract preparation protocols. Figure taken from Krinsky et al.

1.4 Bacterial strains

We need a potential host for preparation of cell free extract. Bacterial strains are designed differently to achieve desired purpose. Like DH5α carries truncated Endonuclease A and Rec1 and End1 mutation which reduces the frequency of crossing over and recombination of plasmid DNA to genomic DNA. Thus DH5α serves as an optimal system for stable incorporation of plasmid DNA. BL21 is deficient of lon and omp-t proteases gene that increases protein stability which makes the system optimal for protein expression. BL21 (DE3) contains DE3 lysogen that carries the gene for T7 RNA polymerase unde lac UV5 promoter, hence IPTG ineducable. Rosetta strain is a lacZY derivative designed to enhance expression of eukaryotic proteins that uses codons rarely used in *E Coli.* Also supply tRNAs for the codons AUG, AGG, AGA, AUA, CCC, GGA on a compatible chloramphenicol resistant plasmid. Rosetta* additionally carry a mutated rne gene (rne131) which encodes for truncated RNAse E enzyme that lacks the ability to degrade mRNA,resulting in additional mRNA stability. This strain

is suitable for heterologous gene expression. Rosetta * pLysS in addition produces T7 lysozyme to minimise basal level expression of T7 RNA polymerase prior to induction. Leads to tight control of expression. Strain description is presented in (Fig. 5). The main purpose of this project was to express protein in our homemade cell free extract therefore BL21 DE3 which provides additional stability to expressed proteins was a potential candidate for extract preparation. Also, in the later part of the project we expressed lacZ gene which was under the control of T7 promoter, as BL21 DE3 also carries T7 RNA Polymerase gene which makes the strain suitable for this project. However, BL21 DE3 doesn't carry delta lacZ therefore it shows native expression of lacZ which is a major problem for quantification of expression of β-galactosidase only from the constructed plasmids. But we didn't have any other suitable strain, we used BL21 DE3 for preparation of cell free extract.

DH5α	*F– endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK–mK+), λ–*
BL21	*B F– ompT gal dcm lon hsdSB(rB–mB–) [malB+]K-12(λS)*
BL21(DE3)	*B F– ompT gal dcm lon hsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS)*
Rosetta*(DE3)pLysS	*B F– ompT gal dcm lon? hsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS) pLysSRARE[T7p20 ileX argU thrU tyrU glyT thrT argW metT leuW proL orip15A](CmR)*
MG16 55	*K-12 F– λ– ilvG– rfb-50 rph-1*

Figure 5: Strain description of various *E Coli* strains

1.5 Comparison of protocol for CFE Energy solution

Sun.et al. sep,16,2013		**Silverman et al. june,19,2019**		**Jewett et al. march 2, 2015**		**Krinsky et al, Oct, 2016**	
						ENERGY MIX	70% (v/v)
Mg-glutamate	14 mM	Mg-glutamate	10-20 mM	Mg-glutamate	12 mM	HEPES-KOH	(pH= 8)55mM
K-glutamate	40-160 mM	Ammonium-gluta	10 mM	Ammonium-gluta	10mM	Magnesium acetate	14mM
DTT	0.33-3.3mM	K-glutamate	130mM	K-glutamate	130mM	Potassium acetate	50mM
HEPES	50mM	HEPES	57mM			Ammonium acetate	155mM
Nucleotide Mix	1.5 mM ATP and GTP, 0.9 mM CTP and UTP	Nucleotide Mix	1.2 mM ATP and 0.850mM GTP, CTP and	Nucleotide Mix	1.2 mM ATP and 0.850mM GTP, CTP and	Polyethyleneglycol (PEG) 4000	3%(v/v)
tRNA	0.2 mg/ml	yeast tRNA	0.171 mg/ml	E. coli tRNA	0.171 mg/ml	3-phosphoglycerate (3-PGA)	40mM
CoA	0.26 mM	CoA	0.27 mM	CoA	0.27 mM	20 amino acids	2.5mM
NAD	0.33 mM	NAD	0.33 mM	NAD	0.33 mM	Adenine triphosphate (ATP)	1.2mM
cAMP	0.75 mM	PEP	30 mM	PEP	30 mM	Guanidine triphosphate(GTP)	1 mM
Folinic Acid	0.068 mM	Folinic Acid	0.034 mg/mL	Folinic Acid	0.034 mg/mL	Uridine triphosphate (UTP)	0.8mM
Spermidine	1 mM	Spermidine	1.5 mM	Spermidine	1.5 mM	Cytidine triphosphate (CTP)	0.8mM
3-PGA	30 mM	Putrescine	1 mM	Putrescine	1 mM	DNA template	10μg/mL
Amino acids	1.5mM each, leucine 1.25mM	Amino acids	2mM each	Amino acids	2mM each, leucine 10 micro M	S30-T7 lysate	30% (v/v)
		Oxhalic acid	4mM	Sodium oxalate	4mM		
						DNase, RNase free H2O to reaction volume	
8.9-9.9 mg/mL		1000 nano g/micro L		~1 mg/mL			

Figure 6: Comparison of reagent for CFE energy mix solution preparation used by different authors.

The CFE system is very popular and widely used for various expression systems. The CFE system is non-living and incapable of producing its own energy and other necessary reagents for performing transcription and translation and protein production. Therefore supplementing the CFE with appropriate and adequate substrates is crucial for efficient protein production. Hence over time researchers

developed different methods by which the transcription-translation system can be activated in CFE. We carefully looked into all possible approaches and selected the best and minimal approach for our system. The primary and the most important difference between the approaches is the energy substrate. Glucose is the primary carbon source for *E Coli* as they use glycolytic cycle and downstream TCA cycle to generate energy from glucose. Therefore people tried to find the most stable and efficient intermediate from the glycolytic cycle as an energy substrate along with glucose. It was reported that PEP PhosphoenolPyruvate was a more efficient energy substrate for protein production in CFE than glucose. However, PEP is relatively unstable in CFE as it degrades quickly and produces inorganic phosphate which reduces the protein production when present in excess. Therefore a new intermediate 3-PGA (3-Phosphoglyceric acid) was tested and found that 3-PGA is relatively more stable in CFE and more efficient energy substrate for protein production in CFE than PEP (Chatarjee et al) (Fig. 1). Apart from energy substrate NTPs are required for optimal mRNA production. Basic amino acids are required for production of proteins. HEPES, potassium, ammonium, PEG are required for providing stability to the transcription-translation machinery. Magnesium is required for stabilizing the ATP driven reactions. CoA and NAD act as cofactor when PEP is used as an energy substrate. Therefore carefully compared all these protocols (Fig. 6). The main criteria of selection here was the energy substrate that is PEP and 3-PGA. Hence we choose the Krinsky protocol for our energy mixture preparation as it uses 3-PGA and overall the protocol uses minimal reagents and is more optimised. Ue also used PEG 4000 instead of PEG 6000 in this project.

1.6 β-galactosidase assay

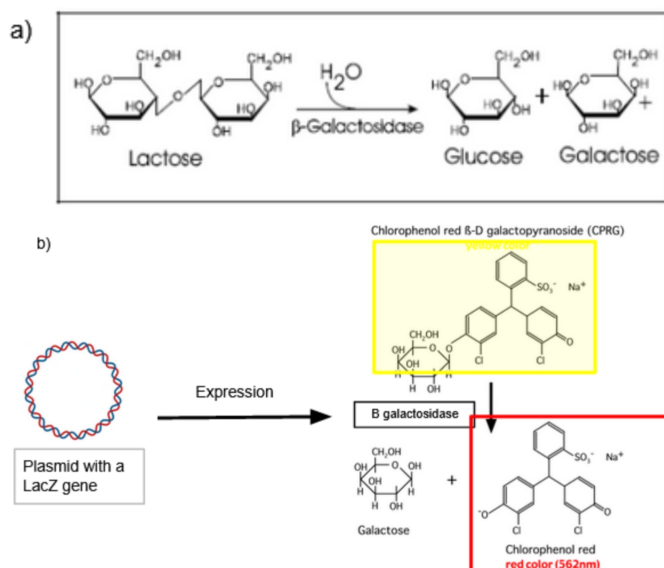

Figure 7: The schematic represents the activity of β-galactosidase. a) Conversion of lactose to glucose and galactose in presence of β-galactosidase. b) Conversion of CPRG to chlorophenol red in presence of β-galactosidase. Figure taken from http://seroudelab.biology.queensu.ca/GAL4/CPRG.html

In the later part of the project after testing our homemade cell free extract by expressing gfp, we wanted to construct and express lacZ in our homemade cell free extract. We needed to quantify the expression of β-galactosidase in our homemade extract for which a potential reporter system is required. The lacZ gene codes for the β-galactosidase enzyme which is traditionally known for breaking lactose into glucose. *E Coli* cells cannot use lactose directly as energy source, for that need to convert lactose to glucose and β-galactosidase does the work. Basically β-galactosidase breaks the 1,4 glycosidic linkage between two glucose molecules. The idea was used to synthesise compounds that resemble the structure and additionally photo-active. CPRG Chlorophenol red-β-D-galactopyranoside is a long-wavelength dye which is commonly used for colorimetric assays. CPRG similarly contains a sugar molecule linked to three phenol groups. The phenol groups are in resonance and the sugar molecule increases the resonance energy. Due to the resonating structure, CPRG dye is colored yellow. Accordingly, when we add CPRG to β-galactosidase, β-galactosidase breaks the bond between sugar molecules and phenol rings. When the bond is broken the resonance energy of the phenol rings decreases. We know that energy is inversely proportional to wavelength ($E=hc/\lambda$). Therefore as the energy decreases the wavelength increases and correspondingly the broken CPRG molecule emits red light instead of yellow (Fig. 7). Hence we thought of using the dye CPRG for quantification of expression. This system is widely used as a reporter for β-galactosidase expression. We wanted to make homemade cell free extract and test it. A colorimetric assay would be a perfect way to test our extract. Therefore we planned to construct a lacZ expression plasmid and test the β-galactosidase expression in our CFE with the help of the dye CPRG.

2 Materials and Methods

2.1 Bacterial strains and plasmids

Escherichia Coli DH5α strains were used for stable incorporation of pSB1C3-mRFP (from iGEM) plasmid. Plasmid containing lac promoter, cap binding site, lac operator, RBS, mRFP and transcription stop site. BL21 (DE3) strains were used for optimal expression of pSB1C3-mRFP (from iGEM) plasmid. Similarly DH5α strains were used for stable incorporation of pEt15B-HP-GFP (designed by Yash Jhawale) plasmid. Plasmid containing lac and araC dual promoter, cap binding site, lac operator, RBS, GFP and transcription stop site. BL21 (DE3) strains were used for optimal expression of And-gate GFP pEt15B-HP-GFP (designed by Yash Jhawale) plasmid. MG16 55 strain was used for expression of lacZ.

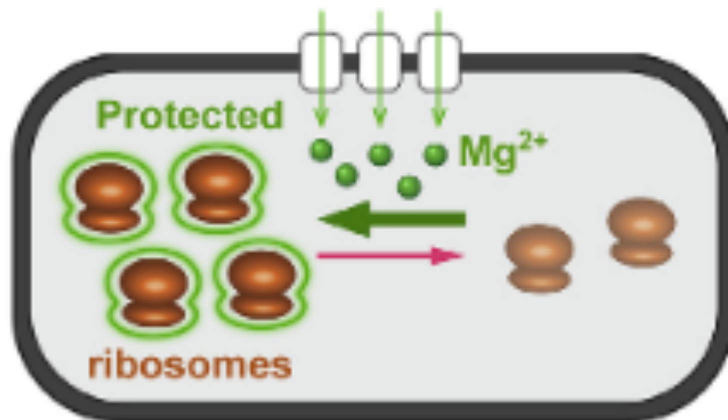

Figure 8: The Schematic represents action of magnesium in cells. Influx of magnesium in cells provides stability to ribosomes which keep them alive and active in extract. Figure taken from Gurol et al, April 4, 2019 Elsevier Inc.

