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BLaTM: A novel method to measure transmembrane domain interactions

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Für Miriam

Zusammenfassung

Membranproteine sind der Hauptangriffspunkt von Medikamenten. 20% bis 30% aller offenen Leserahmen auf dem Genom kodieren für diese Proteine, welche über eine oder mehrere hydrophobe Transmembransegmente (TMSe) in zelluläre Membranen eingebettet sind. Membranproteine können über diese Segmente sehr spezifisch oligomerisieren. Diese Interaktionen sind häufig essentiell für die Bildung von tertiären Proteinkomplexen und damit die Funktion dieser Proteine. Obwohl diese Proteine von großer biologischer Relevanz sind, sind sie im Gegensatz zu löslichen Proteinen schlecht erforscht. Um die Funktion von TMSen bei den Wechselwirkungen zwischen (unterschiedlichen) Untereinheiten eines Komplexes zu verstehen, benötigt man einen sensitiven Assay, welcher sowohl homo- als auch heterotypische TMS-TMS Interaktionen messen kann. Die Entwicklung eines derartigen, neuen Assays war das Ziel dieser Forschungsarbeit. Der neue Assay sollte bewährte Assays wie ToxR, GALLEX oder BACTH übertreffen und dabei dennoch weiterhin auf E. coli basieren. Um die komplexen Mechanismen, die anderen Assays zu Grunde liegen, wie etwa die Gentranskriptionsaktivierung zu umgehen, beruht der neue Assay BLaTM auf der direkten Aktivitätsbestimmung eines "Split-Proteins". Als Split-Protein wurde hierbei rekonstituierten das Antibiotikumresistenzprotein β-Lactamase TEM-1 ausgewählt, welches β-Lactame wie Ampicillin enzymatisch hydrolysieren kann. Die Fragmente des Split-Proteins werden auf DNA Ebene an zwei zu untersuchende TMSe fusioniert. Der Anteil an rekonstituierten Enzym ist hierbei proportional zur Affinität der beiden TMSe. Die Vorteile dieser Methode sind die gut quantifizierbare enzymatische Aktivität und der gleichzeitige Nachweis der Membraninsertion. Der BLaTM Assay wurde mit Hilfe der TMSe von Glycophorin A (GpA), von Sulfhydryl Oxidase 2 (QSOX2) und des hochaffinen LS46 TMS optimiert und verifiziert. Die TMS-Affinität korreliert mit dem zuverlässigen und genau bestimmbaren LD₅₀ Wert, also der Ampicillinkonzentration welche für 50% aller E. coli letal ist. Es konnte hier gezeigt werden, dass mit diesem Assay mit hoher Reproduzierbarkeit sowohl homo- als auch heterotypische TMS-TMS Interaktionen vermessen werden können. Außerdem konnten die bereits bekannten Effekte bestimmter Mutationen, welche zuvor mit anderen Methoden wie ToxR, GALLEX oder NMR nachgewiesen worden waren, erfolgreich reproduziert werden. Zusätzlich kann der Assay an verschiedene TMS-

Affinitäten angepasst werden, wodurch sowohl die Identifizierung von hochaffinen TMSen, als auch der Nachweis von schwächeren Dimeren möglich ist. Zusammenfassend ist BLaTM eine neue Methode für die Erforschung von intramembranen Protein-Protein Wechselwirkungen durch die Bestimmung von homo- und heterotypischen TMS-TMS Affinitäten.

Abstract

The main drug targets in humans are membrane proteins. 20% to 30% of all open reading frames are coding for these proteins which are embedded into cellular membranes by one or several hydrophobic transmembrane domains (TMDs). It is known that these membrane protein regions can form oligomers via distinct amino acid motifs which are very sensitive to mutations. These interactions may be essential for quaternary interactions during the assembly of proteins complexes and thus support protein function. Despite their outstanding biological relevance, membrane proteins are only poorly investigated compared to their soluble counterparts. To uncover the role of TMDs in protein assembly a new assay is needed which enables exact measurements of heterotypic TMD-TMD interactions. This method should surpass previously established assays, such as ToxR, GALLEX or BACTH, but remain E. coli-based. To overcome the complexities of gene transcription activation used in those assays, the new so called BLaTM assay is based on the direct activity-measurement of a reconstituted split-protein. The antibiotic resistance protein β -lactamase TEM-1, which hydrolyses β -lactams such as ampicillin, was chosen because of the ease of its quantification and because it simultaneously works as membrane insertion control. By split protein fusion to TMDs of interest at the DNA-level, the enzyme activity is directly proportional to the TMD-TMD affinity. The BLaTM assay was optimized and verified using the well-investigated TMDs Glycophorin A (GpA), Sulfhydryl oxidase 2 (QSOX2) and the artificial high dimerizing LS46 TMD. TMD affinities were defined by the ampicillin concentration lethal to 50% of expressing E. coli cells - the LD₅₀. It has been successfully demonstrated here that the BLaTM assay can be used with high reproducibility for the quantification of homo- and especially heterotypic TMD-TMD interactions. Disrupting effects of distinct mutations in the GpA and QSOX2 TMDs that were known from ToxR, GALLEX, BACTH or NMR measurements, could be reproduced with BLaTM. Additionally, the assay can be adjusted to different TMD affinities, allowing the identification of strong dimers as well as low affinity. Summarizing, the BLaTM is a novel method for the investigation of intramembranous homo- and heterotypic protein-protein interactions which allows the determination of affinities of low and high affinity TMD dimers.

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1 Introduction

1.1 Cell membranes

Biological membranes are semipermeable bilayers, which separate different compartments in a cell and define its shape. They consist of amphipathic lipids and sometimes also contain sterols, like cholesterol in vertebrates [1]. Depending on the species and compartment, the lipid composition differs highly [1, 2]. The hydrophilic headgroups facing the aqueous surrounding are forming a double layer with a highly hydrophobic core. This core, filled with the acyl chains of the lipids, has a diameter of about 30 Å [3]. In contrast to small hydrophobic molecules, charged and large molecules cannot cross the hydrophobic core, so that it acts as an essential selective barrier. Together with the lipid headgroups on both sides, the membrane double layer has a diameter of about 60 Å. The length and the saturation state of the acyl chains is temperature dependent which ensures a liquid crystalline phase of the membrane [4]. Moreover, it is assumed that the different lengths of the acyl chains also fulfill other functions such as grouping proteins and lipids by hydrophobic mismatch [5, 6]. In addition to the differences in lipid composition, the lipids are not distributed equally between the inner and outer layer of the membrane. This asymmetry is maintained by specific floppases and flippases and is essential for cell survival. The collapse of the asymmetric distribution in mitochondria, for instance, is an apoptotic signal, and thus, lethal for cells [7]. However, biological membranes are more than simple barriers. They hold a place for incorporated membrane proteins that may for example be part of highly enzymatic activities such as the respiratory chain or act as an interface for intercellular communication.

1.2 Membrane proteins

Membrane proteins are not only coded by 20% to 30% of all open reading frames [8, 9], but also represent more than 60% of all drug targets [10], pointing out the importance of this protein class. Nevertheless, soluble proteins are far better investigated than membrane proteins. This disproportion is reflected by the number of entries in the RCSB protein database (PDB) with less than 3% membrane proteins [11]. The concentration of this kind of protein in membranes is highly compartment specific and depends on its enzymatic

activity. Therefore, the membrane protein concentration is no more than about 20% in myelin and 23% in the human red blood cell plasma membrane, but 50% in other plasma membranes and up to 76% in the mitochondrial membrane [12, 13].

1.2.1 Classification

Membrane proteins are classified according to their α -helical and β -barrel secondary structure and their topology. Until today, β -barrel membrane proteins are only found in the outer membranes of Gram-negative bacteria, mitochondria and chloroplasts [14]. The α -helical integral membrane proteins are subdivided by their topology into partial (monotopic) or complete (bitopic or polytopic) membrane penetrating proteins [15]. Furthermore, bitopic proteins (single-pass) are distinguished by their periplasmic terminus (type I: N_{out} or type II: C_{out}), whereas polytopic proteins (multi-pass) are generally termed type III and β -barrel proteins type IV [16]. Examples for bitopic proteins are receptors (e.g. EGFR), cell adhesion proteins (e.g. integrin) or protein anchors. Examples for polytopic proteins are transporters (e.g. ABC-transporter), channels (e.g. voltage-gated ion channel), receptors (e.g. G protein–coupled receptor) or proteases (e.g. rhomboids). The topology of the proteins is characterized by the "positive-inside rule" which implies that positively charged amino acids are more prone to face the cytoplasm [17, 18].

The membrane spanning regions are called transmembrane domains (TMDs) and generally consist of more than 20, mostly hydrophobic, amino acids [3]. As these stretches are longer and more hydrophobic in the membrane than in soluble protein regions, they can be easily predicted as TMDs by simple hydropathy plots as shown by Kyte and Doolittle in 1982 [19-21]. By the introduction of a "Biological hydrophobicity scale", which describes the enthalpy ΔG_{app}^{aa} (apparent free energy of membrane insertion of every single amino acid) of membrane insertion for each amino acid at each TMD position, the prediction was improved more than 20 years later (s. 1.2.3) [22, 23]. Attempts to improve the discrimination efficacy further, are still ongoing. In this context, the terminal hydrophobic helices rule for multi-pass proteins, saying that most C-terminal TMDs are more hydrophobic than internal helices [24, 25], is utilized, for example.

1.2.2 Recognition and translocation

To ensure the specific recognition of membrane or periplasmic proteins, there are complex membrane protein insertion and transportation machineries in every organism. In prokaryotic cells the main protein for membrane protein insertion, embedded in the cytosolic membrane, is the SecYEG translocon which is homologous to the eukaryotic Sec61-type translocon. It acts in concert with several cofactors and is responsible for the actual insertion in most cases. The translocation of proteins through the membrane is mainly realized by two different pathways, which are named after their unique signal peptide recognition proteins SecA or SRP (signal recognition particle), respectively [26]. The membrane proteins are recognized via a pathway specific N-terminal signal peptide [27]. It consists of a positively charged N region, followed by a hydrophobic H region and a polar C region, which also contains the signal peptidase cleavage site [28]. The regions, but not the amino acid sequences for recognition, are conserved over eukaryotes and prokaryotes so that signal peptides are in general interchangeable [29]. Though a wide range of sequences can be recognized as signal peptides [30] it was shown that they can contain additional information regarding for example the translocation pathway [27], translocation efficacy [31, 32] or signal peptide cleavage rate [33, 34]. Furthermore, they may contain post-cleavage functions such as self-antigens on immune cells [35] or cofactors [36].

One of the two possible pathways f or translocation is the co-translational SRP-dependent pathway where the ribosomal mRNA to protein translation occurs in parallel to the translocon-mediated protein translocation and membrane insertion. In this pathway, the very hydrophobic signal peptide is recognized by the SRP which binds the ribosome [37-39]. This pathway is mainly used for integral inner membrane proteins as this process avoids solvent exposure of hydrophobic, membrane embedded protein regions. The signal sequence is usually the first TMD of a multi-pass protein and is also called signal anchor, as it is not cleaved by a signal peptidase [26]. However, there are also well investigated exceptions like the human calcitonin receptor which have a cleavable signal peptide [40]. Hence, it is problematic to use the SRP-dependent pathway for the expression of recombinant membrane proteins, if they must not have a signal anchor. The SRP, together with the ribosome and the nascent polypeptide chain, bind the SRP-receptor FtsY on the cytoplasmic membrane. The hydrolysis of two GTPs (one by FtsY, and one by SecYEG) leads to the release of the nascent chain from the SRP [41]. In the following, this newly synthesized polypeptide is translocated into and through the membrane by the holotranslocon (SecYEG, YidC, SecDF/YajC) or YidC or SecYEG only.

The SecA-dependent pathway on the other hand is preliminary used for secreted and outer membrane proteins. Furthermore, it has been shown that this pathway is efficient for the insertion of inner membrane proteins in artificial systems [42, 43]. Usually, it is a post-translational process that is supported by the ATPase SecA [38], which recognizes the less hydrophobic signal peptide [37, 44]. The two chaperones SecB and TF keep the completely translated nascent polypeptide in a translocation competent unfolded state [45-47]. After signal peptide recognition, SecA binds to the SecYEG complex and drives translocation through the SecYEG channel by ATP hydrolysis and the proton motive force [48]. Subsequently, the signal peptide is cleaved off by a signal peptidase.

Besides the two main pathways the Tat pathway (twin-arginine translocation) is an additional, important post-translational secretion system in bacteria. In contrast to the other two pathways the Tat pathway can transport folded proteins across the inner membrane to the periplasm [49]. These folded proteins are also recognized by a signal peptide. This peptide, however, is less hydrophobic than in the SecA or SRP-dependent pathway and contains a twin-arginine motif in the N region [50, 51]. This system for the export of folded proteins is necessary for proteins which have to bind for example complex redox cofactors in the cytoplasm [52].

1.2.3 Insertion and folding

The mechanism of TMD insertion from the translocon into the membrane is under heavy debate. The central protein of the bacterial translocon, stabilized by SecE, is SecY, which is composed of ten TMDs that form a channel through which nascent polypeptide chains can pass [53, 54]. The SecY protein is U-formed with a lateral gate between the TMD 2b and TMD 7 and a plug inside the channel, which prevents ion leakage through an inactive translocon. The bound signal peptide introduces conformational rearrangements, which widen the channel and remove the plug, so that the nascent polypeptide can enter the translocon [55].

In the relatively simple "in-out" model (Figure 1 A) a passing hydrophobic polypeptide segment is recognized as TMD by a hydrophobic ring [53, 56, 57], so that the lateral gate opens and the TMD enters the phospholipid bilayer [58]. In the "sliding" model (Figure 1 B) the hydrophobic polypeptide segment does not enter the central channel, but slides along the lateral gate - always exposed to the acyl chains of the lipids. The position in the

lateral gate represents the hydrophobicity of the polypeptide. If the region of the polypeptide is hydrophobic enough it enters the lipid bilayer. It is suggested that there are no distinct open and close states. TMD insertion rather is a continuous process, in which all energetic available conformations are explored by the potential TMD [59]. This scanning of conformations could explain why even mutations that are not located in the lateral gate region influence the insertion efficacy or topology.



