MASTER RESEARCH PROJECT 2: REPORT

Investigation of Protein Dynamics Using Genetically Encoded Unnatural Amino Acids

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Abbreviations

µg	microgram
µL	microliter
µM	micromolar
aaRS/RS	aminoacyl –tRNA-synthetase
Amp	ampicillin
APS	ammonium persulfate
Ara	arabinose
AzF	4-azido-L-phenylalanine
BCNK	bicyclo[6.1.0]non-4-yn-9-ylmethanol-L-lysine
BocK	N(ε)-tert.-butyl-oxycarbonyl-L-lysine
bp	base pair(s)
BPA	p-benzoyl-L-phenylalanine
Cm	choramphenicol
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
e.g.	*Exempli. Gratia*
et.al.	*et alii/alia/aliae*
EtOH	ethanol
FRET	Förster/fluorescence resonance energy transfer
g	gram
g	gravitational acceleration
His	histidine
HRP	horseradish peroxidase
i.e.	*Id est*
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kan	kanamycin

L	liter
LB	lysogeny broth
Lib.	library
M	molar
M.bakeri (Mb)	*Methanosarcina barkeri*
M.jannaschii(Mj)	*Methanocaldoccous jannaschii*
MCS	multiple cloning site
MeOH	methanol
mg	milligram
min	minute
MbPylT	*Methanosarcina barkeri* pyrrolysine tRNA
MjYRS	*Methanocaldoccous jannaschii* tyrosine aminoacyl-tRNA synthetase
mL	milliliter
mM	mill molar
nt	nucleotides
OD_{600}	optical density at 600 nm wavelength
O-mRNA	orthogonal messenger RNA
ORBS	orthogonal ribosome binding site
O-Ribo-Q	evoloved orthogonal ribosome
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
Pfu	*Pyroccocus furiosus*
PVDF	polyvinylidene difluoride
PylT	pyrrolysine tRNA
QC	QuickChange
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
sfGFP	super folded green fluorescence protein
sm	single-molecule
Spec	spectinomycin
TBE	TRIS-Borate-EDTA-buffer
TEMED	N,N,N’,N’-tetramethylethylenediamine
Tet	tetracycline
TRIS	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
uAA	unnatural amino acid
UV	ultraviolet
v	volume
w	weight
WB	western Blot
WT	wild type

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Abstract

Protein engineering has become an extensively used tool in many fields which allow us to study protein functions and characterize proteins using range of available biophysical methods. With the genetic code expansion, it is now possible to incorporate functional groups and other properties in the proteins which are naturally not present. Site specific incorporation of unnatural amino acid (uAA) is widely used in protein engineering. However, incorporation of multiple uAAs remains a significant challenge. Tunability of protein expression for multiple uAAs incorporation using orthogonal ribosome remains another important challenge. In this study we try to investigate the role of inducible promoter in tunability of protein expression using orthogonal ribosome and orthogonal ribosome binding sites along with tRNA/amino acyl RNA synthetase pair. Six different o-RBS were designed based on the work of Rackham and Chin [26] during the study on an inducible promoter. 4 best fit o-RBS were selected after screening experiment. Expression of protein incorporating uAA was done with orthogonal ribosome. Unfortunately, the experiment failed. Further analysis on failure concluded that the functionality of orthogonal ribosome was lost due to some unexplained reason.

Keywords: Orthogonal ribosome, o-RBS, tRNA/aaRS, uAA and orthogonal translation machinery.

1. Introduction

All living organisms from prokaryotes to eukaryotes share many similarities at molecular level. They all are built with simple organic macromolecules like nucleotides and proteins. Proteins are involved in range of activities like cellular growth to cellular metabolism to signal transduction and play an essential role in the survival of living organisms, making them one of the most studied subjects in life science [1]. They are made up from 20 different canonical amino acids. Unique arrangements of these natural amino acids determine the structure and function of the proteins. The individual amino acid arrangement is governed by the nucleotide sequence of a protein's gene, encoded by non-coinciding triplet codons, made up of a combination of the four nucleotide bases adenine (A), cytosine (C), guanine (G) and thymine (T). Triplet codon system (4^3) facilitate 64 possible codons, out of which 61 are assigned for decoding the 20 different amino acids and the remaining three for the termination of protein synthesis. [2]

1.1 Genetic Code Expansion

Genetic code is conserved through all kingdoms of life from archea to mamalia. In 1976, it was discovered that nonstandard amino acid selenocysteine (Sec) is directly encoded in *Clostridium* leading to the speculation of genetic code expansion [3]. It was in 1986, when two independent research groups proved that Sec is incorporated into the selenoproteins directly in response to in-frame opal stop codon (UGA) [4, 5]. This was the first event of genetic code expansion present in both prokaryotes and eukaryotes. Sec was given status of 21st amino acid [6]. In 2002, pyrrolysine (Pyl) was found to be the 22nd genetically encoded amino acid, this time in response to the amber stop codon (UAG). The allocation of Pyl appears restricted to the *Methanosarcinacea* (Methanogenic archea) and Gram-positive *Desulfitobacterium hafniense* [7, 8, 9]. The aminoacylation mechanism for both amino acids was different; incorporation of Sec in selenoproteins is done via an enzymatically modified serine that was charged to a special selenocysteinyl-tRNA. On the other hand, Pyl is directly paired to pyrrolysyl-tRNA (PylT) by the cognate aminoacyl-tRNA synthetase PylS [10].

Synthetic biologist started looking to take advantage of the degeneracy of genetic code, for the purpose of genetically encoded incorporation of amino acids with new functionalities in proteins by artificial means. There are around 70 different unnatural amino acids (uAAs) now available today [1]. Each having distinct functional groups, which can be used as, UV-inducible photo crosslinkers, post translational modification, spectroscopic and NMR probes, chemical handles that can be modified even in the living cells [11]. In order to achieve the artificial genetic code expansion, it is required to introduce an exogenous tRNAs and their cognate amino acyl RNA Synthetase (aaRS) into the host cell; which have to function absolutely orthogonal to the endogenous components [1, 11, 12]. To put it briefly, the endogenous aaRS should not aminoacylate the external tRNAs with any canonical amino acid present in the host cell and, in turn, the internal tRNAs should not be charged with uAAs by the orthogonal aaRS [12]. Therefore, blank (nonsense, frameshift, or otherwise unused) codons are used for complementing the anticodon of the orthogonal tRNA notably the seldom used amber stop codon (TAG), acknowledging the movement of the applicable codon to the amino acid used as a substrate by the orthogonal aaRS [13] (Figure 1). Using this principle, gentic code expansion was performed in *E.coli* by using yeast PheRS/$tRNA^{Phe}_{CUA}$ pair in 1998 by Furter [14]. The ability to evolve aaRS specificities towards new uAAs played a critical role in success of this approach. This was first accomplished in 2001 by the lab of Peter Schultz using TyrRS/$tRNA^{Tyr}_{CUA}$ pair from *M. jannaschii* [15]. Their approach can be divided into two main steps, firstly, site directed mutagenesis was performed on a set of active sites residues of the aaRS, which generated a large library of variants (usually $>10^9$). In second step after multiple rounds of positive and negative selection specific aaRSs was isolated for the amino acid. To achieve this *E. coli* cells are transformed together with the library plasmids and a reporter plasmid encoding an antibiotic resistance gene disrupted by an amber codon. In the presence of the uAA cells accommodating an active synthetase (which can identify the uAA or a natural amino acid) will suppress the amber codon, eventually becoming resistant to the antibiotic. Cells having aaRSs that recognize natural amino acids are eliminated in a following round of negative selection in the absence of the uAA. In repetitive rounds of positive and negative selection the aaRSs of interest is finally isolated from the library [12, 15 - 17].

An expanded genetic code for incorporation of 5 distinct uAA was shown for eukaryotic system using the similar approach (Figure 1. 1) of positive and negative selection with reporter gene in *Saccharomyces cervisae* by the lab of Peter Schultz [18]. Thus, establishing a new field of protein

biochemistry, where proteins can be labeled with desired functionalities using amber stop codon, which opened new possibilities. This new technique is known as the amber suppression technology [19].

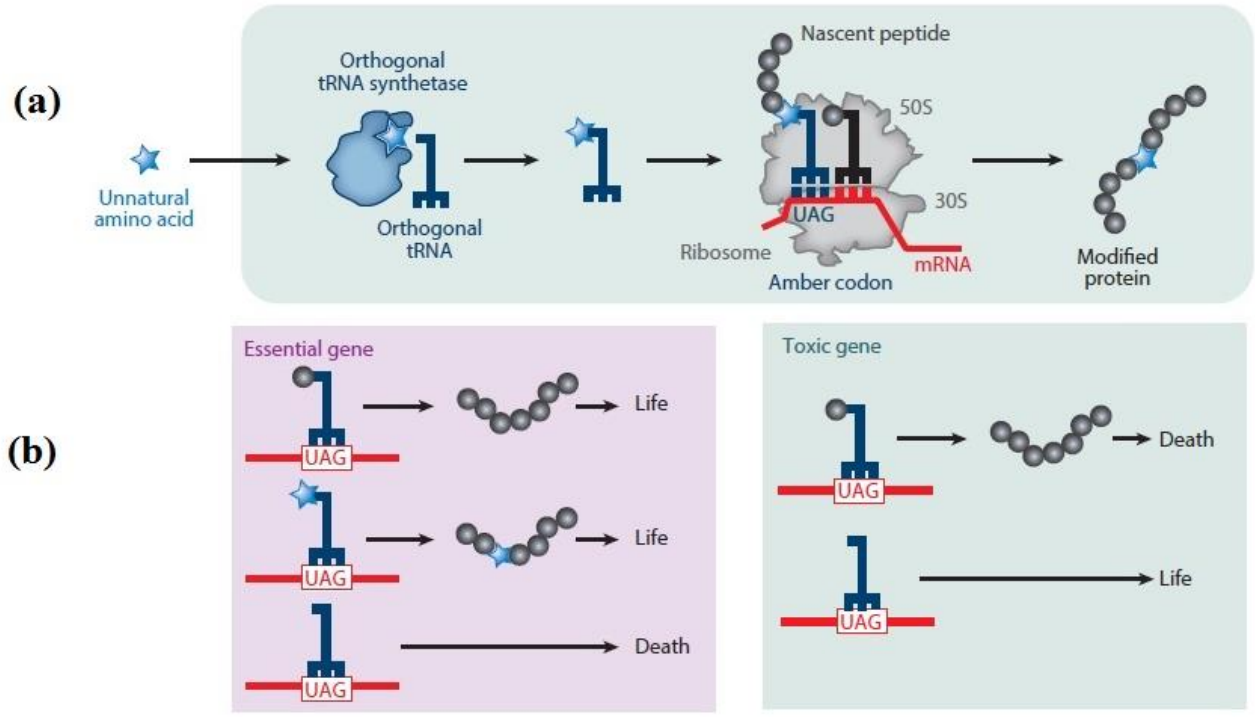

Figure 1. 1: Schematic representation of Genetic Code Expansion using amber codon: (a) Genetic code expansion enables the site-specific incorporation of an unnatural amino acid into a protein via cellular translation. (b) Sequential positive and negative selections enable the discovery of synthetase/tRNA pairs that direct the incorporation of unnatural amino acids (Adapted from Chin 2014) [11]

In protein synthesis, when an in-frame amber stop codon (UAG) at the aminoacyl-site (A-site) of the ribosome is encountered, release factor (release factor 1 (RF1) in prokaryotes and eukaryotic release factor (eRF) in eukaryotes) binds at the A-site of ribosome and leads to the termination of protein synthesis and releasing the nascent polypeptide chain [20]. Genetic code expansion exploits amber codon suppression by orthogonal $tRNA_{CUA}$ binding to an amber codon at the A-site of ribosome and incorporate uAA into the growing polypeptide chain [21]. In bacteria, one of the major bottlenecks of this approach is the competition between RF1 and orthogonal $tRNA_{CUA}$ for binding to an amber codon at the A-site of ribosome. This might results in premature truncation of peptide chain, affecting the yield of full length protein [22]. To improve the incorporation efficiency removal of RF1 was done which found to be lethal to the bacteria [23].