2.2 Growth media and conditions

The cells were grown in Luria-Bertani (LB) broth (HiMedia, Mumbai, India). The media were made in deionized water and autoclaved. All cultures were grown at 37°C in Panasonic MIR 154 Cooled Incubator, unless stated otherwise. Cells containing plasmid were grown in media containing 20 ug/ml Chloramphenicol, Kanamycin, Ampicillin antibiotic (Sigma, USA) for pSB1C3-mRFP, T7 lacZ, pEt15B-HP-GFP constructs respectively. For protein expression, secondary cultures were made with single colony inoculation in desirable culture volumes, grown overnight and OD 0.6 to achieve desirable culture and then induced with reported concentrations of IPTG and arabinose (SRL Chemicals, India) for various constructs used, and grown at 37°C.

2.3 Transformation

For expression, mRFP plasmids were transformed using CaCl2 method (SHAMBROOK J. and Russel, D.W. (2001) Molecular Cloning A Laboratory Manual 2001, ISBN-10 0-87969). DH5α competent cells were removed from -80°C and thawed on ice. 1 ul of mRFP DNA was added to 50 ul competent cells followed by incubation on ice for 30 minutes. The cells were then heat shocked at 42°C for 1 minute in a water bath. Then the cells were immediately transferred and incubated on ice for 5 minutes. 200 ul of LB was then added to cells and incubated at 37°C, 180 rpm for 1 hour. The cells were then plated on a chloramphenicol plate and incubated for 16 hours. Transformation efficiency is given by Colony Forming Unit (CFU) divided by DNA spread on the plate in ng.

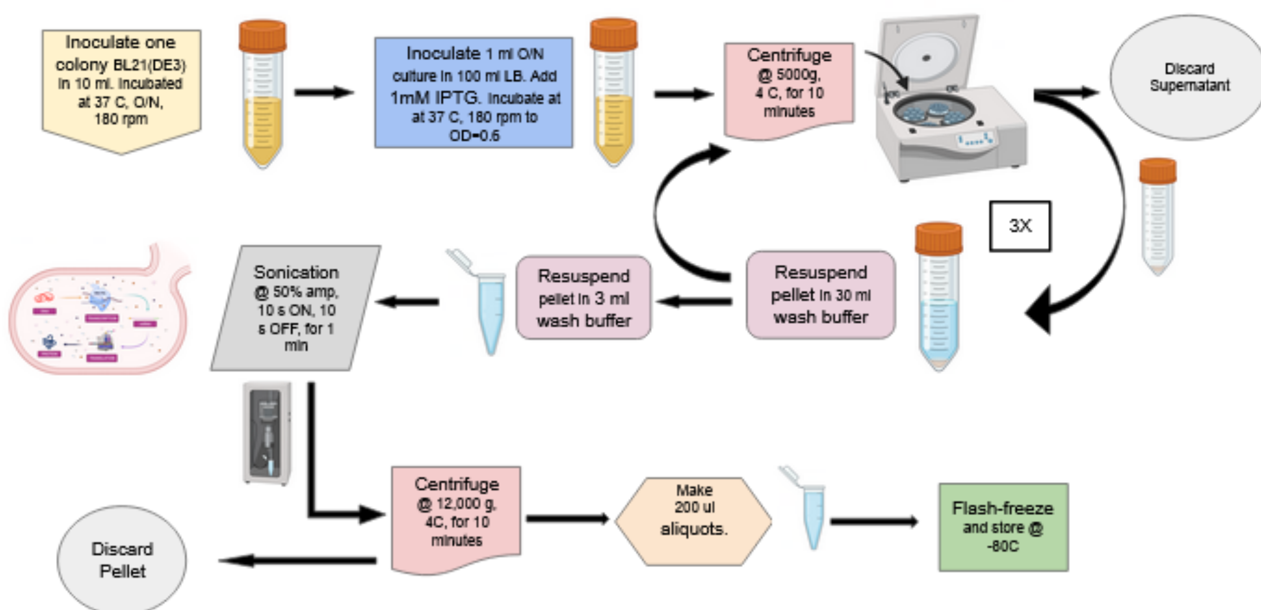

Figure 9: The Schematic represents BL21 (DE3) Extract preperation protocol.

2.4 Cell Free Extract Preparation

Cells were grown from a single colony in LB media over-night (18 hours). Then from that culture 1% inoculum was added to 100ml LB media and grown to OD 0.6. Cells were then harvested using centrifugation 5810R centrifuge (Eppendorf, Inc Germany) at 5000g, 4° C for 10 minutes. Cells grow and die in the media which end up toxicating the media. The media was completely removed. The pellet was then resuspended in 30 ml wash buffer (50 mM tris, 14 mM Mg-glutamate, 60 mM k-glutamate, 2 mM DTT) (11) and centrifuged using 5810R centrifuge (Eppendorf, Inc Germany) at 5000g, 4° C, for 10 minutes. The washing step was repeated thrice with final centrifugation at 7000 g. Perturbation in the cell creates ribosomal stress leading to non-functional ribosomes. Magnesium in the wash buffer enters the cell and provides additional stability to the ribosomes (18). As extract is devoid of cell membrane and the process of making extract generates stress on ribosomes and the transcription-

translation machinery. Therefore the presence of magnesium and potassium provides stability to ribosomes and the transcription-translation machinery (Fig. 8). Pellet was then resuspended in 3 ml wash buffer and lysed using VibraCell Ultrasonicator (Sonics, Inc USA). The lysed cells were then centrifuged using table top centrifuge (Eppendorf, Inc Germany) at 13000 g, 4° C, for 10 minutes. Supernatant was collected and made 200 ul aliquots. The aliquots were then flash-frozen and stored at -80° C for further use (Fig. 9). Protocol taken from MICHEL C. JEWETT. Cell-Free Synthetic Biology: Engineering Beyond the Cell. 2016;8:a023853.

2.5 Plasmid DNA isolation and cloning

The plasmid DNA constructs were isolated using the Alkaline Lysis Method. Cells were pelleted and resuspended in solution I (Glucose, TrisCl, EDTA) buffer by vortexing vigorously. Then solution II (NaOH, SDS) buffer was added, mixed by inverting the tube rapidly. Followed by adding solution III (K- acetate, Glacial acetic acid) buffer which was mixed by quick vortexing. The mixture was incubated on ice for five minutes. Next the mixture was centrifuged at 12000g for five minutes and supernatant was collected. 95% ethanol was added to the supernatant and mixed by vortexing. Followed by incubation at room temperature for two minutes and centrifuged at 12000 g. Pellet was collected. The process was repeated with 70% ethanol and the collected pellet was air dried and resuspended in deionized water. Stored at -4° C. For cloning Q5 DNA polymerase was used in a mixture along with mixing 10mM dNTPs, 10uM forward primer, 10 uM reverse primer, 27B-lacZ plasmid and Q5 buffer. The reaction volume was adjusted with nuclease free water. Next the reaction mixture was processed through a thermal cycle. For amplification the thermal cycle was repeated thirty times using Mastercycler X50s (Eppendorf, Inc Germany). For denaturing DNA a temperature of 98°C was used for 10 seconds followed by for primer anealing 60° C for 20 seconds followed by for DNA polymerisation 72° C for 3 minutes 30 seconds. For Gibson assembly of amplified lacZ fragment and both T7 gene 10 enhancer (110 base pairs) and BBa-B0034 (95 base pairs) inserts concentrations of DNA used 0.043 pico molar, 0.083 pico molar and 0.063 pico molar respectively. One insert at a time with lacZ backbone was mixed to 5 ul gibson reagent, volume of 10 ul was adjusted with water and incubated at 50°C for 20 minutes. Another fragment was made similarly just without the gibson reagent for negative control in Mastercycler X50s (Eppendorf, Inc Germany). For DPN1 digestion the gibson mix was added to 1 ul DPN1, 1.5 ul cutsmart buffer and the volume of 15 ul was adjusted with water. The mixture was then incubated for 1 hour at 37°C followed by 80° C for 20 minutes on ThermoMixer (Eppendorf, Inc Germany). The plasmids were then transformed into DH5α cells and poured onto antibiotic agar plate for screening.

2.6 SDS-PAGE Protein Electrophoresis

12% resolving gel (30% acrylamide, 1.5 M Tris (pH-8.8), 10% SDS, 10% APS, TEMED, MQ H2O) was made and poured into 1mm gel casting plates (Bio-Rad, USA) to three fourth of the shorter plate. A layer of isopropanol was added on top. After solidification the isopropanol was drained. 5% stacking gel (30% acrylamide, 1 M Tris (pH-6.8), 10% SDS, 10% APS, TEMED, MQ H2O) was made and gel casting plates were filled. A comb was inserted at the top. After the gel was solidified the comb was removed. Then the gel cast was assembled into the running apparatus (Bio-Rad, USA) and filled with a running buffer. Samples were loaded into the wells of the gel and ran at 120 v. Finally the image

was taken using SYNGENE G-Box (USA).

2.7 Agarose Gel Electrophoresis

8% gel was made by dissolving 0.4 g of agarose in 50 ml 1x TAE in the microwave. 8 ul Ethidium Bromide (EtBr) was added to the solution and mixed well. The mixture then was poured onto the gel cassette with a suitable comb. After the gel was solidified the comb was removed. Then the gel cast was assembled into the running apparatus (Bio-Rad, USA) and filled with a running buffer. The samples were loaded with 6x gel loading dye along with DNA ladder. Followed by running at 110 v. Finally the image was taken using SYNGENE G-Box (USA).

2.8 Absorbance and fluorescence kinetic measurements

In vitro expression of mRFP in a scale of 25 ul, 50 ul and 150 ul was monitored at 10 minutes interval using Varioskan Flash Multimode microplate reader(Thermo Scientific, USA) in a flat bottom 96 well plate (Corning, USA) at 37° C (excitation filter 584 nm; emission filter 602 nm). In vitro expression of and-gate-GFP in a scale of 50 ul was monitored at 10 minutes interval using Varioskan Flash Multimode microplate reader (Thermo Scientific, USA) in a flat bottom 96 well plate (Corning, USA) at 37° C (excitation filter 488 nm; emission filter 509 nm). To measure the expression of lacz plasmid 0.48 mM CPRG was added to the reaction mixture and loaded in triplicates. Absorbance measurement was taken at 577 nm. Reaction mixture was loaded in a scale of 25 ul and was monitored at 10 minutes interval using Varioskan Flash Multimode microplate reader(Thermo Scientific, USA) in a flat bottom 96 well plate (Corning, USA) at 37° C (577 nm).

