CONSTRUCTION OF A GFP CONTAINING RECOMBINANT PLASMID FACILITATING IMMOBILIZATION, VISUALIZATION AND QUANTIFICATION OF FUSION PROTEINS ON INTERACTIVE POLYMERIC SURFACES

by

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CONSTRUCTION OF A GFP CONTAINING RECOMBINANT PLASMID FACILITATING IMMOBILIZATION AND VISUALIZATION OF FUSION PROTEINS ON INTERACTIVE POLYMERIC SURFACES

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CONFIDENTIAL DISCLOSURE AGREEMENT LETTER

I, Erinç Şahin, have disclosed herein certain confidential ideas and information regarding a two component system composed of a recombinant vector facilitating immobilization, visualization and quantification of any proteins and a chemically modified surface, which I refer to as my trade secrets. In partial fulfillment of my Master's degree requirements, I have submitted a thesis, conducted under the guidance of my thesis supervisor and co-supervisors Zehra Sayers and Alpay Taralp. In this thesis, information relevant to my trade secretes have been incorporated, solely with the purpose and belief that you may more easily evaluate the scientific merit of my work and pass judgment on the concept of awarding a degree.

By reading the thesis subject matter hereafter, you agree to treat it as valuable proprietary trade secret information and maintain it in confidence. Accordingly, I have disclosed patentable aspects of this work with the understanding that you wish to have the opportunity to evaluate the subject matter as a whole on the following basis:

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CLAIMS

1) Circular recombinant plasmid DNA construct encoding a) a protein tag, b) a visual marker protein, and containing c) a multiple cloning site suitable for insertion of an additional gene, characterised in that the gene sequence encoding the protein tag and the visual marker protein are specifically designed and engineered at the DNA level for respectively a) immobilisation purposes and b) visualisation and quantification purposes at the protein level.
2) 2) Construct according to claim 1, characterised in that the protein tag is chosen from the group containing lysine (lys), histidine (his), tyrosine (tyr), phenylalanine (phe), arginine (arg), glutamic acid (glu), aspartic acid (asp), glutamate, aspartate, asparagine (asn), glycine (gly), glutamine (gln), alanine (ala), valine (val), tryptophan (trp).
3) Construct according to claim 1 or 2, characterised in that the protein tag is a histidine-tag such as a polyhistidine variant, in particular (6X) histidine.
4) Construct according to any one of the preceding claims, characterised in that the visual marker protein is chosen from the group containing fluorescent or phosphorescent proteins.
5) Construct according to claim 4, characterised in that the fluorescent protein is chosen from the group containing Green Fluorescent Protein (GFP), Red Fluorescent Protein (RFP), Yellow Fluorescent Protein (YFP) and Blue Fluorescent Protein (BFP) as well as their variants and/or mutants.
6) Construct according to any one of the preceding claims, characterised in that the multiple cloning site contains restriction enzyme recognition sites.
7) Construct according to claim 6, characterised in that the restriction enzyme recognition site is chosen from the group containing SacI, SalI, HindIII, EagI, NotI.
8) Construct according to any one of the preceding claims, characterised in that it further contains a frame adapter of variable length between the visual marker and protein tag genes.

9) Protein expressed by circular recombinant plasmid DNA construct according to any one of claims 1 to 8, characterised in that in the MCS adjacent to the visual marker, it further contains an additional target protein and in that the tag is suitable to interact directly with appropriate surface pendant groups of a support material.
10) Protein according to claim 9 characterised in that it is a fusion protein.
11) Use of the protein constructs of claim 9 or 10, in immobilisation and visualisation of proteins on compatible support material.
12) Method for preparing and immobilising a protein on a support material, characterised in that it contains the steps of:
    a) Engineering at the DNA level, in series a protein tag suitable to interact directly with appropriate surface pendant groups of a support material, a fluorescent marker protein for visualisation and quantification purposes at the protein level and a multiple cloning site suitable for insertion of a target protein to be immobilised,
    b) Inserting the corresponding gene of the target protein to be immobilised into the multiple cloning site;
    c) Initiating protein expression.
    d) Optionally pre-treating the support material;
    e) Incubating the protein and support material together, wherein the protein is immobilised to the support via specific tag-surface interactions;
    f) Washing away the non-specific biomolecules;
    g) Optionally quantifying the fluorescence of the visual marker protein;
    h) Optionally desorbing the target protein.
13) Method according to claim 12, characterised in that the support material is chosen from the group containing polymers, biopolymers, glass and composites containing silicone dioxides, metals and metal oxides, as well as any combination thereof on the microscopic, mesoscopic or macroscopic length scale.
14) Method according to any one of claims 12 and 13, characterised in that the support material is chosen from the group containing polymers, silicon dioxides, aluminum oxides, titanium oxides, magnesium oxides, borates, metals and other metal oxides.

15) Method according to any one of claims 13 and 14, characterised in that the polymers are chosen from the group containing polyolefins such as polystyrene, polyacrylates, polymethyacrylates, polybutylene, polyvinylalcohol and related derivatives, polyvinylchlorides, polyisoprene, polypropylene, polyphenols, polyamides, polyesters polysulfones, polyethersulfones, polyethersulfides, polyimines, polyethyleneglycols, polypropyleneglycols, polyimides, polycarbonates, polyurethanes,

16) Method according to claim 15, characterised in that the polymer surface is chemically treated to bear various functional groups chosen between carboxyl groups, hydroxyl groups, amino groups, amide groups, ester groups, imide groups, imine groups, mercapto groups, nitro groups, sulfonate groups, phosphate groups, phosphonate groups, cyano groups, sulfone groups, aldehyde groups, epoxide groups, urethane groups, ketone groups, phenolic groups, aromatic groups, alkyl, alkenyl, alkynyl, acyl and aryl groups, silanol groups, silicon oxide groups, siloxane groups, metal hydroxide groups, metal oxide groups, and elemental metals.

17) Method according to any one of claims 13 to 16, characterised in that the support material is carboxylated polystyrene.

18) Immobilised protein construct obtained by the method according to any one of claims 13 to 17, characterised in that it is covalently or non-covalently bonded to the support material.

19) Immobilised protein construct according to claim 18, characterised in that it is non-covalent and yet freely accessible and leach-free like proteins immobilised in the covalent sense.

20) Use of the immobilised protein constructs according to any one of claims 18 and 19 in applications selected from analysis, diagnosis (like in enzyme based diagnostic kits), incubation, storage, sensing, arraying and orienting, catalysis, stabilisation, binding, signal transduction, chemical transformation, implant passivation and surface biocompatibilization, surface activation, purification, detoxification and scavenging.

ABSTRACT

A two-component system was designed and developed to facilitate immobilization of any peptide such as enzyme or signal peptide onto appropriately modified surfaces. The system was based upon a specialty plasmid that codes a sequence for a poly(6)histidine tag, a GFP (green fluorescent protein) gene, and a multiple cloning site, to which the gene of the target peptide may be inserted, and an interactive surface that was tailored with negatively charged functional groups.

The target plasmid bearing a poly(6)histidine coding region was engineered starting from pETM-11 plasmid, which is a vector optimized for expression purposes. In order to better preserve the traits of pETM-11, the non-essential gene of a protein called MAD was excised and replaced with a frame adaptor, as well as GFP gene of comparable size to MAD. This "frame adaptor" –a frame-adjusting, small oligonucleotide sequence joining the histidine anchors and the fluorescent GFP linker– prevented the frameshift problem that would be introduced after substituting the MAD gene with the GFP gene. In addition, it was designed to code for a highly flexible sequence of amino acids (Gly-Gly-Thr) to best preserve the solution-phase traits of the GFP gene. The finalized “immobilization adapter” protein construct, termed GFPimm, was expressed and isolated using either a Ni^{2+} polyhistidine tag affinity column or ion exchange chromatography. Following isolation, the protein is tested for binding performance using surface-modified polystyrene 96-well plates. In contrast to the typical mode used to bind polyhistidine tags, in which a coordination bond between Ni^{2+} and imidazole anchors the protein to a surface, the strategy proposed herein was to exploit the positive charges of histidine and negative charges on the surface to achieve salt bridging. For this purpose, a polystyrene surface was modified to bear negatively charged surface groups via incubation with a persulfate reagent, ammonium persulfate.

It follows that the recombinant vector designed and constructed in this study is not only amenable to cloning and expression but also for realizing easy purifications, visual tagging of target proteins by fusion methodologies, and performing immobilizations in specialty applications.

ÖZET

Uygun biçimde değişikliğe uğratılmış yüzeyler üzerine enzimler veya sinyal peptitleri gibi her türlü proteini sabitlemek amacıyla iki bileşenli bir sistem tasarlandı ve oluşturuldu. Sistem, çoklu (6'lı) histidine dizisi kodlayan bir kısım, GFP (yeşil parlayan protein) geni, sabitlenmesi hedeflenen proteinin geninin yerleştirilebileceği bir çoklu klonlama bölgesi içeren yüzey sabitlemeye özelleşmiş bir plazmid ve eksi yüke sahip işlevsel gruplar taşımak üzere değişikliğe uğratılmış, etkileşen bir yüzeyden oluşmaktadır.

Çoklu (6) histidine kodlayan kısım içeren hedeflenen plazmid gen klonlama ve ekspresyonu için optimize edilmiş olan pETM-11 ekspresyon vektörü değiştirilerek elde edilmiştir. pETM-11'in optimize edilmiş özelliklerini daha iyi korumak için, uygulamamızda gerekli olmayan MAD geni çıkarılmış, yerine sırasıyla çerçeve kayması engelleyici bir adaptör ve pGFPuv vektöründen elde edilen, MAD geniyle yaklaşık aynı uzunlukta olan GFP geni yerleştirilmiştir. Sözü edilen "çerçeve kayması engelleyici adaptör" –kodon çerçevesini doğru diziye ayarlayan, histidin tutunucu dizisi ile parlayan GFP bağlayıcısını birleştiren küçük nükleotid dizisi– MAD geninin GFP geniyle değiştirilmesi sonucu oluşacak olan çerçeve kayması mutasyonunu engellemiştir. Ek olarak bu adaptör, fiziksel olarak çok esnek bir amino asit dizisini (Gly-Gly-Thr) kodlamak ve böylece GFP'nin solüsyon fazı karakteristiğini mümkün olduğunca korumak üzere tasarlanmıştır. Sonuçta elde edilen, GFPimm olarak adlandırılan "sabitleme adaptörü" protein konstrakt, ekspresyondan sonra hem Ni^{2+} çoklu histidin ilgi kolonu hem de iyon değiştirme kromatografisi kullanılarak izole edildi. İzolasyon sonrasında proteinin yüzeye bağlanma özellikleri, değiştirilmiş yüzeye sahip 96-kuyulu polistiren kaplar kullanılarak denendi. Bu çalışmada, çoklu histidine gruplarını yüzeye bağlamak için genelde kullanılan Ni^{2+} ile imidazol arasındaki koordinasyon bağının yerine histidinin artı yükleri ile yüzeydeki eksi yükler arasında tuz bağı oluşturma stratejisi uygulandı. Buna paralel olarak polistiren yüzey de persülfat kullanılarak eksi yüklü yüzey grupları taşımak üzere değişikliğe uğratıldı.

Bu çalışmada tasarlanan ve oluşturulan rekombinant vektörün sadece gen klonlama ve ekspresyonda değil, aynı zamanda kolay protein izolasyonu, hedef proteinlerin birleştirme yöntemi kullanılarak görsel işaretlemesi ve biyoteknoloji endüstrisinde kullanılabilecek özelleşmiş yüzey sabitleme uygulamalarında da işlevsel olacağı düşünülmektedir.

To my family
for
believing,
respecting,
trusting
and
supporting
my decisions and choices in life.

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ABBREVIATIONS:

APS: ammonium persulfate

Arg: Three letter code for arginine amino acid

Asp: Three letter code for aspartate amino acid

BL-21(DE3): An *E. coli* strain developed for high efficiency expression

BSA: Bovine serum albumin

cDNA: Complimentary DNA (synthesized from mRNA by reverse transcription)

DNA: Deoxyribonucleic acid

EDTA: Ethylene-diamine-tetra-acetic acid

ELISA: Enzyme linked immuno-sorbent assay

EMBL: European Molecular Biology Laboratories

FDA: U.S Food and Drug Administration

FPLC: Fast perfusion liquid chromatography

FT-IR: Fourier transform infrared region spectroscopy

GFP: Green fluorescent protein

GFPimm: In-house constructed green fluorescent protein with poly-histidine tag

GFPuv: A green fluorescent protein variant optimized for enhanced fluorescence (used for in-house purified GFPuv protein)

Gln: Three letter code for glycine amino acid

Glu: Three letter code for glutamate amino acid

Gly: Three letter code for glycine amino acid

GRAS: Generally recognized as safe

His: Three letter code for histidine amino acid

IMAC: Immobilized metal ion affinity chromatography

kDa: Kilodalton

Lys: Three letter code for lysine amino acid

MAb: Monoclonal antibody

MAD: Multiple wavelength anomalous dispersion

MCS: Multiple cloning site

mRNA: Messenger ribonucleic acid

OD_x: Optical density (absorbance) measured at "x" wavelength.

PAGE: Polyacrylamide gel electrophoresis

pETM-11: A poly-histidine fusion, expression vector.

pETM-Adp: In-house constructed intermediate plasmid.

pETM-GFP-Imm: In-house constructed, GFP and poly-histidine tag fusion vector specialized for easy immobilization and quantification of proteins.

pGFPuv: Commercially available recombinant vector coding for a GFPuv protein.

rGFPuv: Commercially available GFPuv. Used as standard in quantification measurements.

SDS: Sodium dodecyl sulfate

TAE: Tris-acetic acid-EDTA buffer

TBE: Tris-boric acid-EDTA buffer

Thr: Three letter code for threonine amino acid

Tyr: Three letter code for tyrosine amino acid

XL1 Blue: An *E. coli* strain developed for gene cloning

1. INTRODUCTION

Immobilization, as a strategy of general scope, has been incorporated into many established facets of biotechnology, particularly in situations where enzymes are used to mediate synthetic transformations in industry or facilitate diagnosis of some clinical cases by the help of certain kits used in medical examinations. For immobilizing the proteins the following technologies are currently being used:

Adsorption technology has the advantage that the protein-surface interaction is non-covalent and thus precludes any potentially disruptive chemical modifications associated with covalent immobilization, affording enzymes that often retain good, albeit perhaps altered biological activity, but with the common disadvantage of leaching of adsorbed enzymes from the surface and being lost in time. Another disadvantage, which is common in all non-site-specific immobilization methods described below is the low number of enzymes in the whole immobilized population having the correct orientation to leave the active site available. Although adsorption has the mentioned disadvantages, other major advantages include rapid and facile preparation of the material (Bickerstaff, 1997).

Encapsulation technology, like the above said adsorption technology, features many advantages associated with non-covalent protein-surface interactions, particularly the very native-like properties of matrix-enclosed enzymes. In addition, enzymes are easily encapsulated and well retained by the support. A related disadvantage however may be poor substrate accessibility both due to encapsulation and being randomly oriented as described in adsorption technology, leading to slowed kinetics or lost apparent activity in the case of large substrates (Bickerstaff, 1997).

Cross-linking technology features the advantages and disadvantages normally associated with covalent modification, one advantage being that protein is well retained on the support. A typical disadvantage of covalent modification is the possibility of loss of biological function as a result of chemical reaction or conditions imposed during the immobilization protocol. A potential advantage/disadvantage defined by context is the

alteration of biological function. In the case of cross-linking technology, another disadvantage is the waste of useful biocatalyst since clumps of enzymes are often bound together, intertwined with the support. Under such conditions, diffusional limitations may define the effective enzyme concentration to only those residing near the surface. Furthermore, the dynamic mobility of one enzyme may be impeded by virtue of being anchored to a neighbouring enzyme (Bickerstaff, 1997).

Covalent bonding, like above mentioned cross-linking technology, features the advantage of binding enzyme irreversibly to a surface. The observation of poor or altered activity has introduced modifications to the original method, in which a linker strand separating enzyme and surface imposes a distance constraint, thus restoring much native-like activity with possible exception to site-specific alterations normally associated with covalent modification of protein groups (edited by Gebelein, 1990). More often than non-covalent methods, the conditions required to achieve covalent bonding may require harsh conditions that lead to enzyme instability and deactivation (Bickerstaff, 1997).

Site specific immobilization using antibodies, uses the advantage of recombinant DNA technology for fusing the enzyme of interest to an antibody while the surface is coated with the specific anti-antibody. Mostly, biotin-streptavidin couple is used for this purpose. As an advantage over non-site-specific methods of immobilization described above, in this method the enzyme is properly oriented so that active site accessibility is higher (reviewed by Butterfield *et al.*, 2001). Although this strategy overcomes undesired effects on enzymatic activity caused by the harsh chemical treatment and randomly oriented immobilization, it is rather tedious to form both a fusion protein and an anti-antibody coated surface since the strategy indeed is composed of more than one immobilization together with monoclonal antibody synthesis, just to immobilize one target protein.

Although benefits of immobilization are case specific, some reported advantages include prevention of enzyme loss in continuous systems, increased thermal or pH stability, increased longevity, and under certain circumstances higher activity (Smith, 1996; Hermanson *et al.*, 1992; edited by Cass *et al.*, 1998). Immobilization as a practice has undergone substantial change in response to the demands of the industry. Once considered a relatively crude and non-specific method, modern immobilization protocols strive to realize optimum levels of homogeneity, orientation and loading efficiency of enzyme along

the surface of the support medium. To characterize immobilization efficiency, enzyme assays relating activity to concentration are typically employed and interpreted using the oversimplified approximation that the biomolecule is equally active on a surface as it is in solution. In reality, activity in solution-phase and on a surface may be very different. As might be anticipated, any substantial digression from the native state, such as an over-modification of the protein environment, has strong influence on the apparent kinetic constants that relate to protein function. That being said, enzyme loading is arguably one parameter of paramount importance from which product quality –quantity and purity– of immobilization-based commercial syntheses are gauged. With due consideration to the risk of digressing from native-like activity upon immobilization, the task of achieving an accurate enzyme quantification on the basis of solution-phase literature values is wrought with potential errors and experiment-related difficulties.

Quantification of surface bound protein is a common problem in immobilization practices. Direct and indirect methods -such as activity measurements on surface and quantification of unbound protein in solution- are employed to quantify surface bound proteins (Bickerstaff, et al., 1997; Sardar *et al.*, 1997). In direct methods, the activity of the biomolecule is (over-)assumed to be similar to solution phase in order to measure the surface-bound protein amount by an activity based assay. In indirect methods, unbound protein is subtracted from the protein in the initial load to quantify the bound protein. The indirect method requires very dilute protein surface-loading solutions in order to keep the amount in a range where the correlation between protein amount and the assayed parameter is linear.

In extrapolating the potential problems of protein quantification to medical diagnostic kits, biocompatible blood materials, specialty cell culture surfaces bearing signal peptides, and any scenario where biopolymers are used to improve surface properties of specialty plastics, glasses or other negatively charged surfaces, it is noteworthy that many of these biopolymers bear no convenient means of selective immobilization and unlike enzymes, bear no means of convenient quantification, thus posing increased difficulties in estimating their immobilization efficiency. In order to facilitate protein immobilization and purification and to overcome several immobilization-related problems such as the activity loss due to harsh chemical treatments during immobilization and potentially misleading

assumption of unaltered biological activity when immobilized, we have designed a multi-component immobilization/visual detection system that can be applied to proteins, peptides and enzymes in any facet of bio-surface engineering. The approach is ammenable to any protein and is novel in that proteins are expressed ready-made for immobilization, featuring good surface separation to mimic native-like conditions, and surface retention reminiscent of covalent methods, without necessitating the use of chemical reagents in the presence of the protein. The approach is based on five elements:

1. GFP: Fusion protein strategies incorporating the green fluorescent protein facilitate localization of proteins in situ. In the approach explained here, fusion protein strategy is specifically used to visualize and quantify the immobilized enzyme.

2. His-tag: Multiple histidine residues in series are used for selective purification of recombinant proteins. Unlike established purification methods based on His-tag interactions, here, no metal ion is required to enable the immobilization event. Immobilization is achieved through a direct interaction between the surface-pendent carboxyl groups and the imidazole moieties of a poly(6)histidine tag that has been engineered N-terminal to GFP. It follows that the technique can be extended to any peptide tag as long as the surface is designed to bear affinity for it.

3. Cleavable linker: A linker bearing chemical specificity cleavage upon addition of the appropriate external agent. The system includes a TEV (Tobacco Etch Virus) protease recognition site to permit easy release of immobilized peptide from the surface into the solution phase following proteolytic cleavage.

4. Multiple cloning site: A special sequence that has the property of being cut by sequence specific endonuclease enzymes. MCSs are typically used to insert and clone/express foreign target genes in a host organism. In our recombinant vector, the multiple cloning site of pETM-11 was used for inserting GFPuv gene into the modified pETM11 vector and resulting MCS downstream of the GFPuv gene was preserved in order to provide space for the gene of peptide to be immobilized.

5. Carboxylated polymer support media: Carboxylated surfaces are used in reversible/irreversible surface immobilization of positively charged species as well as for

hydrogen bonding species. Here, binding is achieved using persulfate-modified, carboxylated polymeric surfaces.

Similar studies, which bring GFP and immobilization together, were performed. Among these studies the ones that are closest to the one presented here involve the purification (immobilization) of GFP fusion proteins using poly-histidine tag (Wu *et al.*, 2002), immobilization of GFP using poly-amino acid tags and quantification of bound GFP (Nock *et al.*, 1997). Despite the similarities with these studies, our approach is novel in the way that it defines a modular recombinant vector designed for and principally capable of immobilizing any protein provided that its gene is cloned. In addition to the achieving easy immobilization, immobilized enzyme can be quantified by an activity-independent assay based on fluorescence measurements. This is very important since in activity based quantification strategies; the enzyme is is bravely assumed to be as active as in solution and compared to the activity of same enzyme in solution as the standard. However binding the enzymes onto solid supports have a hard-to-ignore potential in alteration of activity.

Another novelty of the strategy is the flexibility of the system. Each above-mentioned element of the designed vector can be replaced in order to use in modified case specific conditions. GFP can be changed with BFP, YFP, RFP or similar fluorescent peptides to independently quantify different immobilized peptides where more than one is involved. Poly-histidine tag can be replaced with other poly-amino acid tags such as negatively charged or ring containing amino acids in order to use with alternatively tailored surfaces. Frame adapter can be replaced with longer or different sequence containing alternatives. Multiple cloning site can be modified to have additional restriction enzyme recognition sites as well as endless possibilities for proteins for immobilization.

In addition to realizing easy and accurate quantification of immobilized peptides, the approach also provides increased mobility and separation of the target protein from the surface through linkage via GFP and a flexible Gly-Gly-Thr frame adapter peptide. It is also noteworthy that the immobilization event is likely to occur at only one point, thereby minimizing the risk of adverse functional consequences. Although any enzyme gene was not inserted within MCS of pETM-GFP-Imm specialty vector for immobilization in this study, above-indicated factors should promote retention of native-like characteristics rather than altered, surface-bound kinetics, which may restrict enzyme action. A his-tag based

immobilization also promotes uniform orientation along the surface, a definite advantage in comparison to established immobilization technologies.

In this study we aimed to construct a specialty recombinant plasmid coding for fusion proteins for immobilizing any peptide (e.g. signal peptides, enzymes) provided that their genes are cloned. In parallel to the plasmid construction, polystyrene surfaces were modified so that they will interact with the expressed proteins coded by the recombinant plasmid mentioned above. The use of immobilization specialized fusion proteins gives the opportunity to easily visualize and quantify immobilization efficiency using a fluorescence-based method instead of an activity-based assay. In addition, targeted outcome of this project included the development of an enzyme-friendly immobilization system in which the enzymes are not subjected to harsh chemical immobilization methods that result in important loss of enzymatic activity. Instead the peptides and the surfaces would be independently prepared for immobilization process.

Although the system is not characterized in the presence of an enzyme for the criteria of activity, specificity and stability; it follows that by using an optimal combination of the above strategies the drawbacks of classical enzyme and/or protein immobilization and efficiency detection methods can be overcome.

2. OVERVIEW:

In this thesis a new application of well-known tools of molecular biology to solving a problem in the chemical industry is described. The strategy developed for immobilization and quantification of enzymes involves several technologies including construction of a recombinant vector, design and production of a modified surface, usage of an immobilization tag and the modified surface, and the use of a fluorescent probe for visualisation and quantification. This brief overview to different aspects of technologies involved aims to provide background information to the work presented in the later chapters.

2.1 Uses of Enzymes by Mankind: A Brief History Until Their Modern Use as Fixed Machines in Production Lines

Although a great number of synthesis reactions may be achieved only by using information from the field of chemistry, in some cases mankind – knowingly or not – has used an alternative source of information to perform certain chemical reactions: the genetic information coded in the DNA. This particular information is the one necessary to code for efficient and highly selective chemists of nature, namely the enzymes. Advantages and disadvantages of using these different materials and sources of information for synthesis reactions are given in *Table 2.1*.

Table 2. 1 Advantages and disadvantages of biological syntheses in comparison to chemical syntheses.

Advantages	Disadvantages
- Production of complex molecules such as proteins and antibodies is not possible by chemical means. - Bioconversions give higher yields when considered with the product homogeneity - Operate at low temperature, near-neutral pH. - Have much greater catalytic specificity. - Bioprocesses are extremely successful in obtaining necessarily one form of isomeric products.	- Can be easily contaminated with unwanted microorganisms, etc. - Product is usually in a complex mixture requiring separation. - Involve providing, handling and disposing of large volumes of water. - Are generally slower than chemical processes. - Are expensive. - May necessitate advanced protocols for waste treatment.

2.1.1 Use of Enzymes in Human Related Applications: On the Way to Modern Biotechnology

Enzymes, without being recognized, have been used for centuries in applications such as cheese production, brewing and bread making. It is known that methods for the fermentation of milk products, meat and vegetables were described in earliest records dating back to 6000 B.C. by civilizations (e.g. Sumerians, Babylonians) of the Fertile Crescent in the Middle East (reviewed by Caplice *et al.*, 1999; Smith, 1996). These are followed by the records about mold-fermented foods in China and beer brewing and bread making combined in Egypt (reviewed by Colwell, R.R., 2002). In more literary sources, such as the famous Greek epic poems "The Odyssey" and "The Iliad" (~700 B.C.), descriptions of processes involving the use of enzymes in cheese making can be found. These examples show that, well before rationalization of the process, enzymatic

conversions of raw materials were empirically being used for especially food related synthesis reactions.

Industrial use and understanding of enzymes revolved independently around yeast and malt in the west, and around sake production and many food fermentations in the east. In 1896, presumably the first enzyme patenting and an important fermentation related technology transfer from East to West occurred by the marketing of *takadiastase*. This was a crude mixture of starch hydrolysing enzymes obtained by growing the fungus *Aspergillus oryzae* on wheat bran, which was well known and used for thousands of years in Asia. Approximately in the time, between 1857 and 1876, fermentative ability of bacteria was demonstrated by Pasteur (Smith, 1996).

Leather processing, –in particular the softening process (bating) in which structural components such as collagen that give rigidity and stability to the unmodified leather were removed– was a hard-to-tolerate application since dog feces and pigeon droppings were used in large quantities. After Otto Röhm –a distinguished German chemist– has discovered in 1905 that active components of this process are in fact some protein degrading enzymes which can alternatively be found in some animal organs, pig and cow pancreases became more "acceptable" and reliable sources of these enzymes and leather processing became tolerable.

In the 1940s, mass cultivation of microorganisms in contaminant-excluding sterile conditions was introduced to have products such as vaccines, antibiotics, polysaccharides. After the use of microbial enzyme resources around mid-1950's, enzyme technology was revolutionized. The main reasons for this jump were as follows:

- The war industry that fed World War II improved the knowledge about large-scale cultivation of microorganisms together with enzyme and/or product harvesting (e.g. penicillin production).
- The rapidly increasing knowledge on the properties and mechanisms of enzymes led their use in industry for their catalytic properties.

- Recombinant DNA technology provided new materials and methods that either increased production efficiency or changed previously impossible processes to routine practices.
- Microorganisms were convenient sources of most potentially industry-related enzymes.

As a result, enzymes found a wide range of applications such as

- production of biological detergents (primarily proteases and amylases),
- baking industry (e.g. amylases),
- brewing industry (e.g. amylases, glucanases, proteases),
- dairy industry (e.g. proteases such as rennin and chymosin, lipases and lactases),
- starch industry (e.g. amylases, amyloglucosidases, glucoamylases, glucose isomerase),
- textile industry (e.g. amylases),
- leather industry (e.g. trypsin),
- medical, pharmaceutical and diagnostic uses (e.g. ELISA tests) (Smith, 1996).

Rationalization of empirical biotechnological applications by the discovery and effective use of enzymes in industry was followed by development of methods for extraction of them from producer microorganisms. Cell-free enzymes were recognized to have a number of advantages over the use of microorganism containing media (Smith, 1996):

- Avoiding conversion of a large amount of substrate into biomass as in the cases where microorganisms were used.
- Prevention of wasteful side reactions caused by different metabolic events within the same microorganism.
- Overcoming the possibility of incompatibility of condition optima for microorganism growth and product formation.
- Ease of product purification when compared to microorganism containing bulk fermentation liquor.

In addition to the developments mentioned above, recombinant DNA technology and protein chemistry studies led to modification of economically important enzymes in order to

- enhance the enzymatic activity,
- improve the stability,
- permit the enzyme to function in a non-optimum environment,
- change pH and/or temperature optima,
- alter specificity so that different substrates may be used,
- enhance the efficiency of process. (Smith, 1996)

Further improvement in strategy involves applications where immobilized enzymes are used, which is discussed in Section 2.1.2.

2.1.2 Immobilization of Enzymes on Surfaces:

The discovery of the "active factors" of ancient industrial processes was followed by the alternative of using cell-free enzymes rather than whole-cells or enzyme containing complex media. Due to developments in protein purification area, an important progress towards enzyme use in industrial and synthesis related applications is achieved.

In most solution-phase reactions, the enzyme used cannot be recovered, and is wasted at the end of the reaction. Immobilization on insoluble supports such as polymeric membranes or particles is carried out mainly in order not to lose the enzyme in synthesis reactions. Since enzymes are physically restricted in this method, it is possible to reuse the immobilized molecules without losing them into the solution. Some reported common advantages immobilization regardless of the technique used are (Smith, 1996; Hermanson *et al.*, 1992; edited by Cass *et al.*, 1998):

- Permits reuse of biocatalysts used in the reaction,
- Reactions may be run with a small amount of enzyme,
- Ideal for continuous operation,
- No product contamination with the enzyme used,
- Permits better control of catalytic processes,

- Prevents autocatalysis in case the enzyme is a protease,
- Improves stability of enzymes especially in extreme conditions,
- Allows construction of multi-enzyme production lines,
- Has high potential in industrial and medical use,
- Since effluent is enzyme free, product purification from the enzyme containing media and disposal problems are overcome.

Along with its advantages, the strategy also has disadvantages concerning the relatively rigid positioning of enzymes on supports. Even though the bulk properties of the support may not have inhibitory effects, the structural properties may be negatively affected by the surface properties of support material, as it is perfectly stated by Enrico Fermi in the expression: *"God made the solid state. He left the surface to the Devil."* (Cass *et al.*, 1998)

Because the disadvantages of enzyme immobilization studies are rather method specific, those will be discussed within the sections about each immobilization strategy. To highlight generally, the common point in those disadvantageous cases is the possibility of alteration of chemical and/or physical properties of active site of enzymes or its accessibility (Hermanson *et al.*, 1992).

In enzyme immobilization practices, the ideal support material and method of immobilization varies greatly with characteristics of the enzyme, nature of substrates and products and reaction conditions used in biocatalysis. Thus, selection of support material and the method of immobilization is not a standardized procedure; instead, it is done by considering the advantages/disadvantages of properties, limitations and characteristics of several combinations of methods and supports. Some criteria to consider in this selection are provided in *Table 2.2* (edited by Bickerstaff, 1997):

Table 2. 2 Important criteria in selecting support and method of immobilization (edited by Bickerstaff, 1997).

Property	Points for consideration
Physical	Strength, noncompression of particles, available surface area, shape/form (beads/sheets/fibers), degree of porosity, pore volume, permeability, density, space for increased biomass, flow rate, and pressure drop
Chemical	Hydrophilicity (water binding by the support), inertness toward enzyme/cell, available functional groups for modification, and regeneration/reuse of support
Stability	Storage, residual enzyme activity, cell productivity, regeneration of enzyme activity, maintenance of cell viability, and mechanical stability of support material
Resistance	Bacterial/fungal attack, disruption by chemicals, pH, temperature, organic solvents, proteases, and cell defense mechanisms (proteins/cells)
Safety	Biocompatibility (invokes an immune response), toxicity of component reagents, health and safety for process workers and end-product users, specification of immobilized preparation (GRAS list requirements for FDA approval) for food, pharmaceutical, and medical applications
Economic	Availability and cost of support, chemicals, special equipment, reagents, technical skill required, environmental impact, industrial-scale chemical preparation, feasibility for scale-up, continuous processing, effective working life, reuseable support, and CRL or zero contamination (enzyme/cell-free product)
Reaction	Flow rate, enzyme/cell loading and catalytic productivity, reaction kinetics, side reactions, multiple enzyme and/or cell systems, batch, CSTR, PBR, FBR, ALR, and so on; diffusion limitations on mass transfer of cofactors, substrates, and products

CRL: calculated risk level, CSTR: continuous stirred tank reactor, PBR: packed bed reactor, FBR: fluidized bed reactor, ALR: air lift reactor.

Considering the above criteria, which are widely branched within themselves, mainly four methods (based only on chemical and/or physical phenomena) are used for random immobilization of enzymes (edited by Bickerstaff, 1997). As schematically indicated in *Figure 2.1*, these are:

1. adsorption,
2. covalent binding,
3. entrapment/encapsulation,
4. crosslinking.

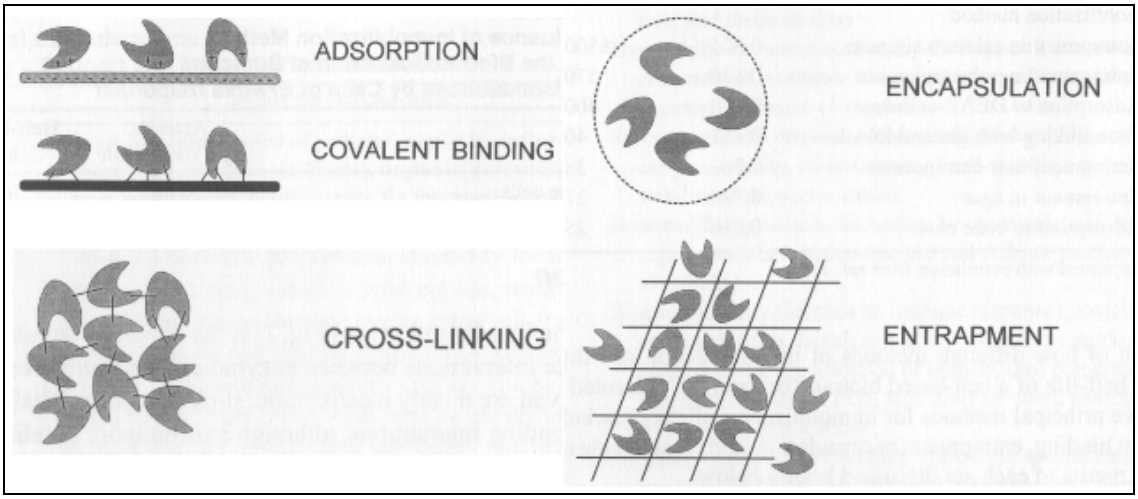


Figure 2. 1 Random immobilization methods using only chemical and/or physical phenomena (edited by Bickerstaff, 1997).

In addition to these techniques in which enzymes are immobilized regardless of their active site orientation, there are applications where immobilization takes place directionally. Such site-specific immobilization studies will also be discussed together (as 5th method of immobilization) with random immobilization techniques in the following sections.

2.1.2.1 Adsorption:

Adsorption is the simplest method of immobilization that uses reversible surface interactions such as electrostatic (i.e. van der Waals, ionic and hydrogen bonding) and hydrophobic (e.g. base stacking) bonding. Although these forces are individually weak for effective binding, they are sufficiently large in number within a large protein molecule and on a practically infinite surface. In this strategy no activation or modification of the enzyme is used; instead, the natural interaction between the enzyme and support results in little or no damage to the biological material. The binding efficiency is typically under the influence of parameters such as pH and ionic strength of the buffer in which the enzyme and surface are incubated together (edited by Bickerstaff, 1997; edited by Mosbach, 1987).

The basic advantages of the technique are (Smith, 1996; edited by Bickerstaff, 1997):

- Little if not none damage is given to the biological component of the composite system,
- The technique is easy and cheap as well as the application procedure being fast.
- Neither the enzyme's nor the support material's properties are altered by chemical reactions.
- Provided the appropriate buffer properties, binding is reversible so that the support can be reloaded with fresh enzymes.