Figure 1: Two models for membrane insertion of TMDs. Left side: colored cylinders: TMDs of a translocon (10 TMDs of SecY and 2 TMDs of SecE). Black: translocated polypeptide. Black cylinder: TMD of a translocated polypeptide. Right side: schematic illustration of the two models. Blue: translocated polypeptide. A) "inout" model: the TMD moves through the central channel and exits the translocon sideways through the lateral gate. **B**) "sliding" model: the TMD does not enter the central channel but slides along the lateral gate until it enters the lipid bilayer in an equilibrium. Adapted from Cymer et al. 2015 [59].

In this context the question arises how and when a sequence is recognized as a TMD to be inserted into the lipid bilayer or transported through the translocon into the periplasm. For this purpose, Hessa et al. [22] defined a biological hydrophobicity scale using ΔG_{app} (apparent free energy of membrane insertion) by measuring the impact of each amino acid at each TMD position ΔG_{app}^{aa} to the insertion efficacy [23]. To calculate ΔG_{app} for a TMD all ΔG_{app}^{aa} values are summed up. Hence, apolar amino acids can compensate for unfavored amino acids and thus, facilitate membrane insertion. It was, however, shown, that this value only gives a hint, as ionic amino acids can be overestimated and the sequence context plays a role in membrane insertion efficacy, too [23, 60].

1.3 TMD-TMD Interaction

Interactions between TMDs play an important role in many cellular processes. First, they are essential for the correct folding of multi-pass membrane proteins. The interactions of the membrane embedded α -helices ensure the correct tertiary structure which is crucial for the function. Furthermore, they are responsible for quaternary structures, such as the formation of translocons or the respiratory chain. Additionally, intermolecular interactions can be responsible for receptor activation, which is for example the case for the oncogene HER2 in breast cancer [61, 62]. The importance of TMD-TMD interaction was shown for instance by the determination of the complete membrane protein interactome of *Arabidopsis thaliana*, where 12102 interactions between membrane proteins and soluble or membrane proteins could be found with a split-ubiquitin yeast two-hybrid screen [63]. Babu et al. determined membrane protein complexes in *Saccharomyces cerevisiae* were determined by copurification followed by mass spectroscopy [64]. Here, 1726 membrane protein - protein interactions and 501 putative heteromeric complexes were found.

1.3.1 Interaction forces

The geometry of interhelical interactions is defined by the crossing angle Ω and the direction of the two α -helices (parallel or anti-parallel). In parallel formation the conformation is called left-handed if $\Omega > 0^{\circ}$, or right-handed if $\Omega < 0^{\circ}$ [65]. In anti-parallel conformations the crossing angle of left-handed pairs is about -155°, for right-handed about 145°. Left-handed conformations are more abundant and have a heptad motif [66], the right-handed have a tetrad motif [65] which terms the periodic appearance of small amino acids (G, A, S). The heptad motif is derived from the "knobs into holes" packing, which is found in soluble coiled-coil structures and is also characteristic for "leucine-zippers" [67, 68]. These idealized motifs define the interaction interface of α -helices and hence, the position of all other interacting amino acids.

Inter- and intramolecular interactions of soluble proteins are well investigated and the impacts of the different forces on the protein stability are well defined. Even though the environment in a lipid bilayer is completely different, the same fundamental forces including van der Waals packing, hydrogen bonding, salt-bridge and aromatic π

interactions can be found. The main difference is the absence of water for what reason (partially) charged atoms cannot be complexed and stabilized by hydrogen bonds. In soluble proteins the buried amino acids are more apolar than the solvent exposed ones, which causes the hydrophobic collapse during protein folding [12, 69]. In contrast to this, in TMDs it is the other way around since the non-polar lipid bilayer is the solvent and the interfacial and buried amino acids are more polar [70-72].

The most common and best investigated interaction motif is the GxxxG, or more general (small)xxx(small) (x = any amino acid, small = G, A, S) [73, 74]. The small amino acids can maximize van der Waals interactions and enable intermolecular hydrogen bonds between the backbones (C α -H \cdots O=C) or the backbone and the side chains of some amino acids, such as cysteine, threonine or phenylalanine (-SH/-OH ··· O=C) [75]. For example the TMD of Glycophorin A [76, 77], the homo- and heterotypic interacting TMD of Integrin aIIb [78-81] or the homotypic interacting TMD 5 of the multi-pass protein TXA₂ [82] are proven to interact via GxxxG motifs. ~12% of all single-pass TMDs contain at least one GxxxG motif [74], which means an overrepresentation of only 32% compared to a random distribution of glycines [83]. Hence, it is not surprising that there are also GxxxG independent interactions such as it is the case for DAP12, which instead interacts homotypically via an aspartic acid and a threonine, though it also consists of a GxxxG motif [84, 85]. Even though, the specificity of GxxxG dependent interacting TMDs can be explained by the sequence context and additional interacting amino acids. Serine for example does not only work as a small amino acid facilitating close contact between two TMDs, but can also form hydrogen bonds to other amino acids, as well as other polar or ionizable amino acids can do. They are the basis for distinct motifs, such as serine zippers or polar clamps [70]. Though the serines can form hydrogen bonds, the interaction providing motif is probably more related to the small side chain at every seventh position as described for the leucine-zipper motif [86, 87]. The existence and function of saltbridges in TMDs is widely discussed as the membrane integration of ionized amino acids is energetically unfavored due to high desolvation energies. In the proposed two-step model [88], which separates TMD membrane integration and TMD-TMD interaction into different processes, salt-bridges should be very stable, once the ionized amino acids are integrated. This can lead to irreversible interactions, such as the T cell receptor-CD3 complex [89, 90]. Furthermore, it is hard to distinguish hydrogen-bonds and salt-bridges in membranes, even in high resolution structures [91]. Therefore, other methods are necessary

to analyze distinct bonds [92], which dramatically limits the dataset. Nevertheless, it was shown in a library screening that homotypic interacting TMDs containing ionizable amino acids can have a very high dimerizing propensity [93]. Additionally, to their stabilizing function, salt-bridges are necessary for various functions of membrane proteins, such as transporter activity of the lactose carrier LacY [94], activation of rhodopsin [95] or gating of the viral voltage-gated potassium channel K_{cv} [96]. The aromatic side chains of phenylalanine, tyrosine and tryptophan can interact with themselves ($\pi - \pi$) and with basic residues such as lysine, arginine or histidine (cation - π) due to their quadrupole moment which was already shown in the TOXCAT assay [97]. Furthermore, homotypic interacting TMDs with $\pi - \pi$ interacting residues were enriched in several library screenings using degenerated codons [93, 98, 99]. In summary, the correct folding of membrane proteins is governed by various weak interactions [100] including a complementary shape of the interacting TMDs.

TMD-TMD interaction interfaces can overlap with TMD-lipid interfaces [5]. This is for example the case for C99, the cleavage product of the amyloid precursor protein (APP) [101]. On the one hand it was shown that the C99 forms a homodimer mediated by a GxxxG motif [102] and on the other hand the same motif is involved in cholesterol binding [103, 104]. As the equilibrium dissociation constant for C99 homodimerization is ~ 0.5 mol%, which is about 1000 times higher than in natural membranes [101, 105], the importance of homodimerization in vivo is under discussion [5]. Especially, as the equilibrium constant for cholesterol binding is ~ 3 mol%, which is at the lower end of physiological cholesterol concentrations in mammalian cells [106]. Thus, most APP or C99 proteins are binding cholesterol. As dimeric complexes are very unlikely, it is not surprising that cholesterol containing lipid rafts and cholesterol concentration are correlated with Alzheimer's Disease [107]. Hence, multifunctional interfaces can cause false results in TMD-TMD interaction measurements at non-physiological concentrations or without competitive binding partners, although for this special case in Alzheimer's Disease it is not proven in vivo yet.

1.3.2 In vivo TMD-TMD interaction assays

All genetic TMD-TMD interaction assays are based on transcription activation of an reporter protein like β -galactosidase (ToxR [77], GALLEX [108], BACTH [42, 109]), chloramphenicol acetyltransferase (TOXCAT [110], POSSYCCAT [111]) or GFP (AraTM

[43]). The DNA-binding domain is fused to the cytoplasmic end of the TMD and thus, directly or indirectly influences the transcription. It was demonstrated for the ToxR [112], GALLEX [113] and BACTH [114] assays that they work with full length membrane proteins as well.

Homotypic ToxR-related, GALLEX and AraTM assays

The principle of the homotypic interaction assays ToxR [77], TOXCAT [110], POSSYCCAT [111], GALLEX [108] and AraTM [43] is very similar (Figure 2). The main difference is the sensor domain (ToxR, LexA or AraC) which influences the gene transcription (lacZ: β-galactosidase, gfp: GFP, cat: chloramphenicol acetyltransferase). In all cases, the fusion domain has to form a dimer to bind the promotor/operator region (ctx, op + / op + or p_{BAD}) and alter the reporter gene transcription [115]. In the ToxR and the AraTM system the transcription is activated, in the GALLEX system it is repressed. Hence, the mRNA level is assumed to be proportional to the amount of expressed reporter protein. The β-galactosidase activity can be quantified by ortho-Nitrophenyl-β-galactoside (ONPG) turnover, which is measured colorimetrically [116]. The activity is measured in Miller units [117] and has to be normalized either to the positive control Glycophorin A wild type (GpA wt) which dimerizes well in the ToxR/TOXCAT/POSSYCCAT assay or to the negative control GpA G83I, which dimerizes poorly in the GALLEX assay, respectively. The ToxR and the TOXCAT/POSSYCCAT systems differ in the reporter protein, which is β -galactosidase in the ToxR system, or chloramphenicol acetyltransferase (CAT) in the other systems, respectively [77, 110, 111]. CAT can be quantified by measuring the resistance against the antibiotic chloramphenicol. In the TOXCAT assay the promotor/reporter gene ctx::cat is plasmid coded, whereas in the POSSYCCAT it is chromosomally coded. The sensitivity for dimerized ToxR proteins is higher in the TOXCAT assay, as there are more available binding sites in the cell and no interference with the chromosomal DNA [115].



Figure 2: Schematic illustration of the homotypic ToxR/TOXCAT/POSSYCCAT [77, 110, 111], GALLEX [108] and AraTM [43] assay. Green: Lipid bilayer of the E. coli inner membrane. Cylinder: TMD. Square: sensor domain (ToxR/TOXCAT/POSSYCCAT: ToxR, GALLEX: LexA, AraTM: AraC). Periplasmic domains are not shown. The amount of dimeric sensor domains depends on the affinity of the TMDs. (left Monomeric sensor domains side) cannot bind to the promotor/operator region (ToxR/TOXCAT/POSSYCCAT: ctx, GALLEX: op+/op+, AraTM: pBAD). Dimeric sensor domains (right side) bind to the promotor/operator region and alter reporter gene transcription (*lacZ*, *gfp* or *cat*).

The principle of these dimerization assays has proven its worth in the last 20 years since the ToxR assay was published in 1996 [77]. The ToxR assay was the template for the newer TOXCAT [110], GALLEX [108], AraTM [43] or CadC based [118] assays whereas mainly ToxR, TOXCAT and GALLEX are widely used. They are suitable for the determination of the impact of single amino acids to the affinity by e.g. alanine or leucine scanning. The TOXCAT [73, 97, 110, 119], as well as the POSSYCCAT [93, 98, 111, 120] system, were successfully used for library screening. The randomized amino acid positions have to be chosen carefully as it was shown that the GALLEX and the ToxR-like assays are TMD orientation and TMD length dependent [108, 121]. Hence, usually four different orientations of each TMD have to be tested to get reliable results. All proteins contain a periplasmic MalE (Maltose-binding periplasmic protein, UniProtKB [122] accession number POAEX9) which has a molecular weight of about 41 kDa. On the one hand, this large domain could sterically inhibit, or influence the interaction of two proteins and lead to false negative result. On the other hand, it ensures proper topology and facilitates insertion into the membrane [123, 124]. The ToxR and GALLEX proteins especially profit from this as they are type II membrane proteins and thus, do not contain a signal peptide. The insertion efficacy and the topology can be proven in a maltose complementation assay [125, 126]. The ToxR and GALLEX assays are using the GpA TMD for the necessary normalization though it is a type I membrane protein in human erythrocytes [127]. The influence of this opposite topology in the E. coli membrane to for

example the dimerization propensity is not investigated, yet. The AraTM protein is a type I membrane protein and although it was emphasized that this topology is an advantage over the previous established assays, no results are published for GpA [43]. One problem of all these systems is that they are transcription-based and thus, many "dark" steps lie in between the translation, the actual interaction and the output signal. First, the cytoplasmically translated proteins have to be inserted into the membrane. This step might be TMD-dependent but can be checked by maltose complementation. Nevertheless, nonintegrated proteins can persist in the cytoplasm as inclusion bodies or soluble complexes. As DNA binding also occurs in this compartment, false-positive signals are possible but have not been observed, so far. Second, the dimerization of the TMDs results in the formation of an active dimeric transcription factor (ToxR, LexA, AraC) which binds its specific promotor region. After recognition, the reporter gene first has to be transcribed and translated into the reporter protein, whereby the efficacy of both steps is cell and environment dependent. For β -galactosidase detection the cells have to be lysed first and the ONPG turnover is measured in the following. The Miller units are normalized to a GpA positive control, which then gives the final result.

Heterotypic GALLEX, DN-ToxR and DN-AraTM

Originally developed for the measurement of homotypic TMD-TMD interactions, all three assays were modified to measure heterotypic interactions, too. In the GALLEX assay the binding domain and the DNA binding site were modified (Figure 3 A) [108] in a way that only a heterodimer, but no homodimers of LexA wt and LexA 408 can bind to the new promotor/operator region (op408/op+) on the genome and thus repress the transcription. The measurable output is the reduction of the β -galactosidase expression caused by the heterodimer.

In the dominant-negative ToxR system (DN-ToxR) [128] and the dominant-negative AraTM system (DN-AraTM) [129] (Figure 3 B) only the DNA binding domains were modified. Here one TMD is fused to the wild type domain and the other to an inactive mutant. Only the wild type homodimer can activate the transcription by binding the operator region (*ctx* or p_{BAD}). When the wild type and a nonfunctional mutant proteins (*: ToxR S87H, AraC R210A) are coexpressed some wild type proteins form inactive heterodimers. So these proteins are not available for homodimerization anymore and there is less active wild type homodimer. The signal decreases if the homotypic interaction is

suppressed by the heterotypic interaction. As an output the difference of the signal between the wt/mut cells and the wild type only expressing cells is calculated.