Further works to improve the efficiency of incorporation of uAA by experimenting with RF1 lead to the construction of strains containing temperature sensitive allele of RF1, which can be grown at the restrictive temperature upon induction of protein expression. This alternative way, but, is of limited use since many recombinant proteins would not tolerate the elevated temperature and extended induction times would result into cell death [24]. A new approach to tackle this problem is engineering an orthogonal ribosome for more efficient decoding of amber suppression codon [22, 24]

1.2 Evolution of Orthogonal Ribosome

Designing of orthogonal ribosome was first accomplished by the lab group of Jason Chin in 2005 [25, 26]. They started with duplicating the ribosome**.**mRNA pair and checking its evolutionary fate doing random mutations, and finding an orthogonal ribosome.orthogonal ribosome binding site on mRNA (o-Ribo.o-RBS). For search or o-Ribo-o-RBS, the Shine Dalgarno (SD) sequence (-7 to -13 from AUG initiation codons) on mRNA was removed and mRNA library was created containing all possible SD sequences *i.e* theoretically 4^7 = 16,384. Library was selected with antibiotics; cells which grew on the resistance were discarded, as they are the substrate for endogenous ribosome and those who didn't survive were selected for screening with o-Ribo. Ribsome library was created by doing eight mutations in 16S rRNA sequence. Five of them are responsible for binding the SD sequence; one is responsible for allowing additional flexibility in spacing between SD and A-site of ribosome, and concluding two forms a bulge proximal minor groove of the SD helix built between ribosome and mRNA. A new functional 16S-rRNA/mRNA pair was found that would no longer interact with the natural counterpart. This was done with reiterative rounds of positive and negative selection (Figure 1. 2) [24-27].

Orthogonal ribosome unlike the parental ribosome is not responsible for coding the whole proteome in natural cell, thus it is possible to encode the protein of interest without affecting the natural translation system. In order to make orthogonal translational machinery more specific and unique, in 2010 the workgroup of Jason Chin came up with quadruplet (frameshift) codon, which can be read only by the orthogonal ribosome and not by the natural ribosome [28]. Thus, the main components of orthogonal translation machinery are evolved orthogonal ribosome,

orthogonal ribosome binding site and evolved orthogonal tRNA/amino acyl tRNA synthetase pairs for incorporating uAAs for TAG/AGGA codon [29].

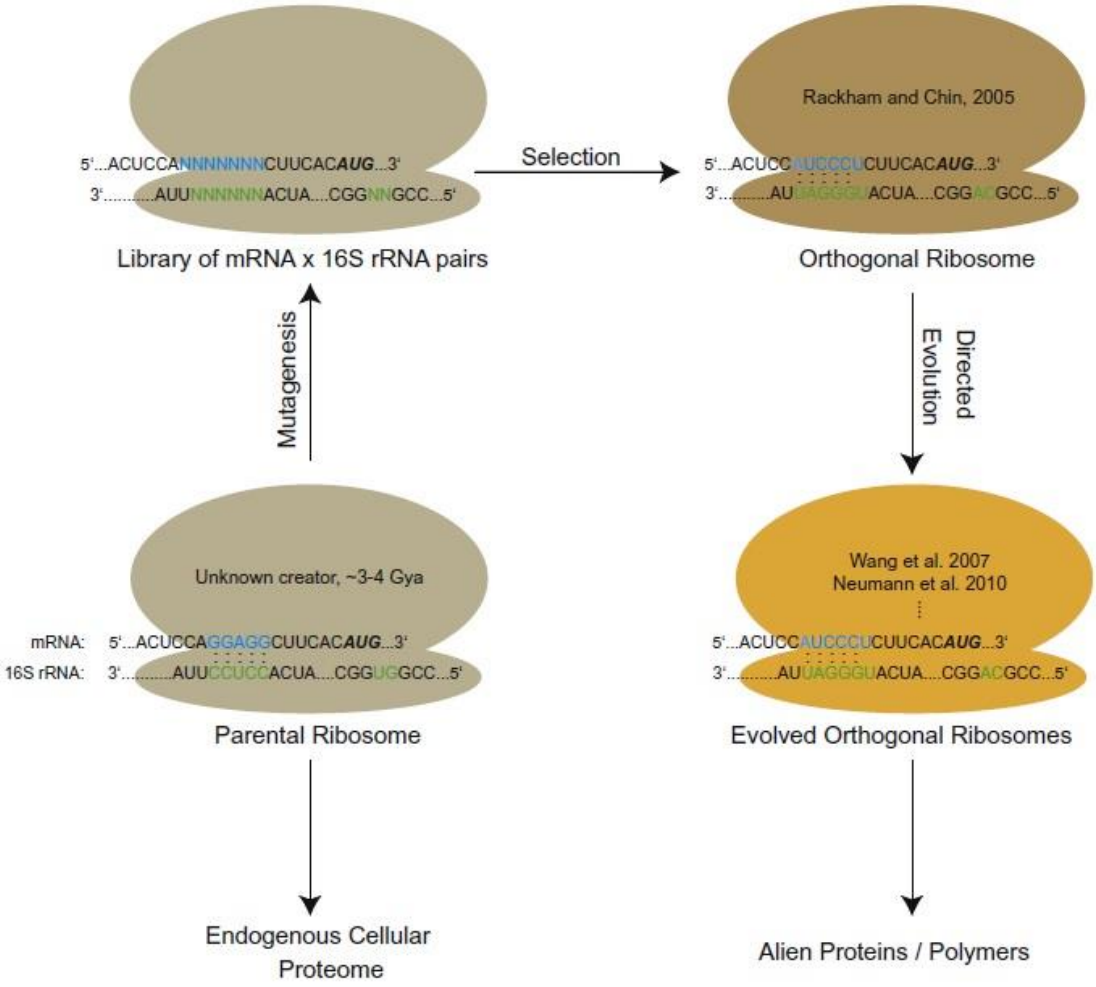

Figure 1. 2 Engineered Orthogonal Ribosome. Orthogonal ribosomes form a parallel translational apparatus that reads only one specific mRNA. This liberates them from evolutionary constraints allowing their evolution towards new function, such as the enhanced suppression of stop and quadruplet codon (Adapted from Neumann 2012) [24]

1.3 Labeling of uAA for FRET studies

Theoretically genetically encoded fluorescent uAAs would cause minimal structural perturbation and are improbable to damage a protein's function and localization [30]. Not many fluorophores are cell-permeable or are simply too large to be a substrate for the aaRSs. Consequently, only a small number of fluorescent uAAs have been directly incorporated into proteins [31].

In order to expedite the labeling of proteins with an extensive range of fluorophores, as hundreds of small organic dyes are commercially available [32], one had to find an alternative approach. The genetically encoded installation of a uAA containing a bioorthogonal reactive moiety provides one such avenue because they allow the subsequent site-specific modification of a protein with almost any probe by bioorthogonal "click chemistry" [33] and hence also any fluorophore that is compatible with the installed uAA.

Azide-alkyne cycloaddition "Click" reaction is used to label the uAA in protein [34]. Generally this reaction needs a Cu(I) as catalyst, which is toxic for most of the cells [35]. Staudinger ligation is a Cu(I) free mechanism that has been used to do labeling with probes *in-vivo* [36] In this work, p-Azido-L-phenylalanine (AzF), an azide and bicyclonon-4-yn-9-ylmethanol-L-lysine (BCNK), a terminal alkyne (Figure 1. 3) was used. In case of AzF, the probe has to be on the terminal alkyne and for BCNK; the probe should be on the azide during the Staudinger ligation [34]

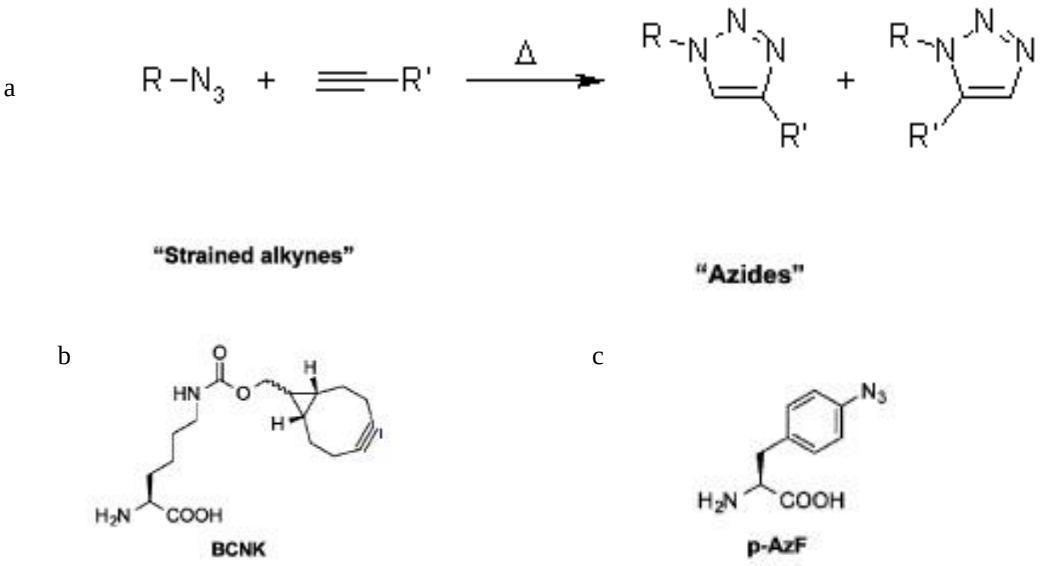

Figure 1. 3: Click chemistry and structure of uAA. (a) Cycloaddition reaction of azide and alkyne. (b) Chemical structure of BCNK (c) Chemical structure of p-AzF (Figure adapted from Lang & Chin 2014) [34]

The concurrent installation of two or more fluorophores in the protein allows the investigation of conformational changes, even on a single-molecule (sm) level, using the powerful technique

Förster/Fluorescence resonance energy transfer (FRET). FRET relies on the energy transfer from a donor to an acceptor fluorophore in a distance-dependent manner and is capable of detecting distances and their changes in 2-10 nanometer scale. Although, the accurate and site-specific labeling of the proteins with apt fluorophores is essential for FRET experiments still a challenging task and is usually the limiting factor [37].

1.4. Challenges faced with Orthogonal Translation

There are two technical obstacles that currently undermine the addition of uAAs in orthogonal translation experiments. Firstly, large-scale experiments can become expensive, as the uAAs traditionally synthesized manually and hence become a costly affair. This problem can be solved if cells are metabolically engineered to produce uAAs [22]. Secondly, incorporation of multiple uAAs in the proteins remains a big challenge. Progress have been made in this field by the lab group of Jason Chin and Heinz Neumann by using orthogonal ribosome and quadruplet codon system, but the results are mostly limited to the test proteins like sfGFP, maltose binding protein (MBP), and glutathione S- transferase (GST) [28, 29]. Multiple uAAs incorporation in substantial protein is still challenging.

Additional challenge faced during the usage of orthogonal ribosome in translation machinery is the use of multiple plasmid system [28], which means that the host cell has to face enormous amount of stress due to presence of multiple antibiotics. Earlier four plasmid system [28] was used to incorporate multiple uAAs in cell, recently it has been modified to a three plasmid system [29], thus reducing the level of stress caused due to different antibiotics. Even more, most of the components of this machinery (orthogonal ribosome, orthogonal ribosome binding site and tRNA/aaRS pair) are present on the plasmids having constitutive promoter. In view of the fact stated above, it is quite difficult to tune the expression level of the protein.

Keeping the abovementioned difficulties in mind this project was designed to make protein expression tunable by using inducible promoters and constructing new orthogonal ribosme binding site based on the work of Rackham and Chin [25, 26].

2. Objectives

- Confirming the functionality of orthogonal ribosome
- Designing of orthogonal ribosome binding site (o-RBS)
- Screening of the best fit o-RBS
- Site directed mutagenesis of amber and quadruplet codon in desired protein having best fit o-RBS
- Incorporation of multiple uAAs in desired protein

3. Materials And Methods

3.1 Materials

3.1.1 Devices And Instruments

Agarose Gel Electrophoresis Chamber	GP-Kuststofftechnik, Kassel
Accu-jet® pro	Brand, Germany
Autoclave HST 4-5-8	Zirbus, Bad Grund
BioPhotometer	Eppendorf, Hamburg
Bunsen Burner Fuego Basis	WLD-Tec, Göttingen
Chemiluminesence Imaging	biostep® GmbH, Jahnsdorf
Centrifuge 5415R.	Eppendorf, Hamburg
Centrifuge Allegra 2IR	Beckman Coulter, Krefeld
Ergonomic High Performance Pipette	VWR International, Darmstadt
FLUOstar Omega	BMG Labtech, Ortenberg
Hoefer miniVE Vertical Electrophoresis System	HoeferInc, USA
Gel Doc 2000	BioRad, München
Gel Shaker Duomax 1030	Heidolph, Schwabch
Gel Shaker Rotamax 1030	Heidolph, Schwabch
Hamilton Syringe 50 µL	Hamilton, USA
Hypercassette 18 x 24 cm	GE Healthcare, München
Incubator Mytron WB 60 k	Mytron,Heiligenstadt
Magnetic Stirrer MR Hei-Standard	Heidolph, Schwabch
Magnetic Stirrer MR3000	Heidolph, Schwabch
Optimax X-Ray Film Processor	Protec, Oberstenfeld
pH Meter PT-15	Sartorius, Göttingen
Pipets Research Plus (10, 100, 1000 µL)	Eppendorf, Hamburg
Power Supply 300V	VWR International, Darmstadt
Power Supply MP-250V	Major Science, USA
Power Supply EV 231	Consort, Belgium
Scanner CanoScan 5600F	Canon Deutschland, Krefeld

Table Top Centrifuge	Eppendorf, Hamburg
Tankblotter Criterion (1.3 L)	BioRad, München
Tankblotter Mini Trans-Blot (0.45 L)	BioRad, München
Thermomixer comfort 1.5 mL	Eppendorf, Hamburg
Thermomixer comfort 2.0 mL	Eppendorf, Hamburg
Vortex Generator VV3	VWR International, Darmstadt
X-Ray Cassette 18 x 24	Rego X-Ray GmbH, Augsburg

3.1.2 Chemicals

All chemicals were bought from those companies recorded below, unless declared otherwise, and fulfilled the purity grade “pro analysis”.