Possible disadvantages of the technique include (Smith, 1996; edited by Bickerstaff, 1997):

- Product contamination by leaked enzymes due to the weak nature of adsorption interactions,
- Nonspecific binding (distinct from orientation),
- Overloading of the support,
- Negative effect on enzymatic activity due to steric hindrance, since the enzyme is directly stuck on the support.

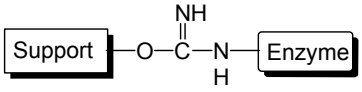
The most important problem among those is enzyme loss due to desorption. Desorption can occur due to several reasons such as pH, temperature, ionic strength, flow rate, bubble agitation, particle-caused abrasion and scouring on vessel walls, which are all under the topic of environmental circumstances within the reaction medium. These problems may be due to binding of substrate or contaminant in substrate, formed product or other conditions that cause changes in protein conformation and ionization status of exposed amino acids on the protein.

In case the substrate, the product or any contaminants are charged, non-specific binding can be a problem. Non-specific binding can lead to diffusional and reaction kinetics problems. In addition, binding of protons (or acids or bases of different definitions) can result in alteration of pH microenvironment near the surface resulting in pH shifts of 1-2 units. Enzymes having strict and narrow pH optima can be affected by the situation (edited by Bickerstaff, 1997). Overloading and absence of a spacer in adsorption can result in activity problems due to steric hindrance.

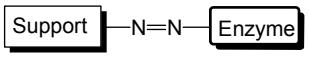
2.1.2.2 Covalent binding:

In this strategy, functional groups on the support and on the amino acids at the surface of the enzyme to be immobilized are covalently bound to each other. Commonly used surface functional groups of enzymes are amino (NH_2) group of lysine or arginine, carboxyl (COOH) group of aspartic acid or glutamic acid, the hydroxyl (OH) group of serine or threonine, and the sulfydryl (SH) group of cysteine. Although many factors are worth consideration in support selection for covalent immobilization, hydrophilicity appears to be the most important property, increasing the popularity of polysaccharide polymers (e.g. cellulose, dextran, starch and agarose) (edited by Bickerstaff, 1997, edited by Mosbach, 1987). Due to the presence of hydroxyl residues on sugar groups, polysaccharides are suitable for surface activation by chemical reaction preparative to covalent bonding. Another advantage of the presence of hydroxyl groups is increased hydrophilicity. Hydroxyl groups interact with water molecules via hydrogen bonding, resulting in an aqueous microenvironment on/in the support. However, polysaccharide polymers are susceptible to bacterial/fungal attacks. Some alternative materials for use as supports are different types of porous silica and porous glass. Physical properties (e.g. pore size) of those different types are adjusted by optimizing conditions such as tempering parameters, burning or solving the contaminants that are introduced in production in order to obtain materials with desired characteristics. Most reactions involving the formation of covalent linkage between enzymes and supports can be categorized under the following main groups (edited by Bickerstaff, 1997; edited by Mosbach, 1987):

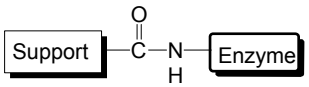
1) Isourea linkage:



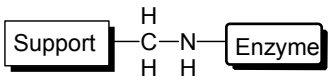
2) Diazo linkage:



3) Peptide bond:



4) Alkylation reaction:



Basically, covalent bonding is performed by a two-step process. In the first step, surface is activated by a specific reagent to form strongly electrophilic (electron-deficient) functional groups. In the binding reaction, preformed electrophilic groups readily react with nucleophilic (electron-donating) amino groups on the surface of the enzyme to form covalent bonds. In order to retain enzymatic activity, it is important to design a coupling that does not involve functional groups of aminoacids that are located at the active site and critical for biocatalysis.

Using the possible activation reactions, a large number of polysaccharide derivatives such as AE-cellulose (aminoethyl: $-C_2H_4-NH_2$), CM-cellulose (carboxymethyl: $-CH_2-COOH$), DEAE-cellulose (diethylaminoethyl: $-C_2H_4-N-(C_2H_5)_2$) were developed and marketed for various applications (edited by Bickerstaff, 1997; edited by Jakoby *et al.*, 1974; edited by Mosbach, 1987).

The most important advantage of the technique is (Smith, 1996; edited by Bickerstaff, 1997; edited by Mosbach, 1987):

- The quality of linkage between biomolecule and surface is not affected by environmental conditions such as pH or ionic strength.

The main disadvantages of covalent binding strategy are (Smith, 1996; edited by Bickerstaff, 1997; edited by Mosbach, 1987):

- The technique may be destructive in terms of enzymatic activity since the functional groups of the enzyme are altered.
- Should be very well designed in order not to alter functional groups at the active site.
- Irreversibility of the immobilization strategy results in difficulty in replacement of exhausted enzyme with fresh enzyme.
- Expensive, time consuming and risky when compared to adsorptive method.

2.1.2.3 Entrapment/Encapsulation:

In this method of immobilization, enzymes are free in solution and their movement is only restricted by the presence of a surrounding network, differing this method from adsorption and covalent binding. The pore size of the network is optimized to a value that is large enough to allow free substrate and product movement but tight enough to prevent leakage of enzymes (edited by Mosbach, 1987). The major methods of entrapment are (edited by Bickerstaff, 1997):

1) Ionotropic gelation of polyionic macromolecules with multivalent cations (e.g. alginate)
2) Temperature induced gelation (e.g. agarose, gelatin)
3) Organic polymerization by chemical/photochemical reaction (e.g. polyacrylamide)
4) Precipitation from an immiscible solvent (e.g. polystyrene)

Encapsulation is identical to entrapment except that the envelope surrounding the enzyme is also free in solution itself. Many materials such as nylon and cellulose nitrate are used to construct microcapsules around 10-100 μm in diameter. Use of biological structures such as liposomes and even cells as capsules are also reported. As an example where cells are used, erythrocytes are swollen in enzyme containing hypotonic solution allowing diffusion of enzymes into the erythrocyte, followed by transfer into hypertonic medium, thereby trapping enzymes in the cell (edited by Mosbach, 1987; edited by Bickerstaff, 1997).

In case of entrapment, the main advantages are (Smith, 1996):

- The enzyme is not the target of a chemical modification (although it may be subjected to).
- Enzyme is relatively free in motion when compared to adsorption and covalent binding.
- Enzyme is kept away from microbial and proteolytic action (increased enzyme stability and half life)

While, the disadvantages are (Smith, 1996; edited by Bickerstaff, 1997; edited by Mosbach, 1987):

- Diffusion of substrate to, and product from the active site; thus not effective for macromolecular substrates and products. In applications where microcapsules are used, this problem may even result in rupture of semipermeable membrane that forms the capsule.
- Preparation is tedious and can result in enzyme inactivation depending on the entrapment technique employed. Extensive effort is needed for optimization of pore size necessary to allow diffusion of substrates and products but prevent leakage of enzymes.
- Enzymes are kept away from microbial and proteolytic action (replacement of exhausted biocatalyst with fresh batch without disintegration of support is not feasible)
- In case of ionotropic gelation, the presence of multivalent cations may affect activity of enzymes that are members of protein families using multivalent metal cations as cofactors.
- In case of temperature driven gelation, the gels formed are mostly soft and unstable and this method introduces the possibility of enzyme inactivation due to incubation in variable temperature environment, which is necessary for gelation.
- In case of organic (chemical/photochemical) polymerization, although they are not main targets of reaction, the enzymes are subject to treatments with harsh chemicals that are either highly reactive monomers or strong oxidizing/reducing agents for initiation of reaction by free radical formation. Such an exposure can result in inactivation of enzymes with considerable possibility.
- In case of precipitation by phase separation, the enzymes are brought in contact with organic solvents. Mostly enzymes are not tolerant to such solvents resulting in enzyme inactivation. Thus, usage of this method is limited to highly stable or artificially stabilized enzymes.

2.1.2.4 Crosslinking:

Crosslinking does not involve the use of a support of any kind. Instead, enzyme movement is restricted by binding enzymes to one another, resulting in a bulk cluster of enzymes that are not free in solution. Both chemical and physical methods can be used to achieve crosslinking (edited by Bickerstaff, 1997).

Chemical crosslinking involves the use of bi- or multi-functional reagents such as glutaraldehyde and toluene diisocyanate forming a covalent linkage between enzymes (edited by Bickerstaff, 1997; edited by Mosbach, 1987).

Physical crosslinking can be achieved by the use of flocculating agents that introduce tangling of macromolecules, meanwhile trapping the enzymes. Polyamines, polyethyleneimine, polystyrene sulfonates and various phosphates are reported to be successful flocculating agents (edited by Bickerstaff, 1997).

Chemical crosslinking is not generally selected as an immobilization strategy alone, but mildly used as a supportive tool for enhancement of enzyme retainment in entrapment/encapsulation procedures. Main disadvantages resulting in this limited use are (edited by Bickerstaff, 1997):

- Enzymatic activity loss due to highly toxic reagents used in crosslinking strategy.
- Severe diffusional limitations for the molecules at the core of the enzyme clot formed by crosslinking.
- High levels of enzyme inactivation due to very restricted molecular movement of enzymes, which is essential for some enzymes where conformational transformation is needed for catalysis.
- Poor mechanical stability as a result of absence of polymeric support.

2.1.2.5 Site specific immobilization:

A major disadvantage of random immobilization techniques mentioned above is that the activity of the immobilized enzyme is often significantly decreased. Reasons for this decrease are mainly the blockage of active site, multiple-point binding causing limitations to the conformational flexibility of enzyme, or denaturation of the enzyme. These disadvantages can be overcome by employing site-specific immobilization (*Figure 2.2*).

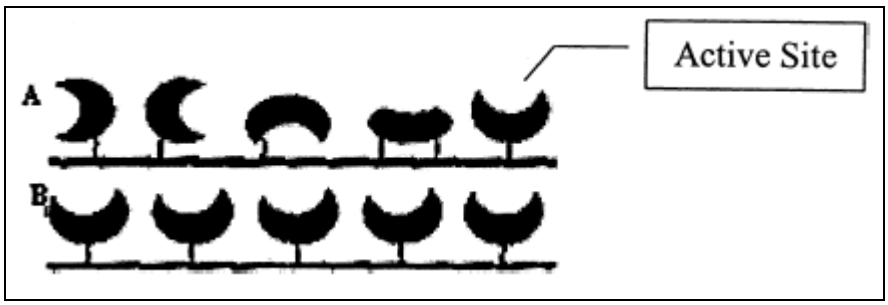


Figure 2. 2 (A) Random immobilization of enzymes. (B) Site-specific immobilization of enzymes where an enzyme coated surface with active sites properly oriented for access of substrates is achieved (Butterfield *et al.*, 2001).

Strategies employing tools of molecular biology may be used to engineer recombinant enzymes which can be oriented with easy access of the active site on surfaces.

Three approaches to site-specific immobilization of enzymes are:

a) Gene fusion to incorporate a peptide affinity tag at the N- or C-terminus of the enzyme. The enzymes are then attached from this affinity tag to anti-tag antibodies on membranes (*Figure 2.3*);

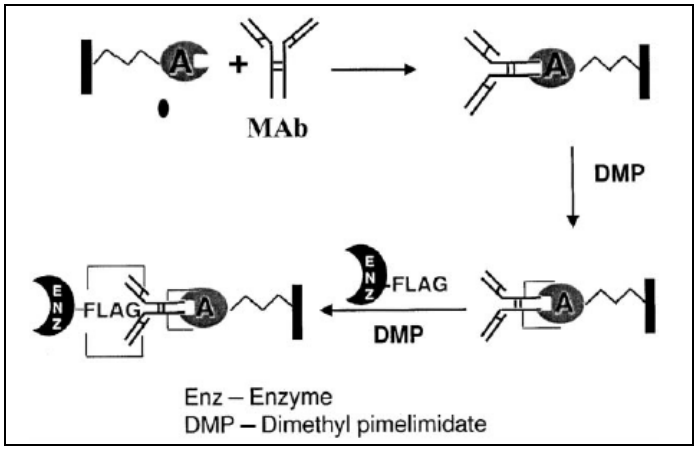


Figure 2. 3 Site-specific immobilization of enzymes using fusion protein technology. The DNA sequence specific for the octapeptide FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) is added to the target enzyme at N- or C-terminus. After expression and purification, followed by addition of the complex to membrane, protein A with a high affinity for IgG antibodies, is immobilized. MAb specific to FLAG is added in turn. In the next step, dimethyl pimelimidate (DMP) is added to stabilize the anti-FLAG-protein A complex. Addition of the FLAG-enzyme fusion complex results in proper orientation of the enzyme with accessible active site (Butterfield *et al.*, 2001).

b) Post-translational modification to incorporate a single biotin moiety on enzymes. Enzymes can be attached to membranes through a (strept)avidin bridge (*Figure 2.4*);

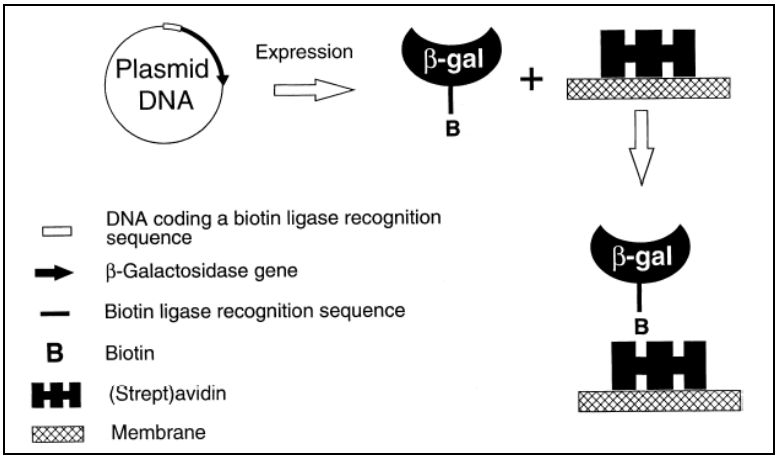


Figure 2. 4 Site-specific immobilization of enzymes (in this case β-galactosidase) using recombinant DNA technology. A biotin ligase recognition sequence is added to the gene of target enzyme in the plasmid. Subsequent expression and purification of the enzyme containing a N-terminal domain biotin allows specific addition of this complex to (strept)avidin previously immobilized onto the membrane (Butterfield *et al.*, 2001).

c) Site-directed mutagenesis to introduce unique cysteines to enzymes:

Enzymes are attached on thiol-reactive surfaces through the sulfhydryl group on the side chain of the introduced cysteine, which is located on the opposite side of the protein from the active site (*Figure 2.5*).

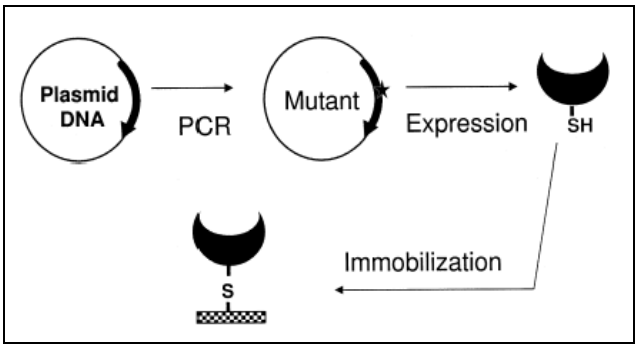


Figure 2. 5 Site-specific immobilization of enzymes using site-directed mutagenesis. Addition of a cysteine amino acid opposite to the active site results in a functional group that is convenient for a subsequent binding reaction with the surface containing a SH functional group (Butterfield *et al.*, 2001).

In all these methods, the active sites of the immobilized enzymes face away from the polymeric surface and resulting in higher activity (Butterfield *et al.*, 2001).

2.1.3 Applications of Immobilized Enzymes/Proteins/Cells:

Although all immobilization practices were reviewed from an enzyme-centered perspective, above-mentioned strategies are applicable to any protein (e.g. antibodies, antigens) or whole-cells (production of biofilms) for applications involving (edited by Cass *et al.*, 1998; edited by Mosbach, 1988, edited by Doyle, 1999):

- synthesis,
- biosensors,
- biochips,
- analysis,
- diagnosis (like in enzyme based diagnostic kits such as ELISA),
- arraying and orienting,
- stabilisation,
- binding,
- signal transduction,
- implant passivation and surface biocompatibilization,
- surface activation,
- separation/purification,
- detoxification and scavenging.

2.2 Recombinant Vector Construction: Ordering Cells What To Synthesize

The discovery of standardized method for transformability of bacteria with nonchromosomal genetic information (Cohen *et al.*, 1972) followed by convenient, successful insertion of target genes into those nonchromosomal information carriers – plasmids – (Cohen *et al.* 1973) signaled the birth of recombinant DNA technology and the start of genetic engineering era (Lander *et al.*, 2000, Singer, 2003). Since then, a large number of plasmids and even artificial chromosomes were constructed *in vitro* and used to

transfer genetic material in order to synthesize recombinant proteins. Plasmid families coming from same ancestors were obtained and new families were formed by novel combinations of preformed families. First recombinant plasmid mediated transformation involved the insertion of ampicillin resistance trait by DNA fragments from p1258 plasmid of *S. aureus* to pSC101 plasmid of *E. coli* and transformation of *E. coli* cells with the modified pSC101 (Chang *et al.*, 1974). This impressive progress in genetics was followed by the successful insertion of a eukaryotic gene fragment from the African clawed toad *Xenopus laevis* into *E. coli* by using a plasmid construct which was the first eukaryotic-prokaryotic "chimaeric" molecule (Morrow *et al.*, 1974), having the name from a beast in Greek mythology, with a lion's head and foreparts, a goat's middle, a dragon's rear, and a tail in the form of a snake; an apparent hybrid of two or more creatures. After the initiation of the use of modified plasmids for gene insertion purposes, some essential properties – such as recombinant selection – were introduced by modifying the vectors using restriction digestion and ligation protocols. Those new assignments started the trend of "vector design and construction for gene cloning". During those studies the pBR322 plasmid, which is the ancestor of a great number of modern plasmids, was constructed by a total of 8 successive steps of restriction cut / ligation processes performed on preexisting and process-formed plasmids (Bolivar *et al.*, 1977). This plasmid then led to the construction of series of modified plasmids, with the following improved functions (Primrose *et al.*, 1994):

- Vectors to facilitate expression of proteins,
- Vectors for the identification of regulatory signals,
- Vectors for the direct selection of recombinants,
- Vectors with additional restriction sites,
- Vectors with different, additional or improved selective markers,
- Vectors with increased stability,
- Vectors with altered copy numbers,
- Plasmids for DNA sequencing,
- Plasmids to permit secretion of proteins,
- Gene fusion vectors to facilitate protein purification and increase stability of mRNA and/or protein of interest,
- Vectors for use in both *E. coli* and unrelated organisms (shuttle vectors)

- Vector couples for investigation of protein-protein interactions by two-hybrid screening.

The progress on construction of gene delivery and expression vectors continued with development of nonplasmid vectors such as (Primrose *et al.*, 1994):

- Phagemids (having phage and plasmid components) particularly for having single stranded DNA for easy dideoxy-sequencing,
- Phage vectors and in vitro packaging into phages, for facile cDNA library construction,
- Cosmids (having plasmid components and *cos* viral sequence enabling in vitro packaging of plasmids), for increasing the available accommodation for insert to 32-47 kb,
- Phasmids (having phage attachment site and plasmid components), especially for effectively infinite shelf life of vector and cleaner recombinant screening results when compared to bacterial colony selection,
- Single stranded viral vectors (such as M13 vector), particularly useful for large insert accommodation, being continuous DNA source (host is not lysed as in phages, growth is retarded), easy dideoxy-sequencing of DNA of interest, being ideal for oligonucleotide directed mutagenesis and probe preparation practices.

As a consequence of specialization in vector designs, gene therapy practices that uses even modified retroviruses (such as human immunodeficiency virus, HIV) as gene delivery agents have emerged (reviewed by Somia et al., 2000).

Recombinant vectors are being designed and constructed not only for cloning and expression but also for specialty purposes such as easy purification, visual tagging by fusion and performing expression in different organisms. In addition to the research oriented uses of recombinant vectors, special-purpose vectors are continuously being constructed especially for biotechnology and healthcare industries.

2.3 Polyhistidine and Similar Affinity Tags: A "Sticky" Business

As a result of developments in the fields of recombinant vector construction and specialty resin synthesis, a new approach emerged in chromatographic separation technology: the use of differences of affinities between molecules as a criterion for separation. In affinity chromatography, a molecule, known as ligand, which specifically binds to the protein of interest, is covalently attached to an inert and porous matrix (Voet *et al.*, 1995). When an impure protein solution is passed through this chromatographic material, the desired protein binds to the immobilized ligand, whereas other substances are washed through the column with the buffer. The desired protein can then be recovered and purified by changing the elution conditions such that the protein is released from the chromatographic matrix. As a prerequisite for such a system for either separation or isolation of proteins, appropriate fusion vector / specialty resin couples had to be designed (e.g. streptavidin/biotin, glutathione-S-transferase/glutathione). Polyhistidine tags, FLAG-tags, thioredoxin, protein A, maltose-binding protein and cellulose-binding domain can be listed as the examples of other most frequently used fusion tags (Dian *et al.*, 2002). In most of those systems, since the resin also had biological molecules as ligands, the storing conditions are limited and the shelf life is relatively short.

Within the context of affinity chromatography, a different approach called immobilized metal ion affinity chromatography (IMAC) was discovered by Everson and Parker in 1974, using the idea of employing immobilized chelating metal ions in order to purify metalloproteins (reviewed by Gaberc-Porekar *et al.*, 2001). As with other forms of affinity chromatography, IMAC is used in cases where rapid and substantial purification of the product are necessary, although compared to other affinity separation technologies it cannot be classified as highly specific. However IMAC has the advantages of higher ligand stability (longer shelf life of resin material), high protein loading, mild elution conditions, simple regeneration and low cost, making the technique an advantageous method especially in large-scale purification procedures for industrial applications (reviewed by Gaberc-Porekar *et al.*, 2001) or a rapid first step of a multi-stepped high purity purification procedure.

The idea behind the most frequently used IMAC protocol, polyhistidine tag, came from the initial use in protein separations with naturally present, exposed histidine residues, which are primarily responsible for binding to immobilized metal ions (*Figure 2.6.a*). Improvement of the strategy was pioneered by the study of Hochuli *et al.* (1987-1988), involving recombinant proteins with engineered histidine affinity handles attached to the N- or C-terminus, especially in combination with the Ni(II)–nitrilotriacetic acid Ni–NTA matrix, which selectively binds adjacent histidines via coordination bonding (*Figure 2.6.b, 2.6.c*). Since numerous neighboring histidine residues are uncommon among naturally occurring proteins, such oligo-histidine affinity handles form the basis for high selectivity and efficiency, often providing a one-step isolation of proteins at over 90% purity (reviewed by Gaberc-Porekar *et al.*, 2001). In fusion vector designs for this purpose, a specific enzymatic or chemical cleavage site is often positioned between the affinity tag and target protein so that the protein can be eventually cleaved off the tag and obtained in its native, unaltered form. This purification strategy has attracted the attention of biotechnology companies and a number of different resin/fusion vector couples together with anti-histidine tag antibodies (shown in *Table 2.3* and *Table 2.4*) were designed and marketed.

In polyhistidine involving purification systems, the affinity should be neither too strong (hard to elute under mild conditions) nor too weak (results in sample loss). Studies showed that optimum purification conditions are obtained in presence of 6-10 closely localized, but not necessarily adjacent (e.g. histidine affinity tag (HAT)) histidines.

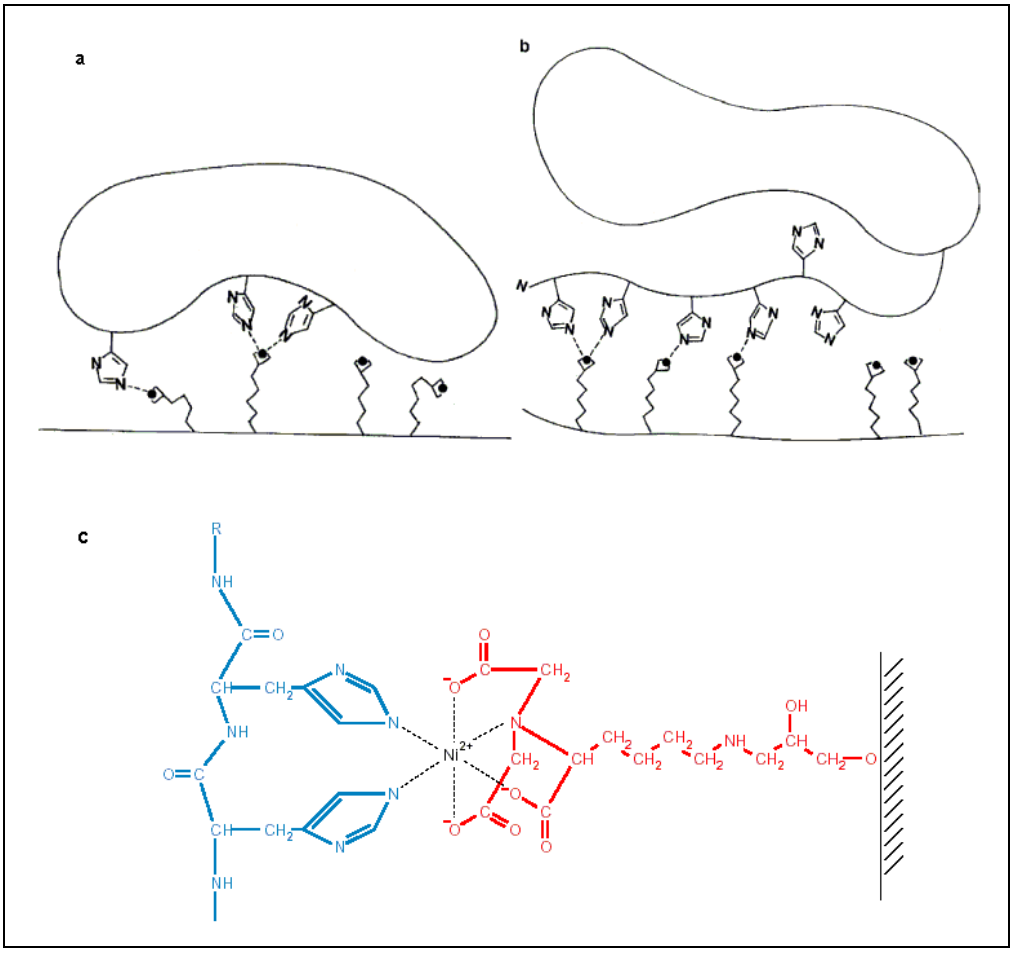


Figure 2. 6 Schematic illustration of the protein binding to a metal-chelated affinity support. Binding of the target protein onto the IMAC matrix is achieved predominantly by: (a) multi-point attachment of native or engineered surface histidines; (b) or by histidine tag added to the N- or C-terminus of the protein (Figures taken from Gaberc-Porekar *et al.*, 2001). In figure (c), the possible interaction between neighboring residues in the 6xHis tag and Ni-NTA matrix is shown (Figures taken from "Qia*express* ®: Detection and Assay Handbook", 2001).

Table 2. 3 Taken from Gaberc-Porekar *et al.*, 2001

Some chelating compounds in use for immobilization in IMAC

Chelating compound	Coordination	Metal ions	Reference	Commercial source
Aminohydroxamic acid	bidentate	Fe(III)	[5]	
Salicylaldehyde	bidentate	Cu(II)	[113]	
8-Hydroxy-quinoline (8-HQ)	bidentate	Al(III), Fe(III), Yb(III)	[114]	
Iminodiacetic acid (IDA)	tridentate	Cu(II), Zn(II), Ni(II), Co(II)	[3,4]	Amersham Pharmacia Biotech, Uppsala; Pierce, Rockford, IL; Sigma, St. Louis, MO; Boehringer Mannheim; Bioprocessing, Princeton, NJ; TosoHaas, Montgomeryville, PA; Merck, Darmstadt
Dipicolylamine (DPA)	tridentate	Zn(II), Ni(II)	[7]	
Ortho-phosphoserine (OPS)	tridentate	Fe(III), Al(III), Ca(II), Yb(III)	[115]	
N-(2-pyridylmethyl) aminoacetate	tridentate	Cu(II)	[35]	
2,6-Diaminomethylpyridine	tridentate	Cu(II)	[36]	
Nitrilotriacetic acid (NTA)	tetradentate	Ni(II)	[13,14]	Qiagen, Chatsworth, CA
Carboxymethylated aspartic acid (CM-Asp)	tetradentate	Ca(II), Co(II)	[7,38,58,116]	Clontech, Palo Alto, CA
N,*N*,*N*′-tris(carboxymethyl) ethylenediamine (TED)	pentadentate	Cu(II), Zn(II)	[7]	

Table 2. 4 Taken from Gaberc-Porekar *et al.*, 2001

Some commercially available expression systems encoding various histidine-rich affinity tags

Expression system	Histidine tag	Cleavage	Immunodetection of the histidine tag	Commercial source
QIAexpress® systems/ *E. coli*	$(H)_6$ extensions at N- or C-terminus	no cleavage at N- or C-terminus	Penta·His™ mAb; RGS·His™ mAb; Tetra·His™ mAb	Qiagen
pcDNA, pEF, etc. series/ mammalian cells	$(H)_6$ extensions at N- or C-terminus	enterokinase at N-terminus	anti-HisG mAb; anti-His(C-term) mAb	Invitrogen, Carlsbad, CA
pMET and pPICZ series/ methylotrophic yeasts	$(H)_6$ extensions at C-terminus	no cleavage	anti-His(C-term) mAb	Invitrogen
pYES series/ classical yeast	$(H)_6$ extensions at C-terminus	no cleavage	anti-His(C-term) mAb	Invitrogen
pTriEx vectors/ *E.coli*, baculovirus and mammalian cells	Protein-$(H)_8$	no cleavage	His·Tag® mAb	Novagen, Madison, WI
pET systems/ *E. coli*	$MG(H)_{10}$SSGHIDDDDK↓H-Protein	enterokinase	His·Tag® mAb against N- or C-terminal His tags	Novagen
	$MG(H)_{10}$SSGHIEGR↓H-Protein	factor X		
	$MGSS(H)_6$SSGLVPRGS↓H-Protein	thrombin		
	Protein-$(H)_6$	no cleavage		
pHAT vectors/ *E. coli*	MKDHLIHDVHKEEHAHAHNKI-DDDDK↓_-Protein	enterokinase	HAT Polyclonal Ab	Clontech
TAGZyme kit/ *E. coli*	$MK(HQ)_6$Q↓-Protein and various, other His tags optimized for this kit	dipeptidyl aminopeptidase I (DPP I) alone or a combination of DPP I, glutamin cyclotransferase (GCT) and pyroglutamyl aminopeptidase (PGAP)	–	UNYZYME Laboratories, Hørsholm

2.4 Green Fluorescent Protein as Location and Expression Reporter: A "Bright" Idea

The green fluorescence of the light producing cells of the jellyfish *Aequorea victoria* under illumination with long-wave ultraviolet light was discovered by Davenport and Nicol in 1955. In 1962, Shimomura et al. reported the extraction of the protein responsible for this fluorescence. In 1971, the protein was isolated independently by Morin and Hastings. The interest in green fluorescent protein (GFP) has grown enormously after its gene was reported to be cloned and the protein was expressed by Prasher *et al.* in 1992 and Chalfie *et al.* in 1994 (edited by Chalfie *et al.*, 1998). As a result of this growing interest, the number of scientific publications that have been published with the term "GFP" in their titles, abstracts or keywords increased from "at least 500" (edited by Chalfie *et al.* 1998) to over 6700 in 2003. The number increases to over 12200 with the addition of results coming from the search of articles having the term "green fluorescent protein".

Since the discovery and cloning of 238 amino acids long wild type *A. victoria* GFP, researchers have gone a long way in understanding and modifying the properties and structure of GFP and assigned various uses to the protein. By using the opportunities given by modern molecular biology techniques, different GFP mutants with shifts in excitation and emission wavelengths (*Figure 2.7*), increase in fluorescence (Heim *et al*., 1995, 1996) or even "humanized" versions (Zolotukhin *et al*., 1996) were produced. Today, certain biotechnology companies are producing and marketing genes and proteins of GFP variants together with other fluorescent proteins for use in diverse applications from protein labeling, quantification (Casey, J.L. *et al.*, 2000) and localization studies to revealing protein-protein interactions by fluorescence resonance energy transfer (Heim *et al.*, 1996 and the references therein).

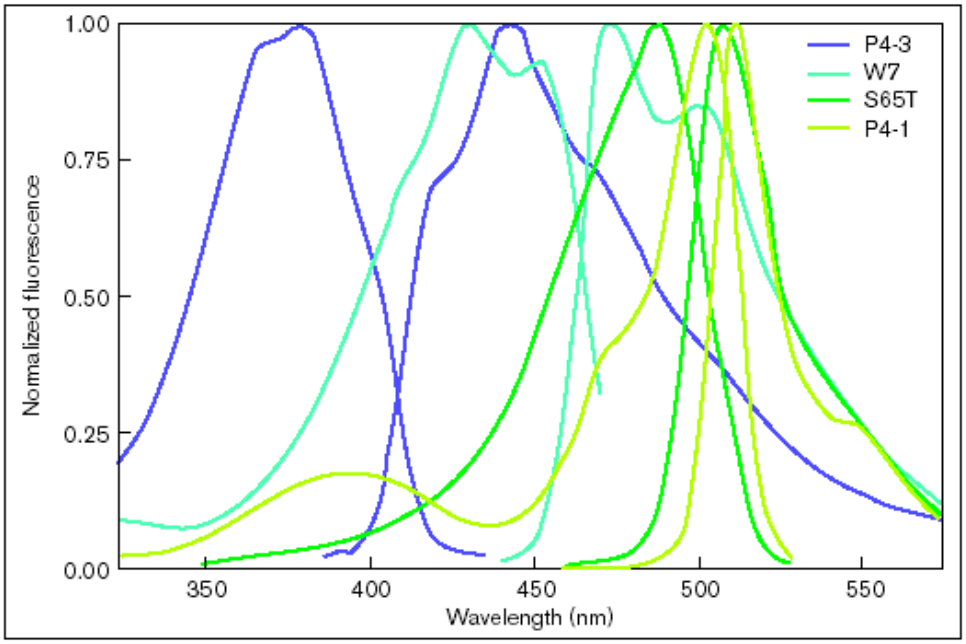


Figure 2. 7 Fluorescence excitation and emission spectra of different GFP mutants. All spectra are normalized to a maximal value of 1. (Heim *et al*., 1996)

2.4.1 Genetic, Physical, and Structural Characteristics of GFP

In studies of GFP, mainly two source organisms were used and compared, one being *A. victoria*, the other *Renilla reniformis* (sea pansy) (edited by Chalfie *et al.*, 1998).