Figure 3: Schematic illustration of the heterotypic variants of the GALLEX [108], dominant-negative ToxR [128] and dominant negative AraTM [129] assays. Green: Lipid bilayer of the *E. coli* inner membrane. Cylinder: TMD. Red square: sensor domain wild type (wt) (GALLEX: LexA, DN-ToxR: ToxR, DN-AraTM: AraC). Blue square: sensor domain mutant (GALLEX: LexA 408, DN-ToxR: ToxR*, DN-AraTM: AraC*). Periplasmic domains are not shown. The amount of dimeric sensor domains depends on the affinity of the TMDs. **A)** GALLEX: the monomeric TMDs (left side), the wild type (wt) homodimer (LexA/LexA) and the mutant (mut) homodimer (LexA 408/LexA 408) cannot bind to the promotor/operator *op408/op*+ (right side). Only the wt/mut heterodimer (LexA/LexA 408) binds to the promotor/operator and represses the transcription of the reporter gene *lacZ*. **B)** Dominant-negative ToxR system and DN-AraTM: the monomeric TMDs (left side), wt/mut heterodimer (ToxR/ToxR* or AraC/AraC*) and mutant homodimer (ToxR/ToxR* or AraC*/AraC*) for AraC*/ToxR* or AraC/AraC*) binds to the promotor (*lacZ* or *gfp*).

In general the variants of the ToxR, GALLEX and AraTM assay used to detect heterotypic interactions have similar advantages and disadvantages as their original versions. It is noteworthy that all assays can only look at one dimer (wild type homodimer or heterodimer) but its amount always depends on the affinity of the other two possible dimer formations, too. The most direct assay is the GALLEX assay as the result is absolute and not influenced by the homotypic interactions. Furthermore, the ToxR DN-system depends on one high- and one low-copy plasmid so that the expression of the two chimeric proteins differs which can influence the result. The DN-AraTM proteins are coded on a low- and a medium-copy plasmid whose copy numbers still differ by a factor of more than five [130, 131]. Even though the GALLEX proteins are coded on two low-copy plasmids and the

copy numbers are more similar, an influence of copy number cannot be excluded, too [124]. Neither the dominant negative assays nor the heterotypic GALLEX could be demonstrated to be suitable for heterotypic screening purposes, although the GALLEX assay should be if using for example MacConkey's selective agar [115]. This agar is selective for Gram-negative bacteria and sensitive to lactose-fermenting bacteria but not sufficiently quantitative to distinguish middle and high affinity TMDs.

BACTH

Another transcription based system is the BACTH assay (Bacterial adenylate cyclase twohybrid) [109]. Originally developed for the measurement of the interaction of soluble proteins it was recently adapted to interactions of transmembrane domains (Figure 4) [42]. In contrast to the previously described assays ToxR, GALLEX and AraTM (Figure 2) the BACTH assay does not depend on a dimerization of transcription domain but on protein complementation (s. 1.4). The *Bordetella pertussis* adenylate cyclase is split on DNA level into an N-terminal fragment (T25) and a C-terminal fragment (T18) which are both fused C-terminal to the TMDs of interest. Each fragment alone is inactive and cannot catalyze cAMP formation from ATP. If the TMDs dimerize, the two fragments T25 and T18 come into proximity and can fold to an active enzyme. The reconstituted adenylate cyclase can now convert ATP to cAMP. The catabolite activator protein (CAP) binds cAMP and thereby activates the *cAMP/CAP dependent* promotor [132] which controls a *lacZ* gene. The expression of β -galactosidase can be quantified colorimetrically by ONPG turnover [117]. As *E. coli* cells express endogenous adenylate cyclase, only adenylate cyclase deficient strains (cya⁻) can be used for this assay.



Figure 4: Schematic illustration of the BACTH assay (bacterial adenylate cyclase two-hybrid) [42, 109]. Green: Lipid bilayer of the *E. coli* inner membrane. Cylinder: TMD. Red semicircle: N-terminal T25 fragment of a bacterial adenylate cyclase (BAC). Blue semicircle: C-terminal T18 fragment of a BAC. The amount of reconstituted BAC depends on the affinity of the TMDs. Monomeric TMDs (left side): The BAC cannot reconstitute thus ATP cannot be circulated to cAMP. Heterodimeric TMDs (right side): The two BAC fragments reconstitute to an active enzyme which converts ATP to cAMP. cAMP and CAP form a complex which binds to the *cAMP/CAP* dependent promotor and activates reporter gene transcription (*lacZ*).

The BACTH assay is optimal for the measurement of heterotypic interactions as only the heterodimer can generate a signal and is in this respect similar to the heterotypic GALLEX. Hence, homotypic dimers are disregarded in this assay, too. It was successfully shown that the BACTH assay is suitable for the screening of interactions of soluble proteins [133, 134] but not yet for membrane proteins. The BACTH assay is not only applicable for parallel dimers but could theoretically be adaptable for antiparallel TMD-TMD interactions. However up to now, this has not been tested systematically. One advantage over the previously described direct transcription based assays is that the DNA does not have to be in spatial proximity to the membrane. The cAMP diffuses to the CAP and the complex then binds to the promotor. On the other hand this means that additionally to the described problems with transcription based methods, another step is introduced. So depending on the growth and stress conditions there may not be enough ATP for the reaction or an altered CAP concentration in the cell. Additionally it is very problematic that the two proteins are coded by one low- and one high copy plasmid which may result in different protein expression levels. As a consequence the results have to be interpreted with great care. Furthermore, the assay is not performed at the physiological temperature of 37 °C but at 30 °C which causes an altered lipid and acyl chain length composition [4] which could indirectly influence the dimerization propensity [135].

MYTH/MaMTH

Other split protein based systems are the MYTH assay (membrane yeast two-hybrid) [136, 137] and the adapted MaMTH assay (mammalian-membrane two-hybrid) [138]. In principle they both operate on a split ubiquitin. The MYTH assay was developed as a yeast two-hybrid system (YTH) for membrane proteins because the original system is only suitable for soluble, cytoplasmic proteins [139]. In the first YTH system the dimerized proteins had to enter the yeast nucleus to activate the reporter gene transcription. Both parts of the split transcription factor GAL4 had to bind simultaneously to the DNA. The fragments were fused to the investigated proteins that consequently had to enter the nucleus, too. Hence, in the next generation (MYTH) the transcription factor is decoupled from the dimerizing proteins (Figure 5). A full-length transcription factor (PLV: protein A-LexA-VP16) is fused C-terminal to the C-terminal ubiquitin fragment (Cub: amino acids 35-76) of a split ubiquitin. This fusion protein is again fused to the first transmembrane protein. The N-terminal ubiquitin fragment (NubG: amino acids 1-34, I13G) is obtained by fusion to the second transmembrane protein. As the transcription factor (PLV) is bound to a membrane protein it cannot enter the nucleus (left side). Due to dimerization of the two transmembrane domains the ubiquitin can reconstitute. Therefore, it can be recognized by endogenous ubiquitin specific proteases (UBPs) which cut off the PLV fragment. This can enter the yeast nucleus and finally activate the transcription of a reporter gene which can be detected. These reporter genes may be *lacZ*, coding for β -galactosidase, or *HIS3*, coding for imidazoleglycerol-phosphate dehydratase which is essential for histidine synthesis [140]. β -Galactosidase activity can be quantified by ONPG turnover [117] or by imidazoleglycerol-phosphate dehydratase activity on histidin deficient agar plates [141].

As there is no nucleus in *E. coli* cells the chromosome is always accessible for the transcription factor. Hence, this assay is not applicable in *E. coli* cells as the transcription would be activated independently from the dimerization and cleavage events. Besides UBPs would have to be expressed additionally to enable the cleavage as they are not endogenous in prokaryotic cells.



Figure 5: Schematic illustration of the MYTH assay (Membrane Yeast Two-Hybrid) [136, 137]. Periplasmatic domains are not shown. The N-terminal ubiquitin fragment (NubG) is fused C-terminally to one transmembrane protein (red), the C-terminal fragment (Cub) to another (blue). A PLV domain (orange; protein A-LexA-VP16) is again fused C-terminally to the Cub domain. After dimerizing of the transmembrane proteins the ubiquitin reconstitutes. Ubiquitin specific proteases (UBPs) recognize the ubiquitin and cut off the PLV domain which can enter the yeast nucleus and activate gene transcription. *lacZ*: β -galactosidase, *HIS3*: imidazoleglycerol-phosphate dehydratase.

Both the MYTH and MaMTH assays are only suitable for yeast and mammalian cells which is more challenging than in vivo experiments in E. coli, but enables dimerization experiments in native membranes and natural environments. Endogenous proteins can be tagged with the split proteins (iMYTH) [142] in the yeast chromosome. So the expression is still driven by the native promotor and ensures unaltered protein expression. The yielded protein concentration is different for each protein, which makes the data analysis difficult and complex. It is hard to distinguish whether two signals are unequal due to varying expression rates or due to different affinities. In the tMYTH variant the proteins are expressed ectopically from a plasmid [137, 143]. For yeast proteins this means that they are overexpressed and could thus influence any aspect in the cell's physiology. The transcription factor is expressed functionally and only due to membrane protein fusion not accessible for DNA binding in the nucleus. If there are Cub proteins left in the cytoplasm, which were not recognized by the translocon machinery, they could independently activate the gene transcription. This could be in particular problematic for overexpressed or nonyeast proteins. Overall the MYTH and MaMTH assays are suited for heterotypic membrane protein interaction measurements, especially because they can measure proteinprotein interaction in their natural environment. Up to now this system was not used systematically for TMD-TMD interactions.

1.3.3 Orientation dependence

A general problem in the ToxR [77, 121] and GALLEX [108] assays is the orientation dependence, which describes the influence of how the TMD interface is geometrically related to the sensor domain on the signal. Up to now there are no reports about this phenomenon in the AraTM or BACTH assay. It is caused by the architecture of α -helical TMDs (Figure 6 A), which is characterized by intramolecular hydrogen bonding of the peptide backbone between the carbonyl (i) and the amino hydrogen four amino acids further (i+4). This results in negative phi (-60°) and psi (-50°) angles which results in a very stable helical structure of 3.6 amino acids per turn and a rise of 1.5 Å per amino acid [12, 144, 145]. The backbone hydrogen bonding shields the polar carbonyl and amino groups from the hydrophobic surrounding area in the lipid bilayer and thus stabilizes the polypeptide. The complete unfolding of a 20 amino acid TMD backbone in a membrane would cost about 80 kcal mol⁻¹ which explains why unfolded polypeptides cannot persist in the bilayer [146]. Nevertheless, about one half of all TMDs are bent or contain disorders such as kinks caused by helix-interrupting prolines [147]. These flexible regions are thought to be important for function or positioning of important amino acids.

Assuming that there is an optimal TMD interface to sensor domain orientation for DNAbinding, which thus generates the highest possible signal, and that a deviation causes falsenegative results, the challenge is to find their interface (Figure 6 B). If there is only one distinct, stiff TMD/TMD interface and a rigid linker between the TMD and the sensor domain, the α -helix architecture should allow pre-defined adjustments of the relative orientation. Addition of each N-terminal amino acid to the TMD helix turns sensor domain located upstream about 100° anticlockwise relative to the TMD-TMD interface [12]. Hence, for a complete coverage of all putative TMD-TMD interface to sensor domain combinations at least three amino acids have to be inserted (Figure 6 B, orientations 0, 1, 2 and 3). To avoid extension of the TMD, the same number of amino acids is deleted at its C-terminus. Thus, the crossing angle of the two TMDs and a potential hydrophobic mismatch are likely to remain constant. Comparing maximal and minimal signals of all orientations yields the "orientation dependence".



Figure 6: Relationship of the molecular structure of an α -helix and orientation dependence. **A)** Schematic illustration of the molecular structure of an α -helix (top to down: N- to C-terminus). Blue band: peptide backbone. Green balls (R): any amino acid side chains. Atoms of peptide bond (O, C, N, H) are labeled. Dotted line: intramolecular hydrogen bond between the carbonyl and i+4 amino hydrogen of the backbone. Each turn consists of 3.6 amino acids (aa) and the rise is 5.4 Å. Adapted and modified from Cruz 2011 [148]. **B)** Schematic illustration of the influence of added amino acids in an α -helical conformation between the TMD and a sensor domain to their relative orientation and the signal (right). Circle: top view of the TMD. Blue arrow: Orientation of the sensor domain (ToxR or LexA). Green: interfacial side of the TMD. Yellow: non-interfacial side of the TMD. Orientation 0: start construct. Orientation 1: one additional amino acid at the N-terminus of the TMD. Orientation 2: two additional amino acids at the N-terminus of the TMD. Orientation 1, 2 and 3) relative to the interfacial side of the TMD.

If the connection between the TMD and the sensor domain is very rigid and if there is one explicit interaction motif, only one or two of the four orientations should generate a signal. The more flexible the linker is the less orientation dependent the signal should be as the sensor domains would adapt to any situation. In general neighboring orientations (0,1; 1,2; 2,3; 3,0) should show more similar signals than opposite ones (0,2; 1,3).

Indeed the affinity of the TMDs could also be altered due to added or deleted amino acids and accordingly change the signal. For example the large cyto- and periplasmic domains could collide in one particular TMD interface to sensor domain orientation so that not all sides of the TMD are available for dimerization. Furthermore, the penetration depth of the interface could influence the signal if the head groups of the lipids or the hydrophobicity, which increases to the center of the membrane, have an impact on the interaction. If so, the first or the last orientation (0 or 3) should show the highest signal because the respective amino acids are in the correct neighborhood. Furthermore, some of the added or deleted amino acids could be part of the interface. This could result in a clear signal shift between two orientations. In reality, any mixture of all these phenomena could lead to a falsenegative measurement. This is not only the case for the ToxR and GALLEX assay but for all experiments which focus on single parts of proteins as for example the TMDs.

A very long and flexible linker between the TMD and the sensor domain should abolish orientation-dependence because it can structurally decouple two domains [149]. On the other hand a too long linker increases the degrees of freedom of the domains thus hindering transcription.