AppliChem, Darmstadt
BioRad, München
Merck, Darmstadt
Roth, Karlsruhe
Sigma-Aldrich, Steinheim
VWR International, Darmstadt

3.1.3 Consumable And Other Materials

96-Well Black Microplates	VWR International, Darmstadt
Amersham ECL Plus WB Detection Reagent	GE Healthcare, München
Amersham ECL Prime WB Detection Reagent	GE Healthcare, München
Amersham ECL Select WB Detection Reagent	GE Healthcare, München
Amersham Hyperfilm ECL	GE Healthcare, München
Eppendorf Tubes (1.5 mL, 2.0 mL)	Eppendorf, Hamburg
Falcon Tubes (15 mL, 50 mL)	Sarstedt, Nümbrecht
Instant Blue	Biozol, Eching
PCR Soft Tubes (0.2 mL)	Biozym, Austria

peqGOLD Universal Agarose	Peqlab, Erlangen
Petri Dishes 92 × 16 mm	Sarstedt, Nümbrecht
Pipet Tips	Sarstedt, Nümbrecht
UV Cuvettes (UVette)	Eppendorf, Hamburg
Whatman filter paper	Whatman, Dassel

3.1.4 DNA, And Protein Size Standards

GeneRuler™ 1 kb DNA Ladder	Thermo Scientific, Schwerte
PageRuler Prestained Protein Ladder	Thermo Scientific, Schwerte
GeneRuler™ 100 bp DNA Ladder	Thermo Scientific, Schwerte

3.1.5. Antibiotics

Table 3. 1 Overview of antibiotics used in growth media/ agar plates

Antibiotic	**Stock Concentration [mg/mL]**	**Company**
Ampicillin (Amp)	100	AppliChem, Darmstadt
Chloramphenicol (Cm)	50	AppliChem, Darmstadt
Kanamycin (Kan)	50	AppliChem, Darmstadt
Spectinomycin (Spec)	50	Sigma-Aldrich, Steinheim
Tetracycline (Tet)	50	AppliChem, Darmstadt

3.1.6 Culture Media And Agar Plates

For agar plates the corresponding medium was supplemented with 1.5% (w/v) agar-agar. LB and 2YT medium were sterilized by autoclaving at 121 °C for 20 min. Antibiotics was added after cooling to at least 55 °C.

Table 3. 2 Growth media used for culturing of *E.Coli.*

LB Medium	**2YT Medium**
10 g tryptone	16 g tryptone
5 g yeast extract	10 g yeast extract
5 g NaCl	5 g NaCl
Adjust to 1 L ddH_2O	Adjust to 1 L ddH_2O

3.1.7 Unnatural Amino Acids

The unnatural amino acids (UAAs) were dissolved with NaOH in ddH_2O (see Table 3.3) just before the addition to the cell culture medium.

Table 3. 3:List of unnatural amino acids used for the genetic code expansion

Amino acid	**Stock Solution [M]**	**Work Conc. [mM]**	**Company**
4-Azido-L-phenylalanine (AzF)	0.5 (in 0.5 M NaOH)	1-5	ChemImpex, USA
Bicyclononynes-L-lysine (BCNK)	0.1 (in 0.2 M NaOH)	2	SynAffix, Nijmegen (NL)

3.1.8 Enzymes

Enzymes were used as recommended by the company's protocol. The following table gives an outline about the enzymes used in this study.

Table 3. 4Overview of the enzymes used

Enzymes	Company
T4 DNA Ligase	Thermo Scientific, Schwerte
Phusion High-Fidelity DNA Polymerase	Thermo Scientific, Schwerte
Restriction Enzymes: ***Apa*I, *BamHI*,*Bsp* T1 *Dpn*I, *Hind*III, *Nco*I *Nde*I, *Xba*I, *Xho*I**	Thermo Scientific, Schwerte

3.1.9 Bacterial Strain

Table 3. 5 Overview of the bacterial strains used

Bacterial strains	Genotype	Company
***E. coli* DH10B**	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15Δ*lac*X74 *rec*A1 *end*A1 *ara*D139Δ (*ara,leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G	Invitrogen, Darmstadt
***E. coli* BL21 (DE3)**	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	NEB, Frankfurt

3.1.10. Buffers And Solutions

All buffers were prepared with ddH_2O

.

Table 3. 6 Overview of buffers and solutions used

Buffers/Solutions	Component
1× PBS	10 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM $KHPO_4$ pH 7.5
$CaCl_2$ Solution for competent cells	60 mM $CaCl_2$ 10 mM Pipes-KOH, pH 7.5 15% glycerol (v/v) Autoclave Store at 4 °C
TBE (1×)	89 mM TRIS base 89 mM boric acid 2 mM EDTA-Na_2
DNA Loading Buffer (1×)	3% glycerol (v/v) 1 mM TRIS base, pH 7.5 1 mM EDTA-Na_2 Bromphenol blue Xylene cyanol
SDS Running Buffer (1×)	25 mM TRIS base 192 mM glycine 0.1% SDS (w/v)
SDS Sample Buffer (1×)	2.5% glycerol (v/v) 12.5 mM TRIS-HCl, pH 6.8 25 mM DTT 0.5% SDS (w/v) 0.025% bromphenol blue (w/v)
WB Transfer Buffer (1×)	1× SDS running buffer 20% Methanol (v/v)
TE buffer (1×)	10 mM TRIS, pH 8.0 1 mM EDTA-Na_2

3.1.11. DNA Kit Systems

Following kits were used for the plasmid purification and gel extraction. Kits were used according to manufacturer's protocol.

peqGOLD Gel Extraction Kit	Peqlab, Erlangen
peqGOLD Plasmid Miniprep Kit	Peqlab, Erlangen

3.1.12. Antibodies

Table 3. 7 Overview of antibodies used for western blotting. His = histidine, HRP = horseradish peroxidase

	Antigen	Host	Conjugate	Diluents (w/v)	Company
Primary (1:5000)	His	Mouse	-	3% BSA-PBS	GE healthcare, München
Secondary (1:10,000)	Mouse	Goat	HRP	5% Milk-PBS	Sigma-Aldrich, Steinheim

3.1.13. Plasmids used in this study

Table 3. 8: List of plasmids used in this study

Plasmid	Description	Source/ Reference
pl048	Orthogonal ribosome (O_RiboQ) on pSC101 backbone with constitutive promoter (Kan resistance)	Neumann's lab plasmid library (Neumann *et.al* 2010)[28]
pl097	Wild type super-folded Green Fluorescent Protein (sfGFP) hexa histidine tag at C-terminus on pBAD backbone plasmid (Amp. resistance) i.e. pBAD_sfGFP_6xHis	Neumann's lab plasmid library
pl117	Orthogonal ribosomal binding site along with constructive promoter and sfGFP_6xHis on pTrc backbone plasmid (Amp resistance)	Neumann's lab plasmid library (Lammers *et.al.*, 2014) [29]
pl175	Aminoacyl tRNA sysnthetase (aaRS)/$tRNA_{CUA}$ pair for incorporating BCN and aaRS/$tRNA_{UCCU}$ pair for incorporation of AzF with constitutive promoter on pCDFDuet-1 backbone (Spec resistance)	Neumann's lab plasmid library (Lammers *et.al.*, 2014) [29]
pl178	Aminoacyl tRNA sysnthetase (aaRS)/$tRNA_{CUA}$ pair for	Neumann's lab plasmid

	incorporating BCN and aaRS/tRNA$_{UCCU}$ pair for incorporation of AzF with lac promoter on pCDFDuet-1 backbone (Spec resistance)	library (Lammers *et.al.*, 2014) [29]
pl237	Orthogonal ribosomal binding site, N-termini hexa histidine TEV cleavage site in H3 (histone 3 optimized for expression along with cysteine mutation) *i.e* pBAD_ORBS 4_6xHis_TEV_H3opt_Q76C (Amp resistance)	Neumann's lab plasmid library (Lammers *et.al.*, 2014) [29]
pl216	Orthogonal ribosome binding site (o-RBS1) in arabinose inducible plasmid system having sfGFP. i.e pBAD_oRBS1_sfGFP_6xHis	This work
pl217	pBAD_oRBS2_sfGFP_6xHis	This work
pl218	pBAD_oRBS3_sfGFP_6xHis	This work
pl219	pBAD_oRBS4_sfGFP_6xHis	This work
pl220	pBAD_oRBS5_sfGFP_6xHis	This work
pl221	pBAD_oRBS6_sfGFP_6xHis	This work
pl226	pBAD_oRBS1_sfGFP-D134AGGA_6xHis	This work
pl227	pBAD_oRBS1_sfGFP-N150TAG_6xHis	This work
pl228	pBAD_oRBS2_sfGFP-D134AGGA_6xHis	This work
pl229	pBAD_oRBS2_sfGFP-N150TAG_6xHis	This work
pl230	pBAD_oRBS3_sfGFP-D134AGGA_6xHis	This work
pl231	pBAD_oRBS3_sfGFP-N150TAG_6xHis	This work
pl232	pBAD_oRBS4_sfGFP-D134AGGA_6xHis	This work
pl233	pBAD_oRBS4_sfGFP-N150TAG_6xHis	This work
pl238	pBAD_oRBS4_6xHis_TEV_H3opt_Q76C_6AGGA	This work
pl239	pBAD_oRBS4_6xHis_TEV_H3opt_Q76C_6AGGA_K9TAG	This work
pl240	pBAD_oRBS4_6xHis_TEV_H3opt_Q76C_6AGGA_K14TAG	This work
pl241	pBAD_oRBS4_6xHis_TEV_H3opt_Q76C_6AGGA_K18TAG	This work
pl242	pBAD_oRBS4_6xHis_TEV_H3opt_Q76C_6AGGA_K23TAG	This work
pl243	pBAD_oRBS4_6xHis_TEV_H3opt_Q76C_6AGGA_K27TAG	This work

3.1.14. Primers used for o-RBS construction

Table 3. 9: Primers used for o-RBS construction

Primer Description	Sequence
pl097_oRBS_forward	cgcaaATGTCCCCTATACTAgttagcaaaggtgaagaactgtttacc
pl097_oRBS_rev._1	CATttgcggAGGGATGtgaaaattgtctcgagCGGGTATGGAGAAACAGTAGAGAG
pl097_oRBS_ rev._2	CATttgcggAGGGATCtgaaaattgtctcgagCGGGTATGGAGAAACAGTAGAGAG
pl097_oRBS_ rev._3	CATttgcggAGGGATTtgaaaattgtctcgagCGGGTATGGAGAAACAGTAGAGAG
pl097_oRBS_ rev._4	CATttgcggAGGGATAtgaaaattgtctcgagCGGGTATGGAGAAACAGTAGAGAG
pl097_oRBS_ rev._5	CATttgcggGAGGGATGtgaaaattgtctcgagCGGGTATGGAGAAACAGTAGAGAG
pl097_oRBS_ rev._6	CATttgcggAGGGATGtgaaaattgtctcgagCGGGTATGGAGAAACAGTAGAGAG

3.2. Methods

3.2.1 Microbiological Methods

3.2.1.1. Chemical Competent Cells

Chemical competent cells were prepared from overnight starting culture by inoculating the fresh 2YT media with 2% inoculums, in the desired volume needed. After incubation at 37˚C, cells were harvested at an OD_{600} of 0.5-0.6 by splitting them into 50 mL aliquots followed by centrifugation (4 °C, 10 min, 4,147 × *g*). All following steps were performed on ice. The supernatant was discarded and pellets were washed with 25 mL of ice cold $CaCl_2$ solution (Table 3. 6). The step of centrifugation and washing of pellet with ice cold $CaCl_2$ solution was repeated two more times. In the final step the pellet were re-suspended with 10 mL of $CaCl_2$ solution. The solutions were made into the 200 µL aliquots and snap frozen in the liquid nitrogen. The competent cells were stored at -80 °C.