The greenish light emitted by *A. victoria* is due to the presence of two closely related proteins: aequorin (21 kDa) and GFP (27 kDa). Aequorin consists of a Ca^{2+} binding apoprotein (apoaequorin), coelenterazine (organic substrate) and molecular oxygen. In presence of Ca^{2+}, coelenterazine is oxidized to coelenteramide resulting in the production of CO_2 and emission of blue (λ_{max} = 470 nm) light, where coelenteramide is the emitter center. In presence of GFP, due to radiationless energy transfer from the blue fluorescent protein to GFP, the wavelength of emitted light shifts from blue (λ_{max} = 470 nm) to green (λ_{max} = 508 nm), identical to *in vivo* fluorescence of the animal. It is discovered that, GFP is not only a helper protein that shifts already occurring fluorescence to a longer wavelength, but also it

is a fluorescent protein that emits light at 508 nm wavelength independently from the presence of other molecules or ions. (Inouye, S. *et al.*, 1994)

First cloned *A. victoria* GFP comprised of 238 amino acids (*Figure 2.8*). Since then, a large number of mutants and fusion peptides of GFP having different amino acid compositions and chain lengths are produced by genetic engineering.

```
  1 ATGAGTAAAG GAGAAGAACT TTTCACTGGA GTTGTCCCAA TTCTTGTTGA
 51 ATTAGATGGT GATGTTAATG GGCACAAATT TTCTGTCAGT GGAGAGGGTG
101 AAGGTGATGC AACATACGGA AAACTTACCC TTAAATTTAT TTGCACTACT
151 GGAAAACTAC CTGTTCCATG GCCAACACTT GTCACTACTT TCTCTTATGG
201 TGTTCAATGC TTTTCAAGAT ACCCAGATCA TATGAAACAG CATGACTTTT
251 TCAAGAGTGC CATGCCCGAA GGTTATGTAC AGGAAAGAAC TATATTTTTC
301 AAAGATGACG GGAACTACAA GACACGTGCT GAAGTCAAGT TTGAAGGTGA
351 TACCCTTGTT AATAGAATCG AGTTAAAAGG TATTGATTTT AAAGAAGATG
401 GAAACATTCT TGGACACAAA TTGGAATACA ACTATAACTC ACACAATGTA
451 TACATCATGG CAGACAAACA AAAGAATGGA ATCAAAGTTA ACTTCAAAAT
501 TAGACACAAC ATTGAAGATG GAAGCGTTCA ACTAGCAGAC CATTATCAAC
551 AAAATACTCC AATTGGCGAT GGCCCTGTCC TTTTACCAGA CAACCATTAC
601 CTGTCCACAC AATCTGCCCT TTCGAAAGAT CCCAACGAAA AGAGAGACCA
651 CATGGTCCTT CTTGAGTTTG TAACAGCTGC TGGGATTACA CATGGCATGG
701 ATGAACTATA CAAATAA
```