3 Results

3.1 Comparison of expression and stability of pSB1C3-mRFP plasmid between BL21 (DE3) vs DH5α

To ease the process of detection of protein expression, a reporter system is very crucial. Detection of protein expression can be done by a complicated process of extraction. A reporter system makes it simple as the molecule is fluorescent hence can be easily detected through fluorimetric analysis. mRFP has a reputation as a reporter because of it's bright fluorescence intensity. There are multiple reporter systems that people use nowadays, however mRFP is famous for its optimal expression and fluorescence in E Coli. Red has the highest wavelength amongst visible lights which makes it the most intense and less scattering. These properties all together makes mRFP one of the best reporters. Therefore we wanted to use the pSB1C3-mRFP plasmid as our reporter system. mRFP has an excitation wavelength of 584 nm and emission 507 nm. We also choose DH5α strain to transform the pSB1C3-mRFP plasmid. DH5α carries truncated Endonuclease A and Rec1 and End1 mutation which reduces the frequency of crossing over and recombination of plasmid DNA to genomic DNA. Thus DH5α serves as an optimal

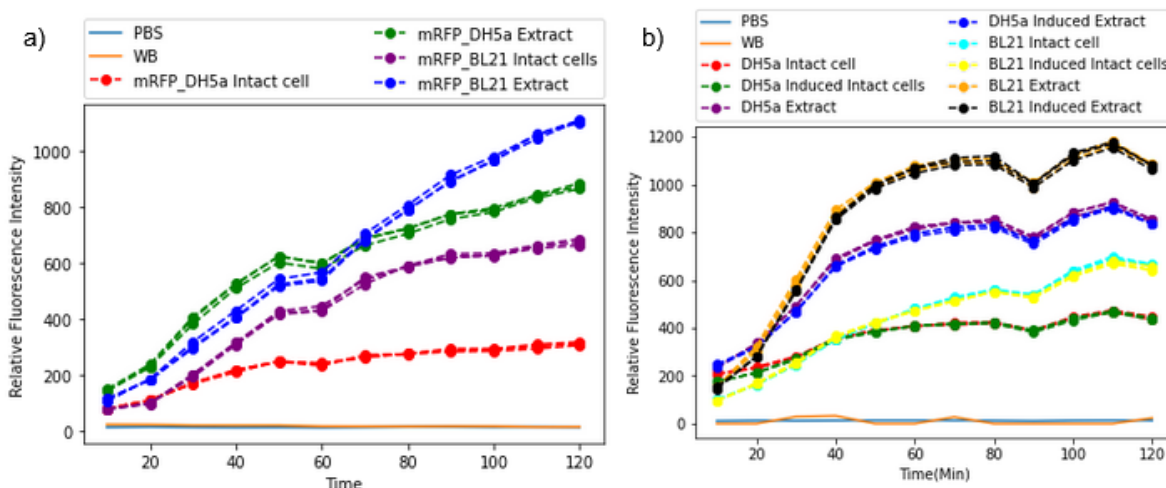

Figure 10: The plots represents comparison of Fluorescence intensity (y-axis) between m-rfp DH5α and BL21 (DE3) intact cell and extract with time in minutes (x-axis). a) Comparison of expression between mRFP DH5α intact cell ,extract and mRFP BL21 intact cell ,extract with PBS and wash budder. b) Comparison of expression between induced mRFP DH5α intact cell ,extract and induced mRFP BL21 intact cell ,extract, PBS and wash buffer.

system for stable incorporation of plasmid DNA. We already found from previous studies that mRFP plasmid is very stable in DH5α intact cells as well as in DH5α extract [Appendix]. Therefore it was the obvious next step to express the mRFP plasmid in BL21 and compare it to DH5α. BL21 carries mutations in Lon and OmpT protease genes which provide additional stability to the proteins, making BL21 one of the best expression systems. We transformed BL21 cells with mRFP plasmid

with transformation efficiency of 54.3. Both mRFP DH5α and mRFP BL21 cells were harvested at their saturation (overnight culture) and washed and lysed as mentioned in the extract preparation protocol. The crude extract was then processed and loaded on 96 well plate along with mRFP DH5α and mRFP BL21 intact cells. The fluorimetric assay clearly indicated that both BL21 intact cell and extract is a better expression system than DH5α (Fig. 10). The comparison was also done between induced with IPTG and uninduced DH5α intact, extract and BL21 intact, extract. Samples were loaded in triplicates onto 96 well flat bottom black plate (corning, USA) and a fluorimetric assay was done. Which again concluded that the pSB1C3-mRFP is constitutively expressing plasmid (Fig. 21). However we wanted to concretize our conformation so we wanted to harvest cells at their early growth (log) phase and lyse them to check if at all the pSB1C3-mRFP is really getting constitutively expressed or not. Hence we induced BL21 cells containing pSB1C3-mRFP with 2mM IPTG and harvested them at OD 0.6. Following the same protocol we washed lysed and made the extract and performed the fluorimetric assay with induced intact cells, uninduced extract and intact cells. It leads to the same result as expected, that holistically confirms that pSB1C3-mRFP is constitutive. We wanted an inducible reporter system for expression in our home made cell free extract, therefore we needed another reporter system in place of mRFP. Despite the advantages with mRFP we had to choose GFP as our reporter for further experiment and finally expression in our home made cell free extract.

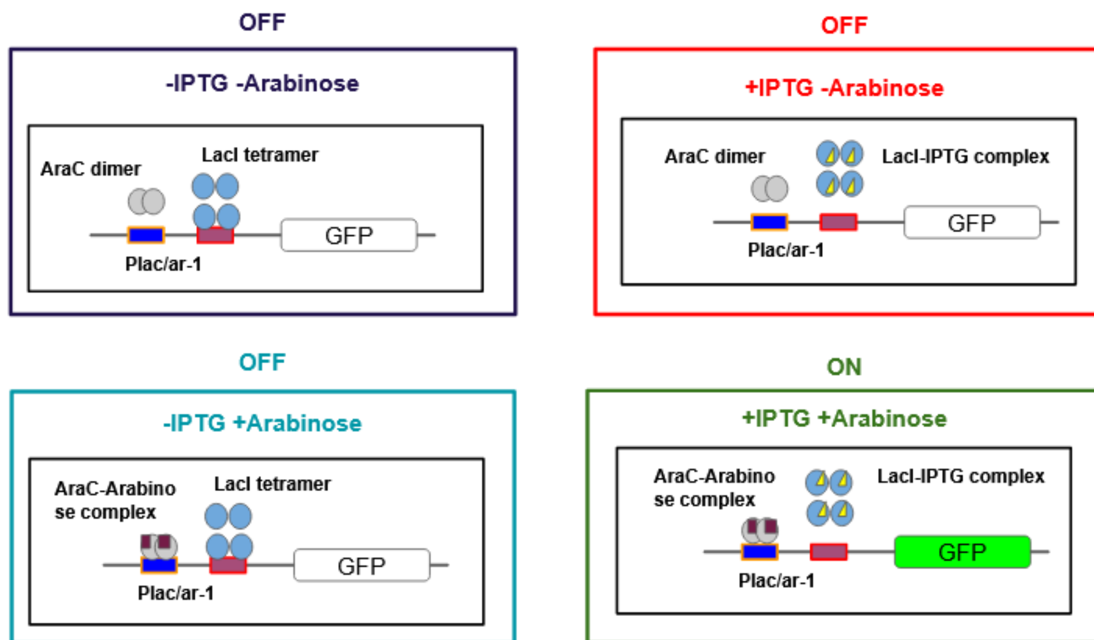

Figure 11: The Schematic represents And-gate-GFP switch. The ON OFF states of pEt15B-HP-GFP plasmid is represented with all possible combinations of IPTG and arabinose.

3.2 Induction and Stability of And-gate GFP pEt15B-HP-GFP in extract

The plasmid pEt15B-HP-GFP carries a GFP gene and two promoters namely LacI and AraC. Therefore the plasmid is designed in such a way that it forms an and-gate system. Which means it acts as a ON/OFF genetic switch, only in presence of both araC and IPTG together the switch is in ON state and GFP is expressed. All other combinations will lead to OFF state and no GFP expression (Fig. 11). The pEt15B-HP-GFP plasmid (Fig. 12a) was constructed by Snehal Kadam (former Masters student from the lab). The pEt15B-HP-GFP plasmid was transformed into BL21 and the concentration of IPTG and araC was optimised by him. The optimum concentration of IPTG he found was 1mM and for araC was 0.7% for this plasmid. We made a culture of Bl21 pEt15B-HP-GFP cells and harvested them at saturation and following the same protocol made extract. We induced the Bl21 pEt15B-HP-GFP extract and intact cells with both araC and IPTG before plating them into the 96 well plate for fluorimetric assay along with Bl21 pEt15B-HP-GFP uninduced intact cells and extract. The excitation wavelength for GFP is 488nm and emission wavelength is 509 nm.

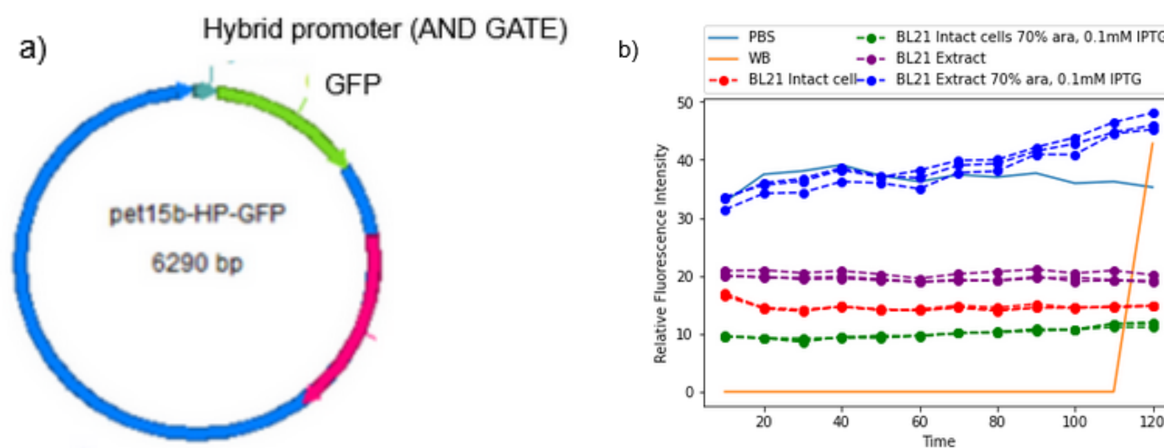

Figure 12: The Schematic represents pEt15B-HP-GFP plasmid and Fluorescence intensity (y-axis) of GFP expression in BL21 (DE3) with time in minutes (x-axis).
a): pEt15B-HP-GFP plasmid map. b): Comparison of expression between induced GFP BL21 (DE3) intact cell ,extract and uninduced GFP BL21 (DE3) intact cell ,extract. Blue: Media, Indigo: S30 buffer, Red: BL21 (DE3) intact cell, Green: Induced BL21 (DE3) intact cell, Purple: BL21 (DE3) extract, Blue: Induced BL21 (DE3) extract.

From the assay it is very clear that BL21 extract induced with 1mM IPTG and 0.7% araC has the highest and increasing expression level. The expression of uninduced intact cells, extract and the induced intact cells are near baseline. That proved that the genetic switch works perfectly and interestingly the switch works in the extract (Fig. 12b). The difference in expression between induced extract and intact cells clearly demonstrated that cell free extract despite being an open system is capable of keeping GFP proteins stable. We wanted to use the pEt15B-HP-GFP plasmid to express it in CFE. High concentration of DNA is required for Cell Free Protein Expression. Hence we made cultures of pEt15B-HP-GFP BL21 cells and extracted the plasmid. There is genetic DNA and plasmid

DNA in *E Coli*, it is crucial to check that if we have collected the right DNA that we desire. There is a EcoRI restriction site and we digested the extracted plasmid with EcoRI. Then we load the digested and undigested DNA onto the 8% agarose gel. We loaded it with a 2-log ladder for reference. It is evident from the gel image that we could extract pEt15B-HP-GFP plasmid successfully.