3.2.1.2.Transformation of Chemical Competent Cells

Chemically competent *E.coli* (Bl21 (DE3)/DH10B) cells were transformed with plasmids using the heat shock transformation method. 200-500 ng of plasmids were mixed with 70-100 µL of chemical competent cells (Ch. 3.2.1.1) and incubated on ice for 10-20 minutes. After 90 seconds at 42 °C, recovery media 1 mL LB (Table 3. 2) was added and the cells were put back on ice for 15-30 minutes. For recovery, the cells were incubated at 37 °C for 45-60 minutes at 700 rpm. Transformants were either plated on the agar plates having suitable antibiotics (Table 3. 1) or used for the inoculation of an overnight culture.

The transformation was mostly suitable for single plasmid transformation. For multiple plasmid transformation, a chemically competent cell was prepared having one or two plasmid inside the cell.

3.2.2. Nucleic Acids Biochemical Methods

3.2.2.1.Preparation of Plasmid DNA

Plasmid DNA was isolated and purified with the help of kit systems (Ch.3.1.11) according to manufacturer's manual. In general, *E.coli (*DH10B*)* was transformed with desired plasmid (Ch.3.2.1.2) and plated on LB-agar plate having the selective antibiotics for overnight incubation. Single colony from the plate was picked and inoculated in 8 mL fresh LB media with proper antibiotics. The culture was incubated at 37°C, 220 rpm for 6-8 hours. Up to 4 mL of this culture were harvested by centrifugation (16,100 × *g*, RT, 5 min) and disrupted by alkaline lysis using the kit buffers. The purification of the DNA was performed over silica columns.

3.2.2.2.Restriction Enzyme Digestion

Restriction enzymes are endonucleases which work by recognizing a precise sequence of nucleotides, varying between four and eight base pairs in length, and frequently paliandromic, followed by producing double strand breaks in the DNA. Some of the restriction enzymes create the overhanging end (Sticky) and some creates ends without overhangs. Digestion of DNA was performed with these enzymes (Table 3. 4) following manufacturer's protocols. The majority of restriction enzymes were either used to clone specific plasmid DNA fragments into a vector backbone (preparative digest) or to perform test digest (Table 3. 10) of purified plasmid.DNA (Ch. 3.2.2.1) from cloning protocol.

Table 3. 10 Pipetting scheme for standard single and double digest

Test Digest		**Preparative Digest**	
1 µL	buffer, 10x	2 µL	buffer, 10x
4-6 µL	DNA	10-12 µL	DNA
3-5 µL	ddH_2O	5-7 µL	ddH_2O

0.25-0.5 µL	enzyme	1-1.5 µL	enzyme mix
2 h, 37 °C		2 h, 37 °C	

3.2.2.3.Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed to analyze size and abundance of DNA fragments from restriction digests (Ch.3.2.2.2) and PCRs (Ch.3.2.2.6,) as well as to purify DNA (Ch.3.2.2.4) as necessary.

0.8% of agarose was melted and completely dissolved in 0.5× TBE buffer in a microwave, casted into an electrophoresis chamber and supplemented with "gel red" in a concentration of 1:50,000 (v/v) after cooling to 50-60 °C

DNA samples were mixed with 10× DNA loading buffer (Table 3. 6) and loaded onto the gel together with a DNA ladder (Ch.3.1.4) to estimate the size of the DNA. Electrophoresis was performed at 100 V, 250-300 mA at RT with 0.5× TBE running buffer (Table 3. 6) until the bromphenol blue dye migrated the length of the gel (40- 60 minutes).

Separated DNA bands were visualized by UV light due to the intercalated "gel red" using a gel documentation machine or a UV table. For cloning purpose (Ch.3.2.2.4), wavelength of the UV light was set to 365 nm instead of 254 nm during visualization of DNA.

3.2.2.4.Extraction of DNA From Agarose Gels

DNA required for cloning products was preparatively digested (Ch. 3.2.2.2) separated via agarose gel electrophoresis (Ch. 3.2.2.3). Visualized on a UV table (365 nm), bands of correct size were sliced out of the gel with a scalpel. Afterwards, the DNA was extracted using a gel extraction kit (Ch. 3.1.11) according to manufacturer's manual. The purified DNA was eluted from the silica columns with max. 20 µL of elution buffer and stored at -20 °C or directly used for ligations (Ch. 3.2.2.5).

3.2.2.5.Ligation of Two Double-Stranded DNA Fragments

A double-stranded plasmid DNA fragment was typically digested with two different restriction enzymes (Ch. 3.2.2.2) and was ligated with another double-stranded DNA fragment, digested with the same enzymes, using the T4 DNA ligase. Therefore, a 3-5 fold molar excess of the smaller fragment, e.g., a gene for a protein, termed as the insert is mixed with the bigger fragment, commonly a vector backbone. For a negative control water is used in place of the insert DNA (Table 3. 11). The ligation was carried out for 2 h, at RT. The product of the ligation reaction (5-10 µL) was directly used for transformation (Ch. 3.2.1.2) and the favorable outcome of the ligation was confirmed by test digests (Ch. 3.2.2.2) and/or by the DNA sequencing (GATC biotech, Germany).

Table 3. 11 Pipetting scheme for a standard ligation reaction mix

	Volume (µL)
T4 Ligase buffer, 10x	1
Vector DNA (big fragment)	2
Insert DNA (small fragment)/H_2O	7
T4 Ligase	0.25

3.2.2.6.Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a simple still delicate enzymatic assay that enables exponential amplification of specific DNA fragment from a complex pool of DNA. PCR machine essentially a thermal cycler has a thermal block which raises and decreases the temperature of the block in distinct, precise and preprogrammed steps. In general, PCR consist of five different steps in which step two to four are repeated for 20-30 cycles.

1. **Initial denaturation**: The reaction first heated above the melting pointing of the two complementary DNA strands (94-96 °C). This step ensures that the template DNA is completely melted, a prerequisite for annealing with the primers (step three of PCR).
2. **Cyclic denaturation**: The goal of this step is similar to that of the first one, melting the DNA (template and newly synthesized strands) by raising the temperature to 94-96 °C to get single-stranded DNA but in cyclic manner.

3. **Cyclic annealing:** The temperature of the reaction is lowered to 60-70 °C, usually 5 °C lower than the melting temperature of the primers, single-stranded oligonucleotides that serve as a starting point for the polymerase. The low temperature allows the primers, which are complementary to the template DNA, flanking the sequence that should be amplified, to hybridize.
4. **Cyclic elongation:** In this step the polymerase synthesizes a new complementary DNA strand starting at a primer by adding dNTPs. Therefore, the temperature is changed to the appropriate optimum for the used polymerase (68-72 °C). The elongation time depends on the length of DNA to be amplified and on the speed of the particular polymerase.
5. **Final elongation:** All remaining single-stranded DNA fragments are fully extended at 72 °C.

The standard course for PCR is shown in Table 3. 12 and the composition for one PCR reaction mix is shown in Table 3. 13

Table 3. 12 Standard course of a PCR

Step	Temprature [°C]	Time	
Initial Denaturing	98	60 sec	
Denaturing	98	10 sec	30 cycles
Annealing	62	30 sec	
Extension	72	3 min	
Final Extension	72	10 min	

Table 3. 13 Pipetting Scheme for the composition of one PCR reaction mix

		Volume [µL]
5x	Pfu Buffer GC	5.00
2 mM	dNTPs	2.50

10 µM	Primer fw	2.50
10 µM	Primer rv	2.50
100 ng/µL	Template	2.00
2 U/ µL	Phusion Polymerase	0.5
	H_2O	11.00

Quick Change PCR (QC-PCR)

A Quick-Change (QC) is a site-directed mutagenesis (developed by Agilent) that allows to easily carry out vector modifications. Primers were designed so that forward and reverse primers both included the desired mutations in a complementary sequence of at least 25 bp. The 3'-ends of each primer were complementary to a minimum of 10-15 bp of the vector backbone to allow annealing to the correct positions. The composition for one QC-PCR reaction mix is shown in Table 3. 14.

Table 3. 14 Pipetting scheme for the composition of one QuikChange PCR reaction mix

		Volume [µL]
5x	Pfu Buffer HF	5.00
2 mM	dNTPs	2.50
10 µM	Primer forward	2.50
10 µM	Primer reverse	2.50
100 ng/µL	Template DNA	2.00
2 U/ µL	Phusion High-Fidelity Polymerase	0.5
	H_2O	11.00

For most of the quick change, a gradient of 8 °C was used for cyclic annealing process. Other stages of quick change remain same as standard course of PCR shown in Table 3. 12. The PCR product was subjected to DpnI digestion for 1-1.5 h. at 37 °C. DpnI, is able to recognize and cleave methylated and hemimethylated DNA. Therefore, it was used to remove parental template DNA from PCR reactions.

Before further usage PCR products were purified with gel extraction kit system (Ch. 3.1.11). The PCR product was used for transformation (Ch. 3.2.1.2) in *E.coli* (DH10B) cells.

3.2.2.7.Measuring Nucleic Acid Concentration

DNA concentrations of aqueous solutions were measured by absorption in UV curettes using a photometer. 5 µL of the nucleic acid containing solution was diluted in 45 µL water. Detection occurred at 260 nm (A_{260}) with pure water (50 µL) as a reference. The purity of the nucleic acid was also given by the photometer, calculated by the quotient of A_{260} to A_{280}.

3.2.3. Protein Biochemical Methods

3.2.3.1. Recombinant Protein Expression

Recombinant protein expressions were prepared from cells transformed with the appropriate plasmids in *E.coli* BL21 (DE3) cells (Table 3. 5).

2% inoculums of overnight precultures were used to inoculate main cultures (20 mL 2YT medium containing the required antibiotics, Table 3. 1). Cells were incubated at 37 °C, 200 rpm, to an OD_{600} of 0.5-0.7. Protein expression was induced by supplementing the media with either, 0.2% arabinose (w/v) or 1 mM IPTG (final concentrations). In some cases no addition of an inducer was necessary since the promoter was constitutively active. For the incorporation of uAAs into proteins of interest, the media was supplemented with the appropriate probe by first dissolving (Table 3. 3) and then added to the main culture. This was performed at inoculation when constitutive promoters were being used or in combination with the inducers for inducible promoters.

Cells were generally harvested at 3-4 h after induction by centrifugation and used for cell lysis. Pellets from whole cell lysate (up to 2 mL medium) were boiled in 1x SDS sample buffer (Table 3. 6) for 10 min at 95 °C. Samples were directly used for SDS-PAGE (Ch. 3.2.3.2.)

3.2.3.2 Discontinuous Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed to analyze the size and purity of proteins. The strong anionic detergent SDS causes the denaturation of proteins and confers a negative charge to them, simultaneously. The discontinuity between stacking and resolving gel relies on different pore sizes and pH values, as well. The pH gradient is responsible for the stacking of the proteins at the border to the resolving gel. Whereas the stacking gels always have the same concentration of acrylamide, those of the resolving gels were varied depending on the expected protein size. During this study 12.5% gel was used (Table 3. 15). Protein ladders (Ch. 3.1.4) helped to estimate the molecular weights of the separated proteins.

Electrophoresis was performed at 300 V, 40 mA for 60 to 80 min in 1x SDS running buffer until the bromophenol blue dye traveled the length of the gel.

After electrophoresis was performed, separated proteins were either visualized by Coomassie Brilliant Blue staining (with Instant Blue, according to manufacturer's manual) or transferred to PVDF, membrane by western blotting (Ch. 3.2.3.3).

Table 3. 15 Composition of polyacrylamide gels for SDS PAGE

Resolving Gel	**Stacking Gel**
12.5%	5%
12.5% Acrylamide	5% Acrylamide
375 mM Tris/Cl, pH 8.8	125 mM Tris/Cl, pH 6.8
0.1% SDS (w/v)	0.1% SDS (w/v)
0.1% APS (w/v)	0.05% APS (w/v)
0.04% TEMED (v/v)	0.1% TEMED (v/v)

3.2.3.3 Western Blot

Immunoblotting was performed to verify the expression of proteins by the direct transfer of proteins from SDS-PAGE (Ch. 3.2.3.2) onto membrane, followed by incubation with specific antibodies against the His_6-tag.

Wet blots were performed with PVDF membrane that was first activated with MeOH and then washed with water followed by soaking in 1× WB transfer buffer. The membrane, SDS gel and Whattman filter papers were assembled according to the instruction manual of the blotter. The transfer was applied at 70 V constant for 45-60 min, at 4 °C in the cold room.The membrane was blocked with 3% BSA-PBS with shaking for 1-2 h at 4°C. Incubation with primary antibody (Table 3. 7) of the membrane was performed overnight in buffer condition similar to the blocking conditions. The membranes were then washed thrice, for 10 min in 1x PBS buffer at RT. Secondary antibody (Table 3. 7) in 5% Milk-PBS was applied and allowed to incubate with shaking for 1-2 h at RT. The membrane was then washed as previously described. Before adding chemiluminescence substrate, the membrane was wished with 1x PBS supplemented with 0.2% Tween 20 (v/v) for 10 min at RT with shaking.