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   MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT 50
   GKLPVPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFF 100
   KDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNV 150
   YIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHY 200
   LSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK*
```

Figure 2. 8 DNA and amino acid sequences from the first entirely cloned *A. victoria* cDNA (Gene bank accession number: M62653; Prasher, D.C. *et al.*, 1992). pDraw32 software was used to properly present the gene and corresponding amino acid sequence.

All naturally occuring GFPs (20 more GFPs are reported) are acidic, globular molecules with a molecular weight of about 27 kDa. *A. victoria* and recombinant GFPs that remain monomeric in dilute solutions being exceptions, GFP is reported to be stable non-dissociable dimers unless denatured. *A. victoria* and *R. reniformis* GFPs and many of the mutants of recombinant wild type GFPs have reported isoelectric points between 4.5 and 5.4 (edited by Chalfie *et al.*, 1998; Narahari, C.R. *et al.*, 2001; Inouye, S. *et al.*, 1994). Although there are engineered mutant variants that have shifted values, native GFP has an excitation maximum around 395 nm, with a smaller absorbance peak at 475 nm, and an

emission maximum around 508 nm when excited at 395nm (Heim, R. *et al.*, 1996; Inouye, S. *et al.*, 1994).

One important property of GFP, which proves advantageous in applications where GFP is used as a reporter of gene expression, is its stability and robust nature against stress conditions of pH, heat and presence of denaturants (Philips, G.N.Jr., 1997, edited by Chalfie *et al.*, 1998). This stability is thought to be due to its unique three-dimensional structure in which the chromophore –color causing region of the molecule– is in the center of a beta-can composed of eleven beta strands at the sides and capped with short loops and distorted alpha helices at both ends (*Figure 2.9*).

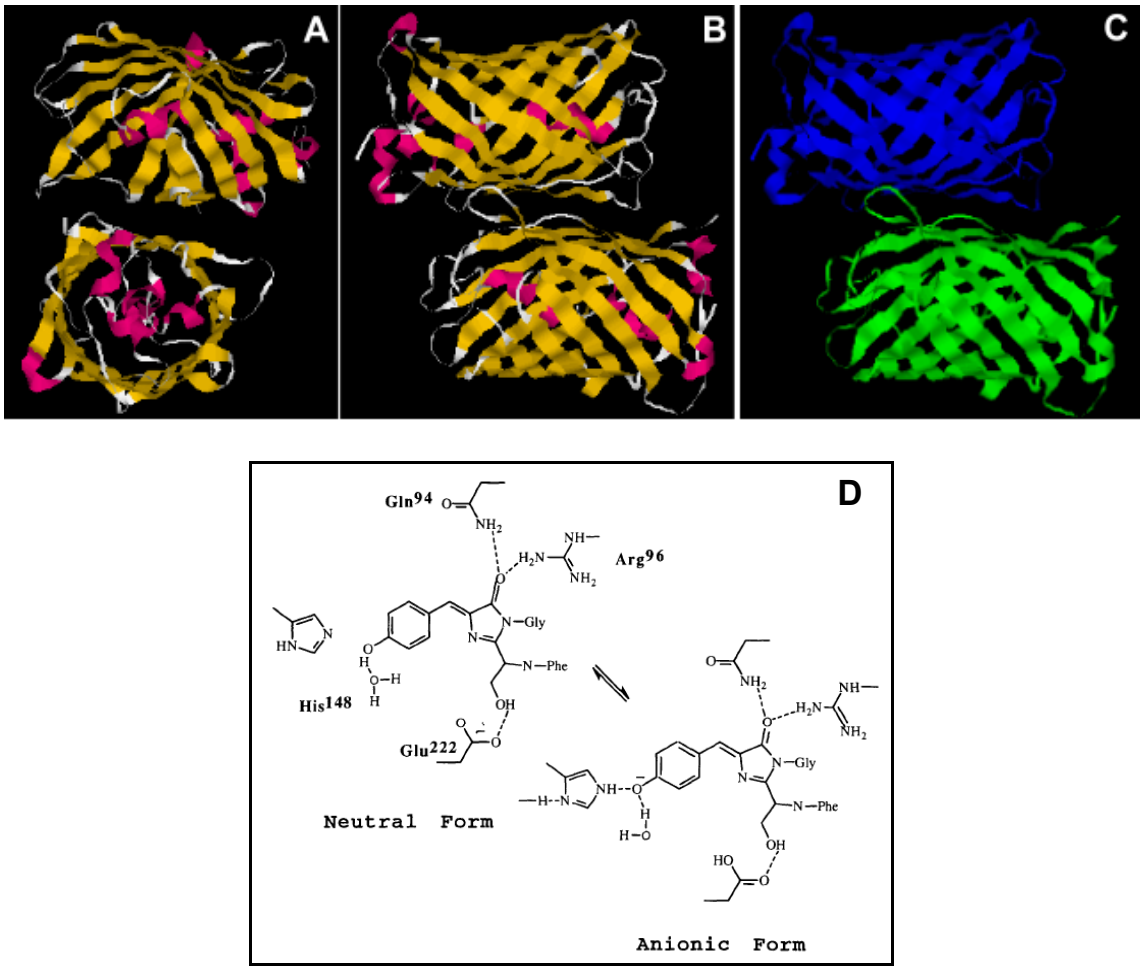


Figure 2. 9 Three dimensional beta-can structure of GFP dimer is shown from different angles, colored by structural component criteria in A and B; colored by independent chain criteria in C. (Protein Data Bank entry: 1GFL [Yang *et al.*, 1996] viewed by RasMol molecular renderer software). D shows the resonant forms of the fluorophore with nearby crucial amino acids His148, Gln94, and Arg96, and the acid Glu222. Interactions between fluorophore and amino acids are shown with dotted lines (Philips, G.N.Jr., 1997).

The three dimensional structure protects the chromophore so well that classical quenching agents such as acrylamide, halides and molecular oxygen have almost no effect on GFP fluorescence (Philips, G.N.Jr., 1997; edited by Chalfie *et al.*, 1998). pH changes between pH 4.5 and pH 12 affect the fluorescence properties of GFP (edited by Chalfie *et al.*, 1998). *Figure 2.10* taken from a study investigating potential use of GFP as an intracellular pH indicator (Kneen *et al.*, 1998), shows the pH dependence of some GFP mutants. It can be clearly seen that pH changes not only alter the fluorescence intensity but also affect its absorption spectrum.

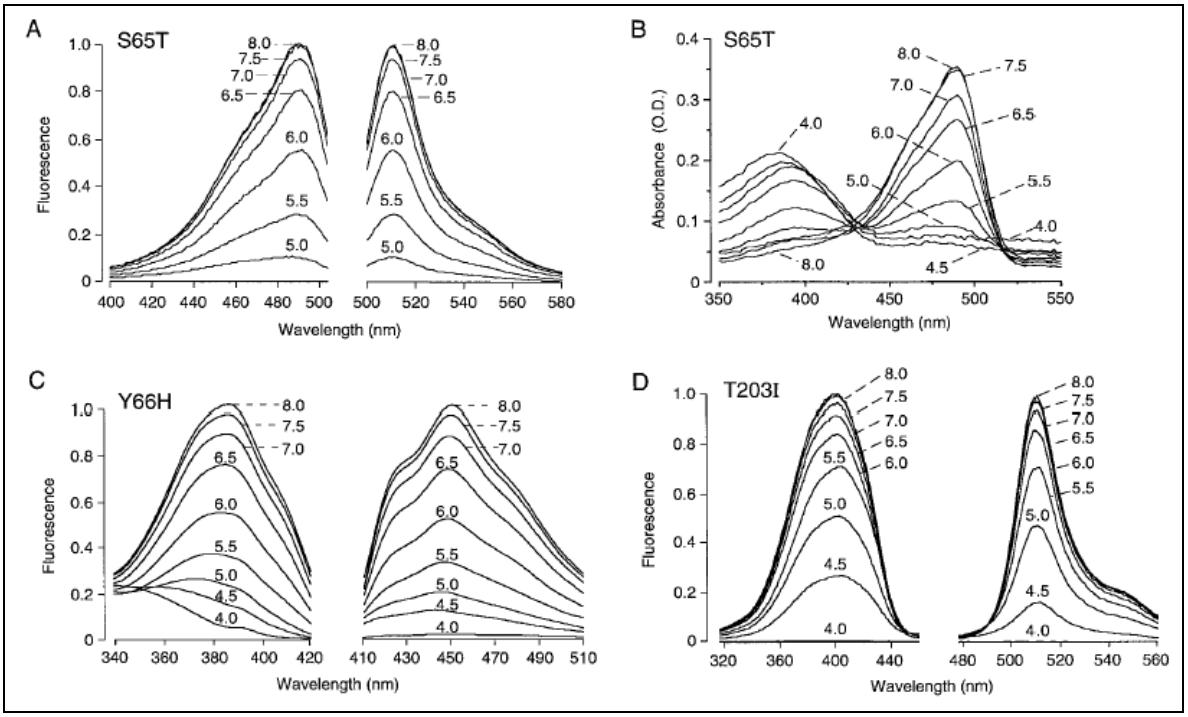


Figure 2. 10 pH-dependent spectral properties of GFPs. (A) Fluorescence excitation and emission spectra of GFP-S65T (~20 mg/mL) at indicated pH. (B) Absorbance spectra of GFP-S65T (0.25 μg/mL). (C and D) Fluorescence spectra of GFP-Y66H and GFP-T203I as in A. (Kneen et al., 1998)

Given the basic information on genetic, physical and structural properties of GFP, various applications are discussed in the following section.

2.4.2 Use of Green Fluorescent Protein Variants:

Having the advantage of being autofluorescent (does not need a substrate or cofactor), wild type GFP and its mutation-derived variants with increased sensitivity and solubility designed and synthesized for *in vivo* studies are being used in a wide spectrum of applications in various model organisms (Chalfie *et al.*, 1994). To name most important applications briefly:

In prokaryotes:

- Noninvasive, endogenous reporter for gene expression using fusion strategy,
- Protein localization studies using fusion strategy,
- Investigations of dynamic processes such as spore formation during bacterial development,
- Tracking of bacteria in complex environments as in the example of bacteria-host interactions (reviewed by Naylor, 1999; edited by Chalfie *et al.*, 1998).

In yeast as simple models of eukaryotic organisms:

- Reporter of gene expression by fusion strategy, within cell cycle of eukaryotes,
- Tracking intracellular protein transport by using fusion strategy,
- Investigation of functions related to compartmentalization within the cell (reviewed by Naylor, 1999; edited by Chalfie *et al.*, 1998).

In *Caenorhabditis elegans*, *Drosophila*, vertebrates, and *Arabidopsis* as models of higher eukaryotic organisms:

- Reporter of gene delivery and expression by fusion strategy,
- Following a marked cell population in time during embryologic development,
- Tracking certain protein molecules (and indirectly their ligands) by fusion strategy and visualizing subcellular dynamics,
- Quantitative analysis of proteins by fusion strategy coupled to flow cytometry,
- Investigating single molecules and protein interactions by fluorescence resonance energy transfer,

- Analyzing effects of mutations or other alterations on protein localization by fusion strategy (Heim *et al.*, 1996; reviewed by Naylor, 1999; edited by Chalfie *et al.*, 1998).

In addition to mentioned uses, using site-directed mutagenesis, scientists and biotechnology companies develop GFP variants having significantly distinct emissions at various wavelengths (Heim *et al.*, 1996). Differential labeling of protein (or indirectly protein-interacting) molecules with different color codes became possible with this improvement, which provided a visual alternative to yeast two-hybrid protein-protein interaction screening.

Although the established uses of GFP are listed very briefly, the only limitation to the variety of applications is the imagination of the scientists, which results in a continuously growing number of uses of GFP in various *in vivo* and *in vitro* practices.

3. MATERIALS & METHODS

3.1 Materials:

The materials used in this project are listed below.

3.1.1 Chemicals

Chemicals that are used are listed in Appendix A.

3.1.2 Molecular Biology Kits

Molecular biology kits that are used for DNA isolation, gel extraction, DNA clean-up/desalting and protein purification are listed in Appendix B.

3.1.3 Biological Components (Cells, Plasmids, Oligonucleotides, Molecular Weight Markers, Enzymes And Enzyme Buffers)

Plasmids, DNA and protein molecular weight markers, enzymes and enzyme buffer systems that are used for DNA modification (i.e. restriction digestion, ligation) and RNA removal are listed in Appendix C. Typical gel electrophoresis photographs carrying characteristic band information of molecular weight markers are also provided in Appendix C. Plasmid maps of utilized plasmids, pETM-11 (EMBL) and pGFPuv (Clontech) were given in Appendix F and Appendix G, respectively.

3.1.4 Equipments

Equipments that are used for overall laboratory experiments are listed in Appendix D.

3.1.5 Bacterial Strains

E. coli bacterial strains XL1 Blue, BL21 (DE3) were kindly provided by European Molecular Biology Laboratories, Hamburg, Germany.

3.1.6 Buffers and Solutions

Standard buffers and solutions used in cloning and molecular manipulations were prepared according to the protocols in Sambrook, J. *et al.*, 1989.

3.1.6.1 Buffer for agarose gel electrophoresis

1 X TAE (Tris-EDTA-Acetate) buffer was used for preparation of 1% agarose gel electrophoresis. 1% agarose gel was used throughout the project. TAE buffer was prepared according to the protocols in Sambrook, J. *et al.*, 1989. DNA was visualized by including 0.005% ethidium bromide in the gel during its preparation.

3.1.6.2 Buffer for denaturing and urea polyacrylamide gel electrophoresis

1 X TBE (Tris-Boric acid-Acetate) and 1X Tris-Glycine-SDS (sodium dodecyl sulfate) buffer was used for polyacrylamide gel electrophoresis. TBE buffer was prepared according to the protocols in Sambrook, J. *et al.*, 1989.

3.1.7 Culture Media

Both solid and liquid culture media were used for growing bacteria. In growth media, 0.1 mg/mL and 0.05 mg/mL antibiotic final concentrations were used for Ampicillin and Kanamycin respectively.

3.1.7.1 Liquid medium

LB Broth from Sigma was used as liquid culture of bacteria. This mixture contains the tryptone, yeast extract, and sodium chloride mixed in appropriate amounts. 20 g of LB Broth was used for preparation of 1 L liquid medium. The liquid medium was autoclaved at 121^{o} C for 30 min. before using.

3.1.7.2 Solid medium

LB Agar from Sigma was used for preparation of solid medium for the growth of bacteria. This mixture contains the tryptone, yeast extract, sodium chloride, and agar, which are mixed in appropriate amounts. 40 g of LB Agar was used for preparation of 1 L solid medium. The appropriate amount of LB Agar is dissolved in correspondent amount deionized water for autoclaving at 121^{o} C for 20 min. Autoclaved medium was poured to petri plates (~20 mL/plate) after cooling down to a temperature at which it is still liquid.

3.1.8 Sequencing

Sequencing service was commercially provided by MWG-The Genomic Company, Germany.

3.2 Methods

All molecular biology protocols were applied according to Sambrook *et al.*, 1989 unless stated otherwise. Enzymatic reactions such as restriction digestion and ligation, and applications that were governed by using molecular biology kits were realized by accepting the supplier information as the base to our application.

3.2.1 Culture Growth

Cells were grown overnight (12-16 hours) in LB Broth medium prior to any application. LB Agar (Miller's LB agar) solid medium was used for the growth of bacteria on both selective and non-selective media.

Liquid cultures were grown at 37^{o}C using shaking incubator set to 300 rpm.

Conventional molecular biology protocols for liquid and solid culture growth and other applications including competent cell preparation, transformation, glycerol stocks and various gel electrophoresis methods were performed according to procedures in Sambrook *et al.*, 1989.

3.2.2 Directional Cloning

Gene cloning steps given below are performed in accordance with protocols in Sambrook *et al.*, 1989. Inserts were directly cloned into modified pETM-11 expression vector and pETM-Adp-Imm expression vector (pETM-11 derived novel plasmid construct) without using a sub-cloning vector.

3.2.2.1 Restriction digestion

pETM-11 (EMBL) (Appendix F) was digested with Nco I and Kpn I in order to remove MAD (multiple wavelength anomalous dispersion) coding fragment and as a preparation to ligation with frame adapter. Frame adapters were also digested with Nco I and Kpn I in preparation to ligation with MAD removed pETM-11. pGFPuv (Clontech) was in turn digested with Kpn I and Sac I in order to get GFPuv gene from the plasmid.

All linearized plasmids were obtained by digestions in which the total volume of enzyme pairs in double digests was limited to 1/10 of total volume of digestion mixes as recommended in Sambrook, *et al.*, 1989, in order to diminish inhibitory activity of glycerol in the enzyme solution.

Sticky end generation at the ends of frame adapters was obtained by double digestions which were strictly optimized case specifically. The efficiency of digestion of frame adapters was checked by running samples of digested adapters in urea gel

electrophoresis with 20% polyacrylamide, 7 M urea concentration in the gel. Parallel-performed digestions of frame adapters were collected and concentrated at the end of digestions.

3.2.2.2 Gel extraction of linearized plasmids

Since they largely affect ligation efficiency, small (digestion product) fragments and the remaining salts from restriction digestion reaction were removed by gel extraction using QIAquick® Gel Extraction Kit.

3.2.2.3 Desalting of digested frame adapters

Frame adapters could not be cleaned from unwanted terminal digestion products since both the adapters and terminal digests were very small and there was only a few bases length difference between them. Despite the failure in removal of terminal digests, salts remained from restriction digestion were successfully removed by using Micro-Spin™ G-25 Columns (Amersham Biosciences) two times for each sample successively.

3.2.2.4 Ligation

Fragments, which were digested to have sticky ends containing Kpn I, Nco I and Sac I recognition sequences, were ligated using T4 DNA Ligase (Promega) by incubating at room temperature overnight. In ligation reactions, series of vector/insert molar ratios were used in order to cover optimum ratio, which was described to be 1:3 (Sambrook *et al*, 1989).

3.2.2.5 Transformation

Transformations were performed using heat shock protocol described in Sambrook *et al.*, 1989. Endonuclease deficient *E. coli* strains XL1-Blue and BL21-DE3 were used as targets of transformation. Transformed cells were plated on Luria agar with appropriate antibiotic,

which was selected according to the antibiotic marker gene on the transformed plasmids and grown overnight at 37^{o}C.

3.2.2.6 Colony selection

Positive colonies were selected on appropriate antibiotic containing plates. Selected colonies were grown in Luria Broth liquid medium containing the selective antibiotic and glycerol stocks were prepared using the liquid cultures.

3.2.2.7 Plasmid isolation

Plasmid isolation was performed either with Qiagen-Qiaprep® Spin Miniprep Kit, or Qiagen Midi-prep Plasmid Purification Kit.

3.2.2.8 Analysis of DNA from transformant colonies

Isolated plasmids were cut with the restriction enzymes specific to the sites located at terminal positions of the insert of interest. Uncut and digested plasmids were analyzed by agarose gel electrophoresis for verification of the presence of insert within the plasmid. Appropriate DNA markers were used for size and concentration determination. In addition, concentration and $OD_{260/280}$ ratio were monitored by absorption measurements.

3.2.2.9 Frozen stock preparation of transformants

Frozen stocks of bacteria containing different plasmids with GFPuv and GFPimm were prepared in 15% sterilized glycerol containing Luria Broth liquid medium with appropriate antibiotics and kept at - 80^{o} C according to the protocol from Sambrook *et al*, 1989.

3.2.2.10 Sequence verification

Qiagen-Qiaprep® Spin Miniprep Kit, or Qiagen Midi-prep Plasmid Purification Kit purified were sent to MWG-The Genomic Company, Germany for sequence analysis. Plasmids were also checked by restriction and electrophoretic analysis before sequencing.

3.2.3 Expression of Target Genes

Both GFPuv in pGFPuv (Clontech) plasmid and GFPimm in pETM-Adp-Imm plasmid were expressed according to the protocol from Sambrook *et al.*, 1989. Different IPTG final concentrations were used ranging from 0.7 mM to 1mM for inducing recombinant protein expression. In expression series, samples were taken with pre-defined time intervals during cellular growth. Sample amount was standardized so that every sample contained same amount of cells necessary to give the OD_{600} absorbance value that was taken at the beginning of cellular growth, which was around 0.5 A. For storage and later analysis, cells were pelleted and frozen at –20 ^{o}C. Pellets were lysed and prepared for SDS-PAGE gel analysis according to the protocol from Sambrook *et al.*, 1989. 10 µl of the samples were loaded on 5%-15% SDS-PAGE gels together with appropriate protein molecular weight marker.

3.2.4 Purification of Proteins

3.2.4.1 Purification of GFPuv

BL-21(DE3) cells transformed with commercial pGFPuv plasmid were grown in 500 mL liquid cultures (with Ampicillin) overnight at 37^{o}C by shaking. Induction was performed using 0.7 mM IPTG as final concentration in liquid medium. Induced protein was purified using MonoQ Ion Exchange Chromatography Column (10/10) (Amersham Biosciences) installed on ÄKTA FPLC (Amersham Biosciences). Ion exchange buffer pair was 30 mM Tris-HCl, pH 7.5 and 30 mM Tris-HCl, pH 7.5 + 1 M NaCl. The gradient used for purification is given in *Figure 3.1*. Total cleared lysate was loaded on the column and 1mL / min flow rate was used for all purification steps.

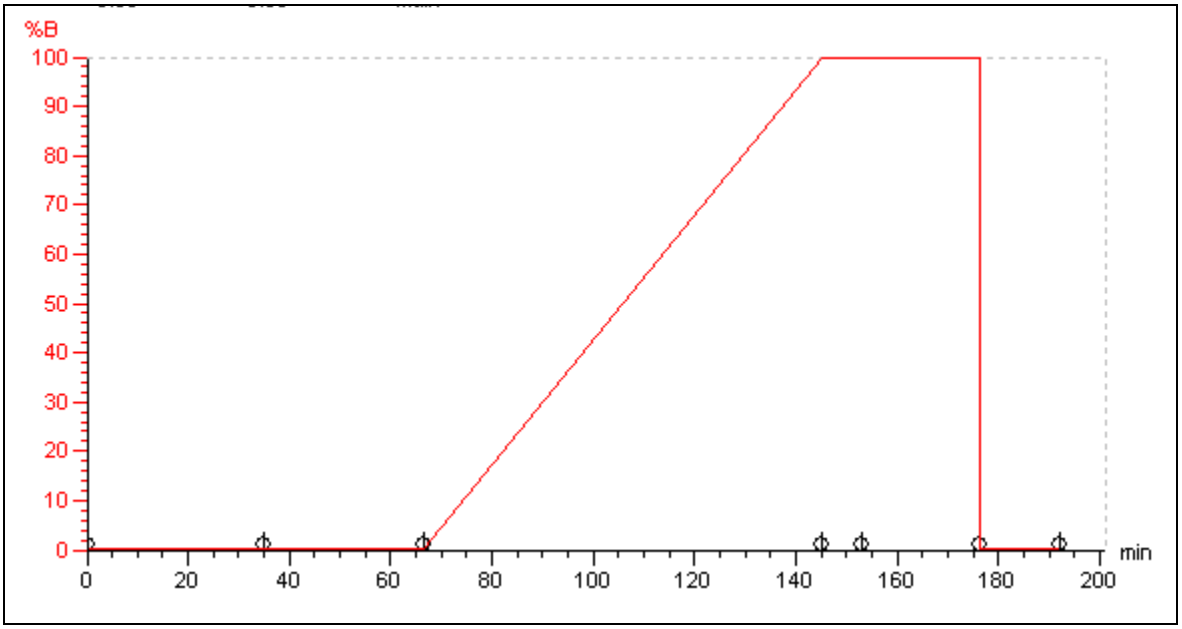


Figure 3. 1 Buffer gradient used for purification of GFP variants. %B indicates the percentage of buffer with higher salt concentration.

3.2.4.2 Purification of GFPimm

BL-21(DE3) cells transformed with constructed pETM-GFP-Imm plasmid were grown in 500 mL liquid cultures (with Kanamycin) overnight at 37^{o}C by shaking. Induction was performed using 0.7 mM IPTG as final concentration in liquid medium.

GFPimm protein was isolated using two different methods alternatively during the progress of study.

In the first method, his-tag based purification system component ProBond™ resin (Invitrogen) was used according to the protocols supplied by the company. The protocol was scaled up so that pellet from 500 mL (OD_{600}: ~ 4A) liquid culture was resuspended in 40 mL binding buffer and applied on 8 mL 50% ProBond™ resin suspension solution. The application was realized in batch type of binding reaction after the resin material is settled and packed to 4 mL, solution phase is discarded and washed as described in kit protocols. During purification, 1X ProBond™ Native Purification Buffer, pH: 8.0 (50 mM NaH_2PO_4, 500 mM NaCl, pH adjusted with NaOH) was used for binding and washing steps while the buffer also contained 250 mM imidazole.

In the second method, GFPimm was obtained exactly in the same protocol with GFPuv in terms of equipment, solutions and automated method file of FPLC software.

3.2.4.3 Quantification of GFP variants in solution

Both GFPuv and GFPimm were quantified relative to a 1 mg/mL rGFPuv protein standard (Clontech) by measuring $OD_{280/260}$ (protein assay) and OD_{398} (characteristic absorption wavelength for GFP) with absorption spectrometry. Relative fluorescence values with fluorescence spectrometry and analysis of polyacrylamide gel electrophoresis results for serial dilutions were also carried out.

3.2.5 Surface Binding Preparation

GFP variants and polystyrene surface was prepared separately for the binding reaction.

3.2.5.1 Preparation of GFPuv and GFPimm

Both GFPuv and GFPimm were dialyzed against 0.5X ProBond™ Native Purification Buffer at pH 6.0, pH 7.0 and pH 8.0 (25mM NaH_2PO_4, 250 mM NaCl solutions, pH adjusted with NaOH) in order to reduce imidazole (applicable when ProBond™ resin is used for GFPimm purification) and excess Na^+ ions which may interfere with surface binding reaction as competitive inhibitors of charge interaction. Dialysis was performed in 1 L volumes with 3 changes, each session was at least four hours at 4^oC. Dialyzed sample volumes ranged from 2 mL to 4 mL throughout the study.

3.2.5.1.1 Removal of precipitate and requantification of solution phase GFP variants

Dialyzed samples were transferred into 15 mL Falcon tubes and centrifuged at 2000g for 10 minutes at 4^oC. Supernatants were transferred into new 15 mL Falcon tubes and short-term stored in order to be used for the rest of the experiments. Supernatants were quantified using the procedure described in section 3.2.4.3.

3.2.5.2 Preparation of 96-well plate and characterization of surface modification

Polystyrene 96-well plates were oxidized using 2M and 3M ammonium persulfate solutions. Oxidation setup was prepared by filling wells of plates with 300 μl 2M and 3M ammonium persulfate solutions, tightly covering the wells with the aid of parafilm and plate cover, applying pressure on the cap. Ammonium persulfate solution loaded plates were incubated at 70^{o}C, 24 hours. The cap was pressed in order to prevent evaporation caused concentration alterations during incubation period. Preparation of 3M APS solution was realized at 45 ^{o}C water bath in order to achieve solubilization. Plates were rinsed several times with flushing warm water and dried at 70^{o}C for 2 hours.

Surface modification was characterized by infrared spectrum analysis of pieces that were cut from bottoms of wells of modified plates.

3.2.6 Surface Binding

Concentrations of GFPuv and GFPimm solutions were adjusted by diluting GFPuv, expression of which is more efficient. Binding reactions were realized by constructing on-plate matrices. Each surface-binding matrix was planned on 96-well plates so that rows and columns represent different GFP variants, different protein concentrations, different binding pHs and different plate modification strengths (APS molarities during surface modification). Binding was performed at appropriate pH values for each protein sample, being the same as pH of the dialysis solutions. During binding studies of GFP variants, 200 μl concentration-standardized protein solutions were filled in the wells of modified 96 well plates and incubated for 2 hours at room temperature. After 2 hours, GFP solutions were replaced with the appropriate dialysis buffer at the binding pH of each well individually.

Final replacement was done after careful and repeated rinsing using appropriate buffers having pH value of each well's binding reaction.

3.2.7 Detection and Quantification of Binding

Binding was detected and quantified by fluorescence measurements in both transparent and opaque (black) 96 well plates. Binding detection was supported by

observation of fluorescence in photographs of ultraviolet light transmitted transparent plates. Quantification of bound proteins was performed by using rGFPuv (Clontech) protein (1mg/mL) as concentration standard both in fluorescence spectrometry and SDS-PAGE electrophoresis.

3.2.8 Analysis

The protein amount was estimated in solution by using $OD_{280/260}$ absorption and fluorescence (398 nm excitation) measurements with respect to rGFPuv standard of known concentration. The mentioned estimations were confirmed by SDS-PAGE analysis by comparing the same standard. Quantification and analysis of surface-bound GFP was performed using fluorescence measurements, standardization of which was done by using above-indicated method using rGFPuv standard. The surface-bound-GFP fluorescence measurements were performed considering the parameters of GFP type, binding concentration, binding pH, measurement pH, measurement state (dry/wet), modification strength on polystyrene surface.

4. RESULTS

4.1 Schematic Description of the Immobilization Specialty Plasmid: pETM-GFP-Imm

Immobilization specialty plasmid – pETM-GFP-Imm – was constructed by a series of double digestion and ligation reactions. Manipulation of different vectors and resultant plasmids after these reactions are given in *Figure 4.1*. Sequences of vectors pETM-11 and pGFPuv are given in Appendix E together with the expected sequences of the vector constructs.

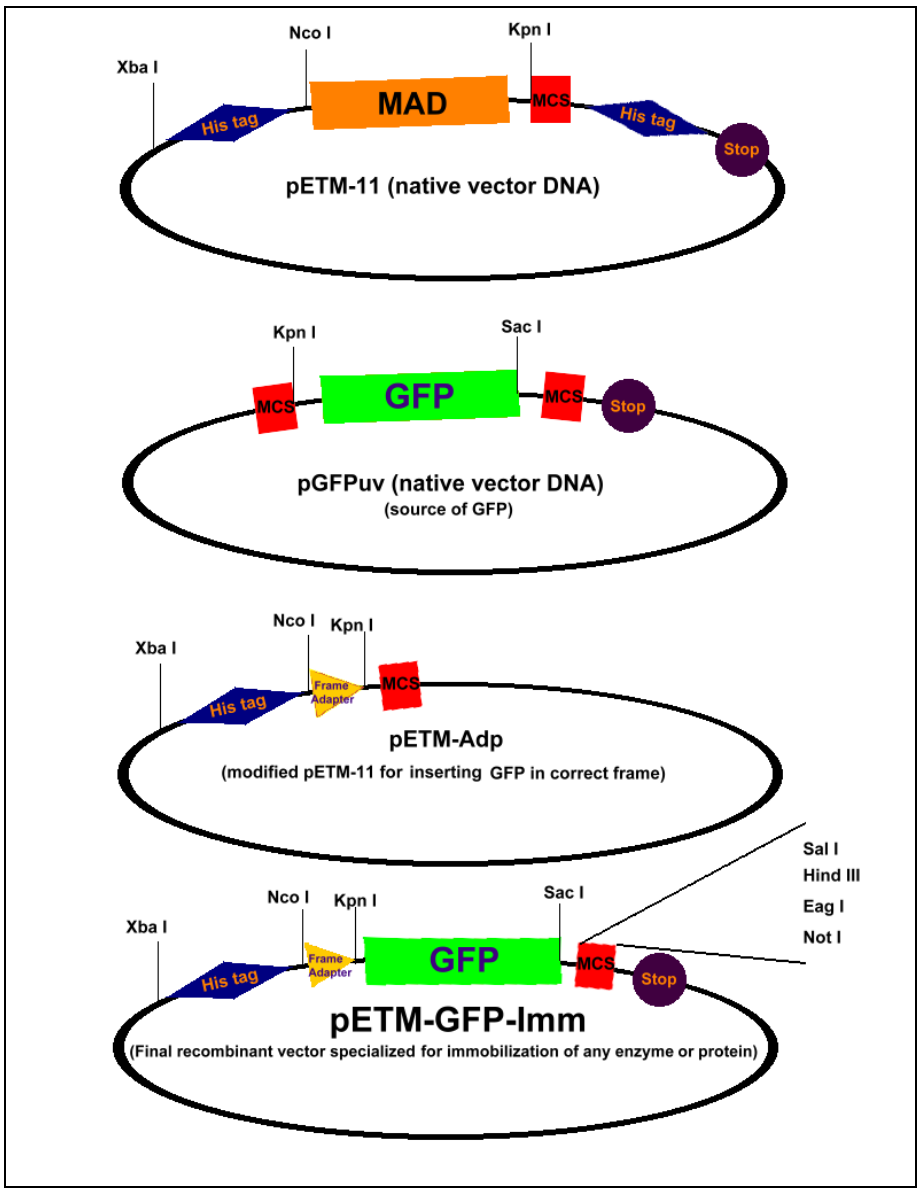


Figure 4. 1. Plasmid construction scheme showing two source plasmids (pETM-11 and pGFPuv), intermediate plasmid (pETM-Adp) and the final immobilization-specialty plasmid (pETM-GFP-Imm).

Final plasmid was designed to be modular and upgradeable simply by digestions and ligations since each component of the plasmid (i.e. affinity tag, frame adapter, fluorescent reporter peptide, multiple cloning site) can be replaced or enhanced with alternatives such as different poly-aminoacid tags, fluorescent peptides and additional restriction enzyme recognition sites. In order to achieve the modularity and easy further modification, every component in the plasmid was inserted by conventional ligation reactions.

Steps in construction of final plasmid pETM-GFP-Imm are discussed in the following sections.

4.2 Digestion of pETM-11 and Frame Adapters in Preparation of Intermediate Plasmid Construction

In order to construct a plasmid specialized for expression of immobilization-facilitated recombinants, poly-histidine tag bearing expression vector – pETM-11 (EMBL) – was used as the main-body plasmid (plasmid map, nucleotide sequence of multiple cloning site and poly-histidine tag coding regions are given in Appendix F).

pGFPuv (Clontech) was utilized as the source of GFP for the final immobilization specialty plasmid. In order to adjust the frame of GFPuv synchronous to the 5' located poly-histidine tag, double stranded "frame adapter" oligonucleotide was designed in-house and synthesized by SeqLab, Germany.

4.2.1 Modification of pETM-11 in Preparation of Frame Adapter Insertion

pETM-11 was purified from 250 mL liquid culture (OD_{600}: ~4A) of carrier *E. coli* clones by using Qiagen Midi-prep Plasmid Purification Kit. The plasmid was quantified by absorption measurement at 260 nm. Based on the absorption measurement, ~15 μg plasmid was double digested with Kpn I and Nco I restriction enzymes in order to excise MAD gene, vacancy of which would be used for insertion of GFPuv gene. The quality of double digestion and plasmid purification were verified by agarose gel electrophoresis analysis. Electrophoresis results showed two major bands that corresponded to MAD-excised pETM-11 (~5352 bp[*]), and MAD gene (~677 bp[*])(*Figure 4.1.A*). Following double digestion, linearized plasmid was gel extracted using QIAquick® Gel Extraction Kit (250). Gel extracted plasmid was checked for purity, and its concentration was estimated with respect to molecular weight marker using agarose gel electrophoresis (*Figure 4.2.B*). Map of linearized plasmid is given in *Figure 4.3*.

[*] Number of base pairs is given as approximate values (~) when there are single stranded restriction digestion products at the terminal regions of long DNA molecule. The values given are the number of nucleotides calculated in case single stranded regions are assumed as double stranded.

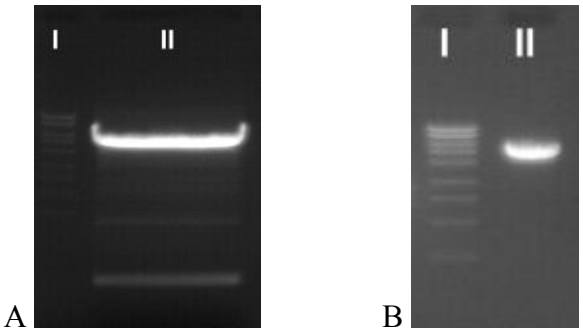


Figure 4. 2 Electrophoretic analysis and preparative digestion of pETM-11 (A-II) and gel extracted (B-II) pETM-11 plasmid. Mass Ruler DNA Ladder™, High Range (Fermentas) was used as molecular weight marker (A-I, B-I).

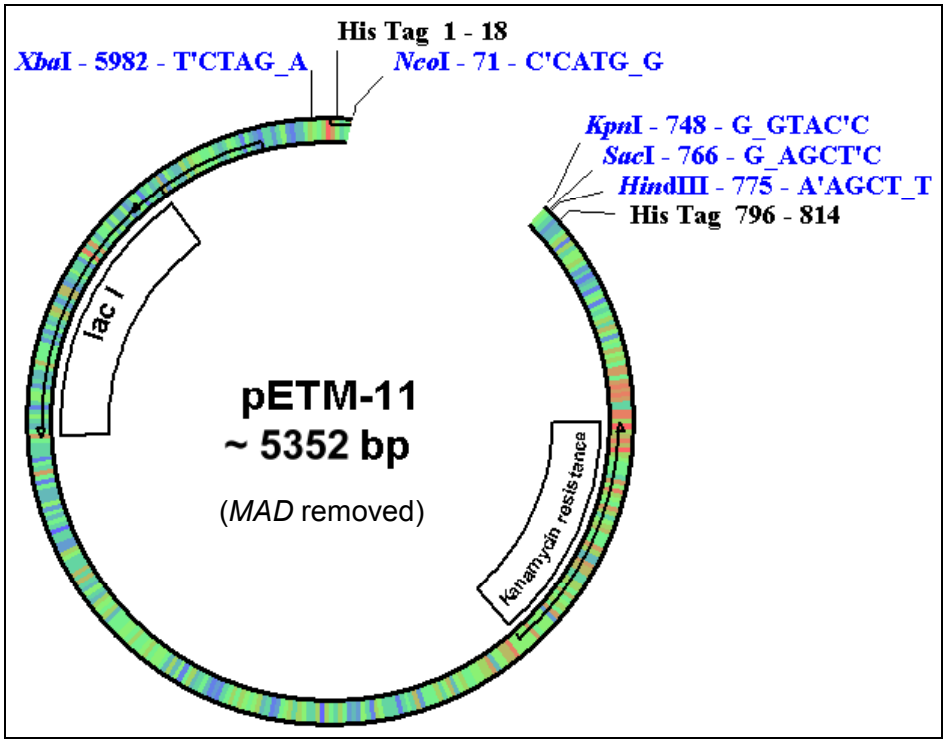


Figure 4. 3. Map of Kpn I and Nco I linearized pETM-11. MAD gene was excised from pETM-11 by the mentioned double digestion. Arrows indicate the directions of open reading frames for the corresponding genes and colors indicate the GC content of local site (showing least GC% to most GC% with blue to red respectively). Map was constructed by plasmid drawing software pDraw32.