1.4 Protein complementation assays

The first split-protein-complementation assay (PCA) was reported in 1994 by Johnsson and Varshavsky [136] and utilized ubiquitin which is only processed by ubiquitin-specific proteases in its quasi-native, reconstituted conformation. Since then many different split proteins for the measurement of soluble protein-protein interactions were designed. All these proteins have to fulfill various requirements: (i) The amount of active protein has to be measureable. It may either be quantified directly (e.g. GFP [150]), by the products of its enzymatic activity (e.g. luciferase [151] or β -lactamase [152, 153]), by gene transcription activation due to educts (e.g. ubiquitin [136], adenylate cyclase [109] or TEV protease [154]) or by another secondary effect such as survival (e.g. DHFR [155]). (ii) There has to be a site where splitting the protein generates two inactive fragments. These fragments should (iii) not dimerize themselves but (iv) forced dimerization via a fused dimerization domain would reconstitute the protein's activity. (v) Smaller proteins are of advantage as the expression is more efficient and the possibility of steric hindrance is smaller. (vi) Optimally the reconstitution is independent of N- or C-terminal fusions of the investigated proteins.

A commonly used PCA is the Bimolecular Fluorescence Complementation (BiFC) assay which was developed for high-throughput applications and is basically a PCA utilizing a fluorescent protein [156-158]. The fluorescence can be quantitatively detected directly or used for library analysis by fluorescence activated cell sorting (FACS). Furthermore, it is also suitable for intracellular localization of interactions [156]. However, the method is critical because there is self-affinity of the fragments and the reconstitution is irreversible [159, 160]. Other common PCAs are based on the complementation of ubiquitin and bacterial adenylate cyclase which were both also adapted for the measurement of membrane protein interactions (s. 1.3.2).

PCAs were used for the measurement of distinct protein-protein interactions in vivo, for library screenings and for in vitro applications. Up to now only the bacterial adenylate cyclase (BACTH [42]) was adapted for the systematical measurement of TMD-TMD interactions (s. 1.3.2). The ubiquitin based MYTH/MaMTH [137, 138] and BiFC [161-168] assays were only used for full length membrane proteins so far.

1.5 β-Lactam antibiotics

The first and most famous β -lactam antibiotic is penicillin which was discovered in 1928 by Alexander Fleming [169] and isolated in 1940 by Ernst Chain [170]. Since then many derivatives were developed to overcome resistant bacteria or to extend their activity spectrum to for example Gram-negative bacteria (e.g. ampicillin) [171]. The members of this antibiotic class differ in their core ring structure which consists of a β -lactam ring and mostly a fused, carboxylated five or six-membered ring which can be unsaturated and which can contain a single oxygen or sulfur atom [172]. Furthermore, they vary in the side groups to avoid binding and degradation by β -lactamases (s. 1.5.2).

1.5.1 Mechanism of antibiotic function

β-Lactam antibiotics inhibit the cell wall synthesis by irreversible binding of penicillinbinding proteins (PBP) due to structural similarity to their natural substrate D-Ala-D-Ala [173-175]. PBPs are a protein group and UniProtKB [122] lists 11 different PBP in the *E. coli* lab-strain K12. The high molecular mass (HMM) proteins (PBPs 1-3) are DD-transpeptidases and essential for cell elongation, shape determination and septation [176]. The low molecular mass (LMM) proteins (PBPs 4-7 and AmpH) are DDcarboxypeptidases whose functions are less clear but which are possibly involved in murein (peptidoglycan) rearrangement. DD-transpeptidases cross-link glycan chains via for example pentapeptides (Figure 7) and build up the murein of the bacterial cell wall [177]. Without these linkages the cell wall becomes unstable and cannot withstand the internal turgor pressure [178]. The common feature among all PBPs is an active-site serine which can be acetylated by β-lactams or by the substrate mimic diacetyl-L-Lys-D-Ala-Dlactate [179-182]. The complete blocking of the active site by this covalent binding explains the high potency of β-lactams as antibiotics.



Figure 7: Structure of murein and cross-linking by DD-transpeptidase. Glycan chains consist of alternating GlcNAc (N-acetylglucosamine) and MurNAc (N-acetylmuramic acid) groups. Some MurNAc contain pentapeptides (L-Ala, D-Glu, m-Dap (meso-diaminopimelic acid), D-Ala, D-Ala). The DD-transpeptidase cross-links the amino group of one m-Dap with the first D-Ala of another peptide by cleavage of the peptide bond to the terminal D-Ala.

1.5.2 β-Lactamase

β-Lactamases (EC 3.5.2.6) are a group of periplasmic enzymes which can cleave β-lactam bonds by hydrolysis. They play an important role in modern medicine as they mediate antibiotic resistance by the degradation of β -lactam antibiotics such as penicillin or ampicillin. β-Lactamases are classified into the Ambler classes A through D, based on amino acid homology [183-188] or into the less common Bush-Jacoby-Medeiros groups 1 through 4, based on substrate and inhibitor profile [189]. The Ambler classes A, C and D are structurally homologous serine hydrolases whereas class B are Zn²⁺ dependent metallo hydrolases [190]. The two main representatives of class A are TEM [191] and SHV [192] which share 68% sequence homology and differ in the size of the active site. The proposed reaction mechanism is shown in Figure 8 A [190]. In brief, the substrate β -lactam antibiotic is bound and positioned by the amino group of Lys₂₃₄, the amide hydrogens of Ser₇₀ and of the amino acid at position 237. Ser₇₀, supported by Glu₁₆₆, forms a high-energy, tetrahedral acylation complex with the β -lactam via a nucleophilic attack to the carbonyl of the amide bond. Due to protonation of the amide nitrogen and regeneration of the Glu₁₆₆ the amide bond is cleaved. An activated catalytic water molecule hydrolyses the ester bond between the β -lactam and the Ser₇₀ and thus regenerates the complete active pocket of the β -lactamase so that the deactivated β -lactam antibiotic can be released.



Figure 8: Catalytic center and reaction mechanism of class A serine β -lactamases. **A**) Reaction mechanism of class A serine β -lactamases. After the hydroxyl group of Ser₇₀ is activated by the protonation of the carboxy group of Glu₁₆₆ it can perform a nucelophile attack on the carbonyl group of the β -lactam bond. The tetrahedral acylation intermediate (\ddagger 1) is unstable and after protonation of the β -lactam nitrogen the β -lactam bond is cleaved and the carboxy group of Glu₁₆₆ regnerated. This group activates a catalytic H₂O molecule which can perform a nucleophile attack on the carboxy carbon of the broken β -lactam bond. The second tetrahedral deacylation intermediate (\ddagger 2) is also unstable and by hydrolysis of the bond between Ser₇₀ and the substrate the complete active center is regenerated. The deactivated β -lactam can be released. Figure adapted and modified from Drawz and Bonomo, 2010 [190]. **B**) Scheme of class A serine β -lactamases amino acid sequence. Catalytic active amino acids (black) [190], stabilizing mutation M182T (blue) [193] and split site for β -lactamase protein complementation assay (red) [153] are emphasized.

Due to high clinical relevance of β -lactamases the protein is well investigated. Originally the active enzyme was quantified colorimetrically by hydrolysis of the β -lactam ring of the chromophore nitrocefin which thus changes its absorption maximum from 390 nm to 492 nm and was used for resistance detection in various bacterial strains [194, 195]. Later the enzyme was utilized for different issues in microbiology and protein chemistry. As nitrocefin and β -lactam antibiotics are not membrane permeable [196] they are perfectly suited for the quantification of periplasmatic proteins [197-199], as membrane integration control and for topology determination [200, 201]. On the other hand nitrocefin has to traverse the outer membrane via porins which can cause detection problems if the permeability is reduced [202, 203]. Another application of this enzyme was the use for
investigation of the haemolysin transport pathway as it also folds in the culture medium [204].

 β -Lactamase is also very suitable for measurements in eukaryotic cells as those contain neither orthologs nor paralogs, which reduces the background activity. As nitrocefin cannot access the cytoplasm, another substrate was needed. Therefore, the cell permeable fluorescent compound CCF2/AM was developed which contains an intrinsic Förster resonance energy transfer (FRET) pair that is coupled by a cephalosporin β -lactam [205]. After hydrolysis of the β -lactam bond by a β -lactamase, the unstable intermediate spontaneously rearranges and the fluorophores separate. Hence, there is no FRET anymore and the donor fluorophore emits the light directly which can be quantified. As the turnover is proportional to the amount of protein, this method was used for protein expression studies [205, 206]. However, for activation the β -lactamase substrate has to be deacetylated after cellular uptake which occurs in eukaryotic cells but not in E. coli where a specific esterase is lacking. Missing esterase activity can be overcome by coexpression of the Fusarium solani pisi esterase cutinase [207]. This adapted method is advantageous for single cell measurements such as FACS, as the fluorophore is trapped in the periplasmic space after deacetylation. Hence, this extended assay was used for the discovery of new amyloid-beta aggregation inhibitors in E. coli [208].

1.5.3 Split β-lactamase complementation assay

The next step of utilizing the TEM-1 β -lactamase in molecular biology was the development of a split variant for protein-protein interaction measurements. The groups of Wehrman and Galarneau developed this protein complementation assay independently in 2002 [152, 153]. In both assays the β -lactamase is split at amino acid Gly₁₉₆/Leu₁₉₈ and generates two inactive fragments. Galarneau added the mutation M182T which stabilizes the enzyme and improves folding but does not influence its activity [193, 209]. Figure 8 B shows a scheme of a class A β -lactamase amino acid sequence with the positions of the enzymatic active amino acids as shown in Figure 8 A, the described M182T mutation and the splitting site. The two fragments show no enzymatic activity as the N-terminal fragment lacks the amino acids Lys₂₃₄ and Ala₂₃₇ (in case of TEM-1) so that the substrate β -lactam cannot be bound and positioned correctly. On the other hand the C-terminal fragment lacks the enzymatic active amino acids Ser₇₀, Lys₇₃, Ser₁₃₀ and Glu₁₆₆, which excludes any enzymatic activity [190]. It was shown that the proteins of interest can be

fused either to the C- or N-terminus of the C-terminal β -lactamase fragment and preferable to the C-terminus of the N-terminal fragment [153].

Hence, this protein complementation assay is suitable for many applications. For example this approach was already used for screening of G-protein-coupled-receptor inhibitors [210] and combined with FACS for toll-like receptor inhibitors [211]. The ability to facilitate ampicillin resistance was used in library screenings for the generation of high affinity TNF- α binding affibodies [212]. Furthermore, it can also be used for in vitro applications such as content mixing assays for liposome fusions to investigate the function of SNARE proteins [213].

2 Motivation

As described in the previous chapter, protein-protein interactions in biological membranes, mediated by TMDs, play an important role in signal transduction, protein folding and assembly. Nevertheless, many interfacial amino acids have not been investigated yet due to the limitation of existing methods. Especially, there is a need for new methods to quantify interactions between pairs of candidate TMDs and to screen for strongly interacting helices.

Hence, the aim of this thesis was the development of a new assay for the measurement of heterotypic TMD-TMD interactions, which could furthermore be adapted for library screenings in the future. All available assays depend on the activation of gene transcription. The new approach should circumvent the problems of these assays by reducing the number of steps between interaction and output signal and enable the measurement of homo- and heterotypic TMD-TMD interactions. To reach this goal, a more direct method, a split protein complementation assay (PCA), was designed. This comprised the use of the well investigated β -lactamase TEM-1, which can be split for PCAs and be colorimetrically quantified [153]. The complete assay was designed as a genetic assay where the TMDs of interest interact in the inner cell membrane of *E. coli* similar to ToxR or GALLEX [77, 108]. This circumvents (i) work intensive protein purification, (ii) increases the throughput, (iii) allows the measurement of mediated ampicillin resistance and (iv) forms the basis for screening set-ups.

Several features, which are necessary for a robust assay, were defined. First, a protein containing a full-length β -lactamase had to be designed which is a template for the following constructs. Second, it had to be proven whether the protein complementation works in the periplasmic space of *E. coli*, which had never been shown before. Therefore, an established soluble dimerization domain was used as a test case to demonstrate the feasibility. Third, a reliable quantification method for reconstituted β -lactamase had to be chosen. Forth, the dimerization has to be reproducibly detected and quantified. Fifth, the complete fusion proteins, containing the split fragments of the β -lactamase and a TMD of choice, have to be detectable was well. For this purpose, the proteins and the assay protocol had to be tested and optimized on the basis of several well investigated TMDs to

achieve a robust, reproducible, reliable and simple new method for the exact measurement of the strength of homo- and heterotypic TMD-TMD interactions.

3 Material and Methods

All buffers were prepared with deionized water (dH₂O) if nothing else is indicated. The compositions of all buffers are described in the corresponding sections. All chemicals were purchased from Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich (St. Louis, MO, US) and all consumables were purchased from Sarstedt (Nürnbrecht, Germany) if nothing else is indicated.

3.1 Preparation of chemical competent cells

Escherichia coli (*E. coli*) cells were made chemically competent using the protocol developed by Chung et al. [214]. 100 mL preheated LB medium (tryptone and yeast extract purchased from Carl Roth (Karlsruhe, Germany) with an appropriate antibiotic (100 μ g/mL streptomycin for JM83, 12.5 μ g/mL tetracyclin for *E. coli* XL1-Blue, none for *E. coli* BL21) were inoculated with 1 mL fresh overnight culture of the required *E. coli* strain and incubated in a 250 mL shaking flask at 37 °C and 140 rpm until a OD₆₀₀ of 0.3 was reached. Then the culture was chilled on ice for 10 min and afterwards pelleted for 10 min at 1000 xg and 4 °C (Hermle Z513K centrifuge, Wehingen, Germany). The supernatant was discharged and the pellet resuspended gently in 10 mL ice cold TSS buffer. The cells were aliquoted in 100 μ L per tube, immediately frozen in liquid nitrogen and stored at -80 °C.