Chemiluminescence detection of protein was performed upon the enzyme conjugated with secondary antibody (Table 3. 7). The membrane was developed either by X-ray cassette method or by chemiluminescence imaging. In X-ray cassette method, the Amersham ECL WB reagents for HRP-conjugates were used. The substrates were incubated on the membranes for 5 min prior to detection. In an X-ray cassette, emitted light was captured on ECL films for several seconds, to minutes, until the desired band intensities were achieved. The films were developed in an automatic X-ray film processor. In Chemiluminescence Imaging® (biostep GmbH), the Amersham ECL WB reagents for HRP-conjugates were used. The substrate was placed on the top of the screen of Chemiluminescence Imaging, the membrane was placed on the screen and the image was recorded with self-written program in Snap and Go® software.

3.2.3.4 Fluorescence Measurement With A Plate Reader

A FLUOstar Omega (BMG LabTech) plate reader was used to measure fluorescence from sfGFP in intact cells. Samples from *E. coli* BL21(DE3) containing the appropriate plasmid combinations to express sfGFP, from genes with WT sequence or harboring an amber and/or a frameshift codon, were taken with intent to being normalized to OD_{600} of 0.5 by pelleting (3 min, 16,100 × *g*) and resuspending in 1 mL 1× PBS. 200 µL of this cell suspension was transferred into one well of a 96-well black micro plate. 200 µL 1× PBS was used as a reference. The fluorescent signals from GFP were measured using the self-written program "GFP_ORBS_KH" (**Plate mode settings:** No. of flashes per well: 10; Scan mode: Orbital averaging; Scan diameter [mm]: 3; **Optic Settings:** Excitation 485 nm; Emission: 520 nm; Gain: variable; **General settings:** Top optic used; Positioning delay [s]: 0.2).

4. Results

4.1 Functional Orthogonal Ribosome

Major objective of this study is to perform double incorporation of uAAs in the protein using orthogonal translation. In orthogonal translation, orthogonal ribosome is the main component as it has to read the orthogonal ribosome binding site (alternate Shine Dalgarno sequences), which can't be read by endogenous ribosome. So to check whether our orthogonal ribosome is functional or not; an experiment was design to check its functionality by doing the protein expression study having the orthogonal ribosome binding site in presence and absence of orthogonal ribosome. Orthogonal ribosome (pl048, Table 3. 8) and orthogonal ribosome binding site (pl117, Table 3. 8) plasmids was acquired from Neumann's lab plasmid library. These plasmids are explained in details in Neumann *et.al*. 2010 and Lammers *et.al.* 2014 [28, 29]. Single and double co-transformation (Ch. 3.2.1.2) was performed in *E.coli* BL21 (DE3) chemically competent cells and was grown in 2YT medium. Cells were lysed in sample buffer and analyzed by SDS-PAGE and Western Blot using anti-His and Coomassie brilliant blue staining. Western blotting was performed as explained in Ch 3.2.3.3. Antibodies used for western blotting are given in Ch.3.1.12. The expression level of sfGFP is negligible in absence of pl048 (fig 4.1 lane 1 and 2), whereas in the presence of pl048 the expression level increases significantly (fig 4.1 lane 3 and 4). Lane 3 and lane 4 of Western Blot shows a band in between ~35 and ~25 kDa (indicated by PageRuler^{TM}, Thermo Scientific), calculated weight of sfGFP_6xHis is ~28.5 kDa. The arrow in the fig 4.1 (right) validates the expression of sfGFP_6xHis in presence of pl048. This experiment confirms that pl048 is functional and can be used for further experiment.

For further experiments on orthogonal translation machinery pl048 was transformed in *E.coli* BL21 (DE3) chemically competent cells and new chemically competent cells were prepared.

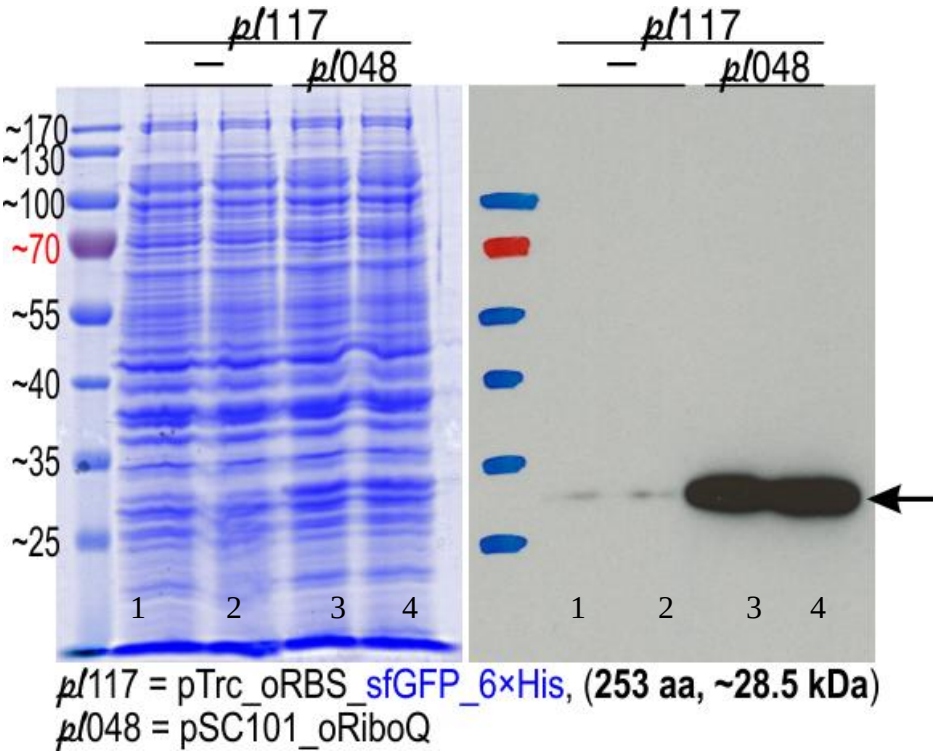

Figure 4. 1: SDS-PAGE and Western Blot of expression of sfGFP_6xHis in presence and absence of orthogonal ribosome (pl048). *E.coli* BL21(DE3) cells were transformed with either pl117 alone or pl117 in combination with pl048. The transformed culture were grown in 2YT medium with antibiotics (Amp for pl117 and Kan for pl048). Cells were lysed in sample buffer and analyzed by SDS-PAGE and Western Blot using anti-His or stained with Coomassie staining. The Western Blot (right) confirms that pl048 is functional. The SDS-PAGE (left) shows a band between 35 and 25 kDa (PageRuler™, ThermoScientific). The arrow indicates the sfGFP band. (Dr. Kangkan helped in preparation of all the figures)

4.2 Expression of recombinant sfGFP_6xHis

As mentioned in Ch. 1.4, one of the challenges faced by orthogonal translation is difficulty in tuning the protein expression as most of the orthogonal ribosome binding site and orthogonal ribosomes are having the constitutive promoter. In order to design orthogonal ribosome binding site having inducible promoter, it is essential to check whether the plasmid is functional in presence of inducer. Thus, an experiment was designed to test the functionality of a wild type sfGFP in presence and absence of the inducer. For this purpose a recombinant sfGFP_6xHis (pl097, Table 3. 8) was used to check the expression level of sfGFP in presence and absence of arabinose. pl097 was transformed by heat shock transformation in *E.coli* BL21 (DE3) (Ch.3.2.1.2), selection was done by using ampicillin. The expression experiment was performed

as explained in Ch.3.2.3.1. Cells were lysed in sample buffer and analyzed by using SDS-PAGE (Coomassie staining) and Western blotting using anti-His was done as explained in Ch. 3.2.3.3. The expression level was insignificant in absence of arabinose induction (fig 4.2, lane 1), however when the cells are induced with arabinose, expression level becomes consequential (fig 4.2, lane 2).

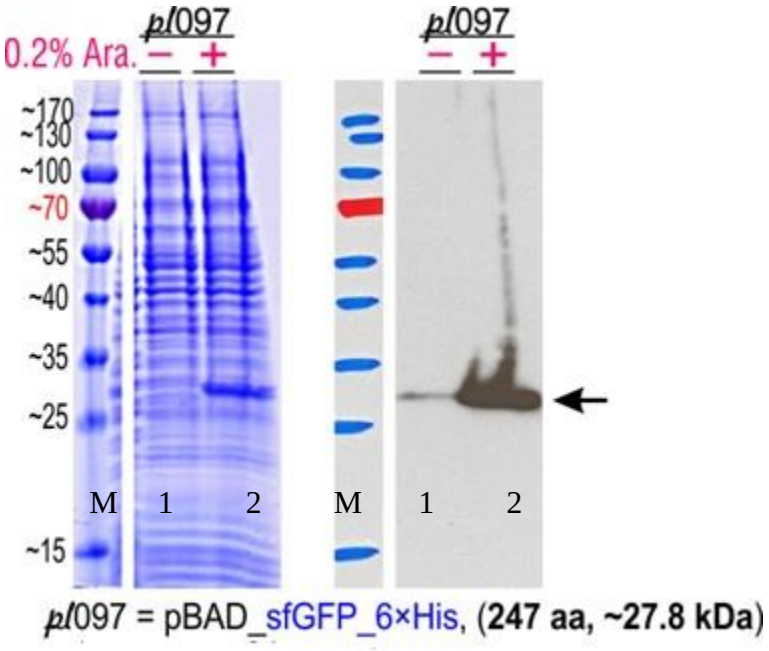

Figure 4. 2 Expression of sfGFP-His in presence and absence of arabinose induction. . *E.coli* BL21 (DE3) cells were transformed with pl097 were grown in 2YT medium with Amp. Cells were lysed in sample buffer and analyzed by SDS-PAGE and Western Blot using anti-His or stained with Coomassie staining. The Western Blot (right) confirms the presence of sfGFP when cells are induced with arabinose (lane 2). The SDS-PAGE (left) shows a thick band between 35 and 25 kDa (PageRuler^{TM}, ThermoScientific) (lane 2). The arrow indicates the sfGFP band. (+) and (-) indicates that cells are induced and non-induced with arabinose.

The SDS-PAGE shows a very thick band between 35 and 25 kDa, suggesting the presence of sfGFP, as its calculated molecular weight is ~28.5 kDa. The same thick band is observed in the Western Blot confirming the presence of sfGFP (fig 4.2, lane 2), leading us to conclude that expression in arabinose system is much better. This experiment proved the robustness and fidelity of inducible (arabinose) expression system. Thus arabinose expression system was selected for incorporating orthogonal ribosome binding sites for orthogonal translation.

4.3 Site-Degeneracy of Orthogonal Ribosome Binding Site (o-RBS)

o-RBS was selected from the work of Rackham and Chin [26], and was termed as o-RBS 1 (highlighted area in the sequence, Table 4. 1). O-RBS 2 to o-RBS 4 was constructed by mutating the first nucleotide (C→ G/A/T). O-RBS 5 and o-RBS 6 was made by either addition of nucleotide base to o-RBS 1 or by deleting one nucleotide base (Table 4. 1).

Table 4. 1 Designed O-RBS Sequences

O-RBS	Sequences (Highlighted region is o-RBS)	References
o-RBS 1	..aCATCCCTccgcaaATG…	Rackham and Chin 2005 [26]
o-RBS 2	.aGATCCCTccgcaaATG…	This work
o-RBS 3	.aAATCCCTccgcaaATG…	This Work
o-RBS 4	.aTATCCCTccgcaaATG…	This work
o-RBS 5	.aCATCCCTCccgcaaATG…	This work
o-RBS 6	.aCATCCCTcgcaaATG…	This work

O-RBS was incorporated in pl097 having an inducible promoter (arabinose inducible system, expression of sfGFP tested in Figure 4. 2). Incorporation was performed by Quick-Change PCR (QC-PCR) as described in Ch. 3.2.2.6. The primers used for the QC PCR are given in Table 3. 9. QC-PCR products are described in Table 3. 8

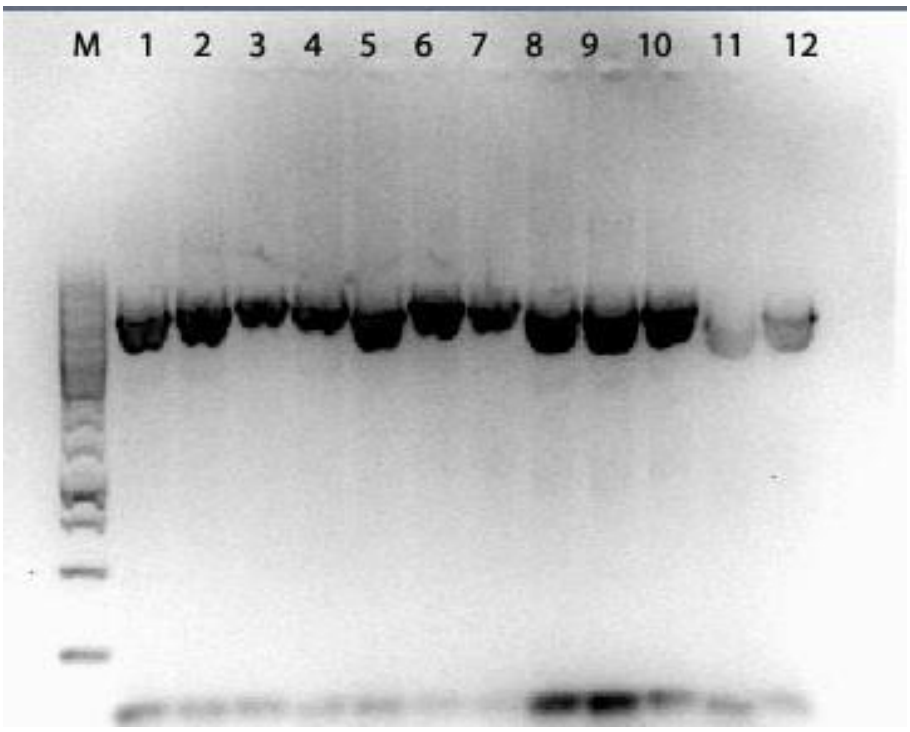

Figure 4. 3: Incorporation of orthogonal ribosome binding site on 0.8% Agarose gel: The final plasmid (pl216-221) having orthogonal ribosome. Lane 1-6 represents pl216-221 respectively in Phusion HF buffer and lane 7-12 represents the same in Phusion GC buffer. Lane M represents GeneRuler 1kb labdder. PCR product was loaded in all the lanes.