As verified by the gel electrophoretic analysis, MAD gene was properly excised from pETM-11 plasmid, leaving a linearized vector bearing Kpn I and Nco I sticky ends for subsequent ligation reaction with frame adapter having the same sticky ends.

4.2.2 Designing the double stranded frame adapters

The product to be synthesized using specialty vector which will be properly expressed and have GFP and histidine as tags only if the GFP sequence is inserted in frame with the start codon of the polyhistidine sequence on pETM-11. This requires insertion of a frame adapter with correct number of nucleotides replacing the MAD gene. The replacement was achieved using sticky ends generated by removal of MAD gene. For using ligation as the strategy, frame adapter was also double digested with Kpn I and Nco I to generate the same sticky ends.

The potential problems with digested oligonucleotides and proposed solutions were as follows:

- Since the oligonucleotides were small targets for restriction enzymes, there was a risk of low digestion efficiency even in presence of correct restriction enzyme recognition sites. This situation was due to DNA modifying enzymes' requirement for available space to "sit" on the DNA in order to perform its function. It is known that, to achieve high efficiency in oligonucleotide digestions, oligonucleotides should be designed with a number of extra nucleotides at the terminal positions and incubation periods should be extended. Although the number of necessary nucleotides varies with the restriction enzyme, generally 2-10 extra bases and around 20 hours of incubation are recommended (New England Biolabs Technical Literature, 2002). 5 extra nucleotides were added at each terminal position on the frame adapters in our adapter designs.
- Digestion products included the fragment that would be inserted into the plasmid together with terminal digests. Since these terminal digests had the same sticky ends (one in each) they also had the potential to ligate with the plasmid, blocking the ligation of frame adapter by competitive inhibition. The wanted and unwanted fragments were so similar and both were so small in size that there was no effective way of separating them. Since very small amount of adapter was used, recovering the DNA from native polyacrylamide gel contained the risk of intolerable sample loss. Although the number of terminal digests would also increase with higher digestion efficiency, having as much double digested frame adapter as possible in

ligation was thought as a potential solution to this problem. Thus, digestion efficiency was strictly optimized to be around ideally near 100%.

Three sets of frame adapters were synthesized throughout the project:

Adp# 1) 5' – GTACG CCATGG ***CATGTACT*** **GGTACC** TTGTG – 3' (30 bp)
3' – CATGC GGTACC ***GTACATGA*** **CCATGG** AACAC – 5'

Adp# 2) 5' – GTACG CCATGG ***CATGACT*** **GGTACC** TTGTG – 3' (29 bp)
3' – CATGC GGTACC ***GTACTGA*** **CCATGG** AACAC – 5'

Adp# 3) 5' – GTACG CCATGG ***GAGGCAC*** **GGTACC** TTGTG – 3' (29 bp)
3' – CATGC GGTACC ***CTCCGTG*** **CCATGG** AACAC – 5'

Normal font: Random nucleotide extensions,
Underlined: Nco I recognition site
Bold, italic: nucleotide additions to plasmid for frame adjustment,
Bold, underlined: Kpn I recognition site

First two adapters were defective either in the number of nucleotides (8 instead of 7, resulting frameshift) necessary to adjust the frame or due to accidental introduction of a premature stop codon (***CATGACT***) within the frame adapter. Yet, since all three frame adapters were optimized for double digestion, remarkable information was gained about the effect of "non-recognition-site sequence specificity" on restriction enzymes' efficiency near the ends of oligonucleotides.

4.2.2.1 Digestion of first frame adapter (Adp#1)

In first double digestion attempts and single digestion controls with Kpn I and Nco I at 37^{o}C for 3 hours, Nco I showed inefficient digestion with recommended buffer system Multi Core™ (Promega) (*Figure 4.4-A, B*). In order to increase efficiency, Y+/Tango™ (Fermentas) buffer system was used alternatively by using Multi Core™ used digestion mixes as controls (*Figure 4.4-B*). Since improvement cannot be achieved by changing the buffer system, longer incubation time (overnight) was applied. Finally, by using

Y+/Tango™ (Fermentas) buffer with overnight (18 hours) incubation, efficient double digestion was obtained. After successful digestion, restriction enzymes and salts in the reaction buffer were removed by application of the reaction mixture into Micro-Spin™ G-25 Columns (Amersham Biosciences). The possibility of DNA loss due to column treatment was checked on urea gel by using untreated digestion mix as control (*Figure 4.4-C*). Although some sample loss was observed, remaining DNA was decided to be sufficient for later experiments.

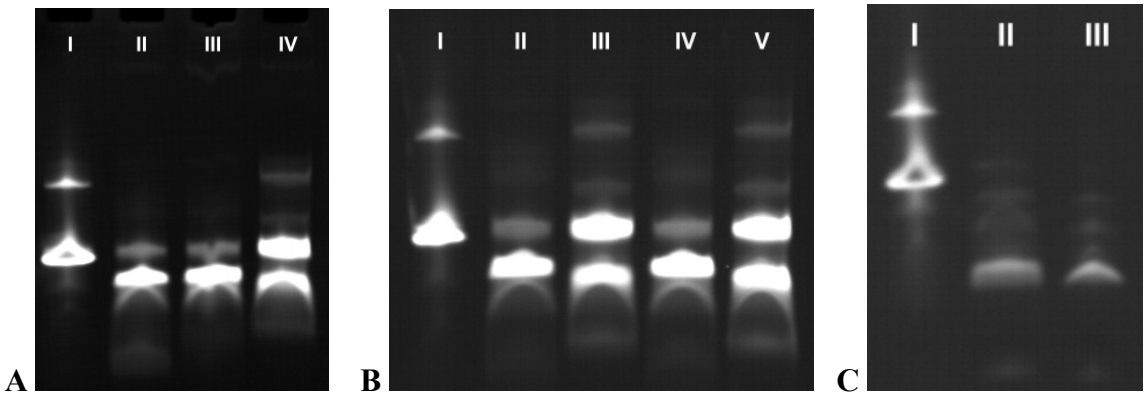


Figure 4. 4 Urea -PAGE analysis of optimization of Adp#1 double digestion. In A, B and C, lane I shows undigested frame adapter. Upper, weaker bands in lanes I in all pictures correspond to nondenatured (double stranded) uncut adapter due to the high loading concentration.

(A) Lane II: double digestion. Lanes III and IV: Kpn I and Nco I single digestions, respectively.
(B) Lanes II and III: Kpn I and Nco I with Multi Core (Promega) buffer system, respectively. Lanes IV and V: Kpn I and Nco I with Y+/Tango™, respectively.
(C) Lanes II and III: double digests without and with Micro-Spin™ G-25 Column (Amersham Biosciences) treatment, respectively, for removal of restriction enzymes and desalting of digestion mix.

4.2.2.2 Digestion of second frame adapter (Adp#2)

After realizing the presence of one extra nucleotide which sets incorrect frame for GFP expression, the second frame adapter was synthesized by removing one nucleotide from the frame adjusting region between the restriction enzyme recognition sites. This random removal accidentally led to the formation of a premature stop codon. Until the realization of the problem, previously optimized digestion conditions were applied to the second adapter and satisfactory results were obtained (*Figure 4.5*). It was also realized that

with the urea gel electrophoresis conditions applied, we were able to distinguish one base difference between first and second frame adapter designs (*Figure 4.5-B*: II-III)

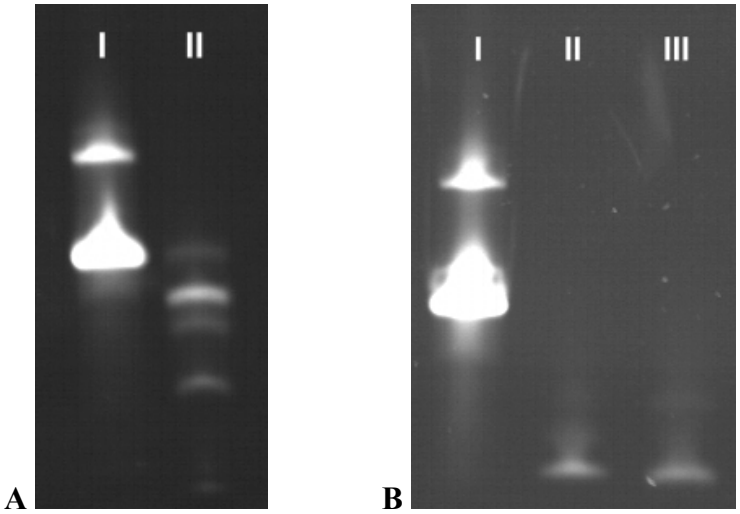


Figure 4. 5 Effect of incubation time on digestion efficiency of Adp#2. Digestion products were analysed on Urea-PAGE. A) Results of double digestion of Adp#2. Lane I: undigested Adp#2. Lane II: after 4 hours of double digestion. B) Successful digestion after overnight (18 hours) incubation. Lane I: undigested Adp#2. Lane II: successfully double digested Adp#1. Lane III: successful double digestion of Adp#2.

4.2.2.3 Design and digestion of third frame adapter (Adp#3)

Third frame adapter was designed so that not only it adjusts the frame of GFP in synchrony with polyhistidine tag, but also connects them flexibly by encoding a freely rotating and bending joint peptide. In order to obtain a joint with relatively free motion (Voet *et al.* 1995), adapter was designed to code for glycine rich oligopeptide (GGA-GGC-ACG: Gly-Gly-Thr). However, the change largely affected digestion efficiency in previously optimized conditions (*Figure 4.6-A*).

The double digestion conditions were optimized *de novo* for the final frame adapter design. During this optimization, frame adapter and bovine serum albumin (BSA) concentrations used for digestion were changed (*Figure 4.6-B*). In accordance with the decreased frame adapter concentration, –helper protein– BSA concentration was increased in order to facilitate temporary binding of the enzymes on DNA for processivity.

Optimizations revealed that double digestion of Adp#3 is more efficient in dilute solutions of the adapter. In order to obtain adequate digested adapter, larger reaction

volumes were used with previously standardized amount of adapter. Double digested adapter was then concentrated by evaporating solution in speed vacuum and enzymes and salts were removed by treatment with Micro-Spin™ G-25 Columns (Amersham Biosciences).

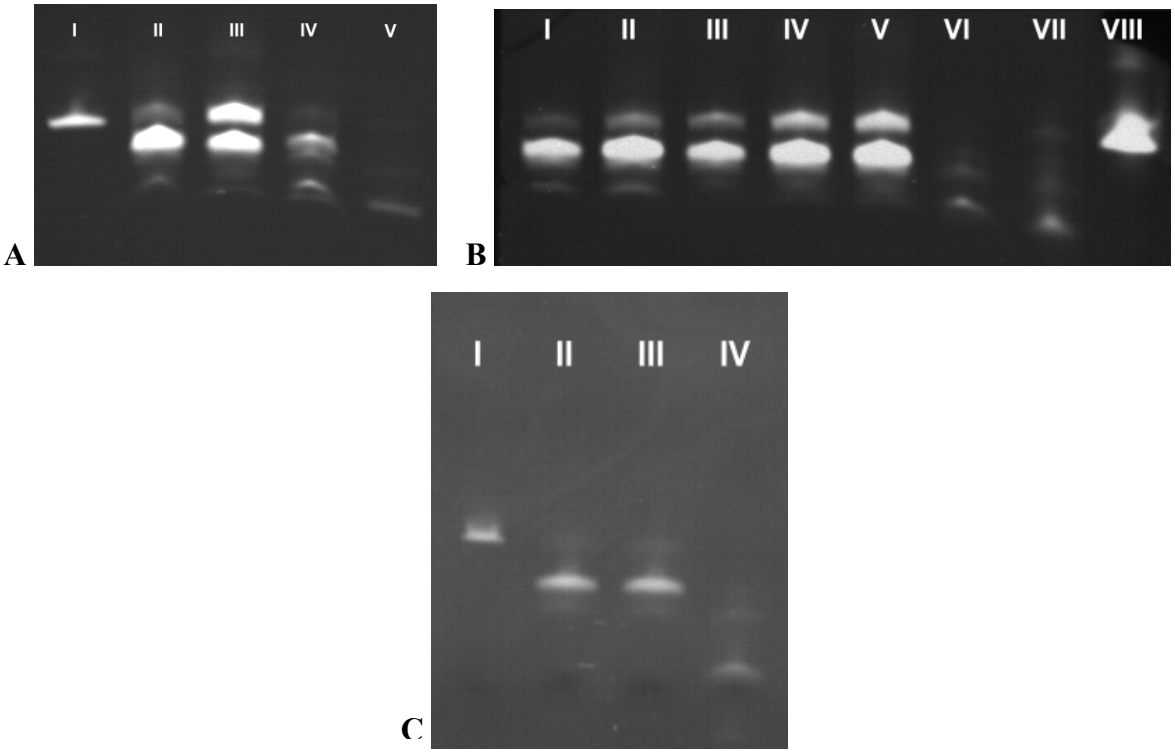


Figure 4. 6 Urea-PAGE analysis of optimization of double digestion of Adp#3. In A and C, Lane I shows undigested frame adapter

A) Unsatisfactory digestions with previously optimized conditions. Lane I: undigested Adp#3. Lane II: KpnI single digestion. Lane III unsatisfactory Nco I single digestion. Lane IV: unsatisfactory double digestion with the conditions optimized for preceding adapters. Lane V: control showing successfully double digested Adp#2.

B) Lanes I and II: 0.025 and 0.05 nmols; respectively, of Adp#3 digested with Kpn I. Lanes III and IV: 0.025 and 0.05 nmols, respectively, of Adp#3 digested with Nco I. Lane V: 0.05 nmol Adp#3 digested with doubled Nco I concentration. Lane VI: 0.025 nmol Adp#3 double digested with Kpn I and Nco I. Lane VII: previous successful digestion as double digestion positive control. Lane VIII: 0.025 nmol undigested Adp#3 as negative control. The concentrations are calculated using loading volumes of the synthesized adapter concentrations, which are reported by Seqlab, Germany.

C) Optimized conditions for Adp#3. Lane I: undigested Adp#3. Lanes II and III: Kpn I and Nco I digested, respectively. Lane IV: Concentrated, double digested sample. The sample is double-desalted using Micro-Spin™ G-25 Columns (Amersham Biosciences) after the concentration step.

4.3 Construction of Intermediate Plasmid: pETM-Adp

pETM-Adp plasmid was constructed twice by using defective second frame adapter (Adp#2) and final correct adapter (Adp#3). Results of the correct adapter used experiments are presented in this thesis.

pETM-11 was double digested with Kpn I and Sac I in preparation to ligation with Adp#3 (*Figure 4.7-A*). Gel extracted double digested plasmid was quantified with respect to Mass Ruler DNA Ladder™, High Range (Fermentas) molecular weight marker and estimated to be 100 ng/µl (*Figure 4.7-B*).

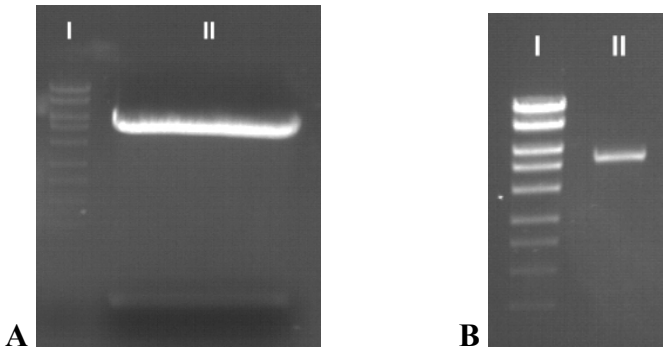


Figure 4. 7 Purification of double digested pETM-11. Lane I: Mass Ruler DNA Ladder™, High Range (Fermentas) in both pictures. A) 80 µl pETM-11 double digest (Kpn I, Nco I) was run on an agarose gel. B) Double digested pETM-11 was gel extracted and the purified fragment was checked and quantified on an agarose gel with respect to the molecular weight marker.

In a few unsuccessful ligation attempts, concentrated frame adapter digestion mixes were used together with double digested pETM-11. It was later suspected that unsuccessful ligations might be due to volume reduction by evaporation resulting in increased salt concentration. As later discussed, that could not be removed by single desalting treatment with Micro-Spin™ G-25 Columns (Amersham Biosciences). Double digested frame adapter was then desalted twice by using Micro-Spin™ G-25 Columns (Amersham Biosciences).

After double-desalting treatment, ligation was successfully achieved. Ligations were carried out in plasmid:insert ratio series as described in Methods section. Transformants were spread on Luria Agar plates with Kanamycin selection. After overnight incubation at 37^{o}C, colonies were picked and grown in 2 mL liquid cultures carrying 0.05 mg/mL

Kanamycin. Minipreps of transformed XL1-Blue colonies were checked by digesting with Kpn I and Nco I. Results were visualized by agarose gel electrophoresis (*Figure 4.8*).

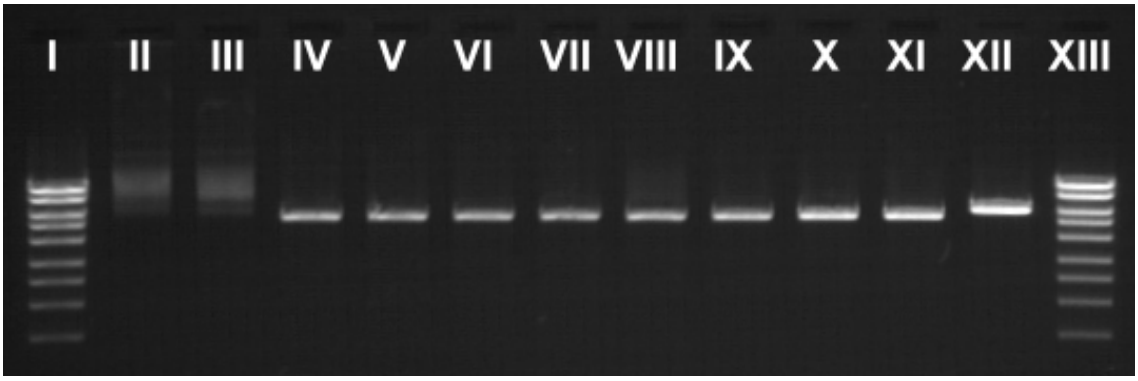


Figure 4. 8 Insertion of frame adapter was checked by digestion with Kpn I and Nco I. Lanes I and XIII: Mass Ruler DNA Ladder™, High Range (Fermentas) molecular weight marker. Lanes II and III: Uncut original pETM-11 and uncut isolated plasmid from colony #4. Lanes IV-XI: Kpn I and Nco I digested colonies #1 - #4 in pairs (i.e. Lanes IV, V: Col#1 Kpn I, Nco I linearized respectively). Lane XII: Kpn I linearized original pETM-11.

Gel results were consistent with theoretical length of pETM-Adp, 5369 bp. The diagram of constructed plasmid is given in *Figure 4.9*.

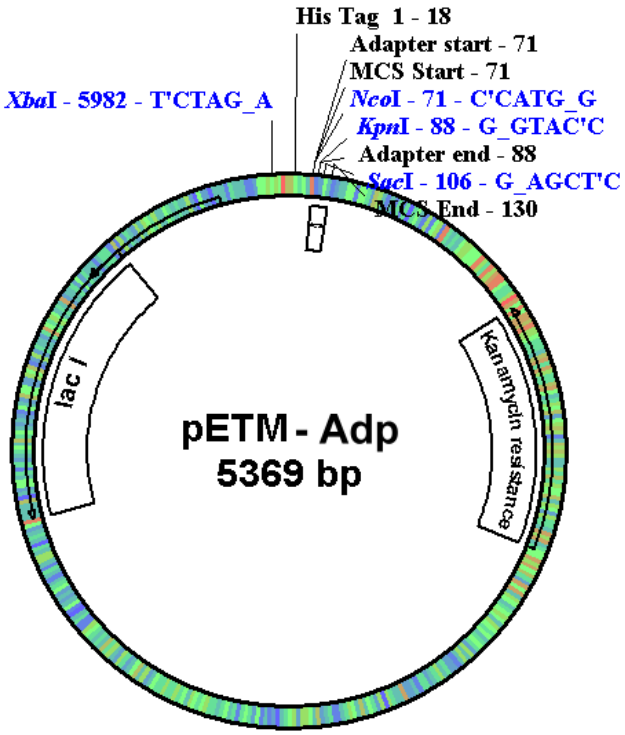


Figure 4. 9 The map of intermediate plasmid construct pETM-Adp. Small box between Nco I and Kpn I recognition sites is the inserted frame adapter. Arrows indicate the directions of open reading frames for the corresponding genes and colors indicate the GC content of local site (showing least GC% to most GC% with blue to red respectively). Map was constructed by plasmid drawing software pDraw32.

4.4 Construction of Immobilization Specialized Plasmid: pETM-GFP-Imm

The plasmid designed to facilitate immobilization was constructed by inserting GFPuv gene into the intermediate plasmid pETM-Adp.

pGFPuv and pETM-Adp plasmids were isolated using midi-prep plasmid isolation protocols from 250 mL liquid culture grown until OD_{600} value was ~ 4A.

pETM-Adp construct was double digested with Kpn I and Sac I in order to generate sticky ends necessary for directional insertion of GFPuv gene (*Figure 4.10-A, B*). DNA amount of pETM-Adp was estimated to be around 100 ng/µl. GFPuv was excised from pGFPuv (Clontech) plasmid using the same restriction enzymes (*Figure 4.10-C, D*). Molecular weight marker based length analyses were consistent with theoretical values of double digestion-linearized pETM-Adp (~5362 bp) and excised GFPuv (~724 bp) fragments.

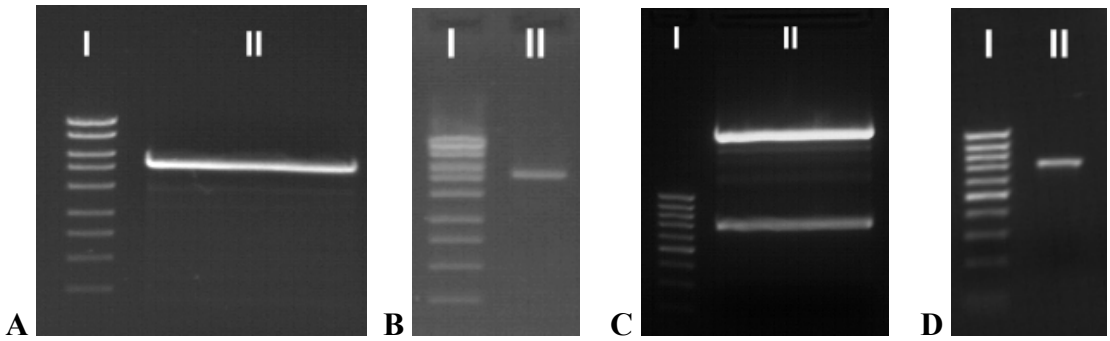


Figure 4. 10 Preparation of pETM-Adp and GFPuv fragments for ligation. Lane II in A and B: Double digested and gel purified pETM-Adp, respectively. C-II and D-II: Double digested pGFPuv and gel purified GFPuv gene, respectively. Lanes A-I and B-I: Mass Ruler DNA Ladder, High Range (Fermentas). Lanes C-I and D-I: Mass Ruler DNA Ladder, Low Range (Fermentas).

Ligations were carried out in plasmid:insert ratio series as described in Methods section. *E. coli* XL1-Blue cloning strain transformants were spread on Luria Agar plates with 0.05 mg/mL Kanamycin selective marker. *E. coli* BL-21 (DE3) expression strain transformants were also spread on Luria Agar with Kanamycin selective marker with the exception that half of the plate surface was pre-spread with 0.75 mM IPTG. After overnight incubation at 37 ^{o}C, colonies were picked and grown in 2 mL liquid cultures carrying 0.05 mg/mL Kanamycin in preparation for plasmid isolation and screening the presence of insert by double digestion with Nco I and Sac I followed by agarose gel electrophoresis (*4.11-C*). Digestion pattern and fragment sizes were consistent with theoretical expectations with the exception of XL1-Blue colony #2 (*Figure 4.11-C(IV)*), which was excluded in the subsequent experiments. In addition, positive colonies were already identified in BL-21 (DE3) spread colonies due to green fluorescence under UV illumination as a result of IPTG induced expression of inserted GFPuv gene (*Figure 4.11-A*).

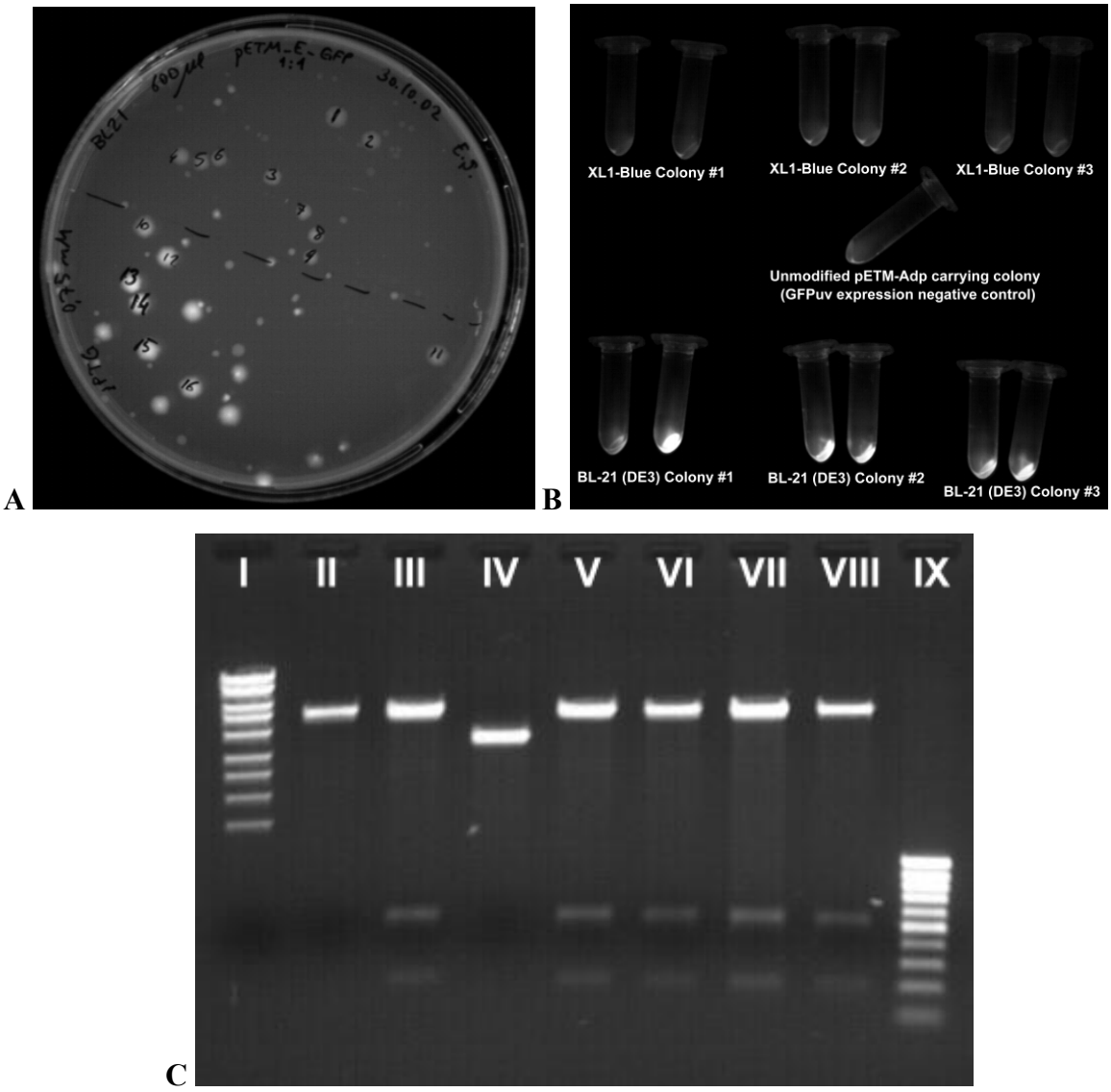


Figure 4. 11 Verification of pETM-GFP-Imm construction by fluorescence under UV illumination. (A) shows the expression of GFP in transformants on Luria Agar + Kanamycin plate. Before inoculation, 0.75 mM IPTG was spread below the dashed line to promote GFPuv protein expression. (B) Pellets of bacterial colonies picked from the plate shown in A. Left and right tubes in each pair shows liquid cultures without and with IPTG respectively. The tube in the middle is the negative control pellet which does not contain GFPuv gene and showing no fluorescence. (C) Electrophoretic analysis of screening results. Lanes I and IX: Mass Ruler DNA Ladder™, High Range and Low Range (Fermentas) molecular weight markers, respectively. Lanes III, IV and V: Plasmids isolated from three different XL1-Blue strain colonies. Lanes VI, VII and VIII: Plasmids isolated from three different BL-21 (DE3) strain colonies. Lane II: The unmodified pETM-Adp construct.

For further verification of the construct, isolated plasmid was sequenced (Seqlab, Göttingen) and the adapter and the insert were proved to be 100% identical to the designed

Adp#3 and the source GFPuv gene (Appendix H). The immobilization specialty plasmid pETM-GFP-Imm was thus constructed as a result of successful ligation and transformations, and frozen glycerol stocks were prepared as described in Methods section. *Figure 4.12* shows the map of the constructed pETM-GFP-Imm plasmid.

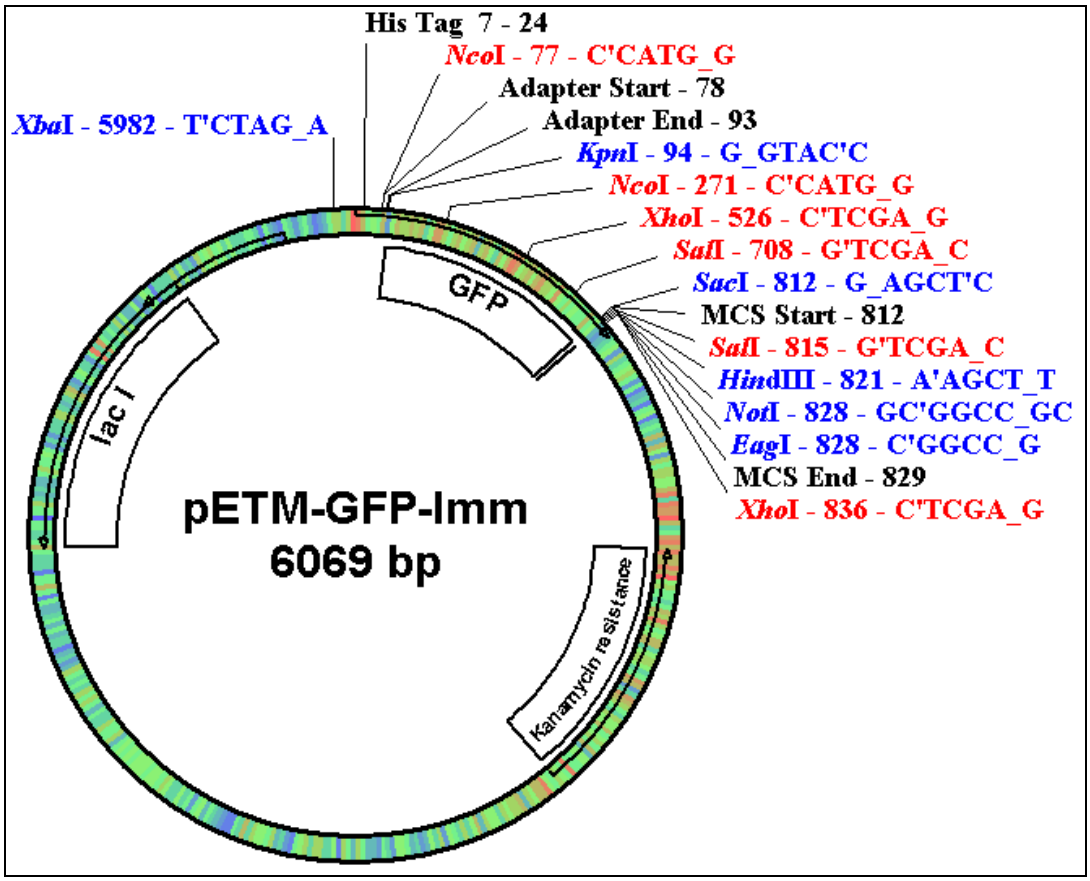


Figure 4. 12 The map of immobilization specialty plasmid: pETM-GFP-Imm. Blue restriction enzyme recognition sites belong to single cutter enzymes. Arrows indicate the directions of open reading frames for the corresponding genes and colors indicate the GC content of local site (showing least GC% to most GC% with blue to red respectively). The map was constructed by plasmid drawing software pDraw32.

4.5 Expression and Large Scale Purification of GFP Variants, GFPuv and GFPimm

BL-21 (DE3) strain colonies containing pGFPuv and pETM-GFP-imm plasmids were used for expression of GFPuv and GFPimm proteins, respectively. Expression was carried out as described in Methods section.

pETM-GFP-Imm containing BL-21(DE3) cells were induced with 0.7 mM IPTG together with untransformed BL-21(DE3) and uninduced pETM-GFP-Imm carrying BL-

21(DE3) as negative controls of expression in terms of transformation and induction, respectively. Cells were harvested with sampling amounts as stated in Methods section. Time and IPTG concentration dependent expression was followed by analysis of cell lysate using PAGE. Presence of GFPimm protein was detected with pETM-GFP-Imm transformed uninduced and pETM-GFP-Imm transformed induced cells as shown in lanes A-VII, B-IV, B-VII and C-IV of SDS-PAGE analyses (*Figure 4.13-A,B,C*).

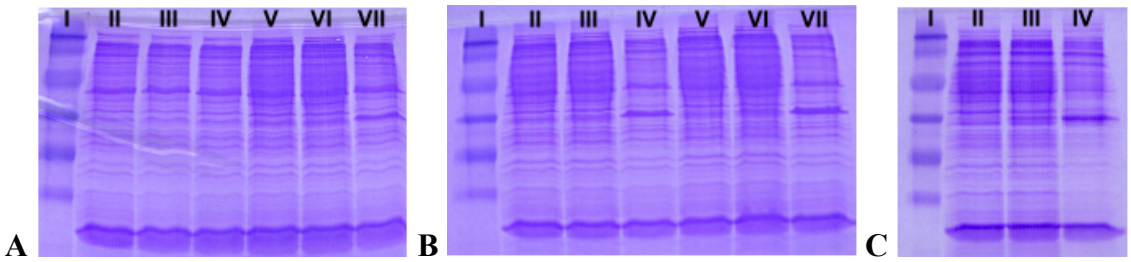


Figure 4. 13 SDS-PAGE analyses of expression of GFPimm protein. In A, B and C lanes I are loaded with Prestained Protein Molecular Weight Marker (Fermentas). A-II, III, IV: t = 0 min.; A-V, VI, VII: t = 90 min.; B-II, III, IV: t = 180 min.; B-V, VI, VII: t = 240 min.; C-II, III, IV: t = 990 min. samplings of untransformed, pETM-GFP-Imm transformed (uninduced), pETM-GFP-Imm transformed (0.7 mM IPTG induced) BL-21(DE3) *E. coli* colonies, respectively.

Isolation of GFPimm protein was carried out using BL21(DE3) cells grown in 500 mL LB liquid culture medium with kanamycin and 0.7 mM IPTG. GFPimm was isolated using ProBond™ resin (Ni^{2+}-Agarose) (Invitrogen) following basically the protocols given with ProBond™ His-tag based protein purification kit. The only exception was that during scaling up, 40 mL 1X native buffer was used for resuspension of cells pelleted from 500 mL liquid culture. Rest of the protocol was applied in accordance with the indicated scale-up.

Figure 4.14-A shows UV illumination of the fractions collected during isolation of GFPimm protein using ProBond™ resin. In Figure *4.14-B* and *C*, PAGE analyses of control samples taken at different stages of isolation and the isolated protein fractions can be seen, respectively. 1 mL fractions were collected during purification of GFPimm. UV illumination results showing that fractions V, VI and VII, eluted at 4 – 10 mL are enriched in GFPimm. The result is also confirmed by the SDS-PAGE analyses. First set of GFPimm

isolates precipitated during dialysis in preparation to surface binding, which is discussed later.

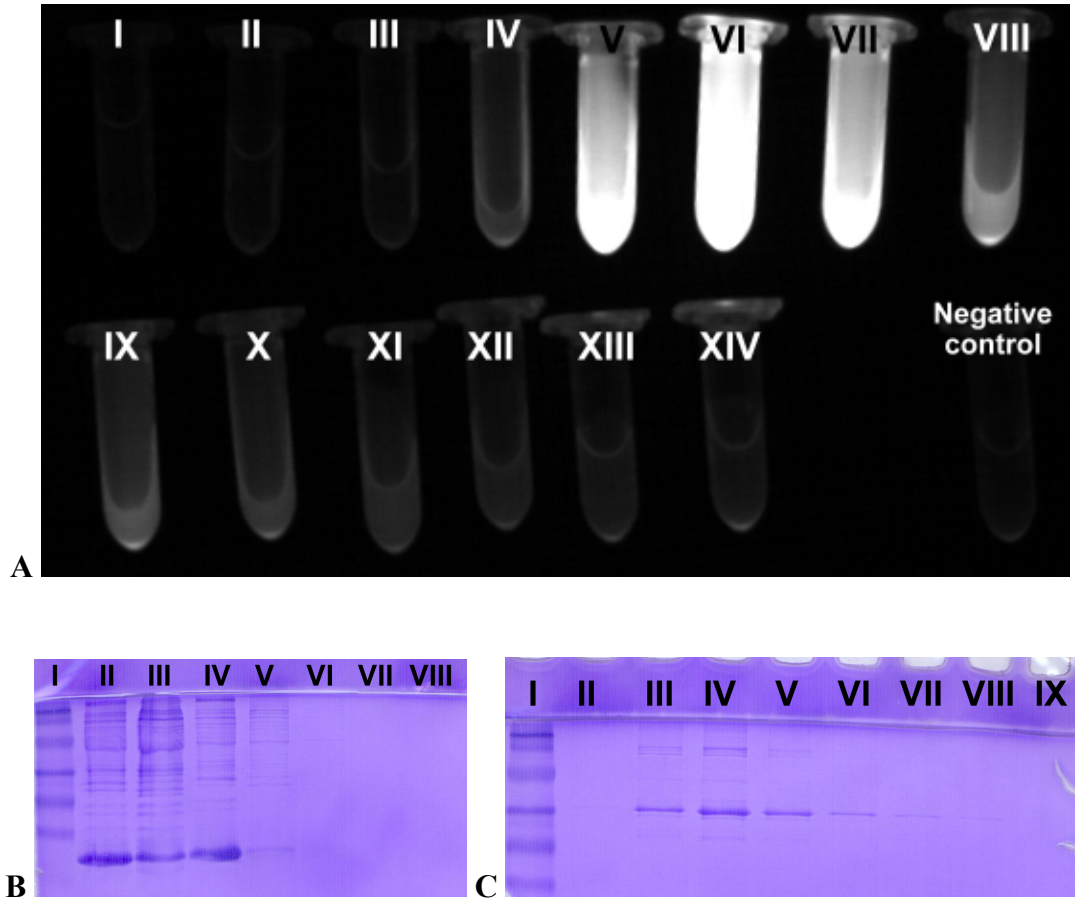
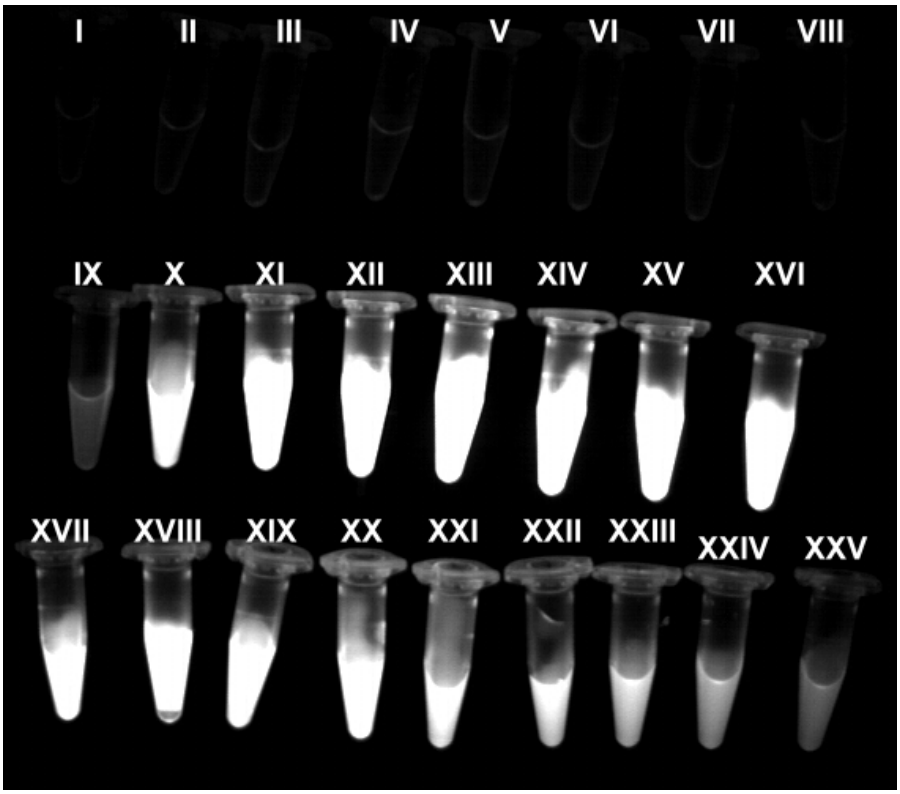


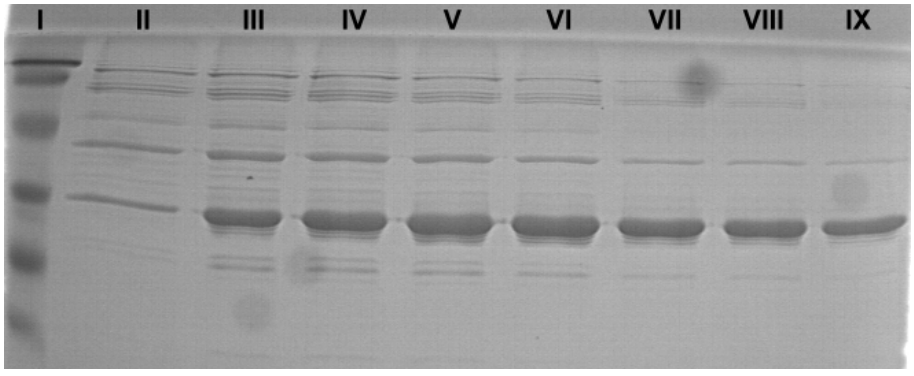
Figure 4. 14 Analyses of results of GFP-Imm purification. (A) UV illumination of fractions collected during isolation of GFPimm protein together with purification buffer filled negative control tube. (B) and (C) SDS-PAGE Analysis of fractions and controls. Lanes I Prestained Protein MW Marker (Fermentas). (B) Lanes II and III samples taken from supernatant and pellet, respectively, after centrifugation of lysed cells. Lane IV the discarded buffer after the Ni^{2+} binding step of histidine tag. Lanes V-VIII wash steps where resin bound recombinant protein is cleared from contaminants. (C) Lanes II-IX electrophoretic analysis of protein in tubes IV-XI in A.

GFPuv protein was expressed and isolated from ampicillin and 0.7 mM IPTG containing 500 mL liquid LB broth medium in parallel to GFPimm to be used as a control in surface binding studies. Protein isolation was carried out on a MonoQ (Amersham Pharmacia) column connected to an Akta-FPLC system, since GFPuv does not have the polyhistidine tag, which is the prerequisite for ProBond™ resin. 0.5 mL fractions were

collected during purification of GFPuv. Details of the isolation procedure are given in Methods section. Photographs of fractions, SDS-PAGE gel and absorption spectra of most concentrated fractions in GFPuv isolation are given in *Figure 4.15-A, B, C, D*. GFPuv expression and purification yielded five to ten times more GFP when compared to GFPimm in all isolation attempts. The possible reasons of this difference are mentioned in Discussion section.

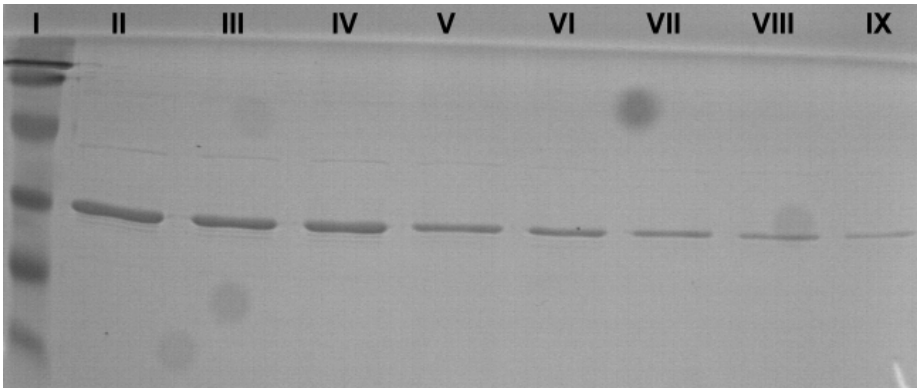


A



B

C



D

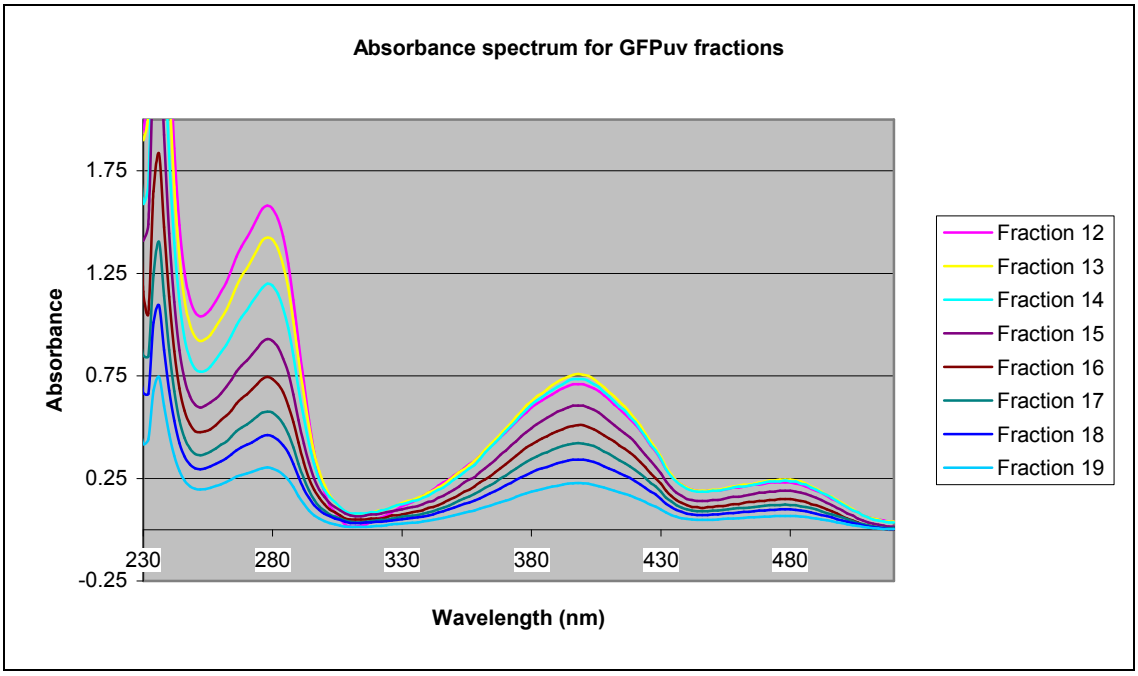


Figure 4. 15 Analyses of results of GFPuv purification. (A) UV illumination of fractions collected during isolation of GFPuv protein. (B) and (C) SDS-PAGE Analysis of fractions. Lanes I: Prestained Protein MW Marker (Fermentas). (B) Lanes II-IX: fractions X-XVII shown in A. (C) Lanes II-IX: fractions XVIII-XXV shown in A. (D) Absorption spectra of most concentrated fractions (fractions XI-XIX in A).

4.6 Surface Binding

As a preparation for surface binding studies GFPuv and GFPimm were standardized in terms of GFP concentration and buffer composition.

4.6.1 Preparation of GFPimm

As the first step of binding preparation for GFPimm, 3 mL total volume was reached by combining fractions V – VII in *Figure 4.14-A*.

Imidazole and excess Na^+ ions coming from the elution buffer, which are potential competitive inhibitors of surface binding reaction, remain present in isolated GFPimm solution unless removed. GFPimm in 1X ProBond™ Native Purification Buffer, pH: 8.0 (50 mM NaH_2PO_4, 500 mM NaCl, pH adjusted with NaOH) was divided into three and dialyzed against 0.1X ProBond™ Native Purification Buffer at pH 6.0, pH 7.0 and pH 8.0 (5 mM NaH_2PO_4, 50 mM NaCl, pH adjusted with NaOH). Details of the dialysis step are given in Methods section.

Dialysis resulted in sample loss by precipitation (*Figure 4.16*), thus after the first trial, newly isolated GFPimm and GFPuv samples were dialysed against 0.5X ProBond™ Native Purification Buffer at pH 6.0, pH 7.0 and pH 8.0 (25 mM NaH_2PO_4, 250 mM NaCl, pH adjusted with NaOH).

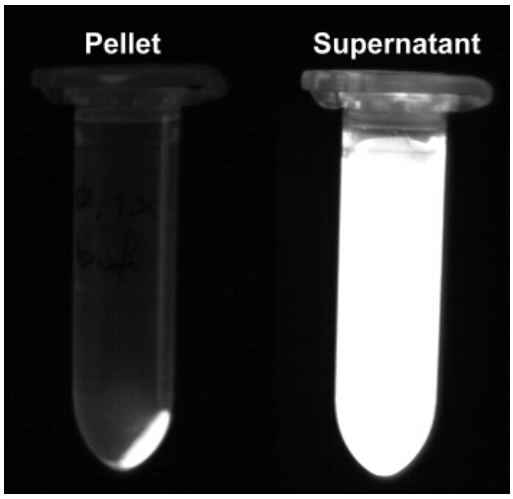


Figure 4. 16 Figure shows the sample loss due to precipitation during dialysis.

4.6.2 Preparation of GFPuv

As the first step of binding preparation for GFPuv, 4 mL total volume was reached by combining fractions XII – XIX in *Figure 4.15-A*.

GFPuv protein was also dialyzed against 0.5X ProBond™ Native Purification Buffer at pH 6.0, pH 7.0 and pH 8.0 in order to equalize binding parameters for GFPuv and

GFPimm even though the former does not have imidazole coming from the purification buffer.

4.6.3 Modification of Polystyrene Surface in Preparation for Binding Reaction

For binding studies, polystyrene 96-well plates were treated with 2 M and 3 M APS as described in Methods section, and characterized by FT-IR. By using the strong oxidizing agent, ammonium persulfate (APS), achievement of a heterogeneous pool of functional groups (containing carboxylic acids, alcohols and ketones on the surface of polystyrene material) was aimed. Fourier transform infrared (FT-IR) spectroscopy results revealed the presence of these groups in the surface-most 1μm layer of polystyrene material (*Figure 4.17*). Details of the preparation of surface modification are given in Methods section.

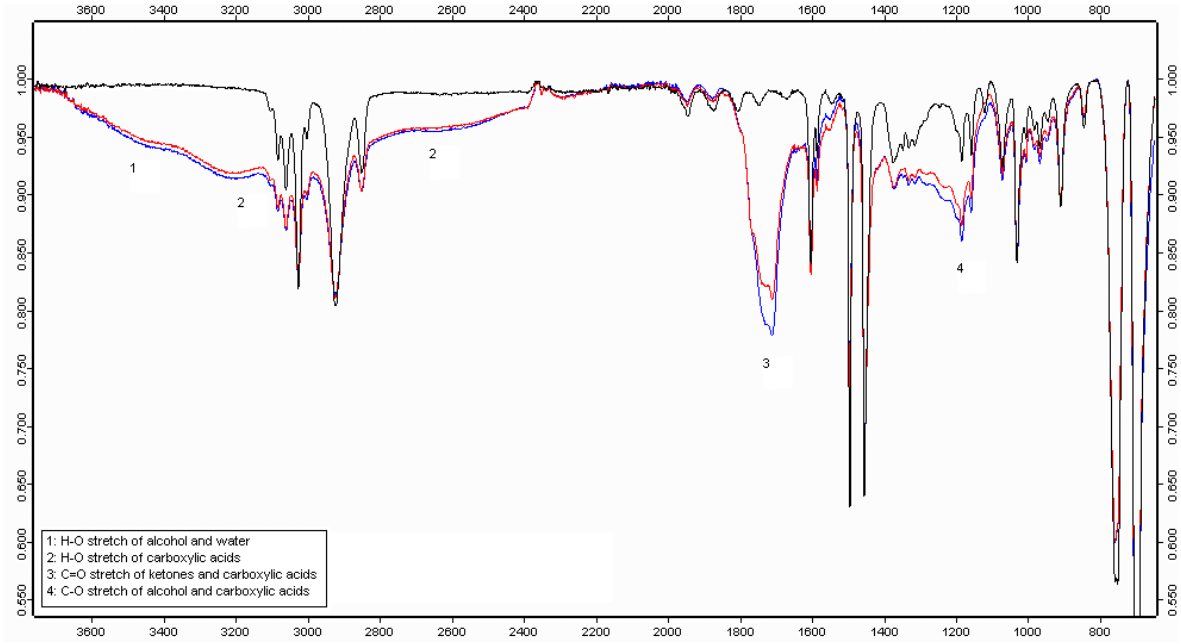


Figure 4. 17 FT-IR spectrum of unmodified, 2M and 3M APS modified polystyrene samples. Black, blue and red lines represent unmodified, 2M and 3M APS modified polystyrene samples respectively. The functional groups and the regions that corresponds to them are shown in the legend at the lower left corner. X-axis and y-axis show wavenumber (cm^{-1}) and transmittance ratio (over 1.0 perfect transmittance), respectively.

4.6.4 Surface Binding

Since first large scale GFPimm isolates were lost due to unwanted precipitation during dialysis, a second isolation was carried out. SDS-PAGE analysis of the most concentrated GFPimm fractions of the second isolation are given in *Figure 4.18-A*. After dialyzing both GFPuv and GFPimm as stated in Methods, amount and purity of previously isolated GFPuv was compared to that of isolated GFPimm by running GFPimm on SDS-PAGE gel together with different dilutions of GFPuv (*Figure 4.18-B*). The absorption values at the characteristic wavelength of GFP, which is 398 nm, were taken into consideration while comparing concentrations of GFPuv and GFPimm. Based on the fact that concentration is directly proportional to absorbance at the molecule-specific absorption wavelength, GFPuv was estimated to be three times concentrated in comparison to GFPimm.

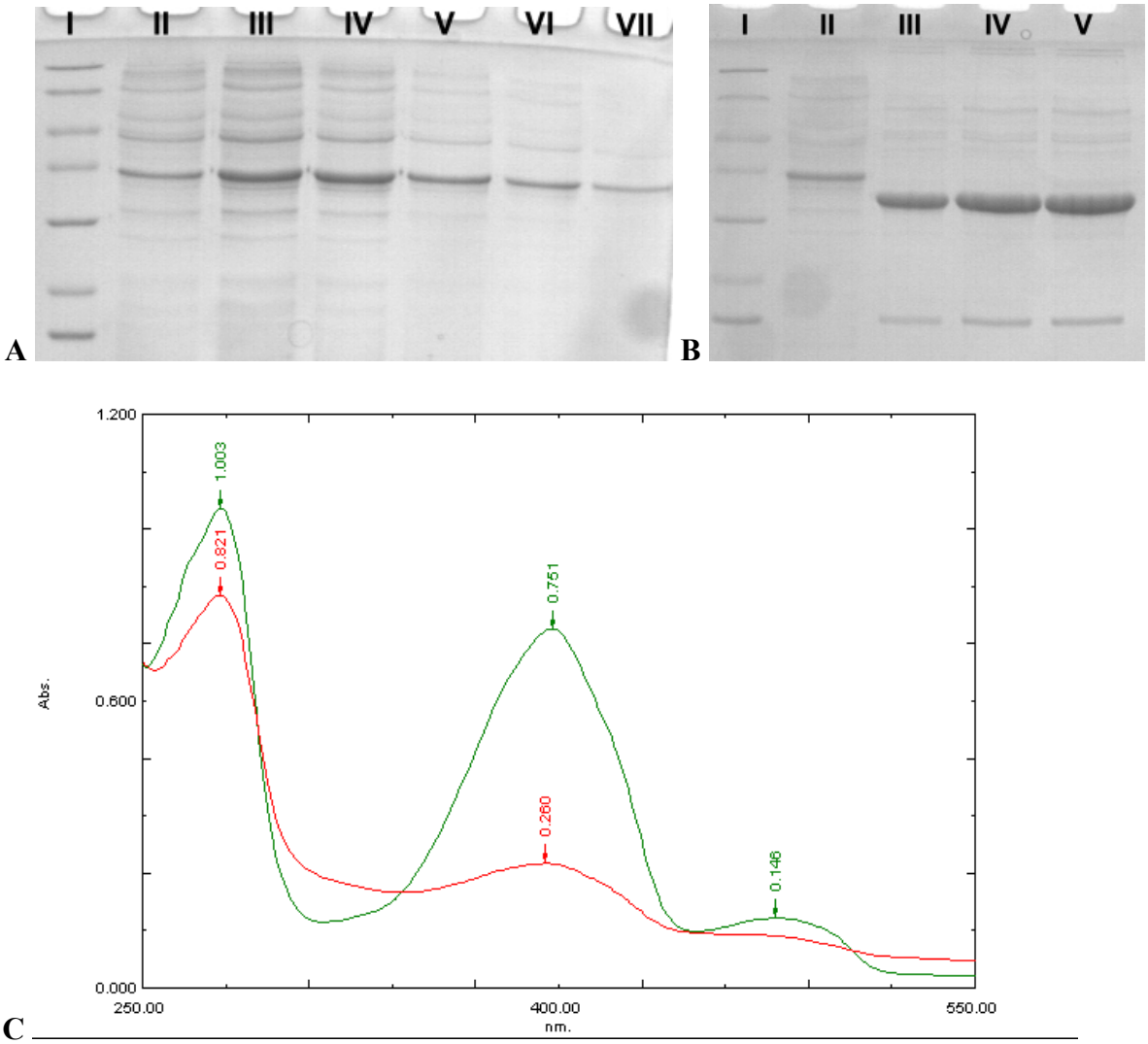
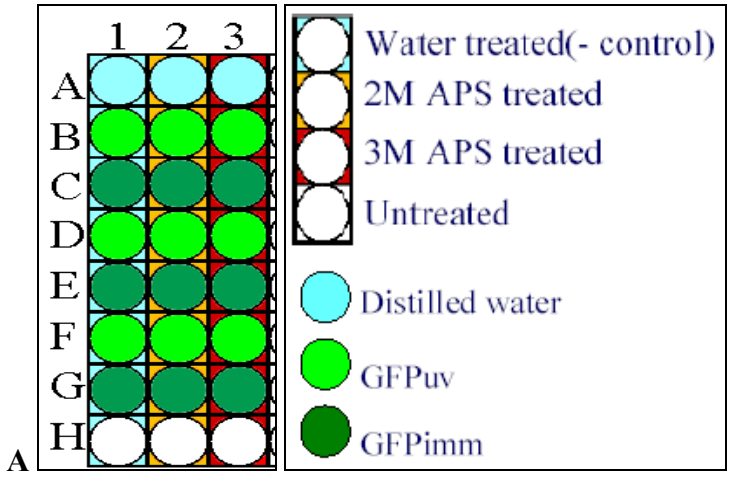


Figure 4. 18 Analysis of GFPimm fractions and GFPimm-GFPuv equalization check. Lane I: Protein Molecular Weight Marker (Fermentas) in both A and B. (A) Lanes II-VII: GFPimm fractions. (B) Lane II: combined and dialyzed GFPimm fractions. Lanes III, IV and V: GFPuv in ratios of 3:1, 2:1 and 1:1 (v:v) when compared to GFPimm loaded on lane II. (C) Absorption spectra of GFPuv (green) and GFPimm (red). Absorption measurements were carried out at 398 nm, the characteristic wavelength for GFP.

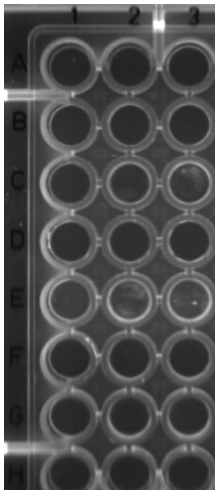
Based on the comparison of absorption measurements of GFPuv and GFPimm, GFPuv was loaded three times diluted and GFPimm was applied undiluted into the wells of modified 96 well plate for binding studies. After incubating the plate with protein solutions for 2 hours, wells were emptied and rinsed repeatedly with appropriate buffers as indicated in Methods section. Chemical (APS) and biological (GFP) modification scheme for the 96 well plate and legends for color codes used in the scheme are given in *Figure 4.19-A*. After rinsing, UV transmitted photographs of plates were taken with and without buffer in the