LB medium (pH 7.0)		TSS buffer		
1% (w/v)	Tryptone		LB medium	
0.5% (w/v)	Yeast extract	5% (v/v)	DMSO	
171 mM	NaCl	10% (w/v)	PEG-3350	
Autoclaved		50 mM	MgCl ₂	
		Freshly prepared, sterile filtered		

3.2 Transformation

The chemical competent cells (s. 3.1) were transformed with plasmid DNA by heat shock. A cell aliquot was thawed on ice for 10 min and up to 10 μ L total volume DNA was added. After 30 min incubation on ice, the cells were incubated in a water bath at 42 °C for 1 min and then again incubated on ice for 2 min. Afterwards 900 μ L LB medium containing

20 mM glucose was added and incubated for 1 h in a turning wheel at 37 °C to allow development of the plasmid coded antibiotic resistance. Subsequently 100 μ L cells were plated on LB-agar plates (LB medium with 1.5% agar) containing an appropriate antibiotic (34 μ g/mL chloramphenicol (Cm) for N-BLa plasmids, 35 μ g/mL kanamycin sulfate (Kan) for C-BLa plasmids).

3.3 Polymerase chain reaction (PCR)

Many molecular methods (s. 0) are strongly dependent on PCR. If not indicated differently, the Phusion High-fidelity DNA Polymerase (cat. no. M0530) from NEB (Ipswich, MA, US) was used. If the PCR product had to be purified by agarose gel purification a 50 μ L reaction was prepared, in other respects 25 μ L. The general protocol for a 25 μ L reaction was as follows.

5 µL	5x Phusion buffer	30 sec	98 °C		Initial denaturation
5 ng	Template DNA	10 sec	98 °C		Denaturation
1.25 μL	Forward primer (10 mM)	30 sec	50-65 °C	30x	Annealing
1.25 μL	Reverse primer (10 mM)	1 min/kb	72 °C		Elongation
0.5 μL	dNTPs (10 mM each)	10 min	72 °C		Final elongation
0 μL - 2.5 μL	DMSO	Hold at	4 °C		
0.25 μL	Phusion polymerase				
Add to 25 µL	dH ₂ O				

The reaction could be optimized by varying the amount of DMSO (standard 3%) and the annealing temperature, depending on primer melting temperature (T_M), which was calculated with the web tool "OligoCalc" (http://biotools.nubic.northwestern.edu/OligoCalc.html) [215] using the "Salt Adjusted" T_M (50 mM Na⁺).

3.4 Molecular cloning

In the section molecular cloning all procedures modifying DNA to create new DNA plasmids with new features are described. Depending on the region on the plasmid and the number of modified base pairs different techniques were used. All restriction enzymes, T4 DNA ligase and T4 DNA polynucleotide kinase (PNK) were purchased from Thermo Fisher Scientific (Waltham, MA, US), the Phusion High-fidelity DNA Polymerase from

North England Biolabs (Ipswich, MA, US) and the PfuUltra II Fusion HS DNA Polymerase from Agilent (Santa Clara, CA, US).

All reactions were performed in a thermocycler (Mastercycler or Mastercycler personal from Eppendorf (Hamburg, Germany)). 10 μ L of the product were transformed into chemical competent *E. coli* XL1-Blue plated on a LB-agar plate containing appropriate antibiotic and incubated for 14 h to 20 h at 37 °C until single colonies were visible on the plates. After that the plates could be stored at 4 °C up to several weeks.

3.4.1 Restriction-based cloning

Transfer of DNA regions longer than approximately 200 bp from one plasmid to another was done by restriction based cloning. A linear vector DNA (plasmid backbone) and a linear insert DNA (DNA region from a donor plasmid) with compatible, usually sticky ends were created and mixed for reaction.

In case of compatible restriction sites on the donor and acceptor plasmid, both plasmids $(1 \ \mu g)$ were digested for 1 h with 10 U suitable restriction endonucleases (Thermo Fisher Scientific) in corresponding buffers. If no compatible restriction site was available, the insert was amplified from the donor plasmid by PCR (s. 3.3) using primers that introduce a new restriction site by 5' extension. To ensure high enzyme efficiency all buffer salts from the PCR reaction were removed by column purification (s. 3.6) before endonuclease digestion. The digested samples were isolated by agarose gel purification (s. 3.6) and merged by T4 DNA ligase. 50 ng vector DNA and the threefold molar mass of insert DNA were used.

2 µL	10x T4 DNA Ligase buffer	20 min	22 °C	
50 ng	Vector DNA	20 min	16 °C	Ligation
$m = \frac{bp(insert)}{bp(vector)} \times 3 \times 50 \ ng$	Insert DNA	20 min	12 °C	
1 U	T4 DNA Ligase	10 min	70 °C	Deactivation
Add to 20 µL	dH ₂ O	Hold at	4 °C	

3.4.2 Cassette cloning

New TMDs, which were meant to be measured in the BLaTM assay, had to be integrated into the N-BLa and C-BLa cloning vectors, which contain an "Integrin α 5 GP" TMD with an *Apa*I restriction site. To perform cassette cloning the vectors were digested using the

two endonuclease restriction enzymes *Nhe*I and *Bam*HI which cut precisely upstream and downstream of the "Integrin α 5 GP" TMD to remove it and thus create two sticky ends (Figure 9, red bases). The new inserts consist of two oligos, whose centers code for the new TMD sequence (Figure 9, black bases, e.g. GpA₁₉-1_wt) and are reverse complement to each other so that they can hybridize. By adding a corresponding set of bases (Figure 9, blue bases) to the ends of the two primers, the same sticky ends as on the digested vector were created on the cassette fragment.

Figure 9: Oligo design for cassette cloning. Each cassette consists of one sense and one reverse complementary antisense primer. Amino acids: capital letters: TMD, lowercase letters: juxtamembrane region. Primer: grey bases: vector backbone, red bases: sticky ends of the vector, blue bases: sticky ends of the insert., black bases: coding sequence for the TMD (e. g. GpA_{19} -1_wt). Restriction sites are indicated.

The cassette was created by hybridizing two complementary oligos due to slowly decreasing temperature. Hence, monomeric oligo nucleotides can specifically dimerize to a double-strand DNA cassette.

10 µL	10x Tango buffer	10 min	90 °C	
1 µL	Sense oligo (100 mM)	45 sec	-1 °C/cycle	70x
1 µL	Antisense oligo (100 mM)	Hold at	4 °C	
88 µL	dH ₂ O			

The cloning vector had to be digested in two steps because of incompatibility of the two restriction enzymes *Nhe*I and *Bam*HI. First 3 μ g DNA were digested with 10 U *Nhe*I in 50 μ L 1x Tango buffer for 1.5 h at 37 °C. Then 10 U *Bam*HI and 5.5 μ L 10x Tango buffer were added to double the buffer concentration. After 1.5 h incubation at 37 °C the linearized vector was isolated by agarose gel purification (s. 3.6). Afterwards the cassette was phosphorylated by T4 DNA polynucleotide kinase (PNK) and ligated into the linearized vector:

2 µL	10x T4 DNA Ligase buffer	30 min	37 °C	Phosphorylation
1 μL	ATP (10 mM)	20 min	22 °C	
35 ng	Linearized vector	20 min	16 °C	Ligation
1 μL	Hybridized cassette	20 min	12 °C	
1 U	T4 DNA Ligase	10 min	70 °C	Deactivation
5 U	PNK	Hold at	4 °C	
Add to 20 µL	dH ₂ O			

To avoid false positive clones, in the last step remaining cloning vector was removed by digestion using 5 U *Apa*I for at least 30 min at 30 °C.

3.4.3 Q5 site directed mutagenesis

For deleting regions of any length, the insertion of short sequences up to 60 bp or substitution of single amino acids Q5[®]site directed mutagenesis, developed by NEB (Ipswich, MA, US), was used. In this method the complete plasmid was amplified by PCR (s. 3.3) and then circularized. The binding sites of the primers define the region on the plasmid for the plasmid. For deletions the corresponding regions are omitted between the 5'binding sites of the primers. For insertions the corresponding regions are introduced by 5' extensions of the primers. For substitutions the corresponding bases are replaced by 5' base exchanges of the primers.

Following, the success of the PCR was checked by agarose gel electrophoresis (0.7% (w/v) agarose). Then the PCR product was circularized:

2 μL	10x T4 DNA Ligase buffer	30 min	37 °C	Phosphorylation
1 μL	ATP (10 mM)	20 min	22 °C	
10 µL	PCR product	20 min	16 °C	Ligation
1 U	T4 DNA Ligase	20 min	12 °C	
5 U	PNK	10 min	70 °C	Deactivation
Add to 20 μ L	dH ₂ O	Hold at	4 °C	

In the last step before transformation the template DNA was removed by digestion for at least 2 h at 37 °C with 10 U *Dpn*I which is specific for methylated DNA and cuts on average every 256 bp.

3.4.4 Quikchange mutagenesis

The Quikchange mutagenesis [216], marketed by Stratagene (La Jolla, CA, US), can be used to mutate sequences between single bases and 60 bases. It is more suitable for shorter insertions or substitutions. In this method two complete reverse complementary oligos were needed, whose centers code for the desired mutation and have downstream and upstream binding sites with annealing temperatures between 55 °C and 60 °C. The PCR will create linear sense and antisense vector DNA fragments, whose 5' region contains the desired mutation. The two fragments can hybridize and form the complete vector with the mutation and two nicked regions. The mutation was inserted by PCR using following conditions:

5 μL	10x PfuUltraII buffer	2 min	95 °C	
20 ng	Template DNA	20 sec	95 °C	
125 ng	Sense oligo	20 sec	58 °C	16x - 18x
125 ng	Antisense oligo	5 min	72 °C	
1.5 μL	DMSO	10 min	72 °C	
1 µL	dNTP (10 mM)	Hold at	4 °C	
1 μL	PfuUltraII DNA Polymerase			
Add to 50 µL	dH ₂ O			

In the last step before transformation the template DNA was removed by digestion for at least 2 h at 37 °C with 10 U DpnI which is specific for methylated DNA and cuts on average every 256 bp. After transformation in *E. coli* the cells will repair these regions and the desired mutation is inserted.

3.4.5 Transfer-PCR

Transfer-PCR (TPCR) is an alternative method to restriction based cloning to transfer large DNA regions from one vector to another without using endonucleases [217]. It combines PCR (s. 3.3) with the Quikchange mutagenesis (s. 3.4.4). The oligos consist of two parts: the 3' parts are binding with an annealing temperature of about 55 °C to the donor plasmid and amplify the desired new sequence. The 5' ends are binding with an annealing temperature of about 65 °C to the acceptor plasmid and enable the new DNA fragment to insert site specific into the acceptor plasmid. A two-step protocol was used in this thesis as it has a higher efficacy then a one-step protocol. Thus first a megaprimer was produced by PCR (s. 3.3) containing the new sequence and extensions on both sites for targeting the

correct position on the acceptor plasmid. Then the megaprimer was purified via agarose gel electrophoresis (s. 3.6) and transferred to the acceptor plasmid following the Quikchange protocol (s. 3.4.4).

10 µL	5x Phusion buffer	30 sec	98 °C
10 ng	Vector DNA	10 sec	98 °C
50 ng	Megaprimer DNA	30 sec	68 °C 18x
1 μL	dNTPs (10 mM)	6 min	72 °C
1.5 μL	DMSO	10 min	72 °C
1 μL	Phusion polymerase	Hold at	4 °C
Add to 50 µL	dH ₂ O		

In the last step before transformation the template DNA was removed by digestion for at least 2 h at 37 °C with 10 U *Dpn*I which is specific for methylated DNA and cuts on average every 256 bp.

3.5 Plasmid propagation

The success of every cloning had to be checked. Therefore, single colonies were picked from LB agar plates with a sterile 200 μ L tip and transferred into a glass tube with 8 mL LB medium containing an appropriate antibiotic. Alternatively overnight cultures were inoculated with fresh transformations directly if homogenous plasmid solution was transformed.

For the preparation of plasmid DNA the NucleoSpin® Plasmid Purification Kit from Macherey-Nagel was used accordingly to the manufacture's protocol for high-copy plasmids (p_{BAD} variants) or for low-copy plasmids (N-BLa and C-BLa plasmids), respectively. All optional washing and heating steps were conducted.

3.6 DNA purification

Depending on the cloning method, DNA fragments had to be separately isolated or purified from buffer salts to ensure high enzyme activity.

If a fragment had to be isolated from a DNA mixture, the sample was separated by agarose gel electrophoresis and stained with ethidium bromide (s. 3.8). The band containing the desired DNA fragment was cut under UV light ($\lambda = 365$ nm) and transferred into a 1.5 mL tube. For the isolation of the DNA from the agarose the NucleoSpin® Gel and PCR clean-

up kit from Macherey-Nagel was used accordingly to the manufacture's protocol. All optional washing and heating steps were conducted.

If no separation but only a desalting was necessary, the sample was loaded directly onto the column according to the manufacture's protocol of the NucleoSpin® Gel and PCR clean-up kit from Macherey-Nagel. All optional washing and heating steps were conducted.

3.7 DNA quantification

The concentration of prepared DNA was determined by measuring the extinction at 260 nm and 280 nm in a quartz cuvette using an Ultrospec 3100*pro* photometer (Amersham Biosciences, Amersham, UK). The DNA was diluted 1:40 in dH₂O and the extinction was measured against a dH₂O blank. The quotient of 260 nm and 280 nm should be around 1.8 for pure DNA. Higher values are indicating RNA contaminations, lower values remaining protein. Values up to 2.0 were accepted for further experiments.

3.8 Agarose gel electrophoresis

Agarose gel electrophoresis was used for DNA purification, quality control of PCR reactions and the analysis of control digestions of cloning products. The used buffer system was Tris-acetate/EDTA (TAE). The agarose concentration defines the optimal separation range. If the desired fragment was shorter than 1000 bp 1.5% agarose was used, if it was longer than 5000 bp 0.7% agarose and otherwise 1% agarose.

TAE	buffer	(pH	8.0)

40 mM	Tris			
20 mM	Acetic acid			
1 mM	EDTA			
Stored at room temperature				

50 mL TAE buffer with the desired amount of agarose low EEO (Applichem, Darmstadt, Germany) was heated in the microwave at maximum power for 100 sec and afterwards the evaporated volume was filled-up with dH₂O. The complete solution was filled in a combined casting and electrophoresis system and 0.03 μ g/mL ethidium bromide was added to visualize the DNA after the beforehand separation. A comb was inserted to form pockets for the samples and after cooling down to room temperature the gel could be used. The

chamber was filled with TAE buffer and the comb and the gel-casting gates were removed carefully. The DNA samples were mixed with DNA Gel Loading Dye (6X) (Thermo Fisher Scientific) and loaded into the pockets. Additionally 6 μ L GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) or 6 μ L GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific) was loaded, depending on the investigated samples. The fragments were separated at 70 V for 50 min and following visualized under UV light ($\lambda = 312$ nm).