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As seen in fig 4.3, the bands for pl216 to pl221 are as expected around 5300 bp. The resultant product was transformed in *E.Coli* DH10B cells for plasmid purification. Purified plasmids were digested with XhoI to test the QC-PCR, as seen in fig. 4.4 sample from lane 3,4,6 14,18 and 20 are showing the expected digest from Xho1. The QC-PCR product was verified by the DNA sequencing (GATC biotech, Germany).

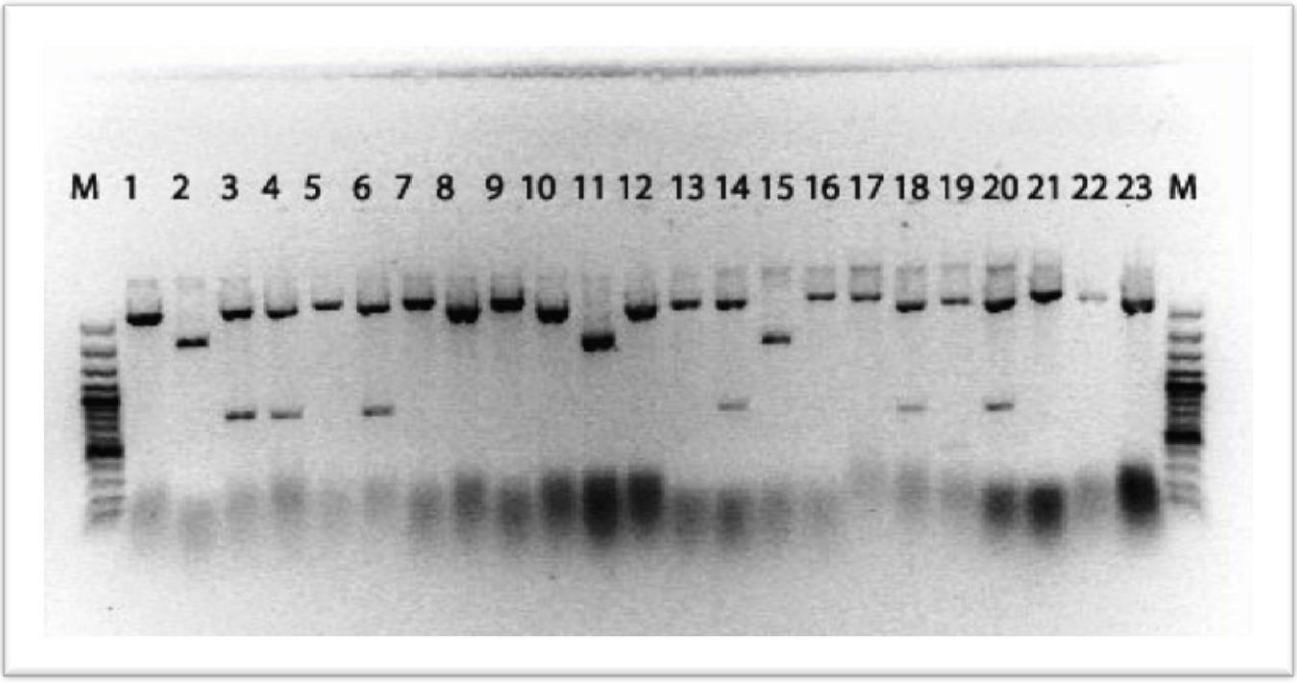

Figure 4. 4 Restriction digestion with Xho1 for o-RBS incorporated plasmids. Lane1-4,5-8 and 9-12 are four different clones of pl216-218 respectively. Lane 13-15,16-19 and 20-23 are clones of pl219-221 respectively. Lane 3,4,6,14,18 and 19 are the correct plasmids as we see two bands (Expected digested band~3kb and ~700bp).

4.4 Screening of o-RBS:

The o-RBS plasmids (pl216-221, table 4.4) were screened by doing transformation with the chemically competent *E.coli* BL21 (DE3) cells harboring pl048 (Ch.3.2.1.2). Expression experiment was performed as mentioned in Ch. 3.2.3.1.Cells were analyzed using western blotting (Ch.3.2.3.3) to screen the best o-RBS plasmid and pl048 combination expressing sfGFP_6xHis for further expression experiments (Figure 4. 5).

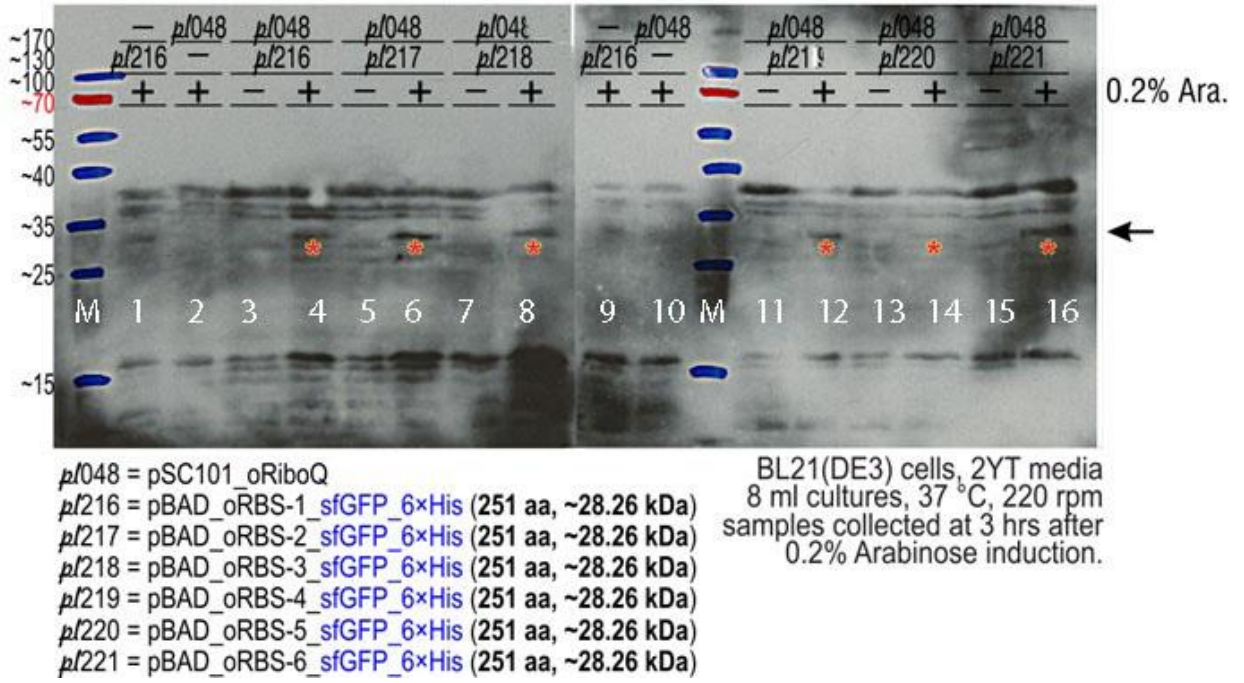

Figure 4. 5: Western Blot of o-RBS screening by expressing sfGFP-6xHis in *E.Coli* BL21 (DE3) cells Chemically competent BL21DE3 cells harboring pl048 was transformed independently with pl216-pl221, cells were grown in 2YT media having Kan and Amp as antibiotics. Cells were lysed in sample buffer and analyzed Western Blot using anti-His. From left to right lane: M: PageRuler™, (ThermoScientific), protein molecular weight marker 1) pl216 (o-RBS 1) without pl048 (orthogonal ribosome) and with 0.2% arabinose induction 2) pl048 without any o-RBS 3) pl216 and pl048 without 0.2% arabinose induction 4) pl216 and pl048 with 0.2% arabinose induction 5) pl217 (o-RBS 2) and pl048 without 0.2% arabinose induction 6) pl217 and pl048 with 0.2% arabinose induction 7) pl218 (o-RBS 3) and pl048 without 0.2% arabinose induction 8) pl218 and pl048 with 0.2% arabinose induction 9) pl216 (o-RBS 1) without pl048 (orthogonal ribosome) and with 0.2% arabinose induction 10) pl048 without any o-RBS M) PageRuler™ , (ThermoScientific) protein molecular weight marker 11) pl219 (o-RBS 4) and pl048 without 0.2% arabinose induction 12) pl219 and pl048 with 0.2% arabinose induction 13) pl220 (o-RBS 5) and pl048 without 0.2% arabinose induction 14) pl220 and pl048 with 0.2% arabinose induction 15) pl221 (o-RBS 6) and pl048 without 0.2% arabinose induction 16) pl221 and pl048 with 0.2% arabinose induction. '*' represents the signal of sfGFP observed on blot. Arrow indicates sfGFP in accordance with its calculated molecular weight (~28.5 kDa) lying in between ~35 and 25 kDa (Indicated by PageRuler™, ThermoScienctific)

o-RBS 1 to o-RBS 4 gives a prominent signals when induced with 0.2% arabinose (Figure 4. 5; lane 4, 6, 8 and 12) compared to the one where there is either an addition of nucleotide base or a deletion of nucleotide base from the original orthogonal ribosome binding site (Figure 4. 5; lane 14 and 16). If a comparison is made between fig 4.2 and figBased on this observation, o-RBS 1 to o-RBS 4 plasmids were selected for inflicting point mutation at amino acid sequence either at 134th position or at 150th position of sfGFP_6xHis by a quadruplet codon (AGGA) or an amber codon (TAG).

4.5 Site-Directed Mutagenesis of sfGFP with TAG and/or AGGA

In order to incorporate uAA (Table 3. 3), we need to include amber stop codon as well as the quadruplet codon in the gene of interest (here sfGFP and Histone H3). To do site directed mutagenesis, QC PCR was performed. An amber codon (TAG) was placed at 150^{th} position in sfGFP by mutating asparagine. A quadruplet codon (AGGA) was incorporated at 134^{th} position in sfGFP by mutating aspartic acid. These codons will be used for incorporating uAAs of our choice.

The larger objective of this project is to study the dynamics of histone with complexes/nucleosome under post translational effect of acetylation in lysine in histone (H3). Therefore two point mutations were done in plasmid having histone (H3.opt) and o-RBS (pl237, Table 3.8) to incorporate both AGGA as well as TAG. QC-PCR products of sfGFP_6xHis and H3.opt were checked with the 0.8% Agarose gel and finally confirmed with the DNA sequencing (GATC biotech, Germany). QC PCR product plasmids for sfGFP_6xHis and H3.opt having amber and/or quadruplet mutations are listed in Table 3. 8.