wells where binding had occurred (*Figure 4.19-B and C*). Similarly fluorescence measurements were carried out on dry and 100 µl appropriate buffer filled plates. Results of fluorescence measurements on dry and buffer filled wells are given in *Figure 4.19-D, E* as histograms, while actual values are supplied in Appendix I. Histograms given in Figure 4.19-D, E show a summary of fluorescence measurements from dry and 100 µl appropriate buffer filled wells respectively. It can be seen that GFPimm binds to the modified polystyrene with higher efficiency in comparison to binding of GFPuv. The results also show that modification efficiency of 3M APS is higher than that of 2M APS. Fluorescence measurements of wells filled with buffer (*Figure 4.19-E*) still yield higher values for GFPimm, however, the difference between binding efficiencies of GFP variants was measured to be less. The reason for the inconsistency between dry and wet measurements may be optical phenomena affecting beam path and detected intensity due to presence/absence of buffer and shape of meniscus it formed within the well as a result of altered hydrophilicity of the wells after APS treatment.

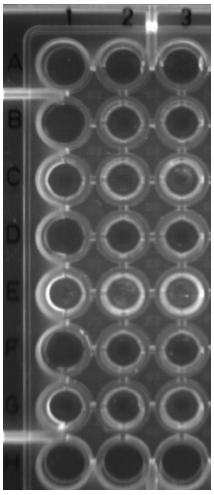
In the histograms and Appendix I, measurements from a column of unmodified wells adjacent to the modified ones are also included. These reflect the level of noise coming from neighboring GFP-bound wells of the transparent plate. Such high level of background prompted the use of black 96-well plates in further experiments.



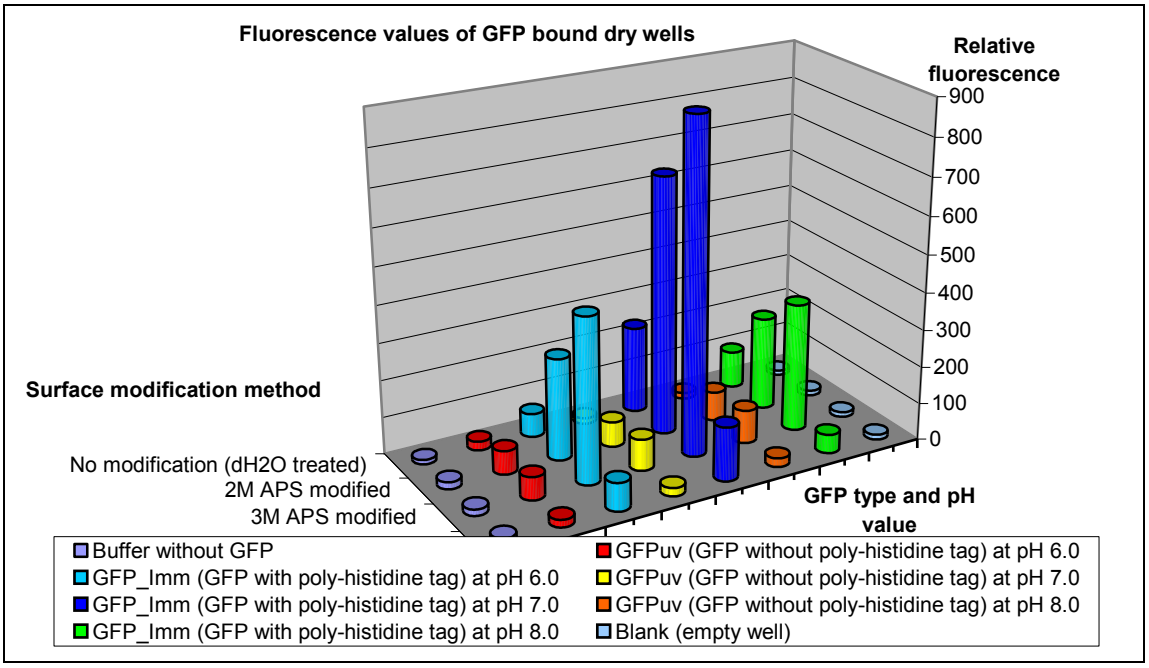
B

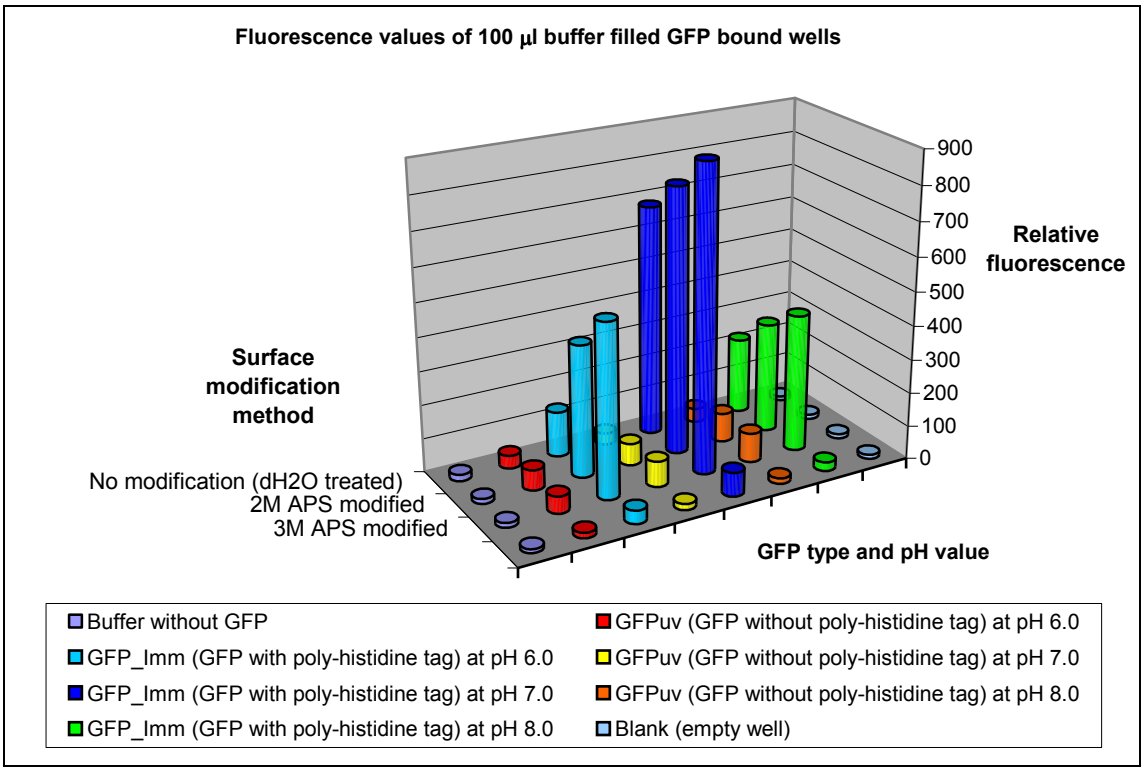

1
2
3
A
B
C
D
E
F
G
H

C


1
2
3
A
B
C
D
E
F
G
H

D


Fluorescence values of GFP bound dry wells
Relative fluorescence
900
800
700
600
500
400
300
200
100
0
Surface modification method
No modification (dH2O treated)
2M APS modified
3M APS modified
GFP type and pH value
Buffer without GFP
GFP_Imm (GFP with poly-histidine tag) at pH 6.0
GFP_Imm (GFP with poly-histidine tag) at pH 7.0
GFP_Imm (GFP with poly-histidine tag) at pH 8.0
GFPuv (GFP without poly-histidine tag) at pH 6.0
GFPuv (GFP without poly-histidine tag) at pH 7.0
GFPuv (GFP without poly-histidine tag) at pH 8.0
Blank (empty well)



E

Figure 4. 19 (A) Modification scheme mapping the 96 well plate in terms of chemical and biological modification. Legends and color codes are indicated in order to display the nature of modifications in each well. (B) and (C) Photographs of UV transmitted dry and 100 µl appropriate buffer (for each well) filled plates respectively. (D) and (E) Measurement of fluorescence from GFP bound 96 well dry and 100 µl appropriate buffer (for each well) filled plates. One additional (empty) column was included in D and E to reveal the effect of neighboring well on fluorescence measurement.

4.6.4.1 Repeating the surface binding experiment using black 96-well plates

Since scattering from adjacent cells seemed to affect fluorescence measurements in transparent polystyrene 96 well plates, black polystyrene 96 well plates were used instead, in a second surface binding experiment. Black 96 well plates were modified with APS as previously stated and nature of modification was checked using FT-IR spectroscopy (*Figure 4.20*).

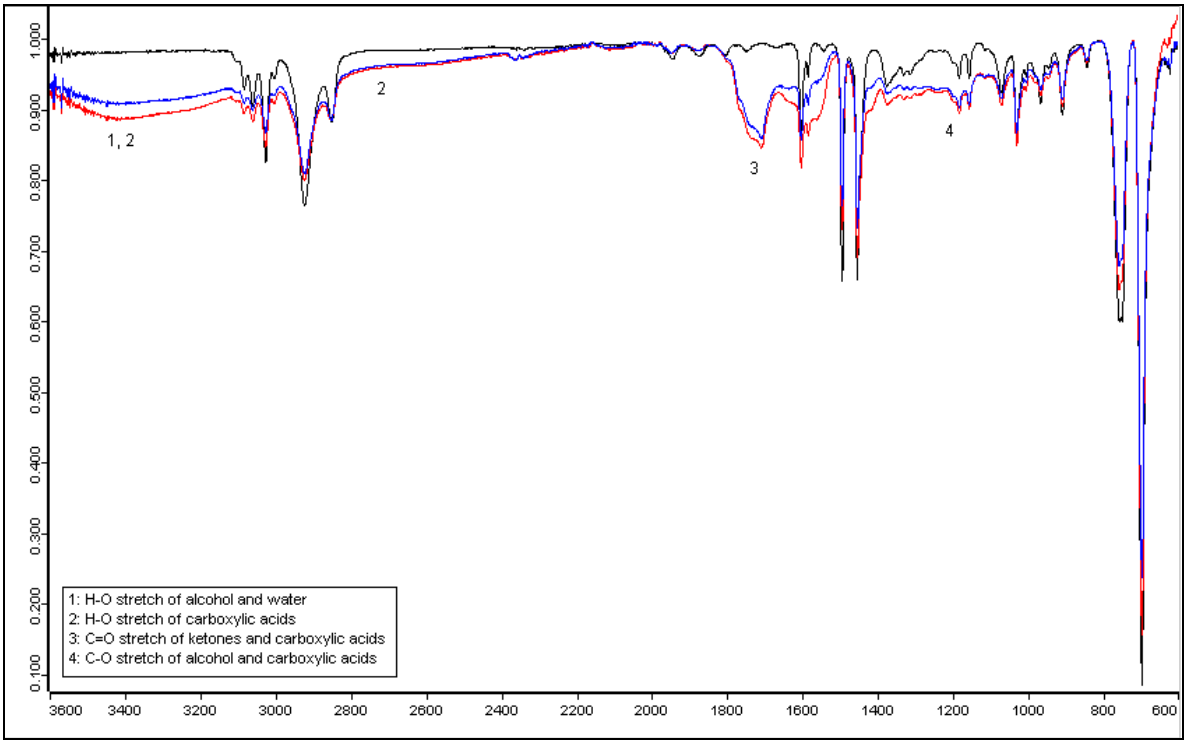


Figure 4. 20 FT-IR spectrum of black polystyrene 96 well plates. Black PS plates were modified using 2 M and 3 M APS solutions and showed the same pattern with previously modified transparent plates. Black, blue and red lines represent unmodified, 2 M and 3 M APS modified samples. X-axis and y-axis show wavenumber (cm^{-1}) and transmittance ratio (over 1.0 perfect transmittance), respectively.

Expression of GFPuv and GFPimm was performed once again in order to prepare samples for black 96-well plate binding measurements. For this set of GFPuv and GFPimm isolations, protocols given in Methods section 3.2.4.1 are applied for both GFP variants. Elution profiles of GFPuv and GFPimm are given in *Figure 4.21-A, B*. Both GFP variants showed similar elution properties but GFPimm separation was not as successful as GFPuv as seen in peak separations in *Figure 4.21-A, B*. By combining the fractions with highest GFP concentration, a total of 4 mL of both GFPuv and GFPimm protein solutions were pooled and dialysed against 0.5X Probond Native Purification Buffer as described in Methods section.

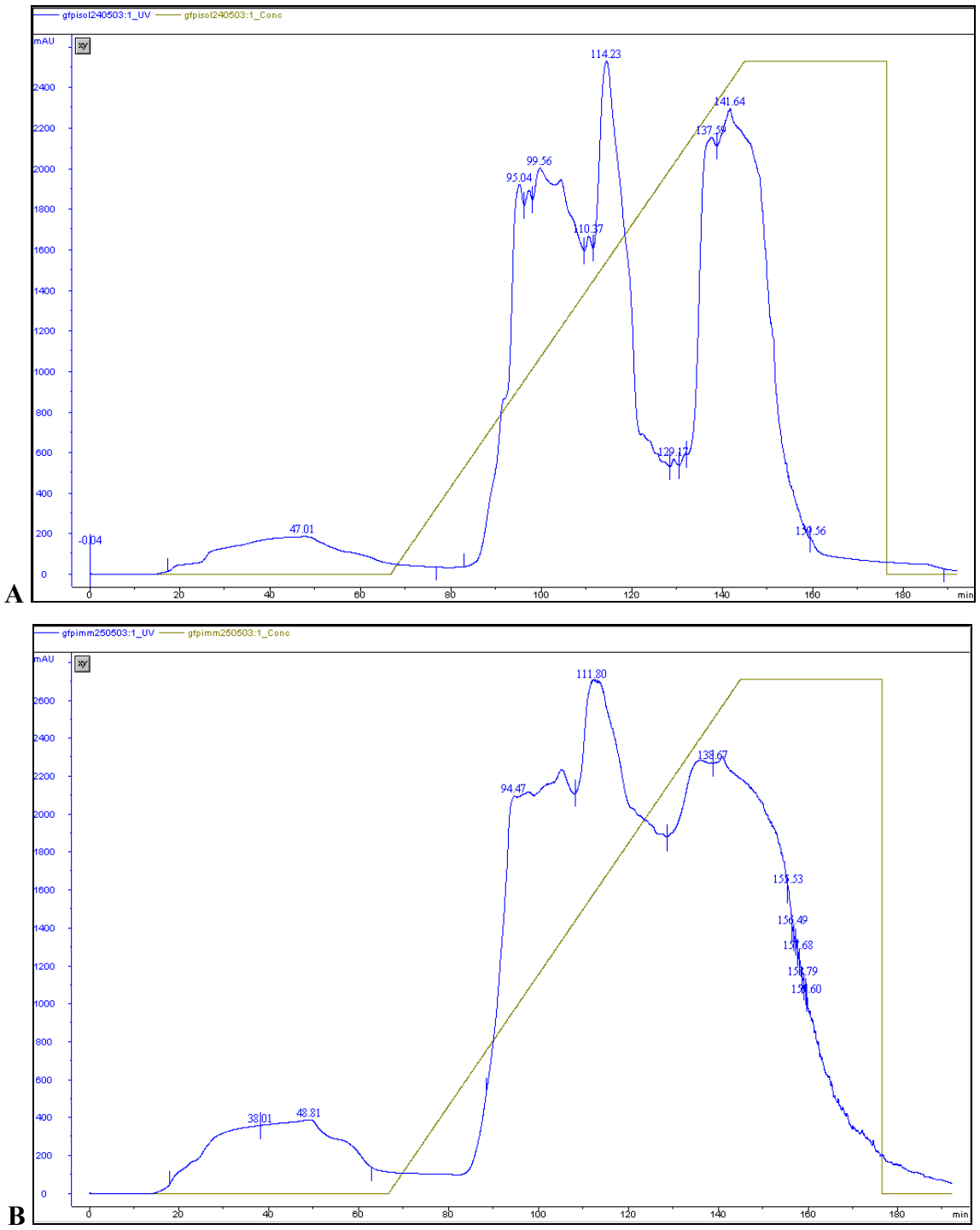


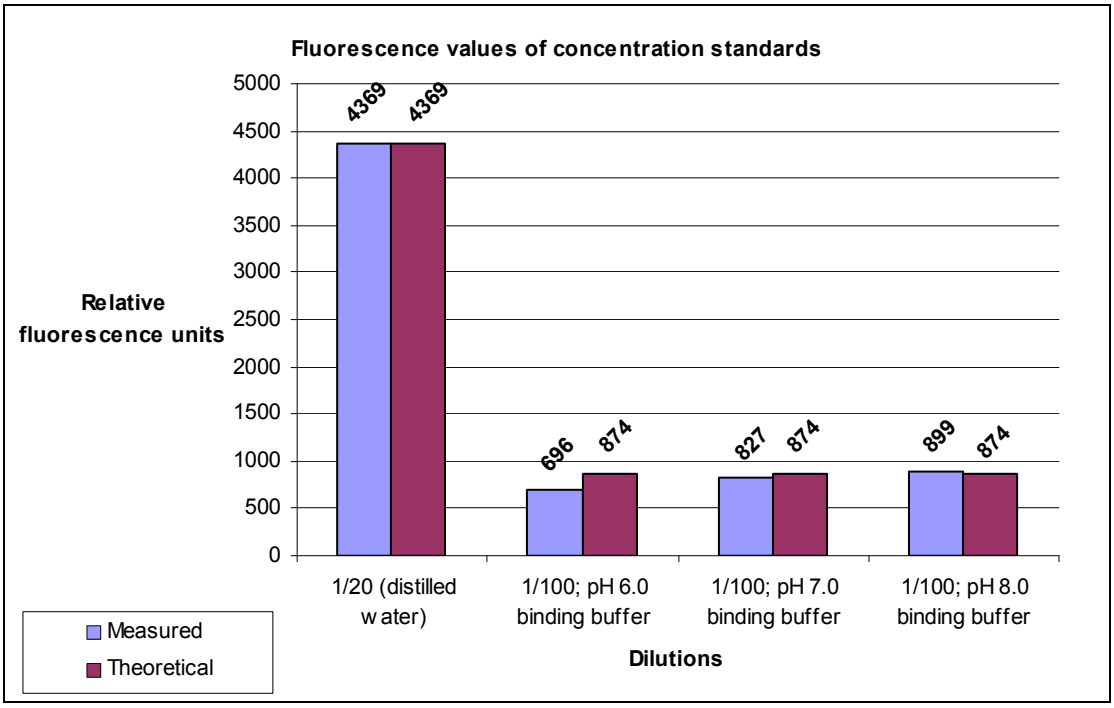
Figure 4. 21 Elution profiles of GFP variants from the Mono Q column. UV absorption at 280 nm (blue) and percentage gradient for buffer pairs (green,100% Buffer A to 100% Buffer B from left to right), during elution are shown. Both GFPuv and GFPimm were eluted the peaks between 90 and 120 minutes on the profiles. (A) Elution profile of GFPuv. (B) Elution profile of GFPimm.

Before binding studies, dilutions of rGFPuv protein (Clontech), which was purchased as a standard, were applied to an unmodified plate in order to find the correlation between the GFP concentration and the relative fluorescence intensity measurement. *Figure 4.22-A* shows fluorescence intensity measurement results of dilutions of rGFPuv protein. 1/100 dilutions at pH 6, 7 and 8 were compared to the theoretical values based on the fluorescence of 1/20 diluted rGFPuv protein. 1/20 diluted sample loaded well contained 10 μg rGFPuv while 1/100 diluted sample loaded well contained 2 μg of the protein. Fluorescence of isolated GFPuv and GFPimm proteins were also measured and values for serial dilutions of isolated GFPuv and GFPimm proteins are given in *Figure 4.22-B*. The measurements were found to be meaningful at 0.005x and 0.0005x dilutions region by comparing with expected values calculated using the applied dilution factors. Fluorescence values measured in this region were considered within the linear range of concentration–fluorescence correlation while very low intensity measurements at higher dilution factors were found to be within the noise level. The inconsistency between fluorescence values of 0.05x and 0.005x diluted GFP samples may be due to the potentially lower pipetting accuracy as a result of significantly high viscosity of concentrated protein solutions.

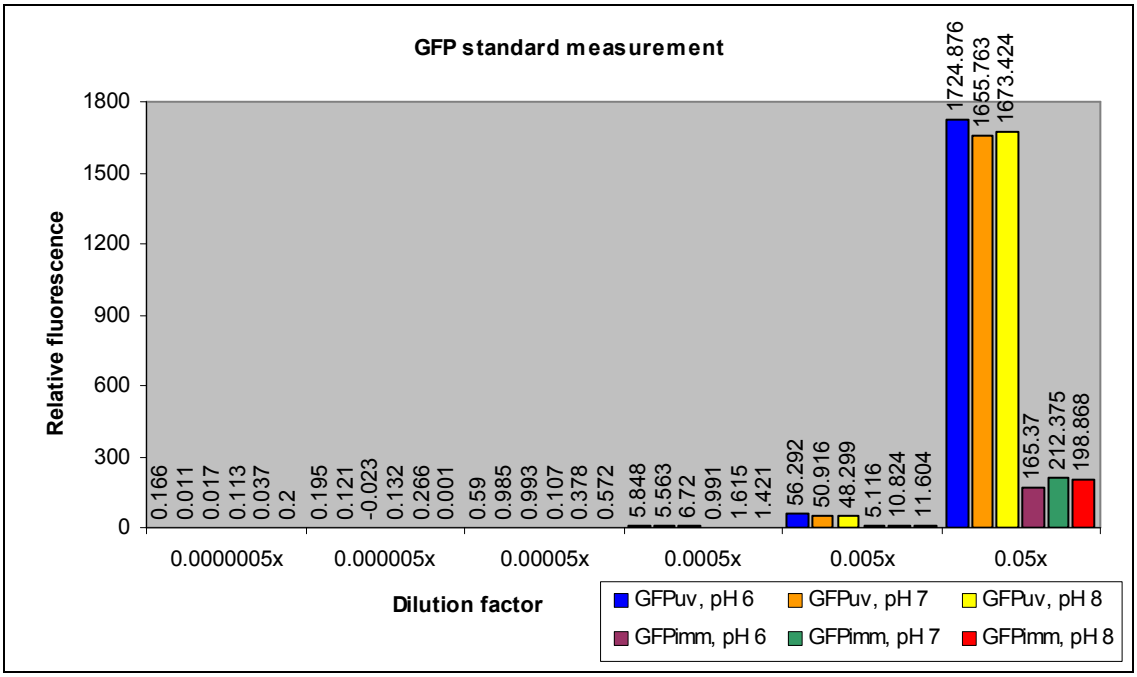
In order to complement estimates on the GFPuv/GFPimm concentration ratio, absorption measurements were also performed in parallel to fluorescence spectroscopy. For these samples absorption values for GFPuv and GFPimm at characteristic wavelength of GFP (398 nm) were measured to be 1.850 and 0.470, respectively. Thus, their concentration ratio was calculated to be approximately 4, which was consistent with fluorescence intensity ratios of 0.005x diluted GFPuv and GFPimm (~ 5) given in *Figure 4.22-B*. Standardization of GFPuv and GFPimm amounts (with respect to rGFPuv standard) in preparation of surface loading, concentrations were thus estimated using absorption, fluorescence measurements and SDS-PAGE results (*Figure 4.22-B, C, D*). As mentioned in in detail in the Discussion section, by quantification of GFP variants with two independent methods, it was also shown that the fluorescence yield of GFPimm was unaltered by the addition of poly-histidine tag. This standardization was done with the assumption that surface bound GFP variants have the same fluorescence characteristics with rGFPuv in solution, which is also discussed in the Discussion section. Based on the measurements of fluorescence intensity of dilutions of rGFPuv standard and 0.005x diluted purified proteins,

purified GFPuv and GFPimm were used at around 400 ng/μl and 40-50 ng/μl, respectively. According to the estimated ratio of GFPuv and GFPimm, same amount of GFP was loaded in wells by 5 times diluting GFPuv.

A



B



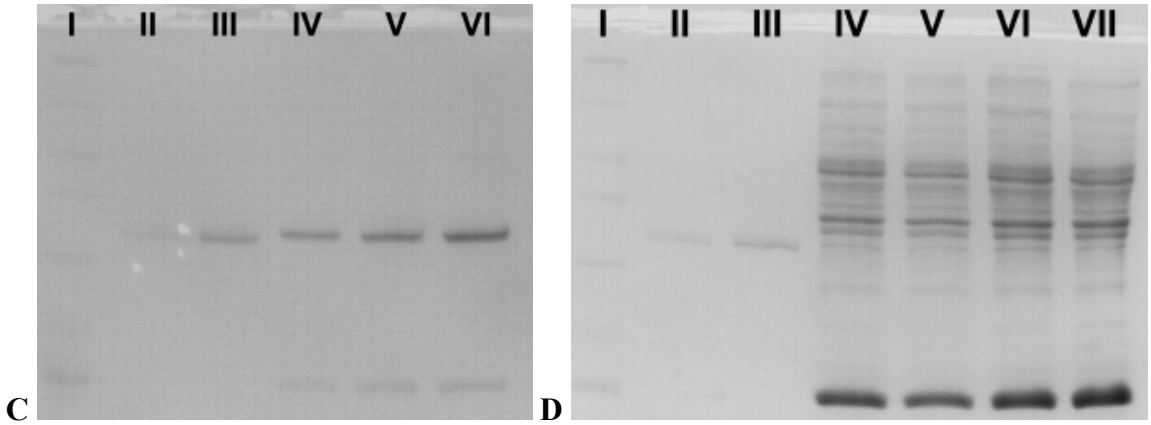


Figure 4. 22 (A) Histogram showing fluorescence values for rGFPuv standard protein. 1/20 dilution well: 10 μg. 1/100 dilution wells: 2 μg of rGFPuv standard. (B) Histogram showing fluorescence values for serial dilutions of isolated GFPuv and GFPimm proteins starting from 20x diluted and ending at 2000000x diluted from right to left. (C) SDS-PAGE analysis of isolated GFPuv protein for checking the purity and quantification. Lanes IV, V and VI: 1, 3 and 5 μl (2x diluted) isolates loaded on the gel. (D) SDS-PAGE analysis of isolated GFPimm protein for checking the purity and quantification. Lanes IV, V, VI and VII: 3, 5, 7 and 9 μl (2x diluted) isolates loaded on the gel. Lane I: Protein MW Marker (Fermentas) in C and D. Lanes II and III were loaded with 500 ng and 1μg rGFPuv protein in both gels shown in C and D.

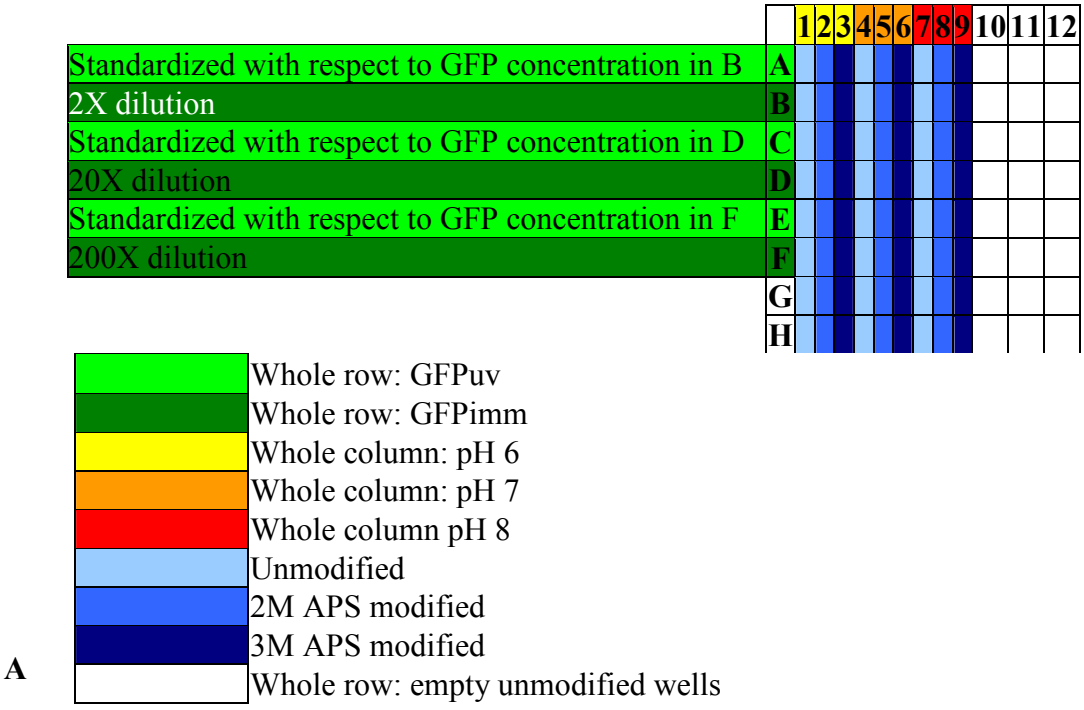
After equalizing the amount of GFPuv and GFPimm by diluting the former, binding reactions were carried out using the modified black 96 well plate according to the configuration given in *Figure 4.23-A*. GFP loaded plates were incubated at room temperature for 2 hours. After 2 hours, fluorescence values were measured from both dry and 200 μl appropriate buffer filled 96 well plates (*Figure 4.23- B, C, D, E, F, G*).

In order to check the possibility to have different fluorescence values with same amount of GFP due to different pH values, the buffer in wells of plate was replaced with pH 7 buffer solution and the plate was incubated at 4 ^{o}C, overnight. Bound GFP fluorescence was re-measured after pH 7 buffer treatment with 200 μl pH 7 buffer loaded in the wells. Fluorescence results of the whole plate at individual binding pHs and pH 7 are given in *Figure 4.24-A, B*, respectively. All fluorescence intensity measurements presented in *Figures 4.22, 4.23* and *4.24* are given as tables in Appendix I.

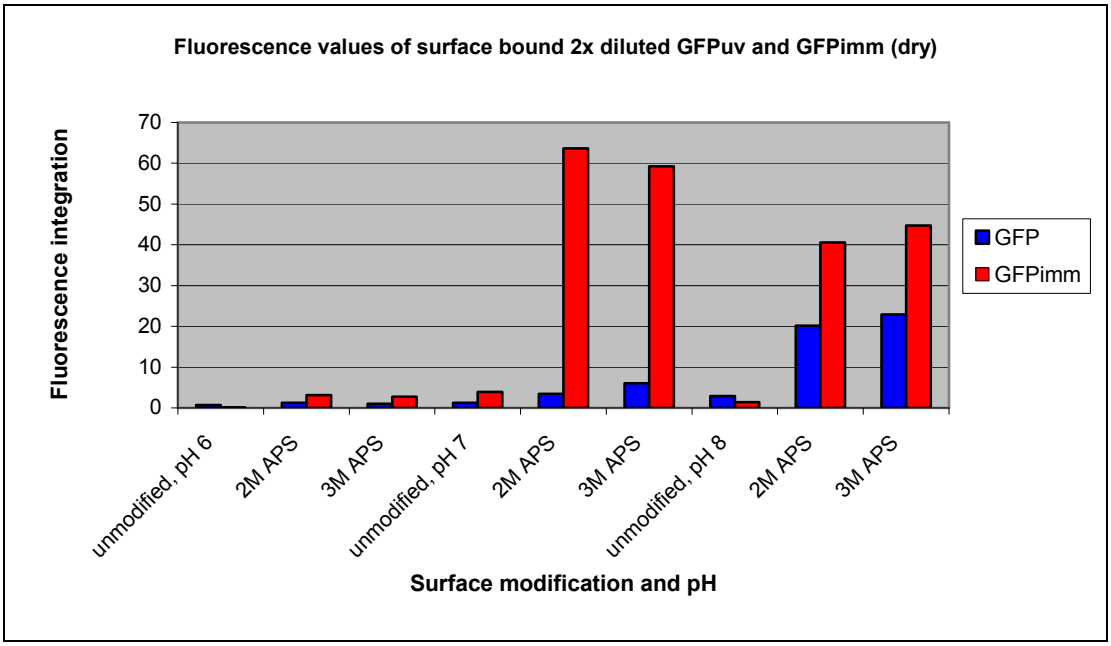
The most meaningful data set was obtained with 2x diluted GFPimm and the equivalent GFPuv, which showed a higher binding of GFPimm at all pH values (the highest being at pH 7) and an increasing binding trend from unmodified to 3M modified wells. In

all measurements, samples with highest dilution factor gave too small fluorescence intensity values that were within the noise range and thus could not be meaningfully interpreted. The figures clearly indicate the difference in binding efficiencies of GFPimm and GFPuv. In general, the highest differential binding occurs at pH 7 while GFPuv seemed to have equal or higher binding efficiency at pH 8 in some of the measurements. Further comments on some unexpected results are included in Discussion section.

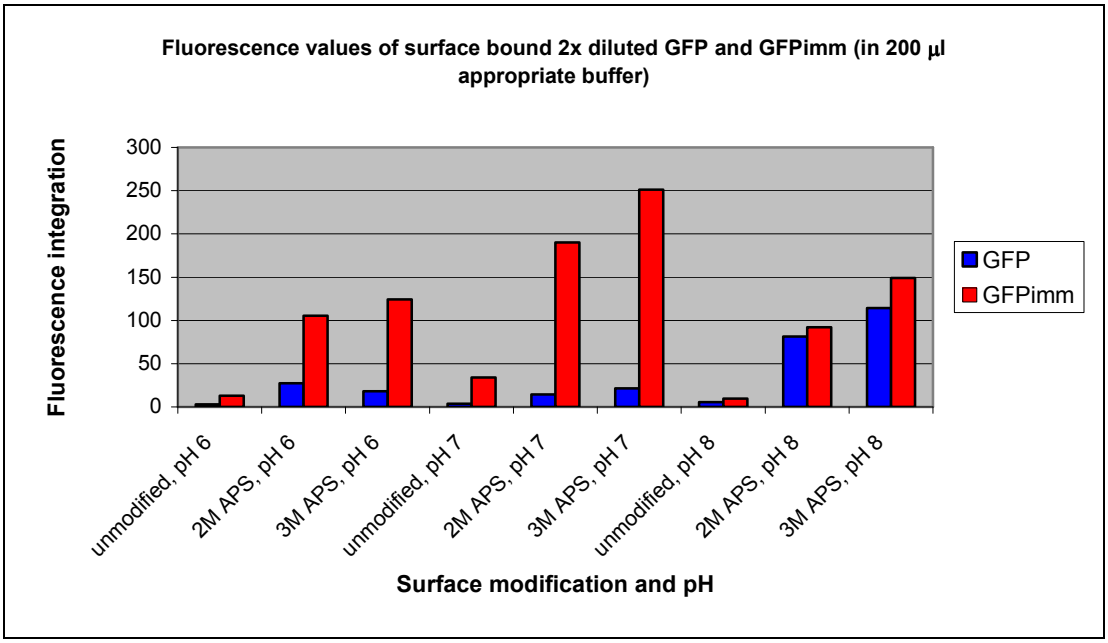
A



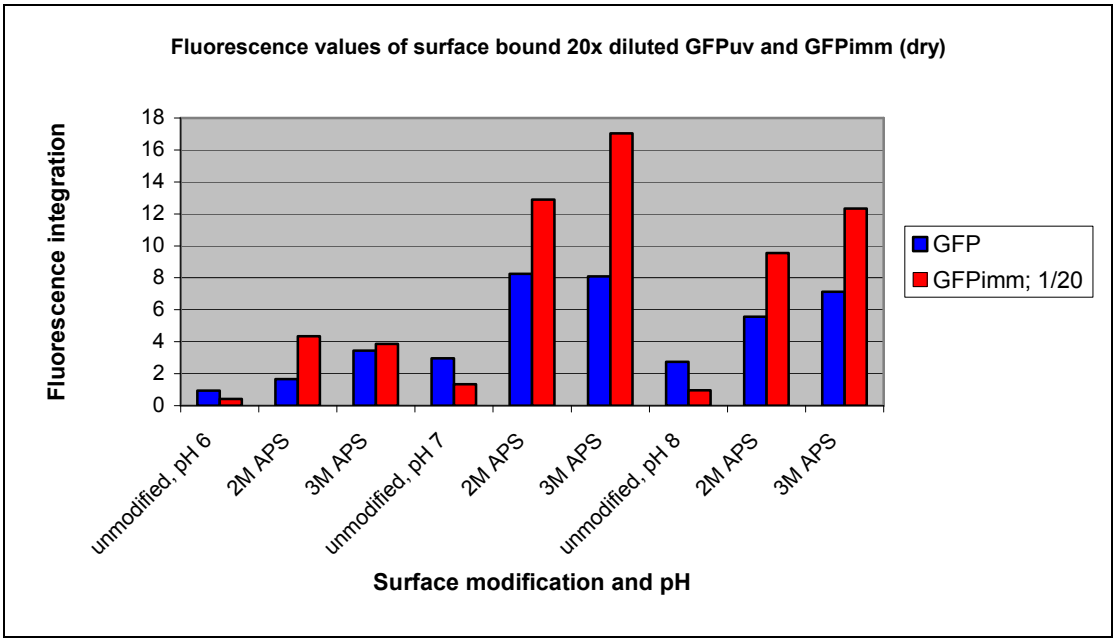
B


Fluorescence values of surface bound 2x diluted GFPuv and GFPimm (dry)
Fluorescence integration
70
60
50
40
30
20
10
0
GFP
GFPimm
unmodified, pH 6
2M APS
3M APS
unmodified, pH 7
2M APS
3M APS
unmodified, pH 8
2M APS
3M APS
Surface modification and pH

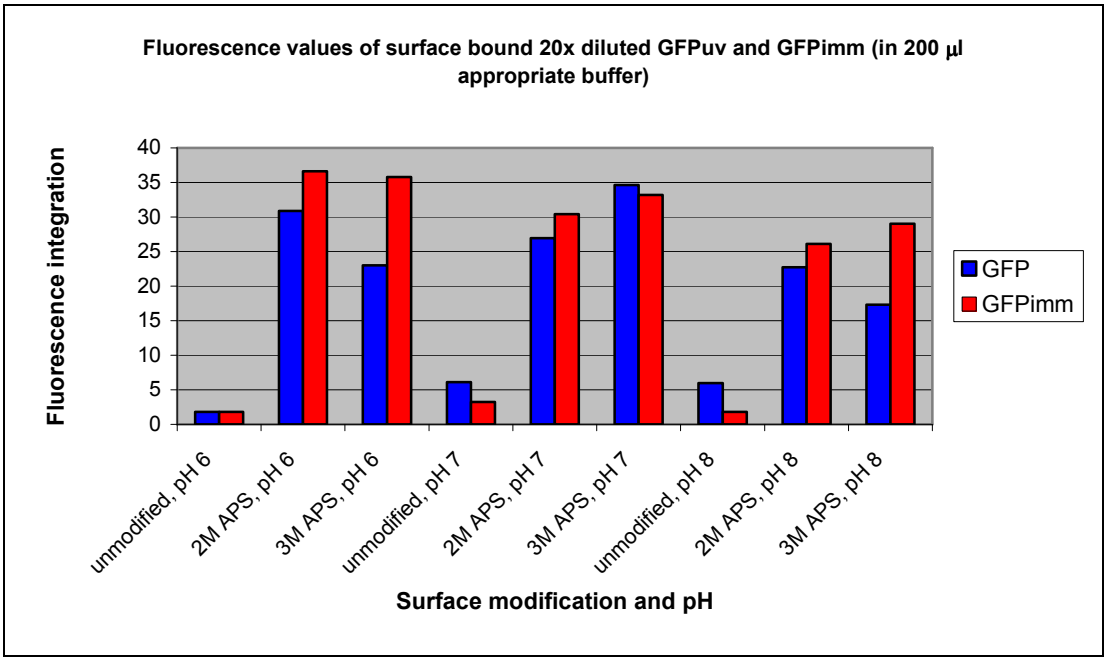
C


Fluorescence values of surface bound 2x diluted GFP and GFPimm (in 200 µl appropriate buffer)
Fluorescence integration
300
250
200
150
100
50
0
GFP
GFPimm
unmodified, pH 6
2M APS, pH 6
3M APS, pH 6
unmodified, pH 7
2M APS, pH 7
3M APS, pH 7
unmodified, pH 8
2M APS, pH 8
3M APS, pH 8
Surface modification and pH

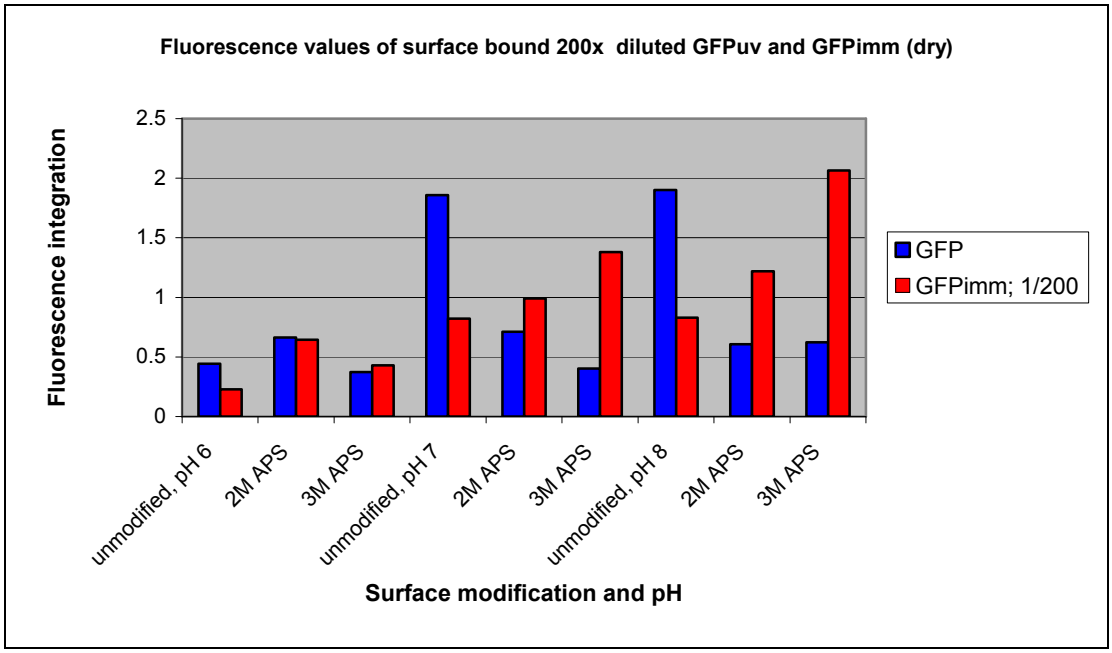
D


Fluorescence values of surface bound 20x diluted GFPuv and GFPimm (dry)
Fluorescence integration
18
16
14
12
10
8
6
4
2
0
GFP
GFPimm; 1/20
unmodified, pH 6
2M APS
3M APS
unmodified, pH 7
2M APS
3M APS
unmodified, pH 8
2M APS
3M APS
Surface modification and pH

E


Fluorescence values of surface bound 20x diluted GFPuv and GFPimm (in 200 µl appropriate buffer)
Fluorescence integration
40
35
30
25
20
15
10
5
0
GFP
GFPimm
unmodified, pH 6
2M APS, pH 6
3M APS, pH 6
unmodified, pH 7
2M APS, pH 7
3M APS, pH 7
unmodified, pH 8
2M APS, pH 8
3M APS, pH 8
Surface modification and pH

F



G

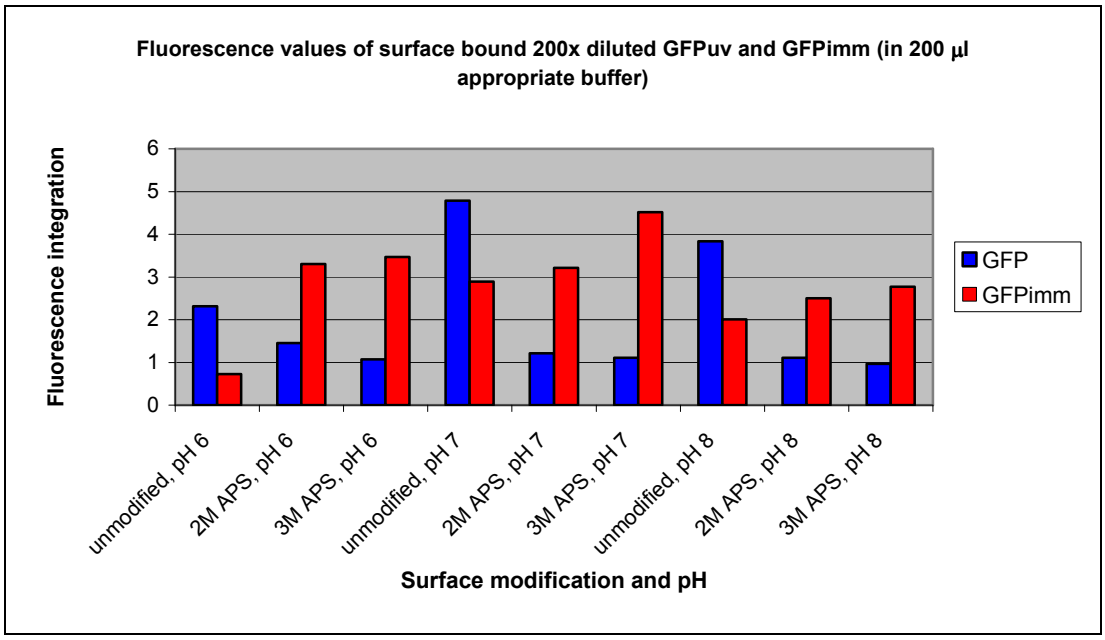
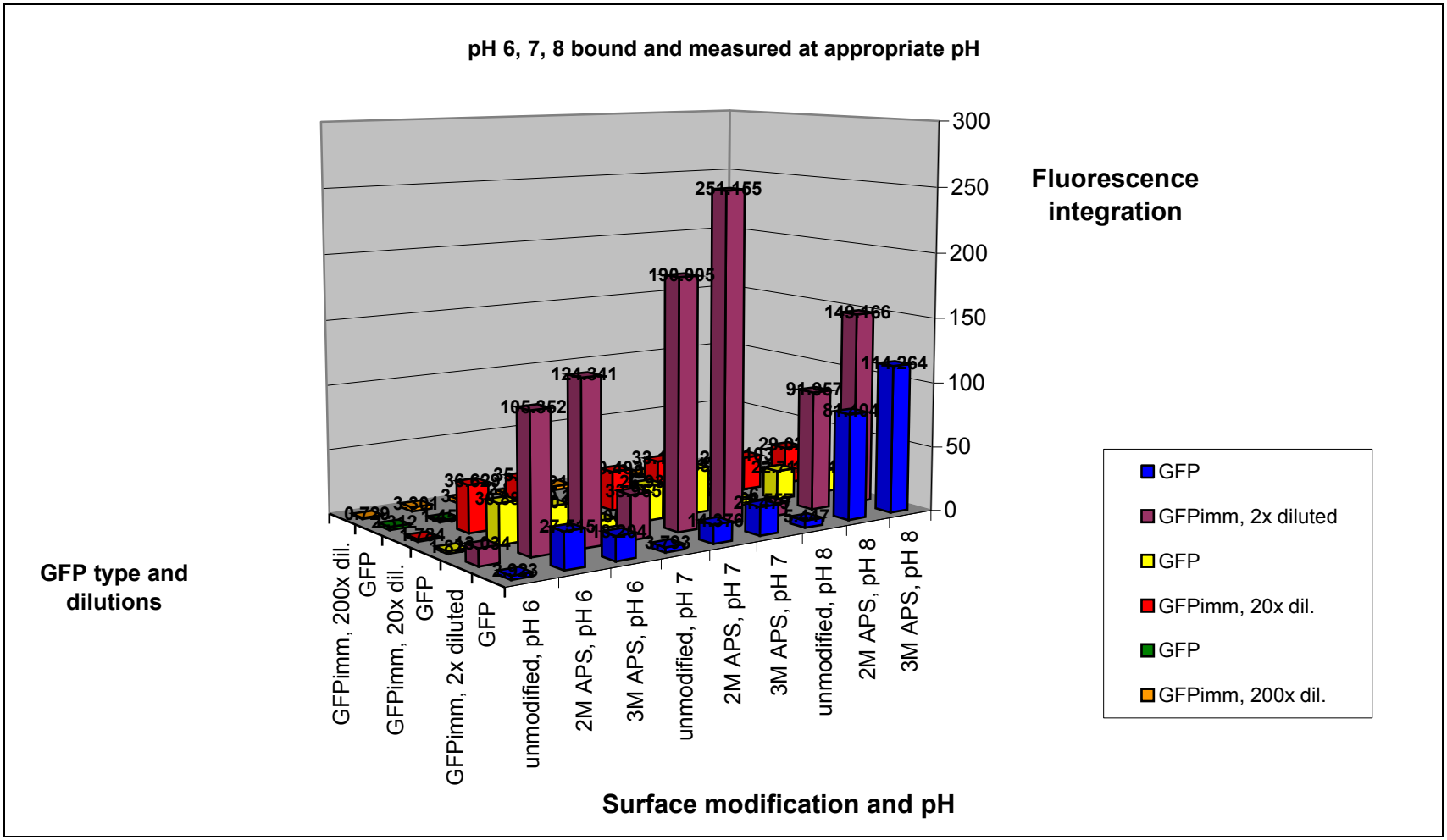
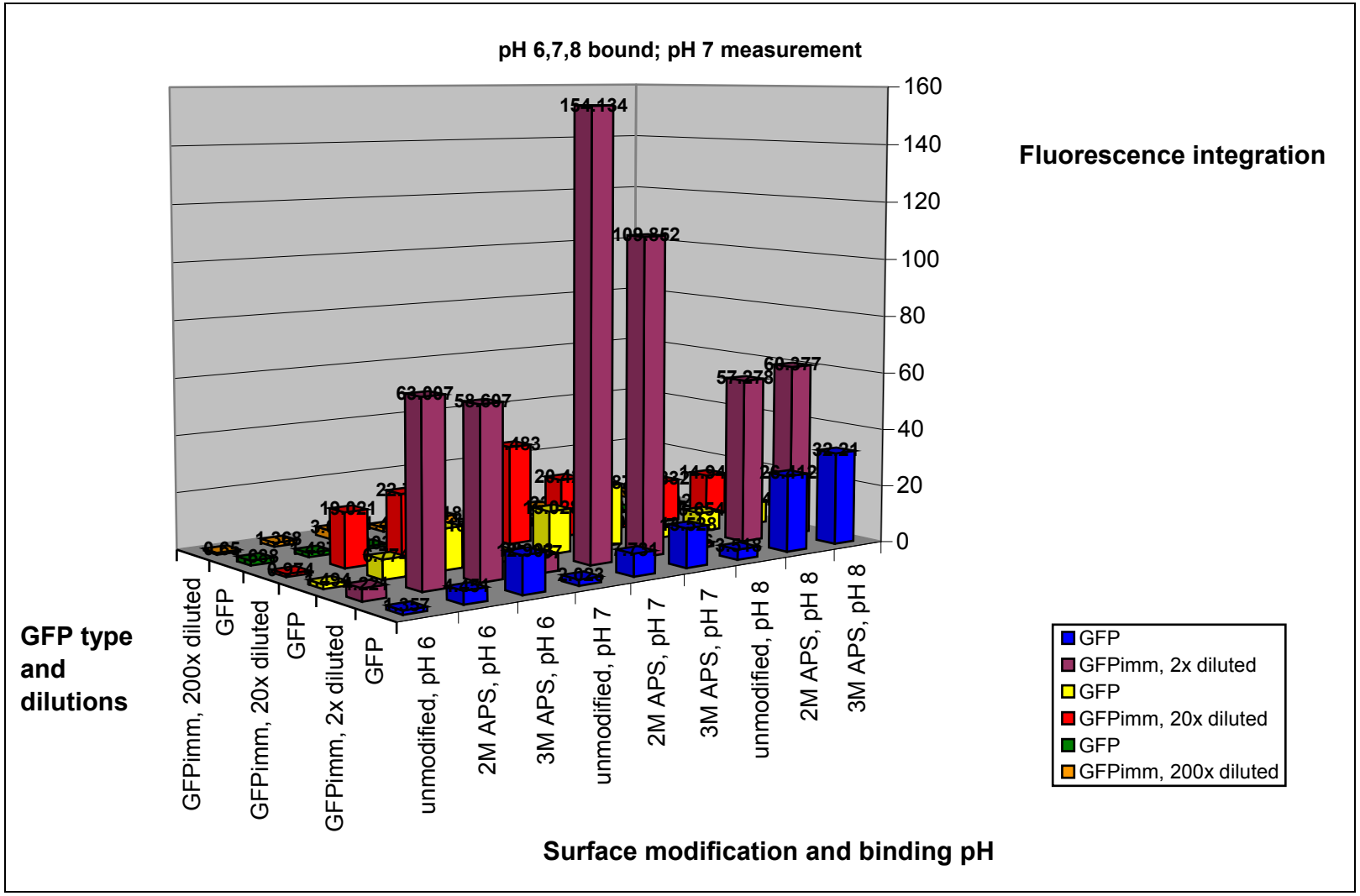


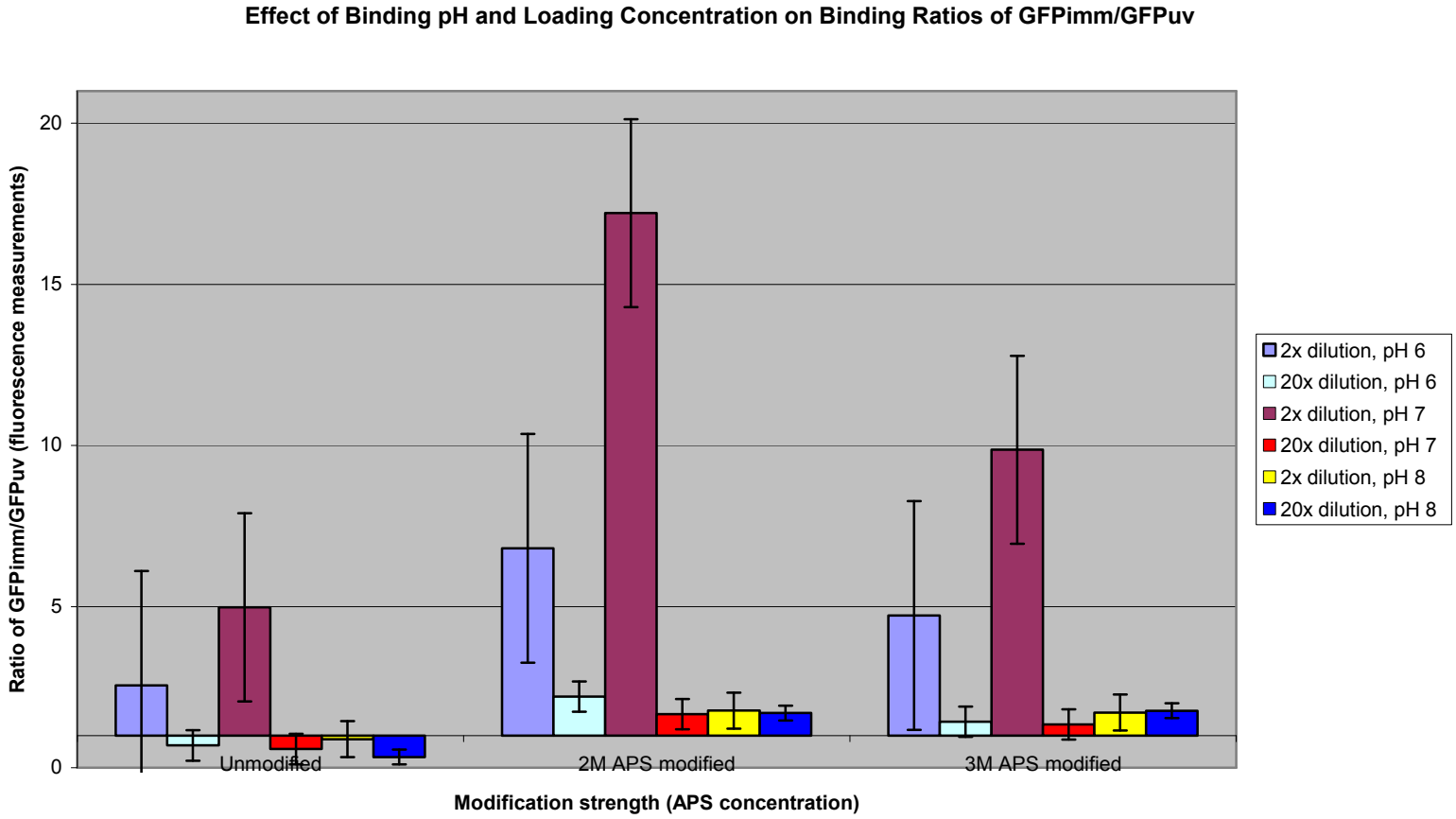
Figure 4. 23 (A) 96 well plate modification and loading configuration is mapped and given with color codes used in the map. (B)-(G) Fluorescence measurements are given in diagrams. B, C, D, E, F and G shows 2x diluted (dry), 2x diluted (appropriate buffer filled); 20x diluted (dry), 20x diluted (appropriate buffer filled); 200x diluted (dry), 200x diluted (appropriate buffer filled) GFP loads, respectively. Numerical values of the measurements are given in Appendix I.


pH 6, 7, 8 bound and measured at appropriate pH
Fluorescence integration
300
250
200
150
100
50
0
251,455
198,005
149,466
124,341
105,352
GFP type and dilutions
GFPimm, 200x dil.
GFP
GFPimm, 20x dil.
GFP
GFPimm, 2x diluted
GFP
unmodified, pH 6
2M APS, pH 6
3M APS, pH 6
unmodified, pH 7
2M APS, pH 7
3M APS, pH 7
unmodified, pH 8
2M APS, pH 8
3M APS, pH 8
Surface modification and pH
GFP
GFPimm, 2x diluted
GFP
GFPimm, 20x dil.
GFP
GFPimm, 200x dil.

A

B


pH 6,7,8 bound; pH 7 measurement
Fluorescence integration
160
140
120
100
80
60
40
20
0
154.134
109.852
63.097
58.607
57.278
60.377
19.021
GFP type and dilutions
GFPimm, 200x diluted
GFP
GFPimm, 20x diluted
GFP
GFPimm, 2x diluted
GFP
unmodified, pH 6
2M APS, pH 6
3M APS, pH 6
unmodified, pH 7
2M APS, pH 7
3M APS, pH 7
unmodified, pH 8
2M APS, pH 8
3M APS, pH 8
Surface modification and binding pH
GFP
GFPimm, 2x diluted
GFP
GFPimm, 20x diluted
GFP
GFPimm, 200x diluted



C

Figure 4. 24 A and B shows measurements of all modified wells together, performed at binding pH and at pH 7 (after overnight pH 7 treatment) respectively. C summarizes the effect of binding pH, loading concentration and strength of surface modification in one histogram by showing GFPimm/GFPuv binding ratios.

5. DISCUSSION

As described in the introduction, the immobilization system is composed of a modular and flexible recombinant vector and a modified surface. On the way to the construction of components of the system, some instructive difficulties were experienced. The inferences from each step of construction of the system are compiled in this section.

5.1 Adapter Design and Preparation

Although last one of the three frame adapters was used for construction of pETM-Adp, three adapters were designed throughout the study (described in 4.1.2). Since all three adapters were optimized for double digestion with Nco I and Kpn I, important observations were made on mechanisms of action of the restriction enzymes.

As recommended in technical reports of restriction enzyme suppliers, 5 bp length extensions after restriction enzyme recognition sites were introduced into the frame adapters' design. However, since two restriction enzyme recognition sites were only 8 and 7 bases away from each other (in Adp#1 and Adp#2/Adp#3, respectively), in addition to the extensions, incubation time was also increased from 4 hours to overnight (16-18 hours).

Since there was one nucleotide difference between Adp#1 and Adp#2, the optimization conditions set for the former also worked with the latter (*Figures 4.4, 4.5*).

The third frame adapter was designed *de novo* changing all the sequence written for Adp#1 and Adp#2. In this design, the adapter was constructed so that it will code a highly flexible (Voet *et al.*, 1995), glycine-rich joint between attachment site and fluorescent reporter in order to achieve kinetics similar to solution phase when bound on the surface. Although the length of Adp#3 was equal to Adp#2, the digestion efficiency decreased significantly due to the changed sequence between the two restriction enzyme recognition sites as explained in Results section (4.2.2.3). The reason for the decrease in efficiency is thought to be as a result of higher GC content of middle part of Adp#3 (5/7: 71.4%) when compared to Adp#2 (3/7: 42.9%). Considering the general reaction mechanisms of type II

restriction enzymes (Voet *et al.*, 1995; reviewed by Perona, 2002), it can be speculated that the local stability increase due to significantly higher GC content may prevent (or make difficult) the bending of DNA by the enzyme. A possibility may be the physical displacement of the enzyme during its bending action. Stress formed on the enzyme during the resistance of local site to bending may result in the displacement from the binding site in accordance with the mechanical equality of acting and reacting forces.

The double digestion reaction was re-optimized to achieve high efficiency digestion. Together with long incubation times, BSA concentration was also increased. BSA is thought to increase enzymatic activity by interacting with both DNA and proteins, thus changing the kinetics from intermolecular to intramolecular reaction by bringing the enzyme and substrate in closer proximity than they are in solution. In order to increase enzyme processivity and stability on DNA, excess (50% higher than recommended concentration) BSA was used to act as molecular glue bringing DNA and the enzyme together. Lower DNA concentration, higher BSA concentration and longer incubation time resulted in an efficiency approaching 100% in double digestion. Since DNA was diluted in optimized reactions, high volumes of digestion mixes were concentrated after the reaction was completed and results were monitored by Urea-PAGE (*Figure 4.6*).

It was important to have digestion efficiency close to 100% since we were not able to isolate double digested frame adapter from the terminal (unwanted) digests which had the potential to interfere with the ligation reaction in construction of pETM-Adp. The problem is mentioned in more details in Results section (4.2.2).

It was seen that by using Urea-PAGE (20%) gels 1 nucleotide base difference in length of the fragments could be detected, however since the fragment of interest was very small, there was diffusional loss of the fragment from the gel during staining with ethidium bromide containing solution. To avoid the loss of double digested frame adapter –for better quantification of the adapter with respect to fragments of known concentration– staining time was decreased while ethidium bromide concentration of staining solution was increased. As a result, the fragment concentration and purity were estimated using Urea-PAGE results. Amount estimation was performed by Urea-PAGE analysis instead of absorption measurements due to the small sample volume and its low concentration (*Figure 4.6*).

5.2 Construction of pETM-Adp and pETM-GFP-Imm

During construction of pETM-Adp by joining Adp#3 and the modified pETM-11, ligation problems were experienced. Since T4 DNA ligase activity is reported to be very sensitive to salt concentration, the source of the problems were likely to be salts remaining from the restriction digestion buffer. The problem is thought to be magnified as a result of concentrating the large volume (dilute DNA containing) digestion mixes to have sufficient amount of digested frame adapter for ligation since salts of the buffer are also concentrated. For desalting process, the digestion mixes of Adp#1 and Adp#2 were passed through Micro-Spin™ G-25 Columns (Amersham Biosciences). Since Adp#3 digestion mix was concentrated after the reaction, it was passed twice through the mentioned column in order to desalt the solution. The first ligation after the double-desalting process resulted in 27 transformant colonies.

During vector constructions, attention was paid not to change distances between functional elements of the backbone plasmid since relative positions of signal sequences and genes on the vector are important in gene expression efficiencies (Primrose *et al.*, 1994). As a result, final plasmid pETM-GFP-Imm was designed to have the same relative positions of fusion genes with original pETM-11 plasmid with the only exception of GFP gene being exactly in place of excised MAD gene (*Figure 4.12*).

5.3 Expression, Purification and Quantification of GFP

During expression of GFP variants, significant difference was observed between the expression levels of GFPuv and GFPimm. The reason of this difference is thought to be the local abundance of histidine coding sequence in poly-histidine tag –in a substrate concentration dependent manner similar to the "ribosome stalling" example in attenuation in tryptophan operon– (Klug *et al.*, 1997). The copy numbers of vectors and the relative positions of promoters with respect to GFP may also be the reason for the difference in the level of expression of the two proteins.

In GFP expression attempts, it was seen that the expression level was largely dependent on aeration. There was significant difference (visually detectable in terms of

fluorescence) in expression levels between the same volume of cultures grown in 500 mL and 1000 mL flasks.

In the second set of purification of GFP variants, which is performed for binding to black 96-well plates, GFPuv and GFPimm showed similar elution behavior in ion exchange column although their ionic properties are very different due to the poly-histidine tag. The reason for this unexpected similarity is the usage of anion exchange column instead of cation exchange while GFPimm is rich in positive charges. The expression and purification steps are not strictly optimized since our focus was mainly on surface binding reaction and detection of its efficiency under the influence of a number of parameters discussed in section 5.4.

Fluorescence intensity and absorbance measurements as well as SDS-PAGE results were used to complement one another to obtain reliable results for quantification of the two GFP variants. Since GFPimm was suspected to have altered fluorescence yield when compared to GFPuv, concentration ratios obtained by absorption and fluorescence measurements were compared. The concentration ratio of GFPuv and GFPimm gave similar results in both techniques, verifying that the fluorescence characteristic of GFPuv was retained after the addition of poly-histidine tag.

In our experiments rGFPuv in solution was assumed to have the same fluorescence characteristics with the surface bound GFP variants. This assumption was based on the position of GFP fluorophore, which is in the core of the protein, away from the surface interactions.

In addition to quantification of GFP variants, the purity of preparations was also monitored by SDS-PAGE analysis (*Figures 4.15, 4.18*). In purifications of GFPimm, two different methods, poly-histidine tag based Ni^{2+} column and standard ion exchange chromatography were used. GFPimm could not be isolated with high degree of homogeneity with either method. Since in the second method GFPuv and GFPimm were purified under exactly the same conditions, it appears that GFPimm elutes either as homo- or hetero-complexes due to intermolecular interactions in the solution. A similar result is obtained by SDS-PAGE analysis where samples are incubated at $95^{o}C$ in presence of 2-mercaptoethanol. Here a series of high molecular weight bands which may be GFPimm homo-complexes or impurities were visualised. This result complicated the use of

electrophoretic analysis in quantification, thus fluorescence measurement was used as alternative quantification method (*Figure 4.22*). Another possibility may be the interaction of the relatively reactive charged tag with a number of proteins in the cell lysate so that they are eluted together from the column. In any case, seemingly lower level of purity of GFPimm was not considered as a problem in comparing surface binding of GFPuv and GFPimm, since this would not cause an artifact that would work in favor of GFPimm. Thus, GFPimm was assumed to be either disadvantageous or equal to GFPuv, which did not cause problem in evaluation of our system.

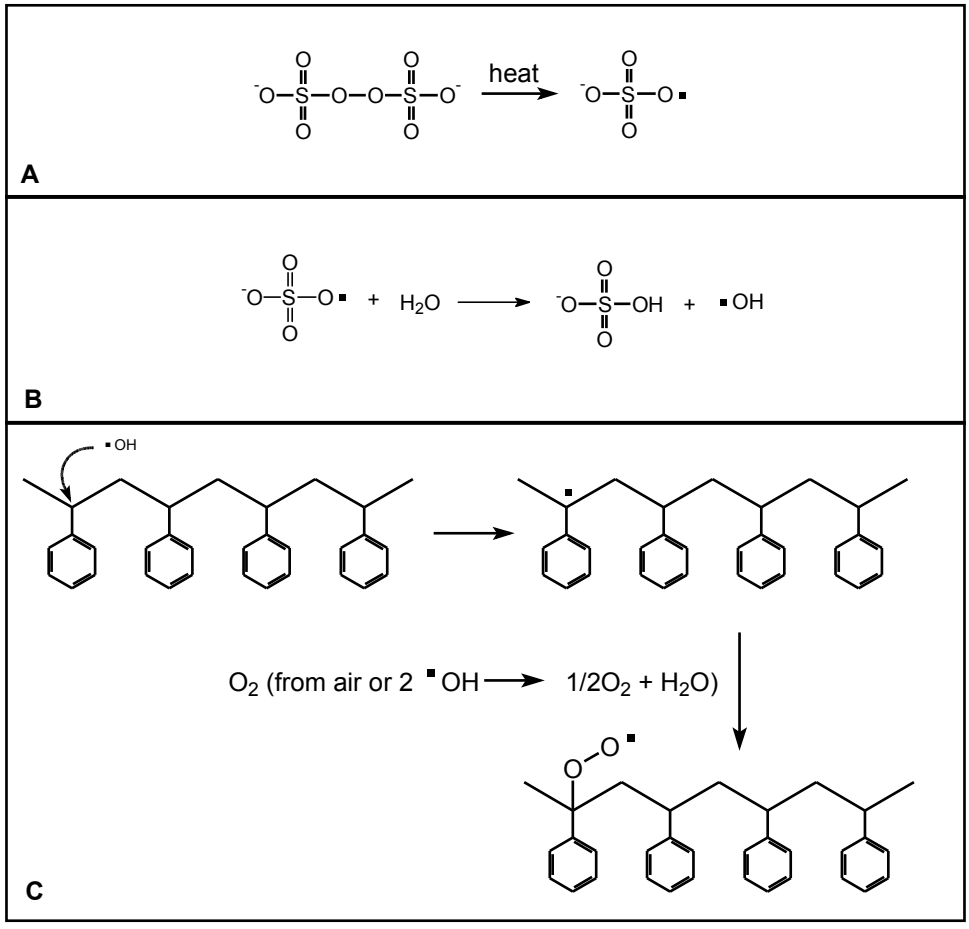
Another possible effect of poly-histidine tag is activity alterations in recombinant enzymes due to the localized charge density of the tag. Since the tag forms an unusually reactive site on the protein it is fused, it may alter the three-dimensional conformation (thus activity in the case of enzymes) reacting with the protein to which it is tagged. Separation of highly charged tag and the enzyme of interest (which is cloned into the multiple cloning site of pETM-GFP-Imm for immobilization) by a linker such as adapter coded joint and GFP is assumed to protect the enzyme from intermolecular poly-histidine tag effect on activity. GFP is thought to be resistant to attacks by poly-histidine tag since the fluorophore is located in the core of the protein with poor accessibility, as in the assumption argument of retaining fluorescence characteristics after binding to surfaces given above.

5.4 Surface Preparation and Binding

In the first round of surface modification experiments, transparent, polystyrene 96-well plates were used. In the fluorescence measurements of GFP bound transparent plates, significant fluorescence was observed in the biologically unmodified (treated with distilled water) wells adjacent to GFP bound wells. Since the GFP fluorescence from neighboring wells were observed to affect each other during quantification in transparent plates, black (opaque) polystyrene 96-well plates were used as alternative.

FT-IR measurements showed that, although both transparent and opaque plates were modified with 2 M and 3 M ammonium persulfate, the difference in surface properties between 2 M and 3 M modified wells of transparent plates is insignificant, while significant in opaque plates. The possible reason may be the concentration loss as a result of APS

crystal formation in 3M solution at room temperature. After recognition of the problem, 3M APS solution was continuously heated in order to be kept over 45°C (at which it is soluble in water) during loading in opaque plates so that the true 3 M concentration could be maintained. By doing so, FT-IR spectra showed that better chemical modification results were obtained. Although the surface modification was assumed to be heterogenous and not fully characterized, a possible reaction scheme with persulfate-modified polymers is given in *Table 5.1*. The reaction mechanisms were predicted using the information from the literature that used polypropylene as the model system (Gugumus, 2002; Kocsis-Karger, 1999; Bamford *et al.*, 1996; Tidjani *et al.*, 1995; Broska *et al.*, 1999; Vaillant *et al.*, 1994; Achimsky *et al.*, 1997; Gugumus, 1998; Rabello *et al.*, 1997; Gijsman *et al.*, 1996), however they may be also applied to polystyrene with the same reactions but different kinetic parameters. Some basic reactions and structures of important intermediates are given in *Figure 5.1*.



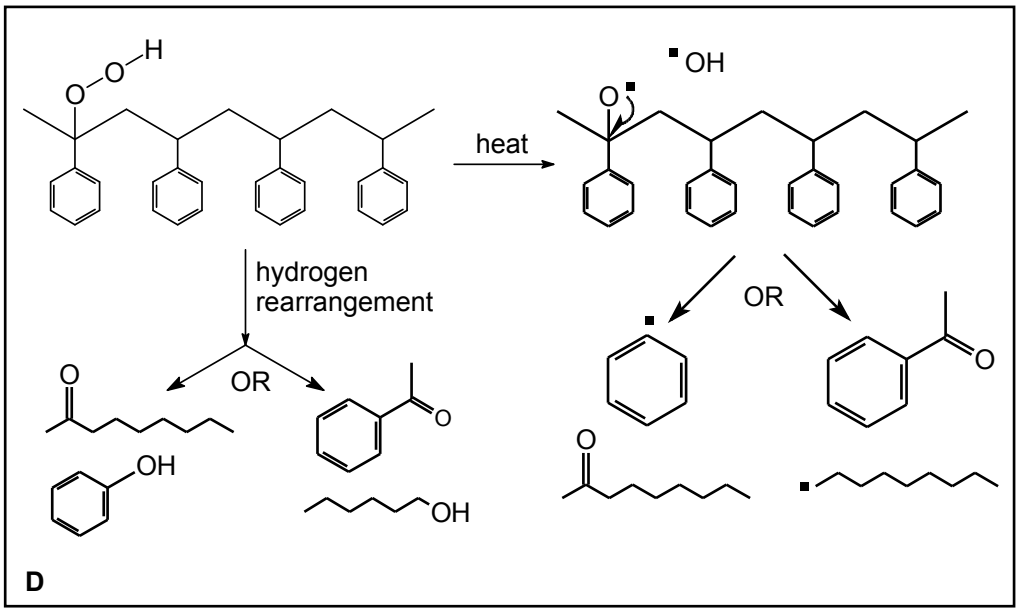