3.9 gDNA extraction from *Saccharomyces cerevisiae*

A few mg baker yeast (Weininger Hefe, Rewe) was resuspended in 25 μ L 20 mM NaOH and incubated at 95 °C for 2 min. To remove cell debris and intact cells the suspension was centrifuged for 5 min at 13000 rpm (Heraeus Biofuge fresco, Thermo Fisher Scientific, Waltham, MA, US). 1 μ L supernatant was used as template DNA for the PCR reaction (s. 3.3).

3.10 DNA Sequencing

DNA plasmids were either sequenced by GATC Biotech (Konstanz, Germany) according to their protocol or in-house using the DNA gel sequencer LONG READIR 4200 (LI-COR Biosciences, Lincoln, NE, US). In the following in-house protocol for each template four sequencing reactions (one different ddNTP each) with a fluorescent dye labeled primer (IRD-700 or IRD-800) were conducted.

Sample preparation

For each sample one mastermix was pipetted. The Tth inorganic pyrophosphatase was purchased from Genecraft (Köln, Germany), the Taq polymerase was expressed and purified in-house. If the plasmid DNA concentration was below $120 \text{ ng/}\mu\text{L}$, $8.2 \mu\text{L}$ DNA solution was used instead of $1 \mu\text{g}$.

3.5x Sequencing buffer (pH 9.0)		Storage buffer (for enzyme dilution)		
175 mM	KC1	10 mM	K ₃ PO ₄ , pH 7.0	
35 mM	Tris	100 mM	NaCl	
0.35%	Triton X-100	0.5 mM	EDTA	
12.3 mM	MgCl ₂	1 mM	DTT	
Stored at 4 °C		0.01%	Tween 20	
		50% (v/v)	Glycerol	
Pyrophosphatas	e dilution (0.5 U/μL)	Stored at 4 °C		
	Storage buffer			
100/(x/y)	Tth inorganic Pyrophosphatase (5 U/μL)	Stop/loading buffer		
10% (V/V)		95% (v/v)	Formamide	
Stored at -20 °C		10 mM	EDTA, pH 9.0	
		0.1% (w/v)	Basic fuchsin	
Master mix		0.01% (w/v)	Bromophenol blue	
7.8 μL	3.5x Sequencing buffer	Stored at -20 °C		
1 μg	Template DNA			
1.1 μL	Labeled primer (2 µM)			
1.1 μL	Pyrophosphatase dilution			
0.3 μL	Taq polymerase			
Add to 18.5 μ L	dH ₂ O			

For the sequencing reaction 2 μ L of each nucleotide mix was pipetted into one PCR tube. Last 4 μ L master mix were added to each tube, gently mixed and the sequencing reaction was started. Before loading on the sequencing gel 3 μ L stop/loading buffer were pipetted to each sample and incubated for 4 min at 95 °C. If the samples were not sequenced immediately they were stored at -20 °C.

Α		С		G		Т	
15 µM	dNTP						
0.21 µM	ddATP	0.21 µM	ddCTP	0.38 µM	ddGTP	0.38 µM	ddTTP

Sequencing protocol

2 min	95 °C	
20 s	95 °C	
20 s	57 °C	30x
1 min	70 °C	
1 min	70 °C	
Hold at	4 °C	

Sequencing gel preparation

All polyacrylamide sequencing gel components were combined except APS and TEMED. Glass plates were washed with 10% (w/v) SDS and isopropanol. Plates, spacers and clamps were assembled. APS and TEMED were added to the sequencing gel mix and the solution was filled between the glass plates with a syringe immediately. Then the comb was inserted with its smooth side, the buffer place holder was fixed on top and the screws were tightened. After polymerization the buffer place holder and the comb were removed, the gel assembled to the sequencing machine and the comb inserted. The two buffer tanks were filled with 1x TBE long run buffer.

Sequencing gel	uencing gelL40% Acrylamide-Bisacrylamide 32:15 gUreamL10x TBE long run buffer5 mLFormamided to 25 mLdH2O	1x TBE loi	1x TBE long run buffer (pH 8.3-8.7)			
5 mL	40% Acrylamide-Bisacrylamide 32:1	134 mM	Tris			
10.5 g	Urea	45 mM	Boric acid			
2.5 mL	10x TBE long run buffer	2.5 mM	EDTA			
3.75 mL	Formamide	Stored at ro	oom temperature			
Add to 25 mL	dH ₂ O					
25 µl	TEMED					
175 µl	10% (w/v) APS					

Electrophoresis

 $0.5 \ \mu$ L of each reaction was loaded onto the gel. During electrophoresis (1200 V, 37 mA, 40 W, 50 °C) the fluorescence was recorded. The sequencing pattern was analyzed with Base ImageIR Image Analysis 4.0 software (LI-COR Biosciences, Lincoln, NE, US) and evaluated with CLC Main Workbench 6.9.1 (CLC Bio, Aarhus, Denmark).

3.11 GFP expression test

As all β -lactamase hybrid protein constructs contain a sfGFP at their C-terminus, the cloning was tested for frameshifts by verifying GFP expression. Emission at 520 nm after

excitation at 485 nm indicated expression of GFP and thus no frameshift mutation in the cassette cloning procedure (s. 3.4.2). For the test of BLaTM 1.1 plasmids two 200 µL aliquots of each overnight culture were prepared - one with 1.33 mM arabinose and another without inducer. Both samples were incubated for 2 h at 37 °C in the turning wheel. 10 µL of each culture were diluted with 90 µL PBS in a black 96-well plate (Nunc, Roskilde, Denmark) and the fluorescence intensity was measured ($\lambda_{Ex} = 485 \text{ nm}$, $\lambda_{Em} =$ 520 nm, PolarStar, BMG Labtech, Ortenberg, Germany). If the induced sample showed an at least three times higher fluorescence than the control, a correct expression and thus the absence of a frameshift was assumed. Because of lower expression levels of BLaTM 1.2 proteins, the overnight cultures had to be diluted 1:10 in LB-medium to ensure higher expression. Again, one sample of each culture was induced with 1.33 mM arabinose and another one used as control. After 4 h expression at 37 °C 200 µL cells were centrifuged for 2 min at 11000 rpm (Biofuge fresco, Heraeus) and resuspended in the same volume low-fluorescent PBS (flow cytometry grade, Thermo Fisher Scientific). Then 100 µL cell suspension was pipetted into a black 96-well plate (Nunc, Roskilde, Denmark) and the fluorescence intensity was measured ($\lambda_{Ex} = 485 \text{ nm}$, $\lambda_{Em} = 520 \text{ nm}$, PolarStar, BMG Labtech, Ortenberg, Germany). If the induced sample showed an at least twofold higher fluorescence than the control, a correct open reading frame was assumed.

For quantitative GFP expression measurements of the BLaTM 1.2 constructs the expression was monitored directly in 12-well plates (Greiner Bio-one, Kremsmünster, Austria). Corresponding to the BLaTM 1.2 protocol (s. 3.15.3) 2 mL of medium were inoculated 1:10 with an overnight culture in the wells and induced with appropriate amounts of arabinose and IPTG (133 μ M arabinose and 0.1 – 0.7 mM IPTG). After 4 h expression the fluorescence intensity ($\lambda_{Ex} = 485$ nm, $\lambda_{Em} = 520$ nm) and the absorption at 544 nm was measured in the plates in a microplate reader (FluoStar, BMG Labtech, Ortenberg, Germany). For analysis the background fluorescence (mean of three non-induced samples) was subtracted from all values. The corrected values were normalized to the cell density by dividing the values by A₅₄₄.

3.12 β-Lactamase activity test

The activity of β -lactamase was tested by the enzymatic cleavage of the β -lactam ring of the chromogenic cyclosporine derivate nitrocefin. After cleavage its absorption maximum switches from 390 nm to 492 nm [153, 194, 199]. The nitrocefin (Merck Millipore,

Darmstadt) was dissolved 10 mM in DMSO and stored at -20 °C. An overnight culture pf JM83 cells containing the desired plasmids was diluted 1:10 in fresh LB medium, induced with 0.2% arabinose and incubated for another 4 h at 37 °C. 200 μ L cells were centrifuged (2 min, 7000 rpm) and resuspended in 200 μ L sodium phosphate buffer (100 mM PO₄³⁻, pH 7.0). 20 μ L of the resuspended *E. coli* culture, 100 μ L sodium phosphate buffer/EDTA (100 mM Na₃PO₄, pH 7.0, 2 mM EDTA), 78 μ L water and 2 μ L nitrocefin (final concentration 100 μ M) were mixed. Then the β-lactamase activity was determined over 20 min by measuring the extinction increase at 492 nm in a 96-well plate in microplate reader (VersaMmax, Molecular Devices, Biberach an der Riss).

3.13 SDS polyacrylamide gel electrophoresis

Proteins were separated depending on their molecular weight by the discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [218]. The combined casting and running system for gels (10 cm x 10 cm x 0.8 mm) "PerfectBlue Dual Gel Twin S" from PEQLAB (Erlangen, Germany) was used.

Gel preparation

The glass plates were cleaned with 70% ethanol, separated with one spacer on each side and fixed with the clamps, notched plate facing to the middle, to the chamber of the electrophoresis device. The position was defined by the gel casting base. The base was turned around so that the gaskets were facing up and the chamber was fixed to seal the glass plates. All resolving gel components were combined except ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). To start the polymerization APS and TEMED were added and the solution was pipetted between the plates immediately to height of about 6 cm. Then the gel was overlaid with isopropanol to protect it from aerial oxygen which inhibits polymerization. 30 min later the gel was completely polymerized and the isopropanol was removed completely. All stacking gel components were combined except APS and TEMED. To start the polymerization APS and TEMED were added and the solution was pipetted between the plates immediately to the solution was polymerized to and the gel was ready for use. It could be stored for several days at 4 °C.

Stacking gel buf	fer (pH 6.8)	Resolving gel bu	ıffer (pH 8.5)			
250 mM	Tris	1 M	Tris			
0.2% (w/v)	SDS	0.26% (w/v)	SDS			
Stored at room ter	mperature	Stored at room te	emperature			
Stacking gel		12.5% Resolving	g gel			
1.6 mL	Stacking gel buffer	2.8 mL	Resolving gel buffer			
0.4 mL	30% Acrylamid- bisacrylamid (37.5:1)	3.0 mL 30% Acrylamid				
10 1		1 <i>6</i> T				
1.2 mL	dH ₂ O	1.5 mL	dH ₂ O			
25 µL	APS (10% (w/v))	50 µL	APS (10% (w/v))			
2.5 μL TEMED		2.5 μL TEMED				

Sample preparation and electrophoresis

The *E. coli* culture samples had to be concentrated and denatured. For concentration 0.5 OD_{600} cell sample was centrifuged for 1 min at 6000 rpm (Biofuge fresco, Heraeus), the supernatant discarded and the pellet resuspended in 20 µL reducing 1x Laemmli loading buffer. Then it was denatured for 5 min at 95 °C.

The gel casting base was removed and if only one gel was used the other site of the chamber was sealed with a blocking plate. The chamber was placed in the buffer tank and the chamber and the tank were filled with Laemmli buffer. The comb was removed carefully and all pockets were washed with buffer. The samples and $4 \,\mu\text{L}$ PageRulerTM Prestained Protein Ladder (Thermo Fischer Scientific) were loaded. The proteins were separated for 80 min at 200 V.

Laemmli buff	er	5x Laemmli loa	ading buffer (pH 6.8)		
20 mM Tris		50 mM	Tris		
192 mM	Glycine	5% (w/v)	SDS		
0.1% (w/v)	SDS	20% (v/v)	Glycerol		
Stored at room temperature		0.02% (w/v)	Bromphenol blue		
		10% (v/v)	β-Mercaptoethanol		
		Stored at 4 °C			

3.14 Western blot

For the specific detection of proteins, separated by SDS-PAGE (s. 3.13), semi-dry Western blotting was used. The colorimetric detection was carried out after a two-step immuno staining of the proteins. Alkaline phosphatase, fused to a secondary antibody, hydrolyzes

5-bromo-4-chloro-3-indolyl phosphate (BCIP). The product 5-bromo-4-chloro-3-indoxyl can be oxidized by nitro blue tetrazolium (NBT) to the blue dye 5,5'-dibromo-4,4'- dichloro-indigo, which is insoluble. The twice reduced NBT forms an insoluble, blue diformazan dye. Both products precipitate on the Western blot nitrocellulose membrane where protein was stained by the antibodies.

Protein transfer

For an efficient transfer of proteins from SDS-PAGE gel to a Western blot nitrocellulose membrane, it was essential that all layers were soaked with blotting buffer and put air bubble-free one on the other. Four filter papers (Munktell & Filtrak, Bärenstein, Germany) were placed on the middle of the anode of the blotting device (Modell SD 1, cti, Idstein, Germany). The stacking gel of the SDS-PAGE gel was removed and the resolving gel with the separated proteins was placed on the filter papers. Then a nitrocellulose blotting membrane (Berrytec, Grünwald, Germany), activated for at least 1 min in blotting buffer, was put on the gel, followed by another four layers of soaked filter papers. Following the cathode was placed on the transfer stack and weighted. The proteins were transferred to the nitrocellulose membrane for 1.5 h at 70 mA (1 mA/cm²).

Protein detection

After that, all proteins on the blotting membrane were stained for 1 min with PonceauS solution to check for transfer efficiency. Then the membrane was washed with water to remove all unbound PonceauS. Afterwards the stained membrane was documented and the bound PonceauS was stripped by washing with alkalized water. Then the membrane was blocked with 10 mL 3% (w/v) skimmed milk powder in TBS for 1 h at room temperature or overnight at 4 °C. Next the membrane was washed for 5 min with TBS and incubated for 1 h at room temperature with 10 mL 3% (w/v) skimmed milk powder in TBS containing 0.01% (v/v) Monoclonal ANTI-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, US) as a primary antibody. The membrane was washed three times for 5 min with TBS-T and following incubated for 1 h at room temperature with 10 mL 3% (w/v) skimmed milk powder in TBS containing 0.01% (v/v) Anti-Mouse IgG AP Conjugate (Promega, Madison, WI, US) as a secondary antibody. Before detection the unbound antibodies were removed by another three 5 min washing steps with TBS-T. The secondary antibodies, and so the FLAG tagged proteins, were detected with the NBT/BCIP

solution until bands were clearly visible. To stop the reaction the membrane was rinsed several times with water. For documentation the membrane was dried at room temperature and scanned.