4.6 Orthogonal Translation in Presence of Amber or Quadruplet Codon:

After the site directed mutagenesis in sfGFP, it was important for us to test the system that whether the incorporation of uAA is taking place or not. If it is happening what is the yield compared to the wild type. Based on this result, incorporation of uAAs in histone will be performed. As we know orthogonal translation machinery needs an orthogonal ribosome (pl048), orthogonal ribosome binding site with protein of interest having amber and/or a quadruplet codon mutation (pl226-pl233, Table 4.4) and evolved orthogonal $tRNA_{CUA}$/amino-acyl-tRNA Synthetase (aaRS) pair or evolved orthogonal $tRNA_{UCCU}$/aaRS (pl175 and pl178, Table 3. 8). Three plasmid systems transformation was done by the heat shock transformation of o-RBS plus sfGFP_6xHis plasmids (pl226-233) in a chemically competent cell of *E.coli* BL21 (DE3) harboring pl048 along with pl175 or pl178 (Table 3. 8) (Ch.3.2.1.2). Expression experiment was performed as explained in 3.2.3.1. Induction with 0.2% arabinose and 1mM IPTG was done when cells OD_{600} reached 0.5-0.6 to induce the o-RBS plasmids (pl226-pl233) and pl178 (lac

inducible promoter) respectively. 1 mM of AzF and 1 mM of BCN-K was also added to the culture at the time of induction. Harvesting was done after 3-4 hour of induction. Whole cell lysate was processed according to Ch.3.2.3.1. The samples were used for loading on 12.5% SDS-PAGE and Western Blotting was done to confirm whether there is an incorporation of unnatural amino acids or not.

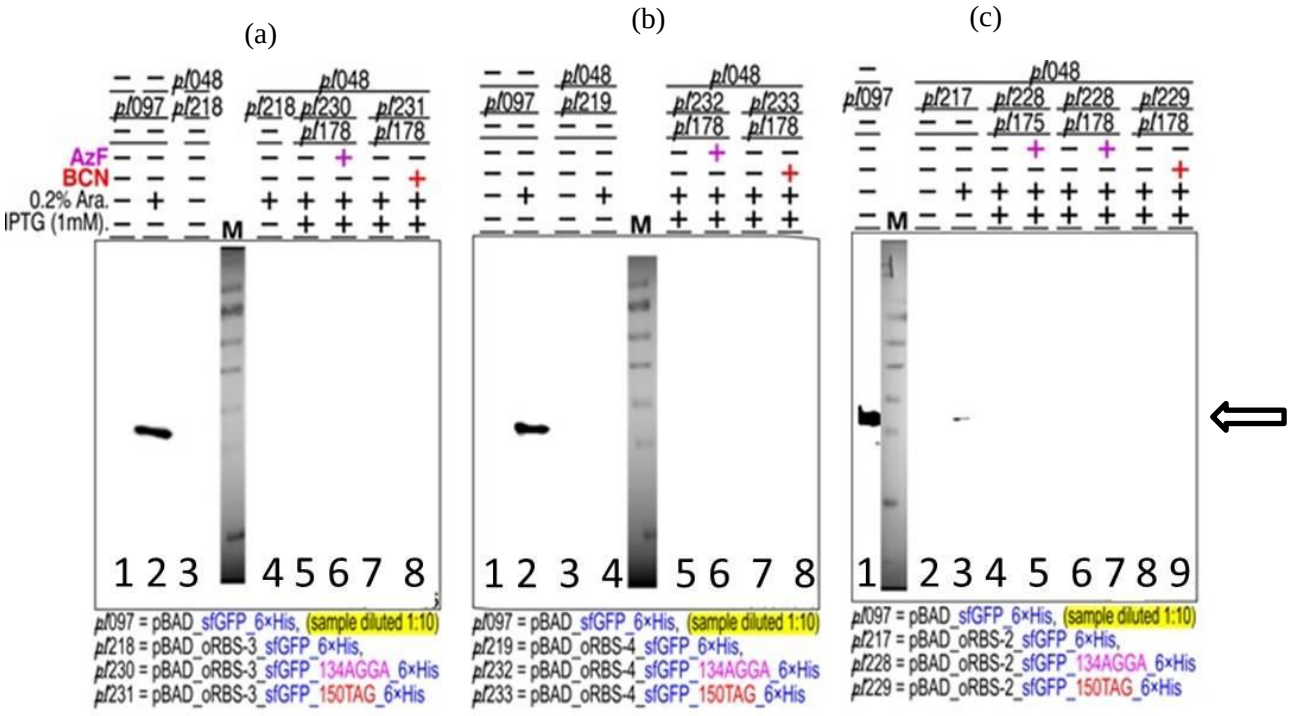

Figure 4. 6 Incorporation of unnatural amino acids in sfGFP-6xHis in *E.Coli* BL21 (DE3) cells: Chemically competent BL21DE3 cells harboring pl048 and pl175/pl178 was transformed independently with plasmids pl216-219, pl226-233, cells were grown in 2YT media having Kan ,Spec and Amp as antibiotics. Cells were lysed in sample buffer and analyzed by Western Blot using anti-His (a) a western blot of 12.5% SDS –PAGE gel showing o-RBS 3 having site specific mutation for incorporating uAAs (AzF and BCN) in sfGFP expression, From left to right lane: 1) wild type sfGFP without induction 2) wild type sfGFP with 0.2% arabinose induction 3) pl218 (o-RBS 3) with pl048 (orthogonal ribosome) without induction M) PageRuler™ marker 4) pl218 (o-RBS 3) with pl048 and with 0.2% arabinose induction 3) pl048 without any o-RBS 4) pl216 and pl048 with 0.2% arabinose induction 5) pl218 and pl048 with 0.2% arabinose induction plus pl178 (tRNA/aaRS pair) ha 6) pl217 (o-RBS 2) and pl048 without 0.2% arabinose induction 7) pl217 and pl048 with 0.2% arabinose induction 8) pl218 (o-RBS 3) and pl048 without 0.2% arabinose induction 9) pl218 and pl048 with 0.2% arabinose induction 10) pl216 (o-RBS 1) without pl048 (orthogonal ribosome) and with 0.2% arabinose induction 11) pl048 without any o-RBS 12) PageRuler™ marker 13) pl219 (o-RBS 4) and pl048 without 0.2% arabinose induction 14) pl219 and pl048 with 0.2% arabinose induction 15) pl220 (o-RBS 5) and pl048 without 0.2% arabinose induction 16) pl220 and pl048 with 0.2% arabinose induction 17) pl221 (o-RBS 6) and pl048 without 0.2% arabinose induction 18) pl221 and pl048 with 0.2% arabinose induction.

Western blot confirms that there is no incorporation of uAAs in sfGFP_6xHis for all the different combinations (Figure 4. 6. (a), (b), and (c)) positive control of recombinant sfGFP_6xHis (pl097, Table 4.1) gives a very strong signal even when it is diluted 10 times (lane 2 Figure 4. 6 (a), lane 2 Figure 4. 6 (b), lane 1 Figure 4. 6 (c)), confirming that the experiment was performed correctly. The samples of pl097 (with arabinose induction) was diluted ten times as the pellet obtained after harvesting was lush green confirming that sfGFP has expressed very well and is present in high amount. The same kind of pellet behavior was not observed in any of other plasmid combination. It is known that efficiency of expression takes a dip, when the protein is expressed orthogonally; the dip in expression efficiency is much more when there is an incorporation of uAA (Table 3. 3).

One of the major suspects for the failure is either the non-functionality or loss of orthoganlity of orthogonal ribosome

4.7 Orthogonal Ribosome (pl048): Functional or Non-Functional?

As seen from fig 4.6, there was no or limited (Figure 4. 6 (c) lane 3) expression of protein in presence of orthogonal ribosome suggesting that there might be some problem associated with pl048. To ascertain that the orthogonal system is working fine, a repeat experiment of Ch 4.1 was performed to check whether pl048 is functional. The experiment was done in 3 different set of conditions. In first set, BL21 (DE3).cells harboring pl048 was transformed with pl117, in second set was a double co-transformation of pl048 and pl117 and in the last set of experiment chemically cometent BL21 (DE3) cells harboring pl048 was transformed with pl219. pl097 was used as a positive control in this experiment. Transformed cells were grown in 2YT media and samples were analyzed with fluorometric measurements of sfGFP expression as mentioned in Ch. 3.2.3.4 and also by Western blotting (Ch 3.2.3.3).

Fluorometric measurement suggests that the orthogonal ribosome is not working as we were unable to see any kind of signal (Figure 4. 7). Wild type sfGFP gives a very strong signal as seen in fig 4.7. Western blotting (Figure 4. 8) of the same experiment confirms that the orthogonal ribosome has become non-functional, as we were unable to observe any signal even in the experimental set which was replica of experiment explained in Ch.4.1 (Figure 4. 1).

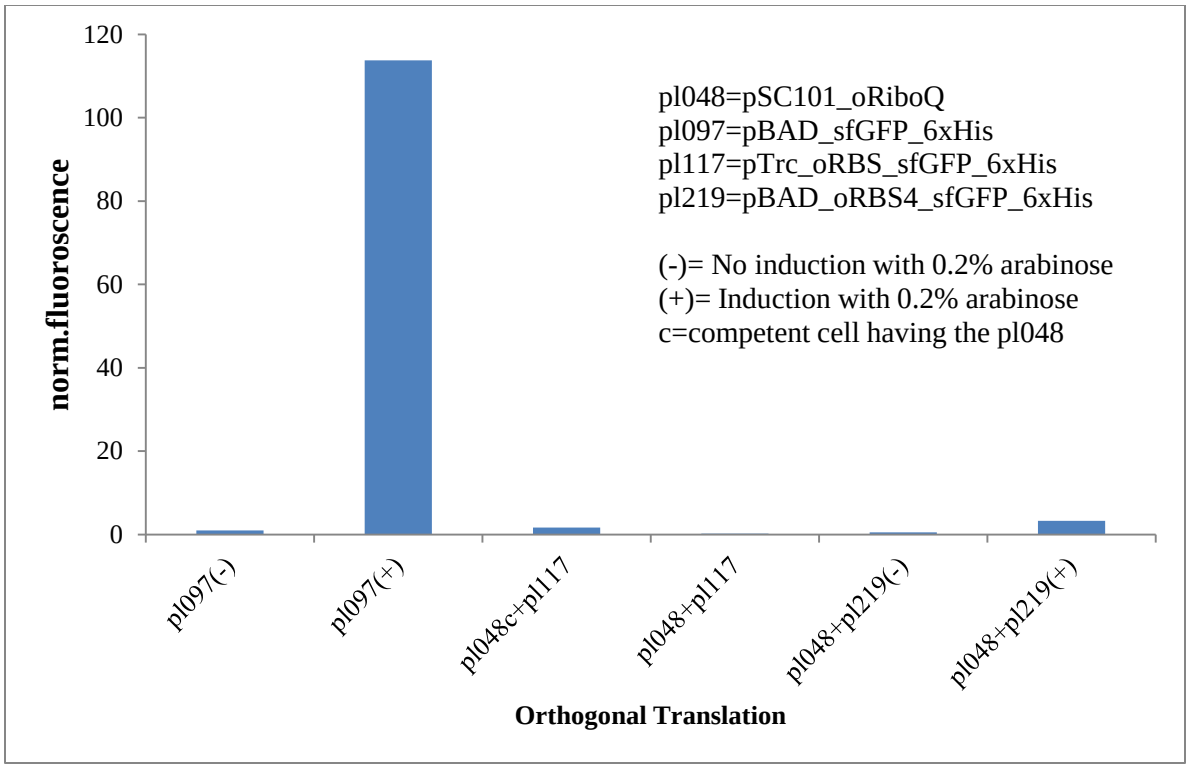

Figure 4. 7: Fluorometric measurements of sfGFP expression: E.coli BL21 (DE) cells were transformed with the independently with plasmids pl097, pl117&048, pl219& 048. Transformed cells were grown in 2YT medium with antibiotics. Cells were analyzed for GFP expression in FlUOrostar..

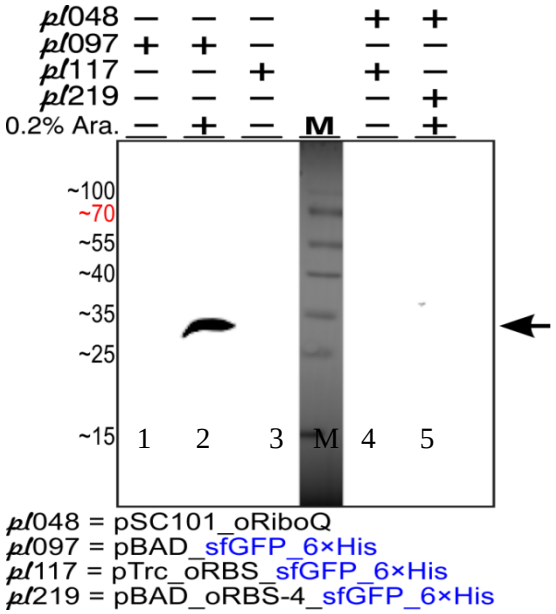

Figure 4. 8: Western Blot of sfGFP_6x expression: E.coli BL21 (DE) cells were transformed with the independently with plasmids pl097, pl117&048, pl219& 048. Transformed cells were grown in 2YT medium with

antibiotics. Cells were analyzed with western blot using anti-His. From left to right lane 1) pl097 without induction 2) pl097 with arabinose induction 3) pl117+pl048 (comp cell in BL21 (DE3)) M) PageRuler^{TM} marker (Thermo Scientific) 4) pl117+pl048 (Double co-transformation) 5) pl219 +pl048 (comp cell in BL21 (DE3)). Western Blot confirms that the functionality of orthogonal ribosome is lost

Discussion

It might be difficult or sometimes impossible to study the complex protein's function and dynamics in the biological process using traditional methods [20]. The expansion of genetic code has become a mighty tool in the field of protein engineering in recent years [22]. New functional groups and properties can be added by unnatural amino acids site specifically into the proteins by this approach [1, 11, 20- 22]. This technique has grown over the period of time which has translated in the expansion of genetic code in many bacteria, yeast, animals and mammals [11, 24]. In contrast, inclusion of multiple uAAs in the protein is seriously restricted by the incorporation of uAA efficiency [22, 24].Additionally, lack of inducible promoters in the components of orthogonal ribosome and orthogonal ribosome binding sites deter the tunability of overexpression of proteins [29]. This thesis focuses on designing of orthogonal ribosome binding sites in an inducible promoter and checking the efficiency of incorporation of multiple uAAs in the proteins.