Figure 5. 1 Proposed models for persulfate oxidation of polymers (shown with polystyrene example): some important reactions, structures of intermediate and final products of the proposed reactions. Free radicals are shown with dots. In the reactions, unshown H sources are mainly neighbouring chains and water. In box D, the polymers were drawn without ring structures for simplicity. (A) Initial formation of free radicals; (B) Formation of hydroxyl radical; (C) Formation of tertiary carbon and peroxyl radical; (D) Proposed product formation starting from hydroperoxide containing polymer molecule.

Table 5. 1 Reaction model illustrating alcohol, ketone, carboxylic acid, ester and ether formation in polystyrene (bold, reactions 9-21). Carbon centers (eg. HR, R·) are generally depicted as tertiary but they may also be secondary, particularly with radical backbiting mechanisms, and primary on occasion. The HR-OOH group specifically depicts a secondary carbon center. An abstractable hydrogen beta to an active center, β-HR'OO·, is depicted in reaction 20. While reactions 3-5 depict two modes of carbon radical formation, hydroxyl attack (reaction 5) is presumed to dominate. Processes leading to bond breakage and eventual mesopatterning are outlined with italics.

$^{-}O_3SO\text{-}OSO_3^{-} \rightarrow 2SO_4^{\cdot -}$	(1) initiation
$SO_4^{\cdot -} + H_2O \rightarrow HSO_4^{-} + \cdot OH$	(2) propagation in aqueous-phase
$RH + SO_4^{\cdot -} \rightarrow R\cdot + HSO_4^{-}$	(3) propagation via surface H-abstraction
$RH + SO_4^{\cdot -} \rightarrow RH^{\cdot +} + SO_4^{2-} \rightarrow R\cdot + H^{+} + SO_4^{2-}$	(4) propagation via surface e^{-} abstraction
$RH + \cdot OH \rightarrow R\cdot + H_2O$	(5) propagation via surface H-abstraction
$R\cdot + O_2 \rightarrow R\text{-}OO\cdot (+ HR') \rightarrow R\text{-}OOH + \cdot R'$	(6) propagation in matrix
$R\text{-}OOH \rightarrow R\text{-}O\cdot + \cdot OH$	(7) thermal decomposition of peroxy groups
$R\text{-}OOH + HOO\text{-}R' \rightarrow R\text{-}O\cdot + \cdot OO\text{-}R' + H_2O$	(8) thermal decomposition of peroxy groups
$R\text{-}O\cdot \rightarrow \mathbf{R'=O} + \cdot R''$	(9) *propagation via scission*
$R\text{-}OOH + H^{+} \rightarrow \mathbf{R'=O} + \mathbf{HO\text{-}R''} + H^{+}$	(10) *acid catalyzed decomposition*
$HR\text{-}OOH + H^{+} \rightarrow \mathbf{R=O} + H_2O + H^{+}$	(11) acid catalyzed decomposition
$HR\text{-}OOH + \cdot OOR' \rightarrow \mathbf{R=O} + \cdot OH + HOO\text{-}R'$	(12) radical decomposition
$HR\text{-}OOH + \cdot OH \rightarrow \mathbf{R=O} + \cdot OH + H_2O$	(13) radical decomposition
$R{=}O + \cdot OH \rightarrow \mathbf{R'(=O)OH} + \cdot R''$	(14) *propagation via transfer*
$R{=}O + \cdot OR' \rightarrow \mathbf{R''(=O)OR'} + \cdot R'''$	(15) *propagation via transfer*
$RH + \cdot O\text{-}R' \rightarrow R\cdot + \mathbf{HO\text{-}R'}$	(16) propagation via transfer
$R\cdot + \cdot O\text{-}R' \rightarrow \mathbf{R\text{-}O\text{-}R'}$	(17) radical termination
$R\cdot + \cdot OH \rightarrow \mathbf{R\text{-}OH}$	(18) radical termination
$R\text{-}OO\cdot + \cdot OO\text{-}R'H \rightarrow \mathbf{R\text{-}OH} + \mathbf{O{=}R'} + O_2$	(19) radical termination
$RO\cdot + \beta\text{-}H_{R'OO\cdot} \rightarrow \mathbf{R\text{-}OH} + \mathbf{O{=}R''} + O{=}R'''$	(20) *radical termination*
$HR\text{-}O\cdot + \cdot OH \rightarrow \mathbf{R=O} + H_2O$	(21) radical termination
$2\ HO\cdot \rightarrow ½\ O_2 + H_2O$	(22) radical termination

It was characterized by FT-IR that the products of the reactions proposed above are consistent with the functional groups formed by APS oxidation of polystyrene. The formation of ketone, alcohol and more importantly carboxylic acid groups are shown in detail in the series of proposed reactions in *Table 5.1*.

Unmodified black polystyrene plates gave the same fingerprint as the unmodified transparent plates when monitored by measuring the FT-IR spectrum. The possible reasons for this are:

- The dye may be an intercalating agent instead of surface coating material and may be effective at very small concentrations that cannot be detected by FT-IR.
- Black dye may be a chemical that is invisible within the IR radiation range.
- Being a weaker possibility, the dye may give weak peaks that are superimposed on the characteristic peaks of polystyrene material, which would make it hard to recognize in the spectra.

As the FT-IR results of the second modification (opaque plate) indicate, differential modification is successfully achieved using 2 M and 3 M APS solutions. In principle, the modification resulted in a heterogeneous population of functional groups on the surface, most significant of which are matrix-residing carboxylic acid residues. It should be noted that the detection penetration limit of the instrument is approximately 1μm resulting in the measurement of bulk properties rather than a surface layer, but it does provide insight on the surface characteristics.

The imidazole and positively charged ions that have the potential to competitively interfere with the surface binding reaction were reduced in the protein solutions by dialysis. The complications that were experienced during dialysis and their solutions were mentioned in Sections 3.2.5.1 and 4.6.1. It was observed that an optimum concentration of ions is necessary at which there would be no interference with surface binding while protein precipitation is also avoided.

Concentrations of GFPuv and GFPimm were estimated using absorption, fluorescence and electrophoretic analysis data. Based on the estimate, GFPuv was diluted to have approximately equal concentrations of both proteins for binding studies. The purities of proteins were not considered as a source of artifact as explained in Section 5.3.

Binding characteristics were checked considering the following parameters:

- GFP type
- Binding concentration
- Binding pH
- Measurement pH
- Measurement state (dry/wet)
- Modification strength on polystyrene surface

The indicated parameters were checked on separate rows and columns of 96-well plates, forming an experimental matrix.

It was seen that in overall, the most efficient binding was achieved at pH 7 although pKa of histidine is near 6. In order to verify that the higher value at pH 7 was not an artifact due to higher fluorescence of GFP at this pH, binding buffers at pH values of 6, 7 and 8 were exchanged with pH 7 buffer and the plate was incubated overnight. pH 7 incubated plates also showed highest fluorescence at pH 7 bound GFPimm. This situation may be explained with the following model. Since around pH 6, a significant percentage of histidine population is ionized, they are in a more reactive state. In their charged state, they might be interacting with charged amino acids on the surface of GFPimm, which competitively inhibits surface binding reaction by blocking the charged histidine residues.

Another unexpected observation was the high affinity of GFPuv (in some cases superior to GFPimm) to modified polystyrene surface at pH 8. This may be a result of a pH dependent alteration in reactivities of amino acids that are naturally located on the surface of GFPuv, such as the one expected at pH 6 for GFPimm (*Figure 4.23-B*).

The difference in 2x and 20x diluted sample loaded wells indicated that the surface is not saturated within this range.

Overnight incubation at pH 7 yielded lower fluorescence values which may be due to protein leakage or denaturation as a result of pH dependent conformational change. Immobilized peptides may be more susceptible to such pH changes after they are bound due to the restricted ability to change into a stable alternative conformation. During the conformational change, they may lose activity or leak into solution due to the stress formed near the bound site.

Modification inhomogeneities on polystyrene plates may be another reason for measurement inconsistencies, especially when the difference of GFPimm and GFPuv is small.

As a summary of binding efficiency comparison, it can be clearly seen that GFPimm has a significantly higher surface binding property when compared to GFPuv (*Figure 4.24-C*). Up to 17 fold difference was achieved between GFPimm and GFPuv by performing the binding reaction at pH 7 with highest surface loading concentration pf proteins on 2M APS modified wells. The error bars indicated in the histogram shown in *Figure 4.24-C* are due to the standard deviation obtained from the measurements at different conditions (dry, binding pH buffer-filled, pH 7 buffer-filled).

The possible interactions between poly-histidine tags and oxidized polystyrene surfaces are summarized in *Figure 5.2-A*. GFPimm was also observed to interact with unmodified polystyrene better than GFPuv. *Figure 5.2-B* explains the model for interaction of GFPimm with unmodified polystyrene. In the case of hydrophobic interaction between imidazole groups of histidines and phenyl groups of unmodified polystyrene, quadripolar interactions are assumed to play an important role. In addition to those interactions, protein molecules have the advantage of being large molecules, thus having the possibility to be effectively immobilized by their own inertia. Since they are large and composed of a large number of reactive groups on surface, they may interact with one group while another one loses interaction with the surface.

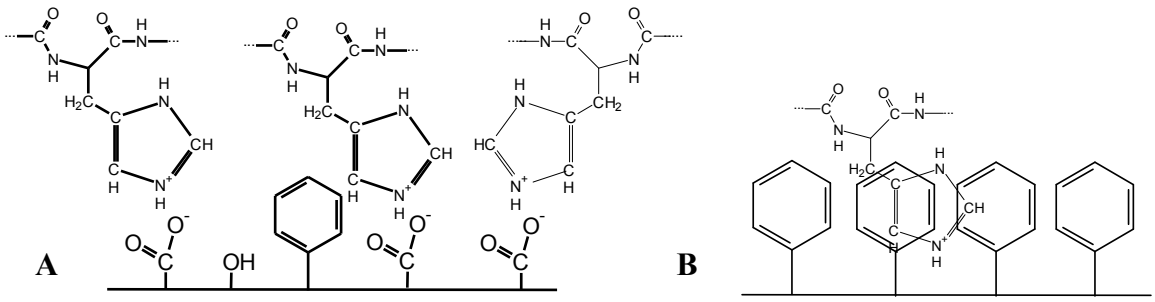


Figure 5. 2 (A) Charge based interaction possibilities between histidines of the poly-histine tag and the functional groups formed on the surface as a result of APS oxidation of polystyrene. (B) Non-covalent, hydrophobic interaction possibility between the electron clouds of imidazole groups of poly-histidine tag and phenyl groups of unmodified polystyrene.

Since the charges formed on the surface increase hydrophilicity of polystyrene surface, the surface is coated with a film of water molecules. In enzyme immobilization practices such an interaction provides an "aqueous environment" for the immobilized enzyme to work as in its natural environment. However, the charges on the surface form an artificial local pH which may negatively affect the enzyme activity (edited by Bickerstaff, 1997; edited by Mosbach, 1987). By using both a hydrophilic surface and a linker peptide, any protein immobilized by using GFPimm based immobilization system will avoid the problem of local pH while being attached to an aqueous environment.

6. CONCLUSION

Although similar studies are present, the immobilization system developed in this study is novel and has a number of advantages over similar systems. The novelties and advantages are described in introduction section.

In this study we have developed a functional two-component immobilization system composed of an activated surface and a recombinant vector encoding a fluorescent immobilization adapter. This immobilization strategy is an alternative to the classical surface linking methods wherein the enzyme is bound to the surface following chemical treatment. By preparing the surface and enzyme to be immobilized separately and encouraging binding in a subsequent step, biological function is likely to be retained. In addition to avoiding the disadvantages of chemical immobilization strategies, our method intrinsically features some benefits associated with classical covalent immobilization such as the preservation of native-like traits by incorporating linker molecules, and improved surface retention in comparison to adsorptive modes of immobilization.

By virtue that the linker contains a fluorescent marker, the system allows for easy visualization and quantification of immobilized target proteins. Although similar studies incorporating affinity tags and GFP markers were performed, this work is novel in the sense that ready-to-immobilize protein is prepared at the DNA level. More importantly, the design allows for expression, immobilization, visualization and quantification of any target protein (*Figure 6.1-A*).

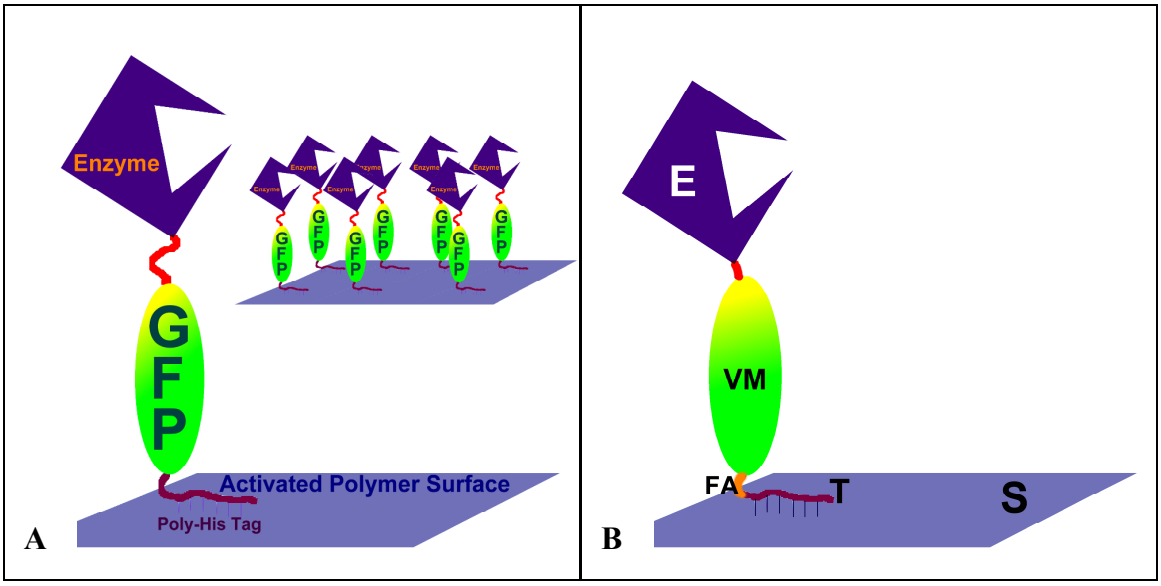


Figure 6. 1 (A) Scheme showing the mechanism of immobilization of any enzyme which is cloned into the multiple cloning site of immobilization specialty vector: pETM-GFP-Imm. (B) The immobilization system is flexible in the sense that every component can be easily changed by single step modifications on pETM-GFP-Imm plasmid. E indicates "enzyme / effector peptide (e.g. attachment signal peptides, antibiotics)", VM represents "visual marker", FA stands for "flexible arm", T indicates "tag of appropriate amino acids" and S stands for "surface" in the figure.

The immobilization system can be used in a wide variety of applications due to its flexible design. Expression of different enzymes or signal peptides, use of different fluorescent proteins instead of GFP, and immobilization via different affinity tags in place of the common His-tag are all possible by manipulating the vector (*Figure 6.1-B*).

The use of different support materials specific to each affinity tag gives the opportunity to develop a large combination of immobilized proteins for application in analysis, enzyme based diagnostic kits, sensing, arraying and orienting, catalysis, stabilisation, binding, signal transduction, chemical transformation, implant biocompatibilization, surface activation, purification, detoxification and scavenging. A provisional patent for the immobilization strategy and its elements has been approved (Application number: PCT/TR03/00019).

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APPENDICES

APPENDIX A
CHEMICALS

(in alphabetical order)

Name of Chemical	Supplier Company	Catalog Number
Acetic Acid	Riedel-de Haén, Germany	27225
Acrylamide/Bis-acrylamide	Sigma, Germany	A3699
Agarose	Sigma, Germany	A-6138
Ammonium persulfate	Carlo-Erba, Italy	420627
Ampicillin	Sigma, Germany	A9518
Boric Acid	Riedel-de Haén, Germany	11607
Distilled water, sterile, MilliQ filtered	Millipore, France	
EDTA (Ethylenediaminetetraacetic acid)	Riedel-de Haén, Germany	27248
Ethanol	Riedel-de Haén, Germany	32221
Ethidium Bromide	Merck, Germany	OCO28942
Glycerol	Riedel-de Haén, Germany	15523
HCl	Merck, Germany	100314
Imidazole	Sigma, Germany	I-2399
IPTG	Promega, Germany	V39517
Isopropanol	Riedel-de Haén, Germany	24137
Kanamycin	Sigma, Germany	K4000
Liquid nitrogen	Karbogaz, Turkey	
Luria Agar	Sigma, Germany	L-3147
Luria Broth	Sigma, Germany	L-3022
2-Mercaptoethanol	Aldrich Chemical Company,	M370-1

	Germany	
NaCl	Riedel-de Haén, Germany	13423
NaH_2PO_4	Merck, Germany	106370
NaOH	Merck, Germany	106462
Nickel Chloride	Merck, Germany	806722
Polystyrene 96-well plate	TPP, SWITZERLAND	TP92696
Polystyrene culture plate	TPP, SWITZERLAND	TP93100
Sodium Dodecyl Sulphate	Sigma, Germany	L-4390
TEMED	Sigma, Germany	T-7029
Tris	Fluka, Switzerland	93349
Urea	Merck, Germany	1.12007.2500

APPENDIX B
MOLECULAR BIOLOGY KITS

(in alphabetical order)

Name of Kit	**Supplier Company**	**Catalog Number**
Micro-Spin™ G-25 Columns	Amersham Biosciences	27-5325-01
ProBond™ resin	Invitrogen, Germany	46-0019
Qiagen Midi-prep Plasmid Purification Kit (100)	Qiagen, Germany	12145 (All buffers in protocols are included)
Qiaprep® Mini-prep plasmid isolation kit (250)	Qiagen, Germany	27106 (All buffers in protocols are included)
QIAquick® Gel Extraction Kit (250)	Qiagen, Germany	28706 (All buffers in protocols are included)

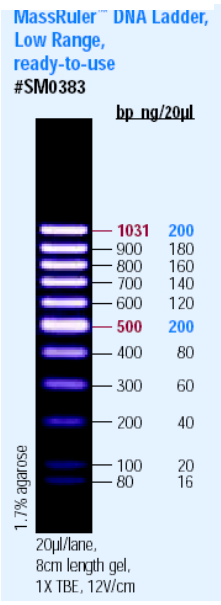
APPENDIX C
BIOLOGICAL COMPONENTS:CELLS, PLASMIDS, OLIGONUCLEOTIDES, ENZYMES AND ENZYME BUFFERS

(in alphabetical order)

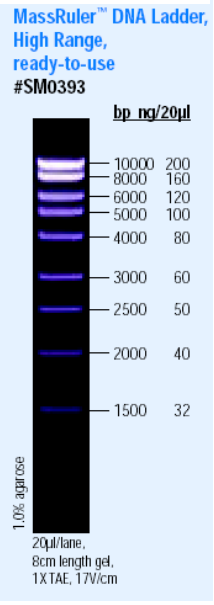
NAME OF ENZYME / BUFFER SYSTEM	Supplier Company	Catalog Number
BSA (Bovine Serum Albumin) (acetylated, 10X concentrated)	Promega, Germany	Supplied with Kpn I, Nco I and Sac I
Kpn I	Promega, Germany	R634A
Multi-Core™ Buffer (10X)	Promega, Germany	supplied with Kpn I, Nco I and Sac I
Lysozyme	Merck, Germany	1.05281
Mass Ruler DNA Ladder, High Range (Agarose gel photograph and MW values of bands are provided below)	Fermentas, Germany	#SM0393
Mass Ruler DNA Ladder, Low Range (Agarose gel photograph and MW values of bands are provided below)	Fermentas, Germany	#SM0383
Nco I	Promega, Germany	R651F
pETM-11	European Molecular Biology Laboratories, Germany	
pETM-Adp	Designed and constructed in-house	
E. coli strains (XL1-Blue, BL21-DE3)	Kindly provided by EMBL, Hamburg, Germany	
Frame Adapters	Designed in-house, synthesized by Seqlab,	

	Germany	
PGFPuv	Clontech, Germany	#6079-1
rGFPuv protein standard	Clontech, Germany	#8366-1
RNAase	Qiagen, Germany	supplied with Qiagen Plasmid Isolation Kits
Sac I	Promega, Germany	R606A
T4 DNA Ligase	Promega, Germany	M180B
T4 DNA Ligase Buffer (10X)	Promega, Germany	Supplied with T4 DNA Ligase
Protein MW Marker (gel photograph and MW values of bands are provided below)	Fermentas, Germany	#SM0431
Prestained Protein MW Marker (gel photograph and MW values of bands are provided below)	Fermentas, Germany	#SM0441
Y+/Tango™	Fermentas, Germany	Supplied with restriction enzymes purchased from Fermentas.

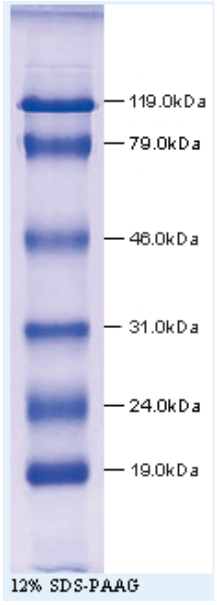
Mass Ruler DNA Ladder, Low Range



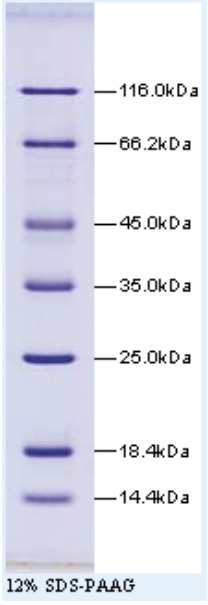
Mass Ruler DNA Ladder, High Range



Prestained Protein MW Marker



Protein MW Marker



APPENDIX D
EQUIPMENTS

Type of instrument	**Supplier Company**
Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA
Balance:	Sartorius, BP221S, GERMANY
	Schimadzu, Libror EB-3200 HU, JAPAN
Centrifuge:	Eppendorf, 5415D, GERMANY
	Hitachi, Sorvall RC5C Plus, USA
Deepfreeze:	-80^{o} C, Kendro Lab. Prod.
	Heraeus Hfu486 Basic, GERMANY
	-20^{o} C, Bosch, TÜRKİYE
Dialysis tubes	CelluSep T3,
	Membrane Filtration Products Inc., USA
Distilled Water:	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA
Filter membranes:	0.2 μm filter: Millipore,
	Catalog# GNWP04700, USA
	0.45 μm filter: Millipore,
	Catalog# HNWP04700, USA
Fluorometer:	SPECTRAmax GEMINI XS, Molecular Devices
	Corporation, USA
FPLC	ÄKTA FPLC, Amersham Biosciences
FPLC Column	MonoQ Ion Exchange Chromatography Column (10/10),
	Amersham Biosciences

Gel Documentation:	UVITEC, UVIdoc Gel Documentation System, UK
	Biorad, UV-Transilluminator 2000, USA
Heater Block:	Bioblock Scientific, FRANCE
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
FT Infrared Spectrophotometer with Attenuated Total Reflectance Accessory:	Equinox 55 TGA-IR, Bruker Optics Inc.
Laminar Flow:	Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY
Microliter Pipette:	Gilson, Pipetman, FRANCE
	Mettler Toledo, Volumate, USA
Microwave Oven:	Bosch, TÜRKİYE
pH meter:	WTW, pH540 GLP MultiCal®, GERMANY
Polystyrene 96-well plate:	TPP, SWITZERLAND
Power Supply:	Biorad, PowerPac 300, USA
	Wealtec, Elite 300, USA
Refrigerator:	$+4^{o}$ C, Bosch, TÜRKİYE
Shaker:	Forma Scientific, Orbital Shaker 4520, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., Innova™ 4330, USA
Sonicator:	Vibracell 75043, Bioblock Scientific, FRANCE

Spectrophotometer:	Schimadzu, UV-1208, JAPAN
	Schimadzu, UV-3150, JAPAN
	Secoman, Anthelie Advanced, ITALY
Speed Vacuum:	Savant, Speed Vac® Plus Sc100A, USA
	Savant, Refrigerated Vapor Trap RVT 400, USA
Thermocycler:	Eppendorf, Mastercycler Gradient, GERMANY
Vacuum:	Heto, MasterJet Sue 300Q, DENMARK
Vortex:	Velp Scientifica, ITALY

APPENDIX E:
Plasmid sequences: pETM-11, pGFPuv, pETM-Adp, pETM-GFP-Imm

Sequence of pETM-11

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   1 CATCACCATC ACCATCACCC CATGAGCGAT TACGACATCC CCACTACTGA
  51 GAATCTTTAT TTTCAGGGCG CCATGGCGGC GGCGGTTCGG ATGAACATCC
 101 AGATGCTGCT GGAGGCGGCC GACTATCTGG AGCGGCGGGA GAGAGAAGCT
 151 GAACATGGTT ATGCCTCCAT GTTACCATAC AAAAACAAGG ACAGAGATGC
 201 CTTAAAACGG AGGAACAAAT CCAAAAAGAA TAACAGCAGT AGCAGATCAA
 251 CTCACAATGA AATGGAGAAG AATAGACGGG CTCATCTTCG CTTGTGCCTG
 301 GAGAAGTTGA AGGGGCTGGT GCCACTGGGA CCCGAATCAA GTCGACACAC
 351 TACGTTGAGT TTATTAACAA AAGCCAAATT GCACATAAAG AAACTTGAAG
 401 ATTGTGACAG AAAAGCCGTT CACCAAATCG ACCAGCTTCA GCGAGAGCAG
 451 CGACACCTGA AGAGGCAGCT GGAGAAGCTG GGCATTGAGA GGATCCGGAT
 501 GGACAGCATC GGCTCCACCG TCTCCTCGGA GCGCTCCGAC TCCGACAGGG
 551 AAGAAATCGA CGTTGACGTG GAGAGCACGG ACTATCTCAC AGGTGATCTG
 601 GACTGGAGCA GCAGCAGTGT GAGCGACTCT GACGAGCGGG GCAGCATGCA
 651 GAGCCTCGGC AGTGATGAGG GCTATTCCAG CACCAGCATC AAGAGAATAA
 701 AGCTGCAGGA CAGTCACAAG GCGTGTCTTG GTCTCTAACT AGTGGTACCG
 751 GATCCGAATT CGAGCTCCGT CGACAAGCTT GCGGCCGCAC TCGAGCACCA
 801 CCACCACCAC CACTGAGATC CGGCTGCTAA CAAAGCCCGA AAGGAAGCTG
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 951 ATTGGCGAAT GGGACGCGCC CTGTAGCGGC GCATTAAGCG CGGCGGGTGT
1001 GGTGGTTACG CGCAGCGTGA CCGCTACACT TGCCAGCGCC CTAGCGCCCG
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1101 CGTCAAGCTC TAAATCGGGG GCTCCCTTTA GGGTTCCGAT TTAGTGCTTT
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5101 ACATCAAGAA ATAACGCCGG AACATTAGTG CAGGCAGCTT CCACAGCAAT
5151 GGCATCCTGG TCATCCAGCG GATAGTTAAT GATCAGCCCA CTGACGCGTT
5201 GCGCGAGAAG ATTGTGCACC GCCGCTTTAC AGGCTTCGAC GCCGCTTCGT

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5251 TCTACCATCG ACACCACCAC GCTGGCACCC AGTTGATCGG CGCGAGATTT
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5401 TTGGGAATGT AATTCAGCTC CGCCATCGCC GCTTCCACTT TTTCCCGCGT
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5751 CCCCGGCCAC GGGGCCTGCC ACCATACCCA CGCCGAAACA AGCGCTCATG
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5851 GGCGCCAGCA ACCGCACCTG TGGCGCCGGT GATGCCGGCC ACGATGCGTC
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6001 TAACTTTAAG AAGGAGATAT ACCATGAAA
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Sequence of pGFPuv (Clontech)

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  51 TGCAGCTGGC ACGACAGGTT TCCCGACTGG AAAGCGGGCA GTGAGCGCAA
 101 CGCAATTAAT GTGAGTTAGC TCACTCATTA GGCACCCCAG GCTTTACACT
 151 TTATGCTTCC GGCTCGTATG TTGTGTGGAA TTGTGAGCGG ATAACAATTT
 201 CACACAGGAA ACAGCTATGA CCATGATTAC GCCAAGCTTG CATGCCTGCA
 251 GGTCGACTCT AGAGGATCCC CGGGTACCGG TAGAAAAAAT GAGTAAAGGA
 301 GAAGAACTTT TCACTGGAGT TGTCCCAATT CTTGTTGAAT TAGATGGTGA
 351 TGTTAATGGG CACAAATTTT CTGTCAGTGG AGAGGGTGAA GGTGATGCAA
 401 CATACGGAAA ACTTACCCTT AAATTTATTT GCACTACTGG AAAACTACCT
 451 GTTCCATGGC CAACACTTGT CACTACTTTC TCTTATGGTG TTCAATGCTT
 501 TTCCCGTTAT CCGGATCATA TGAAACGGCA TGACTTTTTC AAGAGTGCCA
 551 TGCCCGAAGG TTATGTACAG GAACGCACTA TATCTTTCAA AGATGACGGG
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2301 TATCGTAGTT ATCTACACGA CGGGGAGTCA GGCAACTATG GATGAACGAA
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3001 AGGTATCCGG TAAGCGGCAG GGTCGGAACA GGAGAGCGCA CGAGGGAGCT
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3151 TGGAAAAACG CCAGCAACGC GGCCTTTTTA CGGTTCCTGG CCTTTTGCTG
3201 GCCTTTTGCT CACATGTTCT TTCCTGCGTT ATCCCCTGAT TCTGTGGATA
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3301 ACCGAGCGCA GCGAGTCAGT GAGCGAGGAA GCGGAAG
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Sequence of pETM-Adp (intermediate plasmid)

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  51 GAATCTTTAT TTTCAGGGCG CCATGGGAGG CACGGTACCG GATCCGAATT
 101 CGAGCTCCGT CGACAAGCTT GCGGCCGCAC TCGAGCACCA CCACCACCAC
 151 CACTGAGATC CGGCTGCTAA CAAAGCCCGA AAGGAAGCTG AGTTGGCTGC
 201 TGCCACCGCT GAGCAATAAC TAGCATAACC CCTTGGGGCC TCTAAACGGG
 251 TCTTGAGGGG TTTTTTGCTG AAAGGAGGAA CTATATCCGG ATTGGCGAAT
 301 GGGACGCGCC CTGTAGCGGC GCATTAAGCG CGGCGGGTGT GGTGGTTACG
 351 CGCAGCGTGA CCGCTACACT TGCCAGCGCC CTAGCGCCCG CTCCTTTCGC
 401 TTTCTTCCCT TCCTTTCTCG CCACGTTCGC CGGCTTTCCC CGTCAAGCTC
 451 TAAATCGGGG GCTCCCTTTA GGGTTCCGAT TTAGTGCTTT ACGGCACCTC
 501 GACCCCAAAA AACTTGATTA GGGTGATGGT TCACGTAGTG GGCCATCGCC
 551 CTGATAGACG GTTTTTCGCC CTTTGACGTT GGAGTCCACG TTCTTTAATA
 601 GTGGACTCTT GTTCCAAACT GGAACAACAC TCAACCCTAT CTCGGTCTAT
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1701 CAGTTTTATT GTTCATGACC AAAATCCCTT AACGTGAGTT TTCGTTCCAC
1751 TGAGCGTCAG ACCCCGTAGA AAAGATCAAA GGATCTTCTT GAGATCCTTT
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2651 CATAGTTAAG CCAGTATACA CTCCGCTATC GCTACGTGAC TGGGTCATGG
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5351 AAGGAGATAT ACCATGAAA

Sequence of pETM-GFP-Imm (immobilization specialty plasmid)

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 101 AAAAAATGAG TAAAGGAGAA GAACTTTTCA CTGGAGTTGT CCCAATTCTT
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 201 GGGTGAAGGT GATGCAACAT ACGGAAAACT TACCCTTAAA TTTATTTGCA
 251 CTACTGGAAA ACTACCTGTT CCATGGCCAA CACTTGTCAC TACTTTCTCT
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4751 TAACATGAGC TGTCTTCGGT ATCGTCGTAT CCCACTACCG AGATATCCGC
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4851 TCTGATCGTT GGCAACCAGC ATCGCAGTGG GAACGATGCC CTCATTCAGC
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4951 TTCCGCTATC GGCTGAATTT GATTGCGAGT GAGATATTTA TGCCAGCCAG
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5301 CCATCGACAC CACCACGCTG GCACCCAGTT GATCGGCGCG AGATTTAATC
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5401 GCCAATCAGC AACGACTGTT TGCCCGCCAG TTGTTGTGCC ACGCGGTTGG
5451 GAATGTAATT CAGCTCCGCC ATCGCCGCTT CCACTTTTTC CCGCGTTTTC
5501 GCAGAAACGT GGCTGGCCTG GTTCACCACG CGGGAAACGG TCTGATAAGA

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5551 GACACCGGCA TACTCTGCGA CATCGTATAA CGTTACTGGT TTCACATTCA
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5701 ACTCCTGCAT TAGGAAGCAG CCCAGTAGTA GGTTGAGGCC GTTGAGCACC
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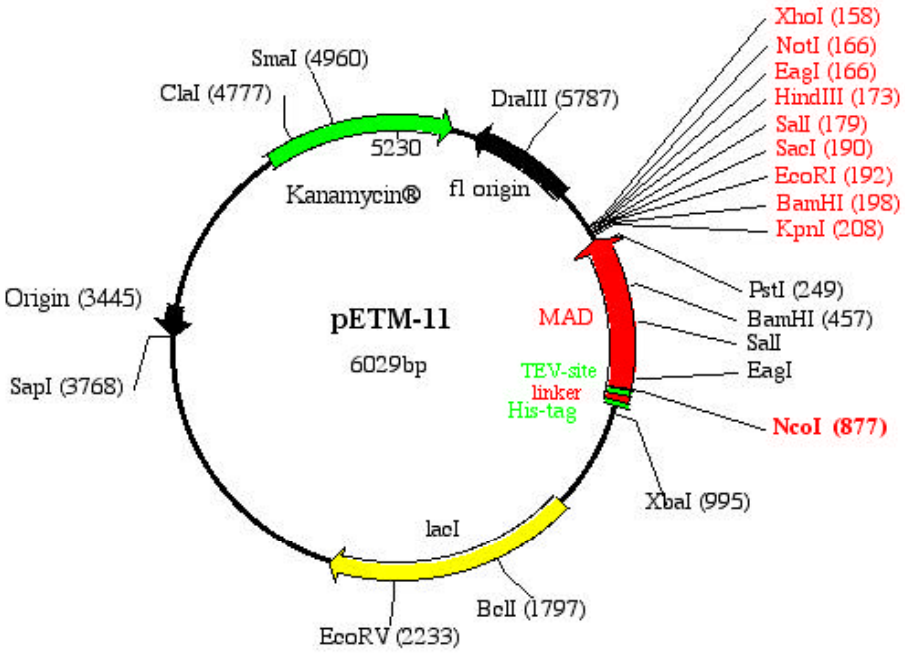
APPENDIX F
pETM-11 (EMBL) Plasmid Map

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CTTTAATTATGCTGAGTGATATCCCCTTAACACTCGCCTATTGTTAAGGGGAGATCTTTA

                    rbs                    His.Tag
AATTTTGATTTAACTTTAAGAAGGAGATATACCATGAAACATCACCATCACCATCACCCC
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                                  METLysHisHisHisHisHisHisPro

                                  TEV site      NcoI
ATGAGCGATTACGACATCCCCACTACTGAGAATCTTTATTTTCAG GGCGCCATGGCGGCG
TACTCGCTAATGCTGTAGGGGTGATGACTCTTAGAAATAAAAGTC CCGCGGTACCGCCGC
MetSerAspTyrAspIleProThrThrGluAsnLeuTyrPheGlu|GlyAlaMETAlaAla


GCGGTTCGGATGAAC..612bp..GACAGTCACAAGGCGTGTCTTGGTCTCTAACTAGTG
CGCCAAGCCTACTTG...MAD...CTGTCAGTGTTCCGCACAGAACCAGAGATTGATCAC
AlaValArgMetAsn..204aa..AspSerHisLysAlaCysLeuGlyLeu***

                                        NotI
      BamHI       SacI                  EagI
KpnI        EcoRI        SalI HindIII         XhoI   His-tag
GTACCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCAC
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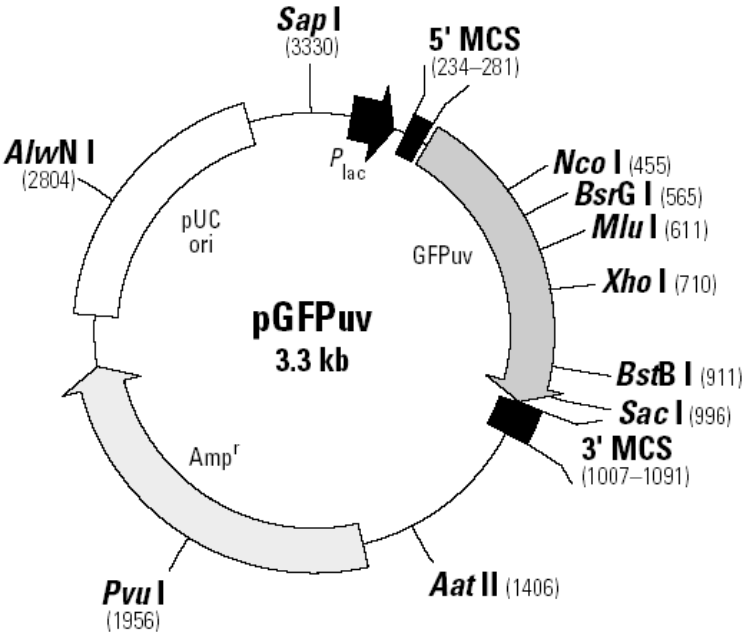
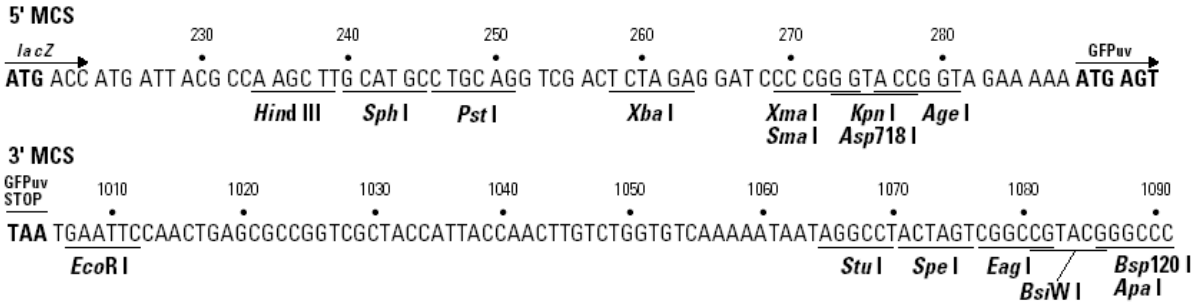
APPENDIX G:
pGFPuv (Clontech) Plasmid Map

pGFPuv Vector Information PT3055-5

GenBank Accession #U62636 Catalog #6079-1





APPENDIX H:
Sequencing result:

Alignment of insertion region of theoretical pETM-GFP-Imm plasmid (BWB8373) with sequenced pETM-GFP-Imm (BWB8207) and GFPuv gene (pGFPuv[GFP])