3.3 Expression of pEt15B-HP-GFP plasmid in CFE

Expression of a plasmid or a genetic switch in cells like *E coli* BL21 is straightforward with successful transformation. On the other hand, expressing a plasmid in extract is very complex and complicated due to the obvious cell free nature of the system. We followed the minimal cell free reaction protocol by Krinsky et al. The reported composition is 70% energy solution and 30% CFE in the reaction mixture. As the CFE is not alive, devoid of growth, reproduction and development hence cannot produce energy substrates on its own. Hence energy supplementation in excess amounts is very essential for the system to potentially express the gene of our interest (Fig. 13). The AND gate plasmid was pre-made and the plasmid induction concentrations (1 mM IPTG and 0.7 % arabinose) were optimized by Kadam, Jawale et al. For testing our homemade cell free extract we then ran two cell free reactions in two separate tubes. In one tube we added all the reagents necessary for the energy mix from the stock solutions along with BL21 DE3 extract, IPTG and arabinose. In this tube we didn't add any DNA. In the other tube we added all the reagents necessary for the energy mix from the stock solutions along with BL21 DE3 extract, IPTG, arabinose and 10 μg/ml concentration of pEt15B-HP-GFP plasmid (Fig. **??**). Finally we incubated both at 30°C for 18 hours in a water bath. We transformed BL21 (DE3) cells with pEt15B-HP-GFP plasmid and made an overnight culture by inducing them with IPTG and arabinose.

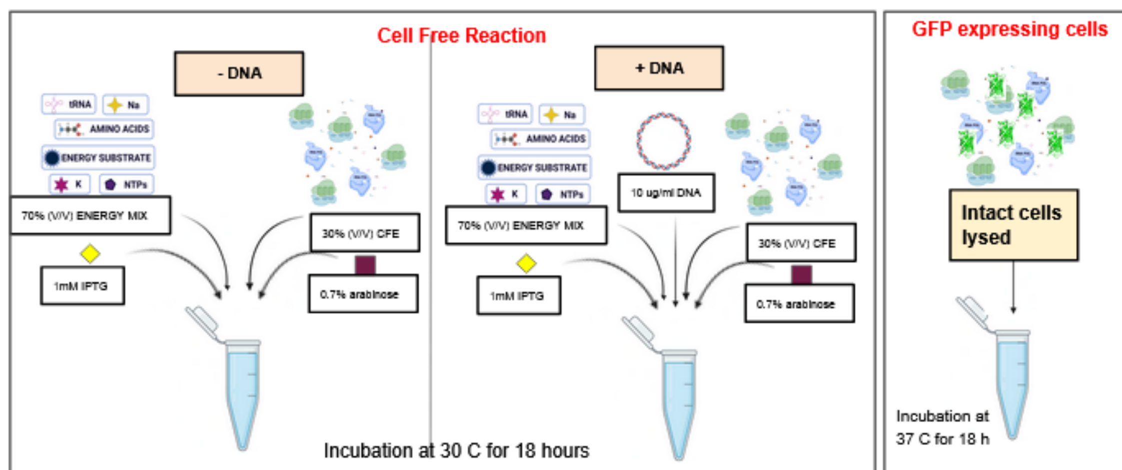

Figure 13: The schematic represents the process of cell free reaction setup for AND gate gfp expression in homemade cell free extract and the intact cells expressing gfp lysed and used for comparison of expression.

The negative control (CFE without DNA), reaction mixture (CFE with DNA) and positive control

(lysed gfp expressing cells) were loaded onto a 96 well half area opaque plate and the relative fluorescence intensity was measured at excitation wavelength 488 nm and emission 509 nm (Fig. 14a). There is a difference in fluorescence emission is observed between the homemade extract expressing GFP and lysed gfp expressing cells.

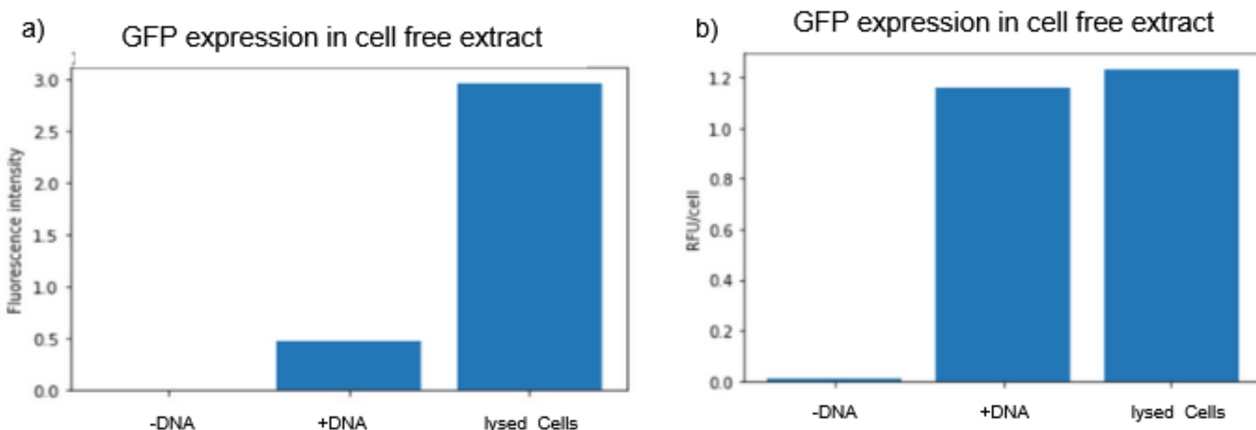

Figure 14: The plots represents Fluorescence intensity (y-axis) of GFP expression in homemade extract. a) Bar plot represents GFP expression in CFE - DNA, CFE + DNA and lysed gfp expressing cells without normalization. b) Bar plot represents GFP expression in CFE - DNA, CFE + DNA and lysed gfp expressing cells. The expressions are normalised with the number of cell equivalent extract present in each reaction and lysed cells (RFU/cell). NC: Extract without DNA, CFE: Extract with DNA, PC: Cells containing GFP plasmid were cultured and then Extracted.

However, we realised that the culture used as positive control was an overnight culture and had an OD of 6 (measured by diluting the samples) and the culture used to prepare the extract had OD 0.6. Which leads us to the difference in fluorescence emission between our homemade extract expression and positive control. It is well known that more the number of cells, the more will be the production and we will end up expressing more number of protein molecules. Therefore in order to compare the expression we need to normalise the fluorescence emission readings we got with the number of cells it contains respectively, so that we can get fluorescence emission per cell. That way we can precisely compare the results. Therefore we proceeded with that and made the calculations, as follows. We made 10 ml MG16 55 culture and harvested at OD(600 nm) - 6 and resuspended in a 500 ul S30 buffer. The OD was too high therefore we measured the OD by diluting the sample 10 fold with LB. We are considering here that OD(600 nm) - 1 represents 10 million cells per ml (Mansi et al). For the calculation of number of cell equivalent extract we used the formula:

$$X = V_2 \times N_{cells}/V_1 \tag{1}$$

N_{cells} = Total number of cells calculated using the OD(600)
V_1 = The final volume of resuspended culture
V_2 = The volume of the extract used for the reaction (CFE)

$$X = \text{The number of cell equivalent extract present in the reaction}$$

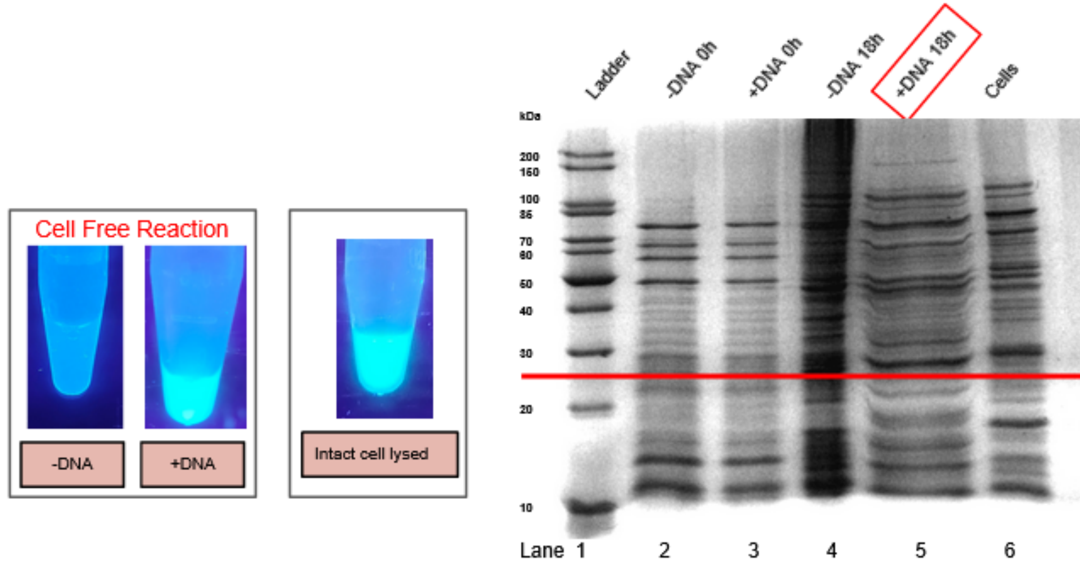

Figure 15: The image represents gfp (27 kDA) expression in homemade extract in tube and on SDS-PAGE. Left: Image of the tubes of cell free reaction without and with AND gate plasmid, other image is of the tube containing the lysate of gfp expressing cells. Right: Image of SDS-PAGE. Lane-1: Unstained protein ladder, Lane-2: Extract without DNA unincubated, Lane-3: Extract with DNA unincubated, Lane-4: Extract without DNA incubated fpr 18 hrs, Lane-5: Extract with DNA incubated fpr 18 hrs, Lane-6: Intact cells expressing gfp were extracted and loaded. The red line indicates 26 kDa region.

The number of cells in turn are determined by the conversion of 1 OD unit to cell density, which has been reported to be 8×10^8 cells/ml (Gangan and Athale, 2017). Therefore the number of cells in 10 ml culture with OD = 6 would be $6x10^9$. The final expected value of the number of cells in the total culture volume of 10 ml would be:

$$N_{cells} = CultureVolume * OD * [Cells/ml - OD] \tag{2}$$

giving us $10 \times 6 \times 10^8 = 6 \times 10^9$ cells. The MG1655 culture was resuspended in 500 μl and we used 20 μl out of the cell suspension, as positive control. Using Equation 2 we find $N_{cells} = 6 \times 10^9$. Then substituting this into Equation 1 we find $X = 2.4 \times 10^8$.This was used to normalize the RFU/X, which resulted in the RFU/cell (Fig. 14)
We normalised the positive control with the number of cells (24 million). Similarly for extract preparation we made 100 ml BL21 (DE3) culture and harvested at OD(600 nm) $\sim$ 0.6 and resuspended in 3 ml S30 bufferGiven the two equations and substituting the volumes of V1=500 μl and V2=20 μl, $X = 2.4 \times 10^8$ cells. We normalised the positive control with this number. A similar treatment was performed for CFE where 100 ml BL21 (DE3) were grown to OD = 0.6 and resuspended in (V1) 3 ml

of S30 buffer. The assay used 20 ul (V2) of extract and therefore $X = 4times10^8$ cells. We similarly normalised our cell free reactions with this value (Fig. 14b).
It is trivial that higher transcription-translation machinery will lead to higher GFP production. Hence the normalization is justified and necessary. Interestingly after normalization we found the CFU per cell homemade extract is comparable to positive control. Which also proves that the protein production efficiency of our homemade cell free extract is satisfactory. We also wanted to make sure of the expression of GFP in our homemade extract on a SDS gel (Fig. 15). We loaded both the unincubated samples (with and without DNA) and both samples from post incubation and extract of BL21 DE3 cell expressing gfp on the SDS gel. From the gel image we confirmed the expression of GFP in our homemade cell free extract with DNA (incubated). A dark band is visible around 27 kDa region in homemade extract with DNA (incubated) lane and extract of BL21 (DE3 cell expressing gfp lane. As the lysate of BL21 DE3 cell expressing gfp sample already contains GFP, it worked as positive control.