Protein expression in presence of orthogonal ribosome in a constitute promoter (fig 4.1) was comparable to the wild type sfGFP expression in pBAD system under the arabinose induction. We can see a very strong signal in both cases on Western blot, but in case of wild type sfGFP expression the thickness of band in Coomassie stained gel (fig 4.2) is almost 3 times of what we see in case of protein expression under orthogonal ribosome. This suggests that protein expression in presence of inducible system is much more compared to the constitutive promoter. Keeping this in mind we expected that when we design orthogonal ribosome binding system on inducible promoter, the expression level should be more than what we observed in fig 4.1.

Results observed in fig 4.6, suggested that something is going wrong as there was no or limited expression of protein under the influence of inducer. The combination of pl048 and pl217 (fig 4.6 (c) lane 3) shows limited expression of the protein and the signal strength is around 10-20 fold less than the wild type protein even though the wild type protein is diluted 10 times before loading on the gel.

In the fluorometric measurement, the expected signal strength for constitute promoter was something around 40-60 as reported by Lammers *et.al*., 2014 [29], but as we see in fig 4.7 there

was no signal or very limited signal corresponding to the constitutive promoter, which again suggest that orthogonal ribosome might have lost its functionality.

Unfortunately, the incorporation efficiency studies could not be completed as the whole system (orthogonal ribosome, o-RBSs and tRNA/aaRS) failed in whole. When this problem was analyzed in detail, it came to the knowledge that orthogonal ribosome might have become non-functional during the course of time (fig 4.8). Even though, the functionality of orthogonal ribosome was checked before starting the designing of o-RBSs (fig4.1). One of the reasons for its sudden change of behavior might be attributed to the first step taken during this project, which is making chemically competent cells harboring the orthogonal ribosome; as the orthogonal ribosome was already present in the host cell giving the host a chance to adjust with this large plasmid (~10 kb) by using the selective regions of plasmid required for the growth of cells and silencing the other region during the expression experiment. As orthogonal plasmid contain a chunk of bacterial genome and mostly made up by mutation of 16S-rRNA [26], it is possible that the orthogonal plasmid DNA might have come in contact with bacterial genome during or after the preparation of competent cell and exchanged the orthogonal part with normal bacterial genome thus losing the orthogonality.

It is safe to conclude that in future experiments orthogonal ribosome should not be included in the host cell by making competent cells. Another reason attributed to the failure of the experiment is the method used to check the functionality of orthogonal ribosome (Figure 4.1), as this is an indirect way thus, chances of errors are more. A better and direct approach would have been selecting them using the reporter assay as explained by Neumann *et. al.* 2010 [28].

It would have been helpful if the positive control, system used to check the working condition of orthogonal ribosome was used in all the expression experiment during this project from the beginning of this work. This would have given us an indication that something is going wrong in the orthogonal translation machinery and corrective measures could have been deployed at earlier stage of the project, which might have helped us in achieving our goal.

Conclusion

Having successfully designed and screened o-RBSs based on the work of Rackham and Chin [26]. Site directed mutagenesis was performed to add amber (TAG) and frameshift (AGGA) codons in the desired protein on the best fit o-RBS (o-RBS 1 to 4). Multiple experimental failures of incorporation studies suggest that orthogonal translation machinery as a whole has failed, which led us to conclude that orthogonal ribosome the major component of this machinery is not working somewhat. Selection of orthogonal ribosome by reporter assay should be performed in future before starting the screening of o-RBS and orthogonal translation.

It was unfortunate that we were not able to meet our larger objective of the project. It would be interesting to see the effect of different promoters (T7, Arabinose, Trc and lac) when encoded with an o-RBS from our study. The next objective should be to check whether the orthogonal translation machinery has different affect with various available protein tags (His tag, Strep tag, GST tag etc.). A better approach would be make a matrix out of this and test all conditions at the same time, then by inserting multiple cloning sites (MCS) make a modular plasmid out of this for extensive usage for protein expressions.

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References

1. Liu, C. C., & Schultz, P. G. (2010). Adding new chemistries to the genetic code.*Annual review of biochemistry*, *79*, 413-444.
2. Ambrogelly, A., Palioura, S., & Söll, D. (2007). Natural expansion of the genetic code. *Nature chemical biology*, *3*(1), 29-35.
3. Cone, J. E., Del Rio, R. M., Davis, J. N., & Stadtman, T. C. (1976). Chemical characterization of the selenoprotein component of clostridial glycine reductase: identification of selenocysteine as the organoselenium moiety. *Proceedings of the National Academy of Sciences*, *73*(8), 2659-2663.
4. Zinoni, F., Birkmann, A., Stadtman, T. C., & Böck, A. (1986). Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from Escherichia coli.*Proceedings of the National Academy of Sciences*, *83*(13), 4650-4654.
5. Chambers, I., Frampton, J., Goldfarb, P., Affara, N., McBain, W., & Harrison, P. R. (1986). The structure of the mouse glutathione peroxidase gene: the selenocysteine in the active site is encoded by the'termination'codon, TGA. *The EMBO journal*, *5*(6), 1221.
6. Böck, A., Forchhammer, K., Heider, J., Leinfelder, W., Sawers, G., Veprek, B., & Zinoni, F. (1991). Selenocysteine: the 21st amino acid. *Molecular microbiology*, *5*(3), 515-520.
7. Srinivasan, G., James, C. M., & Krzycki, J. A. (2002). Pyrrolysine encoded by UAG in Archaea: charging of a UAG-decoding specialized tRNA. *Science*,*296*(5572), 1459-1462.
8. Hao, B., Gong, W., Ferguson, T. K., James, C. M., Krzycki, J. A., & Chan, M. K. (2002). A new UAG-encoded residue in the structure of a methanogen methyltransferase. *Science*, *296*(5572), 1462-1466.
9. Atkins, J. F., & Gesteland, R. (2002). The 22nd amino acid. *Science*,*296*(5572), 1409-1410.
10. Zhang, Y., Baranov, P. V., Atkins, J. F., & Gladyshev, V. N. (2006). Pyrrolysine and selenocysteine use dissimilar decoding strategies. *Journal of Biological Chemistry*, 20740-20751
11. Chin, J. W. (2014). Expanding and reprogramming the genetic code of cells and animals. *Annual review of biochemistry*, *83*, 379-408.
12. Chatterjee, A., Xiao, H., & Schultz, P. G. (2012). Evolution of multiple, mutually orthogonal prolyl-tRNA synthetase/tRNA pairs for unnatural amino acid mutagenesis in Escherichia coli. *Proceedings of the National Academy of Sciences*, *109*(37), 14841-14846.
13. Davis, L., & Chin, J. W. (2012). Designer proteins: applications of genetic code expansion in cell biology. *Nature reviews Molecular cell biology*, *13*(3), 168-182.
14. Furter, R. (1998). Expansion of the genetic code: site-directed p-fluoro-phenylalanine incorporation in Escherichia coli. *Protein science: a publication of the Protein Society*, *7*(2), 419.
15. Wang, L., Brock, A., Herberich, B., & Schultz, P. G. (2001). Expanding the genetic code of Escherichia coli. *Science*, *292*(5516), 498-500.
16. Javahishvili, T., Manibusan, A., Srinagesh, S., Lee, D., Ensari, S., Shimazu, M., & Schultz, P. G. (2014). Role of tRNA orthogonality in an expanded genetic code. *ACS chemical biology*, *9*(4), 874-879.
17. Hu, C., Chan, S. I., Sawyer, E. B., Yu, Y., & Wang, J. (2014). Metalloprotein design using genetic code expansion. *Chemical Society Reviews*, *43*(18), 6498-6510.
18. Chin, J. W., Cropp, T. A., Anderson, J. C., Mukherji, M., Zhang, Z., & Schultz, P. G. (2003). An expanded eukaryotic genetic code. *Science*, *301*(5635), 964-967.

19. Carrico, Z. M., Romanini, D. W., Mehl, R. A., & Francis, M. B. (2008). Oxidative coupling of peptides to a virus capsid containing unnatural amino acids. *Chemical Communications*, (10), 1205-1207.
20. Wals, K., & Ovaa, H. (2014). Unnatural amino acid incorporation in E. coli: current and future applications in the design of therapeutic proteins. *Frontiers in chemistry*,*2*.
21. Ravikumar, Y., Nadarajan, S. P., Yoo, T. H., Lee, C. S., & Yun, H. (2015). Unnatural amino acid mutagenesis-based enzyme engineering. *Trends in biotechnology*.
22. Zhang, W. H., Otting, G., & Jackson, C. J. (2013). Protein engineering with unnatural amino acids. *Current opinion in structural biology*, *23*(4), 581-587.
23. Ryden, S. M., & Isaksson, L. A. (1984). A temperature-sensitive mutant of Escherichia coli that shows enhanced misreading of UAG/A and increased efficiency for tRNA nonsense suppressors. *Molecular and General Genetics MGG*, *193*(1), 38-45.
24. Neumann, H. (2012). Rewiring translation–genetic code expansion and its applications. *FEBS letters*, *586*(15), 2057-2064.
25. Rackham, O., & Chin, J. W. (2005). Cellular logic with orthogonal ribosomes.*Journal of the American Chemical Society*, *127*(50), 17584-17585
26. Rackham, O., & Chin, J. W. (2005). A network of orthogonal ribosome· mRNA pairs. *Nature chemical biology*, *1*(3), 159-166.
27. Wang, K., Neumann, H., Peak-Chew, S. Y., & Chin, J. W. (2007). Evolved orthogonal ribosomes enhance the efficiency of synthetic genetic code expansion. *Nature biotechnology*, *25*(7), 770-777.
28. Neumann, H., Wang, K., Davis, L., Garcia-Alai, M., & Chin, J. W. (2010). Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome. *Nature*, *464*(7287), 441-444.
29. Lammers, C., Hahn, L. E., & Neumann, H. (2014). Optimized plasmid systems for the incorporation of multiple different unnatural amino acids by evolved orthogonal ribosomes. *ChemBioChem*, *15*(12), 1800-1804.
30. Summerer, D., Chen, S., Wu, N., Deiters, A., Chin, J. W., & Schultz, P. G. (2006). A genetically encoded fluorescent amino acid. *Proceedings of the National Academy of Sciences*, *103*(26), 9785-9789.
31. Lee, H. S., Guo, J., Lemke, E. A., Dimla, R. D., & Schultz, P. G. (2009). Genetic incorporation of a small, environmentally sensitive, fluorescent probe into proteins in Saccharomyces cerevisiae. *Journal of the American Chemical Society*, *131*(36), 12921-12923.
32. Giepmans, B. N., Adams, S. R., Ellisman, M. H., & Tsien, R. Y. (2006). The fluorescent toolbox for assessing protein location and function. *Science*,*312*(5771), 217-224.
33. Baskin, J. M., & Bertozzi, C. R. (2007). Bioorthogonal click chemistry: covalent labeling in living systems. *QSAR & Combinatorial Science*, *26*(11-12), 1211-1219.
34. Lang, K., & Chin, J. W. (2014). Cellular incorporation of unnatural amino acids and bioorthogonal labeling of proteins. *Chemical reviews*, *114*(9), 4764-4806.
35. Baskin, J. M., Prescher, J. A., Laughlin, S. T., Agard, N. J., Chang, P. V., Miller, I. A., & Bertozzi, C. R. (2007). Copper-free click chemistry for dynamic in vivo imaging. *Proceedings of the National Academy of Sciences*,*104*(43), 16793-16797.
36. Chakraborty, A., Wang, D., Ebright, Y. W., Korlann, Y., Kortkhonjia, E., Kim, T. & Ebright, R. H. (2012). Opening and closing of the bacterial RNA polymerase clamp. *Science*, *337*(6094), 591-595.
37. Hohlbein, J., Craggs, T. D., & Cordes, T. (2014). Alternating-laser excitation: single-molecule FRET and beyond. *Chemical Society Reviews*, *43*(4), 1156-1171.