```
                    * - single, fully conserved residue
                              - no consensus

                   CLUSTAL W (1.81) multiple sequence alignment

BWB8373            CCATGGGAGGCACGGTACCGGTAGAAAAAATGAGTAAAGGAGAAGAACTTTTCACTGGAG
BWB8207            CCATGGGAGGCACGGTACCGGTAGAAAAAATGAGTAAAGGAGAAGAACTTTTCACTGGAG
pGFPuv[GFP]        ------------GGTACCGGTAGAAAAAATGAGTAAAGGAGAAGAACTTTTCACTGGAG
                             **********************************************

BWB8373            TTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTG
BWB8207            TTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTG
pGFPuv[GFP]        TTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTG
                   ************************************************************

BWB8373            GAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTG
BWB8207            GAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTG
pGFPuv[GFP]        GAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTG
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BWB8373            GAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCT
BWB8207            GAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCT
pGFPuv[GFP]        GAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCT
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BWB8373            TTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAG
BWB8207            TTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAG
pGFPuv[GFP]        TTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAG
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BWB8207            GTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGAACTACAAGACGCGTGCTG
pGFPuv[GFP]        GTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGAACTACAAGACGCGTGCTG
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pGFPuv[GFP]        AAGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTA
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BWB8207            AAGAAGATGGAAACATTCTCGGACACAAACTCGAGTACAACTATAACTCACACAATGTAT
pGFPuv[GFP]        AAGAAGATGGAAACATTCTCGGACACAAACTCGAGTACAACTATAACTCACACAATGTAT
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BWB8373            ACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTCGCCACAACA
BWB8207            ACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTCGCCACAACA
pGFPuv[GFP]        ACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTCGCCACAACA
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BWB8373            TTGAAGATGGATCCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATG
BWB8207            TTGAAGATGGATCCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATG
pGFPuv[GFP]        TTGAAGATGGATCCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATG
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BWB8373            GCCCTGTCCTTTTACCAGACAACCATTACCTGTCGACACAATCTGCCCTTTCGAAAGATC
BWB8207            GCCCTGTCCTTTTACCAGACAACCATTACCTGTCGACACAATCTGCCCTTTCGAAAGATC
pGFPuv[GFP]        GCCCTGTCCTTTTACCAGACAACCATTACCTGTCGACACAATCTGCCCTTTCGAAAGATC
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BWB8373            CCAACGAAAAGCGTGACCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACAC
BWB8207            CCAACGAAAAGCGTGACCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACAC
pGFPuv[GFP]        CCAACGAAAAGCGTGACCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACAC
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BWB8373            ATGGCATGGATGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCAC
BWB8207            ATGGCATGGATGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCAC
pGFPuv[GFP]        ATGGCATGGATGAGCTC-----------------------------------------
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APPENDIX I:
TABLES OF RELATIVE FLUORESCENCE INTENSITY VALUES

GFP bound transparent polystyrene 96 well plate, dry measurement

	No modification (dH_2O treated)	2M APS modified	3M APS modified	Empty
Blank (No GFP)	12.02	19.511	17.073	13.635
GFPuv at pH 6.0	23.96	64.387	62.106	20.444
GFPimm at pH 6.0	63.792	274.97	443.267	76.069
GFPuv at pH 7.0	15.509	67.949	85.149	22.401
GFPimm at pH 7.0	233.672	690.85	888.078	145.186
GFPuv at pH 8.0	16.176	78.776	89.982	23.807
GFPimm at pH 8.0	99.532	250.865	344.894	50.509
No treatment	12.462	13.748	13.114	13.251

GFP bound transparent polystyrene 96 well plate, filled with 100 µl appropriate buffer

	No modification (dH_2O treated)	2M APS modified	3M APS modified	Empty
Blank (No GFP)	19.273	14.676	14.076	11.229
GFPuv at pH 6.0	39.267	61.197	51.422	16.242
GFPimm at pH 6.0	135.422	393.247	514.398	39.453
GFPuv at pH 7.0	34.918	65.44	76.862	18.412
GFPimm at pH 7.0	685.664	787.917	896.646	71.299
GFPuv at pH 8.0	41.757	86.718	87.015	16.624
GFPimm at pH 8.0	226.244	327.216	407.67	26.861
No treatment	12.546	13.605	13.388	12.236

Fluorescence measurements of rGFPuv standard, GFPuv and GFPimm dilutions

rGFPuv dilutions	
20X diluted	4368.524
100X diluted, pH 6	695.942
100X diluted, pH 7	826.601
100X diluted, pH 8	899.446

	GFPuv, pH 6	GFPuv, pH 7	GFPuv, pH 8	GFPimm, pH 6	GFPimm, pH 7	GFPimm, pH 8
0.05X diluted	1724.876	1655.763	1673.424	165.37	212.375	198.868
0.005X diluted	56.292	50.916	48.299	5.116	10.824	11.604
0.0005X diluted	5.848	5.563	6.72	0.991	1.615	1.421
0.00005X diluted	0.59	0.985	0.993	0.107	0.378	0.572
0.000005X diluted	0.195	0.121	-0.023	0.132	0.266	0.001
0.0000005X diluted	0.166	0.011	0.017	0.113	0.037	0.2

GFP bound black polystyrene 96 well plate, dry measurement

	unmodified, pH 6	2M APS, pH 6	3M APS, pH 6	unmodified, pH 7	2M APS, pH 7	3M APS, pH 7	unmodified, pH 8	2M APS, pH 8	3M APS, pH 8
GFPuv	0.737	1.285	0.984	1.268	3.446	6.053	2.902	20.136	22.884
GFPimm; 2X dil.	0.073	3.153	2.752	3.92	63.677	59.241	1.426	40.607	44.747
GFPuv	0.934	1.652	3.442	2.964	8.249	8.091	2.744	5.575	7.121
GFPimm; 20X dil.	0.413	4.338	3.866	1.343	12.888	17.029	0.969	9.55	12.343
GFPuv	0.443	0.663	0.372	1.857	0.711	0.402	1.9	0.608	0.622
GFPimm; 200X dil.	0.228	0.644	0.429	0.821	0.99	1.379	0.829	1.22	2.064

GFP bound black polystyrene 96 well plate, filled with 200 µl appropriate buffer

	unmodified, pH 6	2M APS, pH 6	3M APS, pH 6	unmodified, pH 7	2M APS, pH 7	3M APS, pH 7	unmodified, pH 8	2M APS, pH 8	3M APS, pH 8
GFPuv	2.923	27.515	18.204	3.793	14.376	21.479	5.447	81.404	114.264
GFPimm; 2X dil.	13.034	105.352	124.341	33.965	190.005	251.155	9.557	91.957	149.166
GFPuv	1.826	30.881	23.015	6.105	26.934	34.636	5.956	22.719	17.315
GFPimm; 20X dil.	1.784	36.629	35.803	3.232	30.403	33.177	1.8	26.103	29.028
GFPuv	2.312	1.452	1.073	4.79	1.217	1.107	3.837	1.106	0.97
GFPimm; 200X dil.	0.729	3.301	3.469	2.894	3.211	4.517	2.004	2.501	2.774

GFP bound black polystyrene 96 well plate, filled with 200 µl pH 7.0 buffer (after pH 7.0, overnight incubation)

	unmodified, pH 6	2M APS, pH 6	3M APS, pH 6	unmodified, pH 7	2M APS, pH 7	3M APS, pH 7	unmodified, pH 8	2M APS, pH 8	3M APS, pH 8
GFPuv	1.357	4.451	12.903	2.023	7.731	13.528	3.518	26.412	32.21
GFPimm; 2X dil.	4.221	63.007	58.607	5.857	154.134	109.852	1.46	57.278	60.377
GFPuv	1.494	6.774	14.18	3.096	15.028	21.287	3.517	6.654	7.884
GFPimm; 20X dil.	0.974	19.021	22.753	2.324	34.483	20.422	1.198	14.832	14.949
GFPuv	1.688	1.487	0.903	2.154	0.93	0.975	2.676	0.808	0.502
GFPimm; 200X dil.	0.65	1.368	3.034	1.408	2.518	4.717	1.236	1.342	1.973