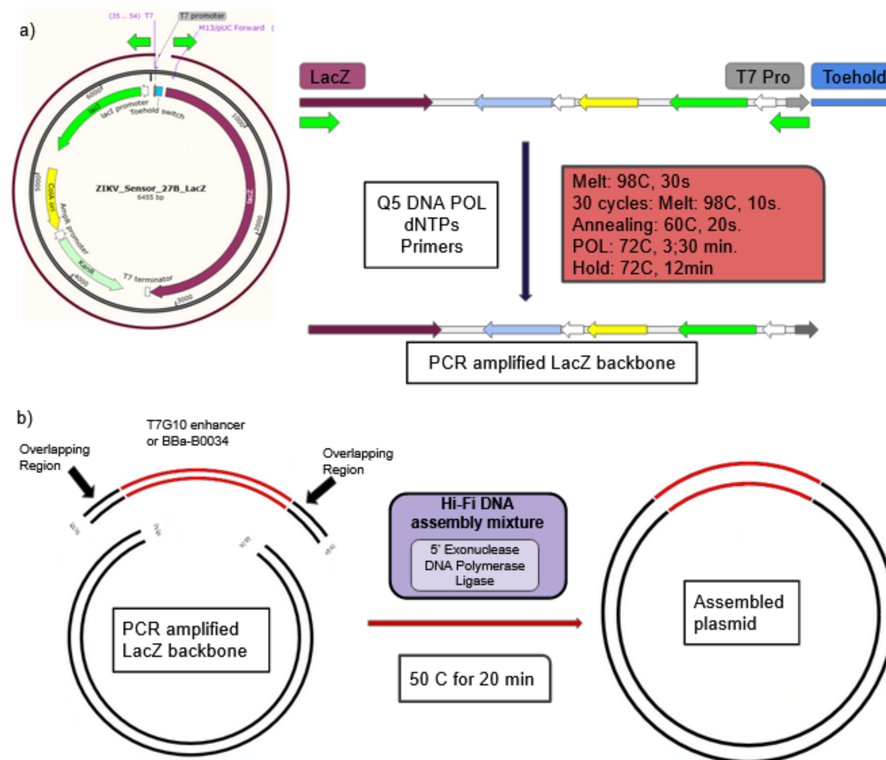

Figure 16: The Schematic represents, a) ZikV-27B-LacZ backbone cloning. b) Gibson assembly of lacZ backbone with inserts T7G10 and BBa-B0034 inserts.

3.4 Plasmid construction for LacZ expression

Along with mRFP and And-gate GFP we thought it would be good to have a gene circuit with T7 promoter which can bind to T7 RNA polymerase. Bacteriophage T7 RNA Polymerase is a

DNA-dependent RNA polymerase that is highly specific for the T7 phage promoter sequence 5'-TAATACGACTCACTATAGGG-3'. The 99 KD enzyme catalyzes in vitro RNA synthesis from a cloned DNA sequence under the T7 promoters. RNA produced using the T7 RNA Polymerase is partly specific and very rapid which makes it the most efficient for protein production. T7 RNA polymerase is also structurally similar to the DNA-directed DNA polymerases of the pol I/pol α family, to the RNA-directed DNA polymerases (reverse transcriptases), and to the RNA-directed RNA polymerases. Hence we choose Zika-27B-LacZ plasmid which has T7 promoter, CoIA origin, Kanamycin resistance, LacZ gene and toehold switch, kind gift from Keith Pardee. We wanted to replace the toehold switch part with T7-G10 enhancer and BBa-B0034 separately.

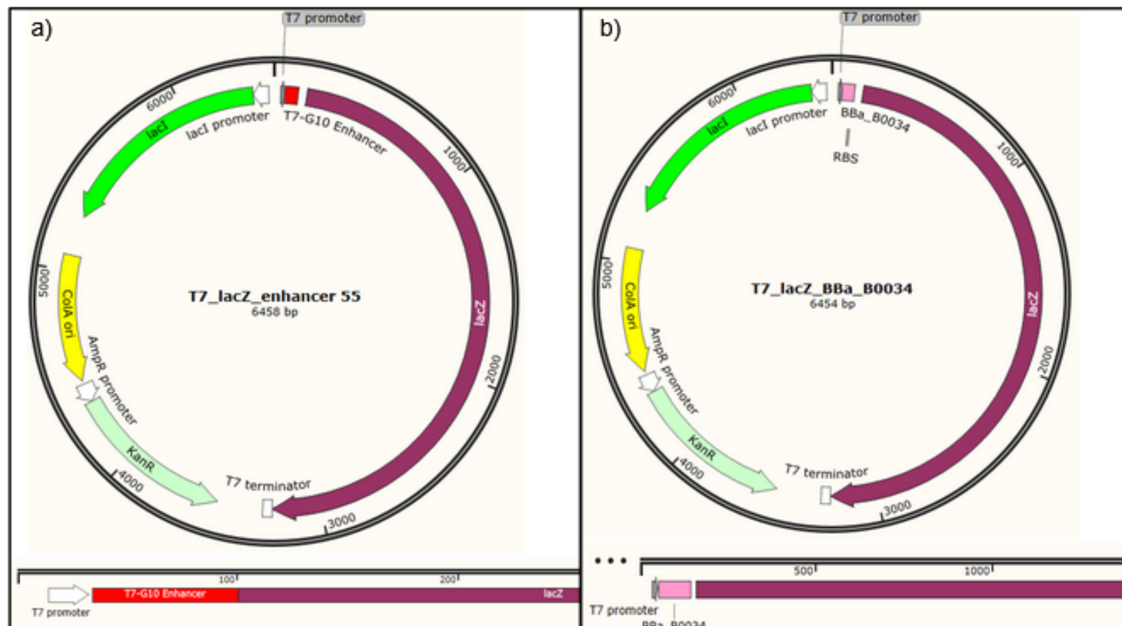

Figure 17: The Schematic represents LacZ backbone with both inserts. a) T7G10 enhancer RBS is inserted into LacZ backbone. b) BBa-B0034 mRFP RBS is inserted into LacZ backbone.

Hence we started with amplification of Zika-27B-LacZ plasmid starting from LacZ to T7 promoter region. For that we choose two promoters, one forward that binds to LacZ and the other reverse that binds to the T7 promoter region. We set up a PCR with Q5 Polymerase and the two primers. Q5 DNA polymerase is a high-fidelity, thermostable DNA polymerase with $3' \rightarrow 5'$ exonuclease activity, fused to a processivity-enhancing Sso7d domain to support robust DNA amplification. We used 30 cycles of PCR for amplification with each cycle having 98 C, 10s for DNA denaturation, 60 C, 20s for primer annealing, 72 C, 3:30 min for DNA polymerization (Optimization was achieved with help from Tanvi Kale). Followed by running it on 8% agarose gel to separate out the correct length fragments from non-specific DNA fragments that produce during a PCR. Then we cut the gel and took the band of interest out with a surgical knife. Then we clean-up the gel on a silica column. Finally elute

the DNA fragment in DEPC treated water. Then we assemble the PCR amplified fragment and the T7-G10-enhancer with gibson mix. The Gibson mix contains 5' exonuclease, DNA polymerase and ligase to join the overlapping reasons of the two fragments (Fig. 16). Followed by DPN1 digestion to get rid of the parental template DNA in the mixture from the PCR. BL21 has methylases that specifically methylated sequences like these and to recognise host DNA versus foreign DNA.

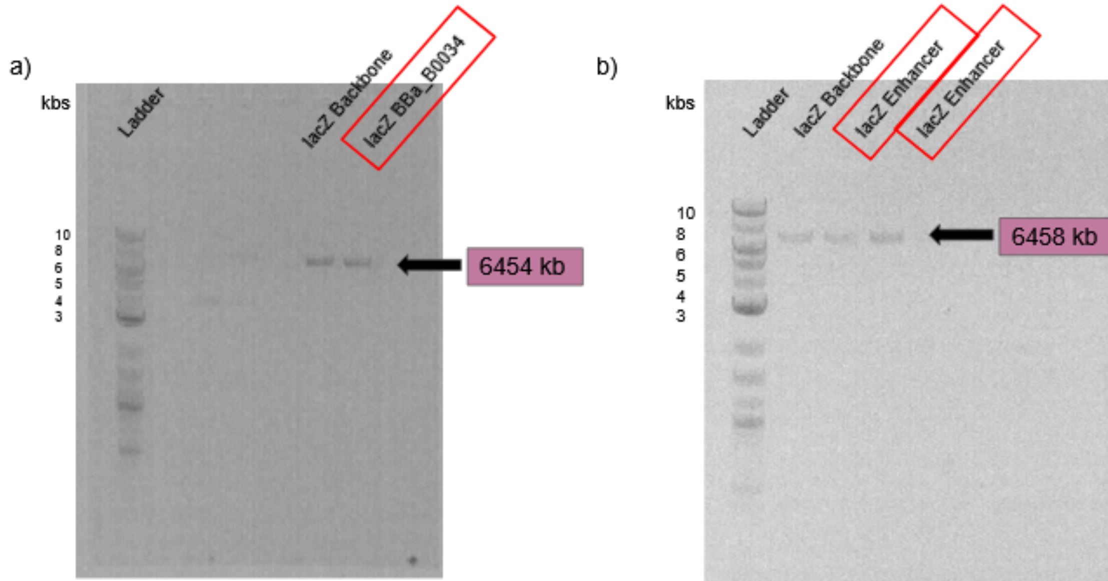

Figure 18: The image represents LacZ backbone with both the inserts on agarose gel. a) Samples were loaded with 2-log ladder, lane 1- ZikV-27B-LacZ backbone digested with EcoRI as control (6455 bp), lane 2- laxZ with BBa-B0034 plasmid digested with EcoRI (6454 bp). b) Samples were loaded with 2-log ladder, lane 1- ZikV-27B-LacZ backbone digested with EcoRI as control (6455 bp), lane 2 & 3- laxZ with T7G10 plasmid digested with EcoRI (6458 bp).

When the sequence is found unmethylated then it is considered foreign and gets digested by host restriction enzymes. DPN1 targets methylated adenine in the sequence GATC. That was how we got both the LacZ expression plasmids and got rid of the parental DNA plasmids. Finally we transformed T7g10 enhancer samples in Dh5α and spread them on agar plate to get colonies with the appropriate antibiotic. We did the same with BBa-B0034 and PCR amplified fragments and finally transformed them into DH5α cells and got colonies on the plate. Similarly we made cultures of both the transformants and then extracted them. Following these processes we expected the constructed plasmids (Fig. 17). There is both genetic DNA and plasmid DNA present in *E Coli*, therefore, it is crucial to check if we have collected the DNA that we desire. There is a EcoRI restriction site and we digested the extracted plasmid with EcoRI. Then we load the digested DNA onto the 8% agarose gel. We loaded it with a 2-log ladder for reference (Fig. 18). It is not clear from the gel image that we have extracted both the lacZ plasmids successfully or not as the backbone and assembled plasmids are very close in length which cannot be distinguished in the gel image. Therefore the best thing to check is by sequencing which we are planning to do in future. We can also check the expression of the plasmids

in our homemade cell free extract. In the next step we wanted to check the expression of both the plasmids.