Blotting Buffer

	Laemmli buffer
20% (v/v)	Methanol
Stored at room	temperature

TBS (pH 7.4)

20 mM 150 mM	Tris
150 mM	NaCl
Stored at room	m temperature

BCIP solution

Dimethylformamid 5% (w/v) BCIP Stored at -20 °C

AP buffer (pH 9.5)

100 mM	Tris
100 mM	NaCl
5 mM	MgCl ₂
Stored at room	m temperature

3.15 BLaTM Assay

3.15.1 Choose of vectors

As there are two different proteins involved in the assay, two plasmids with several important requirements were needed. (i) First the vectors have to be compatible to each other. That is to say they have to use two different replication systems. Otherwise one of the plasmids would get lost during the growth of *E. coli* and after the complete degradation of the antibiotics. (ii) For selection the plasmids have to contain two different antibiotic resistance genes. (iii) Furthermore, the plasmids should have a similar copy number to achieve an equal protein expression. (iv) Low copy plasmids are preferred to accomplish a low protein expression, which is essential for membrane protein interaction assays. (v) The protein expression has to be inducible. That means it can be induced at a defined time point and the strength can be controlled by the amount of inductor. (vi) The vectors have to be

PonceauS solution

3% (w/v)	Trichloroacetic acid
0.3% (w/v)	PonceauS
Stored at room ter	nperature

TBS-T (pH 7.4)

TBS 0.5% (v/v) Tween 20 Stored at room temperature

NBT solution

70% (v/v)	Dimethylformamid
5% (w/v)	NBT
Stored at -20 °C	

Staining solution

20 mL	AP buffer
120 µL	NBT solution
60 µL	BCIP solution
Freshly prepared	

compatible with the cassette cloning technique (s. 3.4.2) which forbids *Bam*HI and *Nhe*I restriction sites on the plasmid backbone.



Figure 10: Illustration of the final vectors containing the BLaTM proteins. Yellow: open reading frames, red: origin of replication, green: operon region. Important restriction sites are indicated. BLaTM proteins (s. 3.15.2) contain a signal peptide (SP), the N- or C-terminal fragment of the split β -lactamase (N-BLa, C-BLa), a spacer between the fragment and the transmembrane domain (TMD), the TMD of interest, a spacer between the TMD and the sfGFP, a sfGFP and a FLAG-epitope for detection. A) N-BLa: pToxRVII [219] based vector containing a p15A origin of replication, a chloramphenicol acetyltransferase gene (Cm^R, chloramphenicol resistance) and a p_{BAD} operator. B) C-BLa: pBAD322K [220] based vector containing a pMB1 origin of replication, a rop gene (copy number repression), an aminoglycoside O-phosphotransferase gene (Kan^R, kanamycin resistance), an AraC gene (regulator protein of the p_{BAD} operon) and a p_{BAD} operator.

The first chosen vector was the pToxRVII vector (Figure 10 A), created by Eric Lindner [219]. The backbone is the pACAY184 plasmid containing the multiple cloning site and the T7 terminator region of the pET22b plasmid. It has a low copy p15A origin of replication [221], a chloramphenicol resistance gene (Cm^R , chloramphenicol acetyltransferase), a p_{BAD} operator [222] and is compatible with the cassette cloning technique.

The second vector was the pBAD322K vector (Figure 10 B), created and provided by John Cronan [220]. It is a pBAD24 vector with an inserted *rop* gene [223, 224] to reduce the copy number and has a pMB1 origin of replication [225] which is compatible to the p15A origin of replication [130]. Furthermore, it contains the p_{BAD} operator, the corresponding *AraC* gene and a kanamycin resistance gene (*Kan^R*, aminoglycoside O-phosphotransferase). As it contained two additional *Nhe*I restriction sites in a non-coding region, these had to be removed first by Q5 mutagenesis (primers: pBAD322_fwd, pBAD322_rev; 55 °C, 3% DMSO), resulting in the pBAD322K_ Δ plasmid. The modified version is compatible to the cassette cloning technique.

3.15.2 Vector construction

This section describes the construction of the plasmids N-BLa 0.1, C-BLa 0.1, N-BLa 1.1, C-BLa 1.1, N-BLa 1.2, C-BLa 1.2 and their variants. Figure 11 gives an overview of the different cloning steps which are explained in detail in the following chapters. For the BLa 1.1 plasmids the flexible linker length is indicated in subscript (5, 9, 13 or 25) whereas BLa 1.2 plasmids always contain a flexible 13 amino acids linker. The TMD of all cloning vectors is "Integrin- α 5-GP" (UniProtKB [122] accession number: P06756; amino acids 996 – 1008, L1002G, A1003P), which contains an *Apa*I restriction site for cloning control.



Figure 11: Overview of the vector construction. Vector backbone and segments of fusion proteins are indicated. Modified parts in bold. MalE: maltose binding protein, BLa: full-length TEM-1 β -lactamase (residues 23 – 286), N-BLa: N-terminal fragment of TEM-1 β -lactamase (residues 23 – 194), C-BLa: C-terminal fragment of TEM-1 β -lactamase (residues 196 – 286), *: β -lactamase M182T mutation, TMD: Integrin- α 5-GP, sfGFP: superfolder GFP, bZip: basic leucine-zipper of GCN4 (residues 235 – 281), signal peptide in brackets (BLa: TEM-1 β -lactamase (residues 1 – 22); PelB: pectate lyase B (residues 1 – 20); OmpA: outer membrane protein A (residues 1 – 23)). Methods: TPCR: transfer-PCR, QC: Quikchange mutagenesis, Q5: Q5-mutagenesis, PCR: insert amplification by PCR, R+L: restriction and ligation based cloning.

Construction of BLaTM full length protein

PelB-SP β-lactamase -	-[flexible linker		TMD		rigid linker		sfGFP	\vdash	FLAG-tag
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Figure 12: Schema of BLaTM hybrid protein

The primary constructs are based on the p_{BAD} ::*mal*E-TMD-sfGFP expression vector designed by Christian Ried [226-228]. The most important modifications were performed in this high copy vector. The MalE and the first rigid linker were replaced by the full length β -lactamase and a five amino acids flexible linker by TPCR (template: pBAD322, primers: MP_AmpR_fwd, MP_AmpR_rev). The M182T mutation was introduced by Quikchange mutagenesis to improve the stability of the enzyme (BLa_M182T_s, BLa_M182T_as; 12 cycles) [153, 193]. The original β -lactamase signal peptide was replaced by a PelB signal peptide (UniProtKB [122] accession number Q00205) by another Quikchange mutagenesis (BLa_PelB_s, BLa_PelB_as; 18 cycles) to achieve a good membrane insertion. This plasmid was termed p_{BAD}::*bla*(PelB)M182T-5x-TMD-sfGFP.

Construction of BLa-bZIP full length protein



Figure 13: Scheme of BLa-bZIP hybrid protein

For the proof-of-principle a construct containing the homodimerizing basic leucine-zipper domain (bZIP, residues 235–281) of the leucine-zipper GCN4 (UniProtKB [122] accession number P03069) [229] was created. The leucine-zipper was amplified from the gDNA of *Saccharomyces cerevisiae* by PCR (primers: GCN4_fwd_15x_speI, GCN4_rev_ft_PstI; 0% DMSO; protocol see below) and replaced the "5x-TMD-sfGFP" fragment of $p_{BAD}::bla(PelB)M182T-5x-TMD-sfGFP$ via the restriction sites *SpeI* and *PstI*. This plasmid was termed $p_{BAD}::bla(PelB)M182T-15x-bZIP$.

3 min	95 °C	
30 sec	95 °C	
20 sec	40 °C	5
+ 0.5 °C/sec		JX
1 min	72 °C	
10 sec	95 °C	
30 sec	55 °C	30x
1 min	72 °C	
5 min	72 °C	
Hold at	4 °C	

Construction of BLa-bZIP split proteins (N-BLa 0.1 and C-BLa 0.1)



Figure 14: Scheme of BLa-bZIP split proteins (N-BLa 0.1 and C-BLa 0.1)

For the final constructs the β -lactamase was split to an N-terminal fragment (residues A23-G194) attended to the PelB signal peptide from Aspergillus niger (Pectin lyase B, UniProtKB [122] accession number Q00205, residues 1 - 20) and a C-terminal fragment (residues L196-W286) attended to the same signal peptide [153]. First the split BLa-bZIP vectors (N-BLa 0.1 and C-BLa 0.1) were constructed by deleting the needless parts of p_{BAD}::*bla*(PelB)M182T-15x-bZIP by Q5 mutagenesis resulting the in vector pBAD::N-bla(PelB)M182T-15x-bZIP and the vector pBAD::C-bla(PelB)-15x- bZIP (N-bla primers: BLa 194G rev, BLa speI-15x fwd, 60 °C, 3% DMSO; C-bla: primers: BLa_PelB+H_rev, BLa_196L_fwd, 56 °C, 3% DMSO). The N-BLa containing insert had to be amplified by PCR first to add one restriction site (pToxRV fwd, pToxRV rev XhoI, 55 °C, 3% DMSO). Subsequently it was inserted into the pToxRVII vector by restriction based cloning using the restriction sites MluI and XhoI. This plasmid was termed N-BLa 0.1. Next the C-BLa containing insert was transferred from p_{BAD}::C-bla(PelB)-15xbZIP vector to the final vectors pBAD322K by restriction based cloning using the restriction sites MluI and PstI. This plasmid was termed C-BLa 0.1.

PelB-SP N-BLa flexible linker TMD rigid linker sfGFP FLAG-tag PelB-SP C-BLa flexible linker TMD rigid linker sfGFP FLAG-tag

Construction of BLaTM 1.1 split proteins

Figure 15: Scheme of BLaTM 1.1 split proteins.

First the split regions were transferred from the p_{BAD} ::N-*bla*(PelB)M182T-15x- bZIP and p_{BAD} ::C-*bla*(PelB)-15x-bZIP vectors to the p_{BAD} ::*bla*(PelB)M182T-5x-TMD-sfGFP vector by restriction based cloning using the restriction sites *Mlu*I and *Spe*I. This lead to the vectors p_{BAD} ::N-*bla*(PelB)M182T-5x-TMD-sfGFP and p_{BAD} ::C-*bla*(PelB)-5x-TMD-sfGFP. Following they were transferred to the target vectors pToxRVII and pBAD322K_ Δ using the same procedure as for the creation of the N-BLa 0.1 and C-BLa 0.1 plasmids. These plasmids were termed N-BLa 1.1₅ and C-BLa 1.1₅ and were used in the BLaTM 1.1 assay. The index indicates the length of the linker between the β -lactamase fragments and the TMD. (For DNA and protein sequences of N-BLA 1.1₁₃ and C-BLA 1.1₁₃ see appendix).

Construction of different linker lengths

For assay optimization the flexible linker between the β -lactamase fragments and the TMD (Figure 15) was extended by Q5 mutagenesis (Table 1). Here the linker was elongated by one, two or four GGGS segments resulting in 9, 13 or 25 amino acids linkers, respectively (Figure 25 A). For the BLa 1.1₉ constructs both Q5 mutagenesis worked (template: BLa 1.1₅, 58 °C, 3% DMSO), but for the BLa 1.1₁₃ and BLa 1.1₂₅ construct, which was a side product of a C-BLa 1.1₁₃ PCR reaction, only the C-BLa PCR reactions worked (template: C-BLa_1.1₅, 55 °C, 3% DMSO). So the extended linkers had to be transferred from the C-BLa 1.1 vectors to the N-BLa 1.1 vectors by restriction based cloning using the restriction sites *Spe*I and *Xho*I.

linker length	N-BLa 1.1	C-BLa 1.1
9 aa	BLa_Spacer_fwd	BLa_Spacer_fwd
	N-BLa_Spacer_GGGS_rev	C-BLa_Spacer_GGGS_rev
13 aa	transfer from C-BLa_1.1 ₁₃	BLa_Spacer_fwd
		C-BLa_2xGGGS_rev
25 aa	transfer from C-BLa_1.1 ₂₅	BLa_Spacer_fwd
		C-BLa_2xGGGS_rev

Table 1: Q5 primers for linker extensions. The C-BLa 1.1_{25} was a side product of a C-BLa 1.1_{13} PCR reaction.

Construction of BLaTM 1.2 split proteins



Figure 16: Scheme of BLaTM 1.2 split proteins.

To obtain less inclusion bodies and a better membrane insertion of the proteins in BLaTM 1.2, the signal peptide PelB of the BLa 1.1 proteins was changed to the one of OmpA (UniProtKB [122] accession number P0A910). The exchange was conducted by Q5 mutagenesis with the plasmids N-BLa 1.1₁₃ and C-BLa 1.1₁₃ as templates. The used primers for the N-BLa vector were BLa_delta_SP_fwd and OmpA_rev (3% DMSO, 55 °C), for the C-BLa vector BLA_196L_fwd and OmpA_rev (3% DMSO, 55 °C). These plasmids were termed N-BLa 1.2 and C-BLa 1.2 and were used in the BLaTM 1.2 assay. (For DNA and protein sequences see appendix).



3.15.3 Assay protocol

Figure 17: Protocol for the BLaTM Assay. Chemical competent *E. coli* cells were cotrashformed with N-BLa and C-BLa plasmids and plated on agar plates. 10 colonies were pooled in 5 mL LB medim and incubated over night. Induction with 1.33 mM arabinose for BLa 0.1 and BLa 1.1 plasmids; 133 μ M arabinose and 0.1 to 0.7 mM IPTG for BLa 1.2 plasmids.