3.5 Expression and comparison of LacZ plasmid in CFE

The phage T7 gene 10 mRNA carries an epsilon sequence element which enhances the translational activity of a lacZ reporter gene construct. Epsilon is partially complementary to the 460 stem–loop of 16S rRNA [19]. The rRNA binding to lacZ mRNA dramatically increases the translation. That was the reason why we chose it to insert between lacZ and T7 promoter fragments and for comparison of expression we inserted BBa-B0034, an synthetically made native *E Coli* RBS to the other. Now in similar fashion to earlier we recompiled the energy solution and added the enhancer lacZ plasmid in 10 μg/ml concentration to one fraction and lacZ BBa-B0034 plasmid of same concentration to another fraction. Then we added BL21 (DE3) extract to both the fractions. We also made another fraction similarly except we didn't add any plasmid to it, remaining exactly the same. We used the cell free

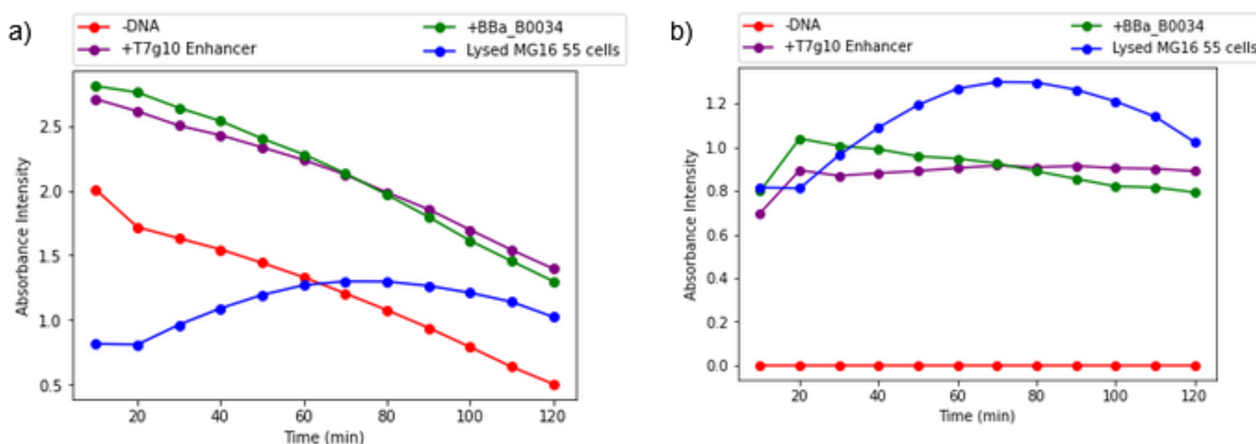

Figure 19: The plots represents absorbance at 577 nm (y-axis) to measure LacZ activity in BL21 (DE3) in home made extract. Absorbance was measured at 577 nm which measures the intensity of red light coming from chlorophenol red, β-galactosidase converts CPRG into chlorophenol red which represents the β-galactosidase expression in the system.
a) The triplicates were averaged and plotted. b) The average plots are plank subtracted and plotted except lysed β-galactosidase expressing cells.

reaction without any DNA as our negative control. We used the overnight culture of MG16 55 cells which carry lacZ gene. These cells were expressing β-galactosidase protein, these cells were lysed using the extract preparation protocol and the lysate containing β-galactosidase protein were used to compare the β-galactosidase expression of our homemade cell free extract. CPRG was added to all the samples for quantification of β-galactosidase expression. The negative control, lacZ enhancer, lacZ without enhancer and positive control were then loaded in triplicates onto 96 well plate and absorbance was measured. The triplicates were averaged and plotted (Fig. 19a). The LacZ expression is clearly observable in the plot. However, We see a decreasing trend in the absorbance over time. The

decreasing trend of absorbance intensity is due to the drying of samples on the plate. The measurement was taken for two hours and the samples were loaded in volumes of 20 ul. Due to which the samples got dried on the plate leading to the decrease in the absorbance intensity.

Figure 20: The plot represents Absorbance intensity (y-axis) of lacZ expression in BL21 (DE3) in home made extract. Absorbance was measured at 577 nm which measures the intensity of red light coming from chlorophenol red, β-galactosidase converts CPRG into chlorophenol red which represents the β-galactosidase expression in the system. The bars represent time average intensity of 120 minutes. Bars prepresents expression of cell free reaction - DNA, Cell free reaction with B0034 plasmid, Cell free reaction with T7g10 enhancer plasmid and lysate of β-galactosidase expressing cells.

BL21 (DE3) is the best protein expression platform as it is deficient of lon and omp-t protease genes that increases protein stability, also it carries T7 RNA polymerase gene which enhances the expression of the lacZ gene that is under the control of T7 promoter. Therefore, we used BL21 (DE3) strain for expression. However, BL21 (DE3) also carries lacZ gene in its genomic DNA hence it inherently produces β-galactosidase. We here expressed lacZ plasmids in BL21 (DE3) extract which already has β-galactosidase inherently expressed in it. As a result it was obvious that we will get a reasonable absorbance reading for the extract without the lacZ plasmid. But here we only wanted to check and compare the expression of our constructed lacZ plasmids. Therefore it was necessary to normalise the absorbance of β-galactosidase in reaction mixtures (CFE with lacZ plasmid) with negative control (CFE without plasmid) to cancel out the inherent β-galactosidase that is already present in the BL21 (DE3) extract. That way we will only account for the β-galactosidase produced by the expression of lacZ expressing plasmids. The negative control, lacZ enhancer, lacZ without enhancer and positive control were then loaded in triplicates onto 96 well plate and fluorimetric analysis was done. The

triplicates were averaged and plotted with normalization Fig. 19b). From the plot we confirmed that both the lacZ plasmids got expressed in our home made CFE. However, the expression of lacZ with enhancer is not higher than without enhancer, they both are in the same range. For further analysis we time averaged the normalized readings for all the samples and plotted to see if we can visualise the difference in expression of β-galactosidase between the two constructed plasmids. We also plotted the bar graph with standard deviation to get an estimate of change in expression over time for all the samples (Fig. 20). From the plot we found out that the expression of B0034 and T7g10 enhancer were comparable and both these expressions were comparable to the expression of lysed β-galactosidase expressing cells. In vitro expression is very complex hence we need to make some more optimisations in order to ger a cleaner expression of both the constructed plasmids.

4 Discussion

For centuries researchers have been using Cells to express desirable proteins. People explored a wide range of strains to express protein. Over time it was realised that proteins that are compatible with the host cell are expressed easily. Strains were modified and enhanced genetically to express eukaryotic protein in prokaryotic host cells. However proteins that are toxic to host cannot be produced by any means. People began to think that it is possible to extract the transcription-translation machinery out of the host keeping it alive, which will allow us to express any protein we desire irrespectively. It might sound simple but keeping alive cellular machinery without keeping the cell intact is extremely challenging. Cells keep their machinery confined within the cell membrane. Through which they regulate internal activity and homeostasis which keeps the host machinery alive and active. The intact cells also keep checks and balances on what enters and exits the cell. Therefore the extract devoid of cell membrane is open and incapable of maintaining internal homeostasis which makes it extremely vulnerable. Collectively it makes cell free extract very challenging to achieve. Finally, the system was developed in the 60's and over time was optimised to achieve efficient expression. People since then have constantly developed the system and has been used in industries for commercial protein production. As we use batch culture and continuous culture of bacteria according to our demand, extract can also be made and used as batch and continuous culture for protein production. Michal Jewett has developed a system where the extract is prepared and enveloped in a dialysis membrane and a continuous flow of energy solution is passed through , which works as a continuous culture and produces the protein of interest continuously.

We here made cell free extract of BL21 (DE3) strain and tested its efficiency. We used three reporters namely mRFP, GFP and β-galactosidase. The mRFP plasmid was constructed by Tanvi Kale (PHD student in lab), GFP plasmid was constructed by Snehal Kadam (former BS-MS student in lab) and the lacZ plasmids were constructed by me. We cloned lacZ part from a ZikV-sensor-27B-lacZ plasmid with a forward and a reverse primer. Then we inserted the insets of our interest in it. We first checked the stability of both mRFP and GFP reporters in extract. This was done by expressing m-rfp and gfp in BL21 (DE3) and DH5-α cells. We then harvested them and made extract and measured fluorescence to check stability. We found that both the reports are quite stable in extract. Hence we proceeded with our experiments and made cell free extract of BL21 (DE3) cells and expressed gfp and lacZ plasmids.We selected E. coli BL21 DE3 cell line for preparation of homemade cell free extract because our primary goal in this project was synthesis and optimization of extract and testing the extract. For which BL21 DE3 is one of the best platforms, as it provides additional stability to the expressed protein. The reviewer is correct in pointing out that BL21DE3 carries lacZYA gene which makes the system not suitable for expression of lacZ plasmid. But the constructed plasmid carrying lacZ gene was under the control of T7 promoter therefore the only strain available which carries native T7-RNA polymerase gene available to us at the time, was BL21(DE3). However, from the experiment we surprisingly got some interesting results. The raw data showed that the expression of lacZ enhancer plasmids are high, compared to extracts without plasmid. By subtracting the readings of the plasmid containing CFE and observing the 2-fold difference between CFE with and without plasmid, we find that relative absorbance in the range of 1 to 1.2 (plasmid – no plasmid), suggesting despite intrinsic lacZ, the overexpression from the plasmid can be measured. In future we hope to use a T7 polymerase expressing cell type without the lacZ gene. To this end we aim to use the commercial E. coli KRX cell line with the following genotype: [F´, traD36, ΔompP, proA+B+, lacIq,

Δ(lacZ)M15] ΔompT, endA1, recA1, gyrA96 (Nalr), thi-1, hsdR17 (rk–, mk+), e14– (McrA–), relA1, supE44, Δ(lac-proAB), Δ(rhaBAD)::T7 RNA polymerase] for the preparation of extract and use the extract for expression of the constructed lacZ plasmids. For quantification of lacZ expression we used CPRG which is converted to chlorophenol red in presence of β-galactosidase. CPRG was added to all the cell free reaction mixture ran for lacZ expression experiment. The absorbance measurement was taken at 577 nm which is the range for red light. Therefore from the intensity of red light emission we could quantify the lacZ expression. We were able to successfully express gfp and β-galactosidase in our home made cell free extract. With these studies we proved that the homemade extract we made is capable and efficient in expression. With further studies it is possible to construct a synthetic gene circuit capable of detecting viral RNA, which can be expressed in our homemade cell free extract for rapid detection. This could be useful for a crisis like the one we all are going through.
Cell free extract carries transcription and translation machinery, which is kept alive to potentially carry out expression of desired gene with addition of required substrates. This can also be transformed into an artificial cell. Liposomes can be defined as nano and microsized colloidal multi-layer vesicles comprising an aqueous compartment enclosed by a bilayer made of either natural or synthetic lipids, as well as the combination of both. Therefore liposomes can be used to capture extract which can function as a cell with added DNA and substrates. The artificial cell might not be capable of performing all the necessary fictions that a typical cell does but it would be capable of performing the basic functions with appropriate DNA. The artificial cell would also be capable of expressing toxins and other complex proteins which is not possible in actual cells. To date the artificial cells have developed such a way that they are capable of division. There are multiple extraordinary scope that can be achieved with cell free extract with rigorous research.

4.1 Conclusions

From all of these experiments conducted we can firstly conclude that reporter proteins like mRFP and GFP are stable in the exact system. We were interested in expressing pSB1C3-mRFP plasmid but the plasmid was not getting induced with the reported IPTG concentration. Therefore we induced the pSB1C3-mRFP plasmid with various IPTG concentrations to check whether it can be induced with any other IPTG concentration. However, it was found that the pSB1C3-mRFP plasmid is constitutive in expression. Hence, we wanted to shift from mRFP to GFP as a reporter. For GFP we used And-gate-GFP plasmid which acts as a switch, therefore we wanted to check the expression and stability in the exact system. We made an extract of cells expressing gfp and found that the And-gate-GFP switch works perfectly and expression is stable as expected in the exact system. We also wanted to make a simple reporter system like β-galactosidase based reporter. The construction of lacZ plasmids worked which was concluded from the agarose gel. We also prepared our homemade cell free extract of BL21 (DE3) cells. The gfp and both the lacZ plasmids were expressed in our homemade cell free extract. We can also conclude that the expression efficiency of our homemade cell free extract is comparable and satisfactory. However, the lacZ enhancer plasmid did not work as expected which can be due to the drying of samples on plate. With some optimization and repeating the experiments the issue may be resolved.

5 References

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6 Appendix

We also conducted some experiments before getting into experiments with cell free extract which I will be discussing in this section.

6.1 Comparing mRFP stability in Extract VS Intact cells

Extract being an open system, it was necessary for us to check the stability of our chosen reporter in it. mRFP is an excellent reporter so we started with it as our reporter. Therefore we started with transforming DH5α cell with pSB1C3-mRFP plasmid. Transformation efficiency is given by Colony Forming Unit (CFU) divided by DNA spread on the plate in ng. We calculated the CFU on the plate to be 22,000 and the amount of DNA added was 611.3 ng. Therefore the efficiency of transformation was 35.9. Protein production in-vivo or ex-vivo requires a reporter system for easy detection and quantification. Before going into protein production in CFE system monitoring the stability of

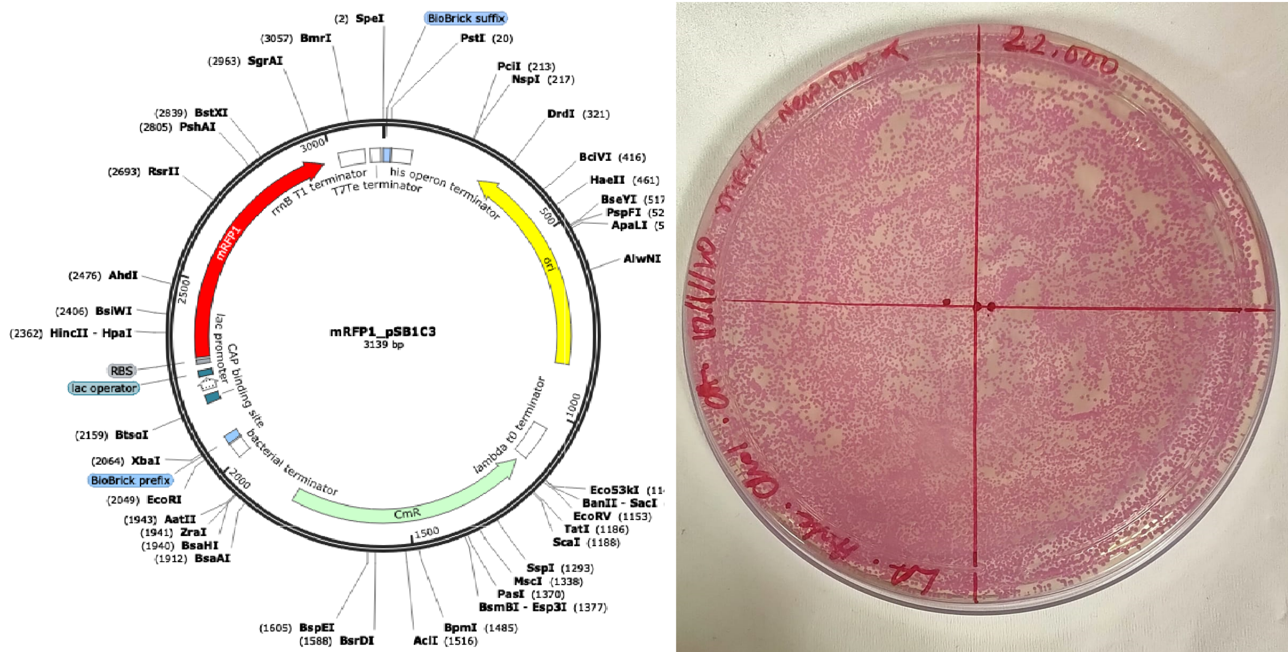

Figure 21: The iGEM plasmids BBa_J04450 (http://parts.igem.org/Part:BBa_J04450) mRFP was inserted into pSB1C3 plasmid (left) and it's transformation in DH5α cells (Right). BBa_J04450 mRFP contains promoter LacI R0010, RBS B0034, mRFP1 E1010, stop B0015. DH5α Competent Cells are a versatile strain of chemically competent cells that can provide a transformation efficiency of > 1 x 10^6 cfu/μg plasmid DNA. 50 μL of competent cells transformed with 1 μL plasmid produced 22000 colonies (right).

protein is crucial, which in our case is the reporter protein. In comparison to intact cells, CFE being an open system has no regulation or control over the reaction (eg- transcription, translation, proteolysis, etc). Hence we began with transforming the DH5α cells with pSB1C3-mRFP plasmid containing chloramphenicol backbone. One colony from the transformed cells (Fig 21) were then

inoculated and incubated at 37°C with LB supplement. The cells at their saturation phase (overnight culture) were further induced with 0.1 M IPTG for six hours. The cells were then harvested and pelted using centrifugation at 5000g, 4°C for ten minutes followed by discarding the supernatant. We then resuspended the pellet in S30 wash (tris, Mg-glutamate, k-glutamate, DTT) buffer by gentle vortexing followed by centrifugation at 5000g, 4°C for ten minutes. This washing step was repeated thrice with the final centrifugation at 7000g. The pellet then was divided into two fractions, one was resuspended in S30 wash buffer and the other in PBS. As we wanted to monitor the stability of mRFP in extract, therefore we used mRFP expressing cells suspended in PBS for comparison. We then extracted the fraction suspended in the wash buffer by sonication at 50% amplitude, 10 s ON/OFF cycle for one minute. The extract then was centrifuged at 12000g, 4°C for ten minutes to pellet down the cell membrane. The extracted supernatant and intact cells were then loaded in triplicates on the 96-well plate along with a wash buffer and PBS. Fluorescence measurement was recorded at ten minutes interval and plotted (Fig. 22). The experimental result suggests that mRFP is more stable in intact cells than in extract. Interestingly we see in case of extract the fluorescence intensity is drastically increasing as compared to intact cells. As mentioned earlier that extract being an open system devoid of compartments and cellular regulation, as a result molecules can be fluid through the mixture. Due to the enhanced fluidity the molecular interaction (kinetics) is faster hence faster protein production and degradation rate.

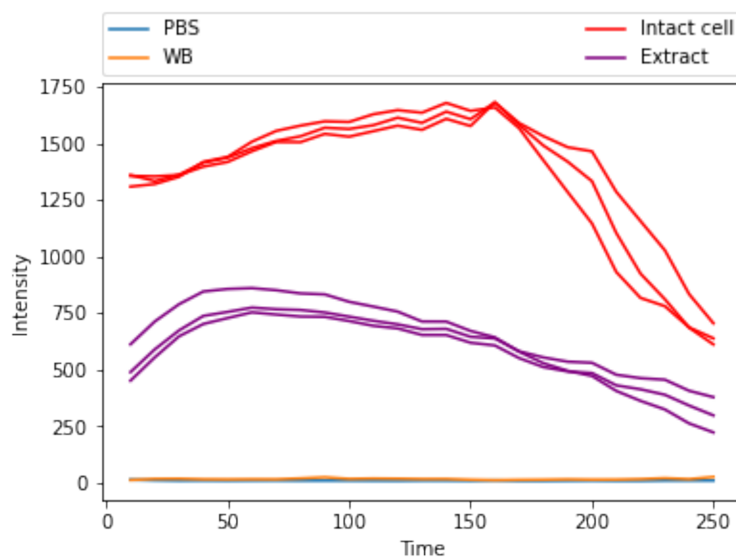

Figure 22: Schematic represents stability of mRFP in intact cells vs extract.
The graph shows fluorescence intensity (y-axis) of pSB1C3-mRFP with time in minutes (x-axis) of intact cells (red) and controls (purple). Multiple curves of the same colour indicate technical replicates (n=3). The measurements were made in a fluorescence plate reader using black plates without shaking.

Conversely in an intact cell the movement is restricted due to the membrane and regulated in order to maintain the cellular processes and homeostasis hence we observe slow and gradual production of

mRFP and increased stability. Another interesting observation is that the overall fluorescence intensity of intact cells is two fold higher than that of extract. As we started from the same culture keeping similar concentration we expect to see similar mRFP production in both intact cells and extract.

6.2 Testing the role of the membrane fraction

The only difference between the extract and intact cell was the membrane which we were getting rid of by pelleting down in the case of extract. Upon careful observation we found a small fraction of the pellet to be red after lysis and subsequent centrifugation. The probable cause could be improper lysis or excessive centrifugation, however, the curiosity persisted and hypothesis emerged. Cell membrane, mostly the inner part, is densely surrounded by cytoskeletal mesh therefore ribosomes and other cellular proteins may get stuck to the membrane. If that is true then in addition the membrane bound mRFP to the extract must show similar fluorescence intensity for extract compared to intact cells.

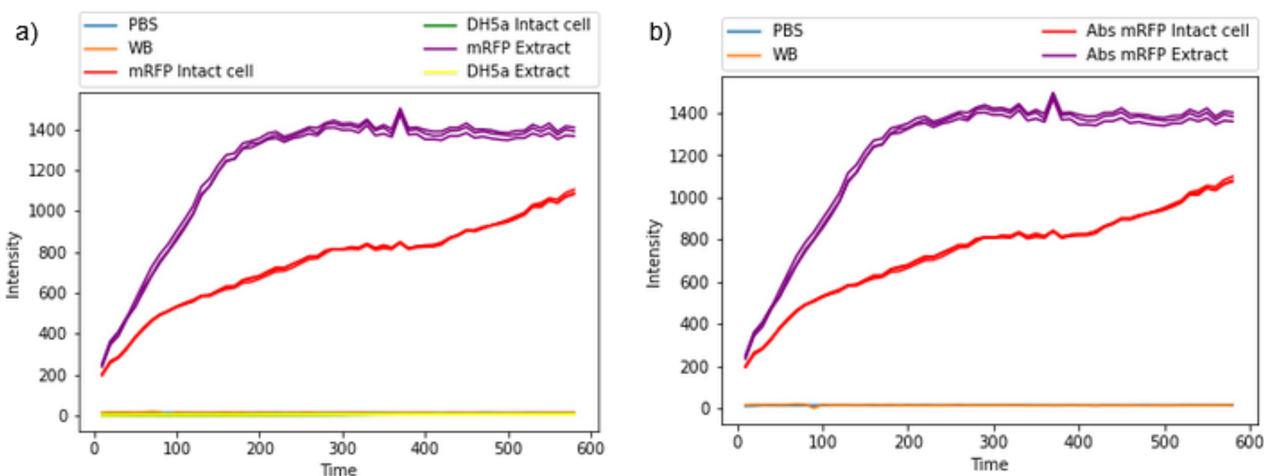

Figure 23: The Schematic represents Fluorescence intensity measurement of pSB1C3-mRFP and control (DH5α).
a) The plot shows mRFP intact cells (red), extract (purple) and DH5α intact cells (green), extract (yellow). The measurements were made in a fluorescence plate reader using black plates without shaking. b) The plot shows mRFP intact cell (red) normalised with DH5α intact cells, mRFP extract (purple) normalised with DH5α crude extract.

Now to test that we made a change to the extract preparation procedure. This time to keep the membrane in the extract we got rid of the centrifugation at 12000g step following lysis. We termed it crude extract as it contains membrane. We again performed a similar experiment as the last one but this time with the crude lysate. Similar to previous experiment the crude extract and intact cells of both DH5α containing pSB1C3-mRFP and DH5α lacking pSB1C3-mRFP were then loaded in triplicates on the 96-well plate along with wash buffer and PBS. Fluorescence measurement was recorded at ten minutes interval and plotted (Fig. 23). Fascinatingly after including the membrane

in the extract we observed that not only the fluorescence intensity of the extract was comparable but surprisingly higher than intact cells.

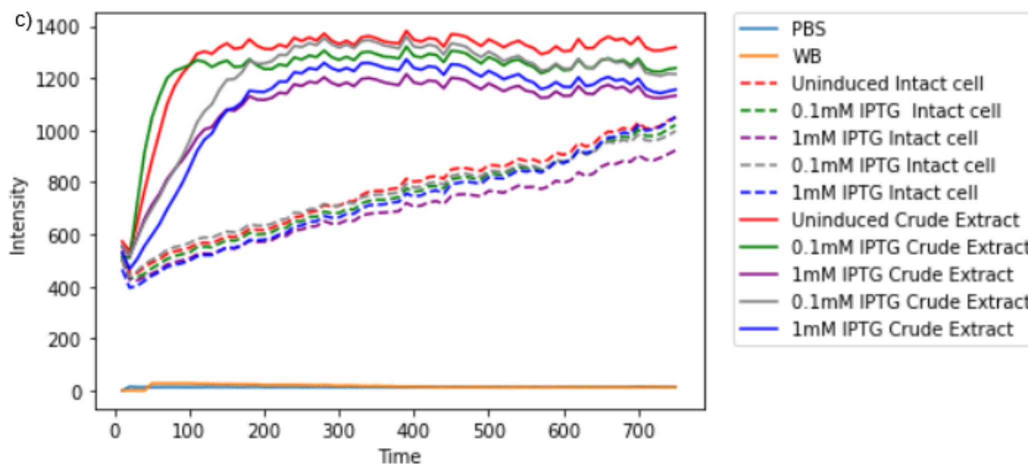

Figure 24: The Schematic represents Fluorescence intensity measurement of pSB1C3-mRFP induced with different concentrations at different time point.
Fluorescence intensity (y-axis) with time in seconds (x-axis) of mRFP intact cells (dashed line), extract (normal line). Fluorescence intensity of uninduced and induced with 0.1 mM, 1 mM IPTG during culture and induced with 0.1 mM, 1 mM IPTG before plating. The triplicates were averaged and plotted.

6.3 Quantification of mRFP production

To understand why there is a higher amount of mRFP production in extract when we added membrane to it we came up with two hypotheses. One is that the extract being non compartmentalised and free from cellular regulations increase kinetics and non targeted TX-TL, altogether just not enhances fluidity (faster protein production) but as well the capability of protein production. The other one is that upon the integration of membrane the overall mRFP count increases which makes it comparable to intact cells and on top as extract is an open system the mRFPs gets diluted thought the mixture in comparison the mRFPs in intact cell are clustered in the cell hence cannot dilute to show crowding effete. To examine which one is the case here we harvested three uninduced cultures and two induced (one with 0.1 mM and other with 1mM IPTG). Following the washing steps and from each culture we prepared one extract and one intact cell fractions. From the three uninduced cultures we induced two of them (one with 0.1 mM and other with 1mM IPTG) just before plating. Similarly the crude extract and intact cells of all five cultures were then loaded in triplicates on the 96-well plate along with a wash buffer and PBS. Fluorescence measurement was recorded at ten minutes interval and plotted (Fig. 24). We observed no difference, all the cultures showed similar results consistent
with the previous experiment. Which indicates there is no effect of induction, after thorough investigation it was found that the plasmid does not contain an inducible promoter (the vector mat

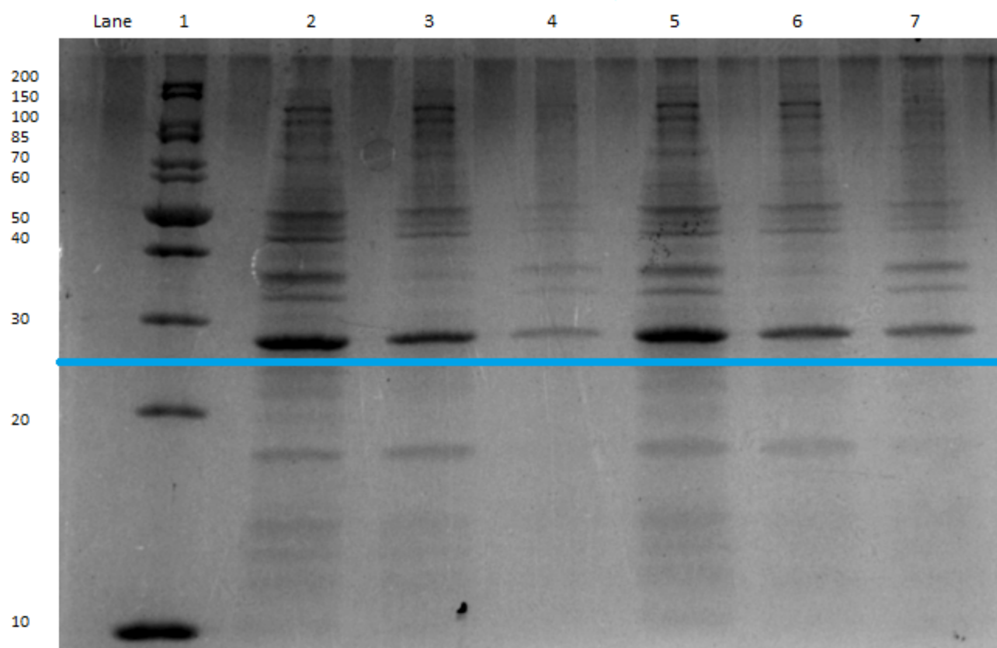

Figure 25: Quantification of mRFP expression in intact cells vs extract.
Unstained protein ladder (1), crude extract at t0 (2), supernatant of crude extract at t0 (3), pellet of crude extract at t0 (4), crude extract at t120 (5), supernatant of crude extract at t120 (6), pellet of crude extract at t120 (7).

was incorrectly uploaded). Hence the experiment was inconclusive, therefore we thought to quantify the amount of mRFP in both extract and intact cells using SDS-PAGE and compare. We made a culture of DH5α containing pSB1C3-mRFP, subsequently washed and lysed following the mentioned protocol. We observed in fluorescence measurement that extract typically reaches peak in two hours (120 min). Therefore we collected one fraction (t0) of the extract and divided it into two, one kept as crude extract and the other was centrifuged at 12000g to collect the supernatant and pellet separately. We then treated all three of them with an SDS buffer and stored at room temperature for landing. The other fraction (t120) was incubated for two hours at 37°C. That too was divided into two, one kept as crude extract and the other was centrifuged at 12000g to collect the supernatant and pellet separately. Similarly we treated all three of them with an SDS buffer and loaded all six samples on SDS gel (Fig. 25). We observed darker bands for all three t120 samples compared to t0 samples. Which confirms production of mRFP in extract, most interestingly we saw a thicker band for pellet t120 compared to pellet t0. Which concretize our hypothesis that indeed ribosomes are embedded into the membrane which irrespectively produce mRFP. This wasn't the real comparison we aimed for as we wanted to compare the protein production between intact cells and extract. Thus we sonicated the intact cells that we had from the same culture at t0 and post incubated t120 and treated them similarly with SDS buffer. We also had a DH5α culture as control that we loaded alone with intact

and crude extract this time. We observed similar bands for both intact cells and extract. Suggesting that both intact cells and extract produce similar amounts of mRFP post incubation. Therefore the difference in intensity we observe in fluorescence experiments might be because of settling down of cells and cellular components over time or could be due to the short exposure time.

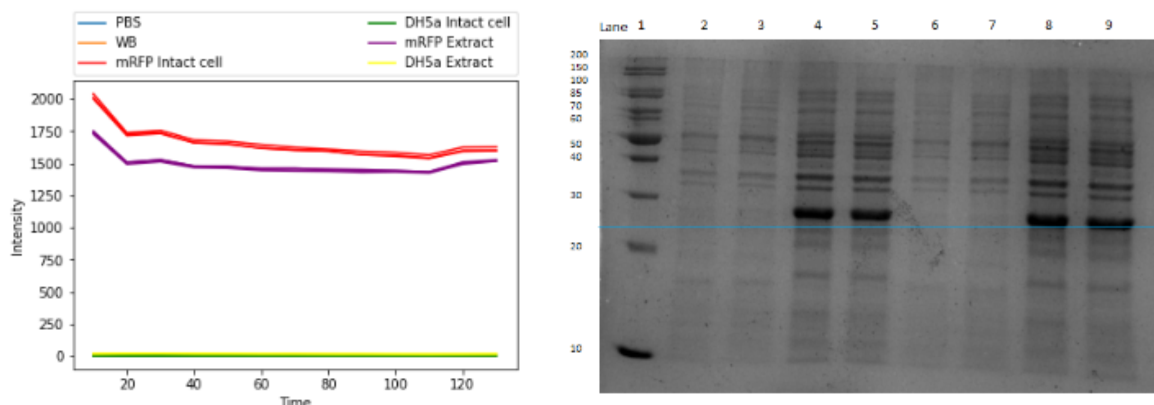

Figure 26: The Schematic represents Fluorescence intensity measurement of pSB1C3-mRFP induced with different concentrations at different time point.
The left plot: The measurements were made in a fluorescence plate reader using black plates with shaking. SDS-PAGE (right), molecular ladder (1), crude extract (control) at t0 (2), intact cells control at t0 (3), crude extract mRFP at t0 (4), intact cells crude extract mRFP at t0 (5), crude extract control at t120 (6), is intact cells control at t120 (7), crude extract mRFP at t120 (8), intact cells mRFP at t120 (9).

We then tested the aforementioned hypothesis and included the shaking step in fluorescence measurement assay. We also increased the exposure time window for the same assay (from 100 to 400 milliseconds). We harvested uninduced saturated mRFP and controlled culture for this experiment. Following the same protocol washed the cells and one fraction from both mRFP and control was finally resuspended in the wash buffer and sonicated to obtain crude extract and the other were resuspended in PBS to keep the cells intact. Then 150 μL of mRFP intact cells, crude extract and DH5α (control) intact cells, crude extract were loaded on plate for fluorescence analysis (Fig. 26), as well as simultaneously 10 microliters of mRFP intact cells, crude extract and DH5α (control) intact cells, crude extract were treated with SDS buffer to load on gel (t0). Then the rest of the crude extract and intact cells were then incubated at 37 C for next two hours while the fluorescence assay was ongoing at 37 C with shaking. After 2 hours (t120), similarly 10 μL of mRFP intact cells, crude extract and DH5α (control) intact cells, crude extract were treated with SDS buffer to load on gel (Fig-6.6). We now with certainty and proof can say that indeed there was no overproduction of mRFP in crude extract and the expression level is the same for extract and intact cells as they follow similar trends and lie in the same order of magnitude in fluorescence measurement and as well see similar bands in SDS gel. Hence we can conclude that the reporter is equally stable in both intact cells and the extracted system.