N-BLa and C-BLa plasmids containing the TMD pair to be measured, were cotransformed into *E. coli* BL21 (BLaTM 1.1) or *E. coli* JM83 (BLaTM 1.1 or BLaTM 1.2), respectively and plated on LB-agar plates containing Cm and Kan for selection for plasmid inheritance. *E. coli* BL21 [230, 231] cells are a protein expression strain, *E. coli* JM83 cells [232] cannot metabolize arabinose and show a higher transformation competence. After incubation for 14 h to 18 h at 37 °C the plates were sealed with Parafilm (Bemis, Oshkosh, WI, US), stored at 4 °C and could be used for inoculation for up to one week. It should be noted that lower plate quality, such as age or moisture, tended to lower the LD50 values.

Hence, the fitness of the cells on the transformation plates is already very crucial for the final measurements. However, if this fact was taken into account, it has been successfully avoided

For the BLaTM assay 10 colony forming units from one agar plate were combined in 5 mL LB-medium (Cm, Kan) and incubated in a turning wheel for 14 h to 18 h at 37 °C. The overnight culture was diluted 1:10 in 5 mL LB-medium (Cm, Kan) containing inducer (BLaTM 0.1 and BLaTM 1.1: 1.33 mM arabinose, BLaTM 1.2: 133 μ M arabinose and 0.1 mM - 0.7 mM IPTG) and incubated again. After 4 h expression the OD₆₀₀ was measured and 25 mL LB-medium (Cm, Kan) of an OD₆₀₀ 0.1 containing the same concentration inducer. 2 mL of the dilution were pipetted in each cavity of a 12-well plate (Greiner Bio-one, Kremsmünster, Austria). Different volumes (Figure 18) of a freshly prepared ampicillin stock solution (concentration depending on the TMD pair: 5 mg/mL – 40 mg/mL) were added resulting in final ampicillin concentration used was adjusted to the LD₅₀ values of the measured TMD to secure high resolution. Applying small concentration steps of ampicillin concentration ensures accurate LD₅₀ determination.

The plates were incubated in a moisturized sealed container for 19 h at 37 °C and 200 rpm on a shaker (shaking amplitude 10 mm, Orbital Shaker 3005, GFL (Burgwedel, Germany)). Then the extinction at $\lambda = 544$ nm was measured directly with a microplate reader (FluoStar, BMG Labtech). For the calculation of the LD₅₀ value, the absorbance data were fitted with the Hill equation (s. 3.15.4). At least two independent cultures for each TMD pair were subjected to the ampicillin gradient in duplicate experiments.



Figure 18: Pipetting scheme for BLaTM assay in a 12-well plate. Volumes given correspond to added ampicillin stock solution per 2 mL of culture (c(Amp) = 5 mg/mL - 40 mg/mL). The final concentration depends on affinity of TMD pair.

3.15.4 Data analysis

LD₅₀ values

The dimerization of a TMD pair was calculated from the cell densities of *E. coli* cultures at different ampicillin concentrations after 19 h growth in a 12-well plate. The relative affinity is described by the LD₅₀ value, which is the ampicillin concentration where half of the cells are dead. This is represented by the half of the maximum cell density. The extinction data at $\lambda = 544$ nm, which are the measured cell densities at different ampicillin concentrations, were fitted with the Hill equation to calculate the LD₅₀ values [233, 234] (equation 1), y: normalized absorption. c: maximum of sigmoidal curve of the fit. k: LD₅₀. x: normalized ampicillin concentration to highest applied concentration. g: Hill coefficient (maximum slope of the sigmoidal curve). The Hill equation fits the growth data to a sigmoidal curve whereas the inflection point is the LD₅₀. For the calculation the data were normalized to the maximum absorption value and to the maximum applied ampicillin concentration. The minimum of the sigmoidal curve was assumed to be 0.

$$y = \frac{c}{1 + \left(\frac{x}{k}\right)^{-g}} \tag{1}$$

The Hill equation was integrated in Python script by Mark Teese and Alexander Götz to simplify the analysis and increase the data throughput. A quality criterion for the fit is the difference between the values of the inflection point and the curve center, which should be 0. Differences of 0.05 were accepted for the y-values and 0.001 for x-values. Failed fits were revised manually and may not be used for further calculations.

Disruption index

The disruption index describes the impact of amino acids mutations of a TMD on the relative affinity defined by the LD_{50} . For comparability of the impact of distinct mutations in different TMDs, the disruption index is normalized to the LD_{50} of the wild type of the investigated TMD (equation 2, [wt]: LD_{50} of the wild type TMD, [M]: LD_{50} of the mutated TMD).

disruption index =
$$\frac{[wt]-[M]}{[wt]}$$
 (2)

If the mutant has a lower affinity than the wild type, the value is between 0 and 1 at which 0 means no effect and 1 complete disruption of the interaction. Negative values indicate a positive impact of a mutation to the ability to dimerize.

4 Results

4.1 Initial experiments

4.1.1 Characterizing the activity of full length β-lactamase

Initial experiments were conducted to optimize split β -lactamase fusion proteins. Therefore, the expression, the localization and the activity of different full-length β -lactamase fusion proteins were monitored and compared. To ensure proper membrane insertion, the native β-lactamase signal peptide was exchanged by the signal peptide of Pectin lyase B (PelB, UniProtKB [122] accession number Q00205) which increased the protein expression to an amount detectable by Western blot (Figure 24). Furthermore, the enhancing β -lactamase stability mutation M182T [193] and the cytoplasmic marker proteins green fluorescent protein (EGFP) and super folder green fluorescent protein (sfGFP) were investigated [227, 235]. Therefore, β -lactamase activity was quantified by the turnover of the chromogenic cephalosporin nitrocefin [194] (Figure 19 A). Due to hydrolysis, the absorption maximum switches from 390 nm to 492 nm. The increase of the absorption at 492 nm is proportional to the β -lactamase activity. The EGFP fluorescence indicates an N_{out} orientation of the fusion protein as GFP is only active under reducing conditions in the cytoplasm but not in the periplasmic oxidizing environment [236, 237]. sfGFP is active in the periplasm as well, but only if it is exported by the post-translational Tat-pathway [238]. This was avoided by a signal peptide specific for the SecYEG-dependent pathway. To ensure diffusion controlled access of nitrocefin to periplasmic β -lactamase, EDTA was added to the buffer, to weaken the E. coli cell wall by complexation of divalent cations which are necessary for cell wall integrity [239]. Combination of both parameters allow topology determination which is aimed to be Nout/Cin. This is the case at high GFP fluorescence and high β-lactamase activity.



Figure 19: β -Lactamase hybrid protein activity and GFP fluorescence. EGFP: enhanced GFP. sfGFP: superfolder GFP. M182T: stabilizing β -lactamase mutation. All constructs contain a PelB signal peptide, full length β -lactamase (BLa), a 5 amino acids linker and the Integrin- α V-GP TMD. A) GFP fluorescence ($\lambda_{Ex} = 485 \text{ nm}, \lambda_{Em} = 520 \text{ nm}$) normalized to OD₆₀₀. Before induction (0 h), 2.5 h and 7 h after induction with 166 μ M arabinose. B) β -lactamase activity measured by nitrocefin turnover. Buffer is supplemented with 1 mM EDTA for cell wall disruption. The nitrocefin cleavage product is quantified at $\lambda = 492 \text{ nm}$. Expression for 5 h and induction with 166 μ M arabinose.

Figure 19 shows that GFP fluorescence (A) of full-length β -lactamase constructs does not correlate with the nitrocefin turnover (B). Western blot analysis had shown that all proteins were equally and correctly expressed (data not shown, because proteins contain different antibody epitopes). EGFP containing proteins (purple and blue) do not show any fluorescence increase after protein expression induction, whereas the superfolder GFP (orange and green) seems to be correctly folded and is fluorescent. In contrast to β -lactamase wild type, only the proteins containing the β -lactamase enhancing M182T mutation [193, 209] can degrade the β -lactam nitrocefin (blue and green). The combination BLa-M182T-sfGFP (green) was used as a base for all subsequent fusion proteins, because (i) Western blot analysis demonstrated full length protein expression, (ii) nitrocefin turnover indicates periplasmic, active β -lactamase and (iii) the high GFP fluorescence level proofs the existence of correctly folded, cytoplasmic GFP. It was previously shown that the M182T mutation support the split-protein-complementation [153].

4.1.2 Complementation of soluble split β-lactamase

After the successful demonstration of membrane anchored β -lactamase showing enzymatic activity, it had to be proven that split β -lactamase complementation also works in the periplasm of *E. coli* [153]. Therefore, two compatible plasmids with a similar low copy numbers and the same p_{BAD} promotor sequence were used as vectors for all

complementation experiments (Figure 10). As published in 2002, the homodimerizing leucine-zipper bZIP from the GCN4 transcription factor conveys nitrocefin hydrolysis by the reconstitution of a split β -lactamase [153, 229]. Hence, the homodimerizing leucine-zipper domain bZIP of the transcription factor GCN4 from *S. cerevisiae* [67, 68, 240] was fused to the C-terminus of both split β -lactamase fragments and periplasmic secretion was enforced by an N-terminal PelB signal peptide. The activity of reconstituted β -lactamase was quantified by nitrocefin turnover and additionally by ampicillin resistance. Furthermore, it should be shown that each fragment alone does not generate any nitrocefin hydrolysis or ampicillin resistance higher than the background.



Figure 20: Initial experiments with split β -lactamase fragments fused to soluble leucine-zipper bZIP. Plasmids transformed in *E. coli* JM83. BLa (blue): native TEM-1 β -lactamase coded on the pBAD322 plasmid (constitutively expressed). JM83 (light blue): *E. coli* JM83 only. NC-BLa_bZIP (green): full length (PelB)BLa_M182T_bZIP. N-BLa 0.1 (brown): N-terminal fragment (PelB)N-BLa_M182T_bZIP. C-BLa 0.1 (brown): C-terminal fragment (PelB)C-BLa_bZIP. Orange: cotransformed N-BLa 0.1 and C-BLa 0.1 plasmids. **A**) β -lactamase activity measured by nitrocefin turnover after 20 min. Induction with 1.33 mM arabinose. Means \pm SEM, n = 1-4. **B**) β -lactamase activity measured by mediated ampicillin resistance (LD₅₀). Induction with 1.33 mM arabinose. LD₅₀ values of full length β -lactamase containing constructs were unquantifiable. Means \pm SEM, n = 3.

Figure 20 A shows the β -lactamase activity of different constructs or cotransformed constructs, respectively. The low signal of the non-transformed *E. coli* JM83 (light blue) indicates only minor background activity due to unspecific hydrolysis of nitrocefin. The activity of the native soluble β -lactamase (blue: BLa) is only one third above that of the soluble β -lactamase bZIP fusion protein (green: NC-BLa_bZIP). This indicates that the protein was successfully transported into the periplasm. Additionally, the C-terminal bZIP domain does not disturb enzymatic activity. The nitrocefin turnover mediated by the individual β -lactamase fragments (brown: N-BLa 0.1 or C-BLa 0.1) is at background level (light blue: JM83 cells only). The cotransformation of these two constructs (orange), which reconstitute the split β -lactamase to an active enzyme after bZIP-mediated dimerization,

leads to a two-fold increased activity. However, this trend is not significant. In a further step, the activity of the negative control, the fragmented β -lactamase alone and coexpressed fragments were quantified by ampicillin resistance (Figure 20 B). For the quantification of β -lactamase activity by ampicillin resistance (LD₅₀-values) the density of bacterial cells grown at increasing ampicillin concentrations was monitored after 19 h. It was measured as absorption at 544 nm directly in a 12-well plate. For each LD₅₀ calculation the growth at 12 different ampicillin concentrations was determined in parallel (Figure 21). *E. coli* JM83 transformed with only one of the two β -lactamase fragments (brown) showed no enhanced resistance to ampicillin relative to the *E. coli* JM83 without any plasmid (light blue). By contrast, the *E. coli* cells which were cotransformed with both plasmids (orange) and contain both parts of the soluble β -lactamase are resistant to ampicillin concentrations by more than 200 µg/mL which exceeds the negative controls for more than 40 times. The LD₅₀ values of the full length β -lactamase constructs could not be quantified using the same set-up because the ampicillin resistance was too high (> 1000 µg/mL).

4.1.3 Evaluation of ampicillin resistance data

The data for the calculation of the LD_{50} -value was directly obtained in 12-well plates as cell density, measured as absorption at 544 nm. The absorption data are plotted against the ampicillin concentrations and the LD_{50} -value is the ampicillin concentration where the absorption is half maximal.



Figure 21: Illustration of fitting absorption raw data of the BLaTM 1.1 assay with Hill equation. A) Absorption data of 6 independent measurements each of QSOX2₂ wt and QSOX2₂ S8A. B) Averaged absorption data of four different TMDs. Single data points of QSOX2 wt and QSOX2 S8A are shown in A). Means \pm SEM, n = 4 - 6.

Figure 21 A shows the absorption data of six independent measurements each of the model TMDs QSOX2₂ wt (orange) and QSOX2₂ S8A (blue) in the BLaTM 1.1 assay. The sigmoidal behavior is the basis for the LD₅₀ calculation. Each data set was separately fitted with the Hill equation (Equation 1, p. 52) for LD₅₀ calculation. It illustrates that in all, except of one mutant outlier, measurements of the QSOX2₂ wild type TMD (orange) enables growth up to higher ampicillin concentrations than its non-dimerizing mutant S8A (blue). The absorption data of all measurements for four different exemplary TMDs were combined (Figure 21 B). It clearly shows TMD-dependent survival of *E. coli* at different ampicillin concentrations. The high error bars at certain data points are always at medium absorption levels because small differences of the resistance cause large absorbance differences.

4.2 BLaTM 1.1

In the following the protocol was optimized with respect to (i) *E. coli* strain, (ii) cotransformation, (iii) inoculation strategy, (iv) incubation time, (v) strength of induction, (vi) density of the cells, (vii) type of shaker and (viii) shaker speed. The final protocol is described in chapter 3.15.3.

Although the system is perfectly suited for the measurement of heterotypically interactions, the optimization experiments were conducted with homotypic interacting TMDs. These model TMDs are very well investigated and their interfaces are known. Furthermore, heterotypic measurements are more complex by inactive homotypic dimers of the two TMDs which may reduce the amounts of protein available for heterotypic dimers (Figure 33). To prove the generality of the findings, two strongly dimerizing TMDs with known interfaces were used for assay implementation. The TMD of the human Glycophorin A (GpA; UniProtKB [122] accession number P02724) was chosen as a test construct, as it is intensively investigated and a very well understood right-handed homodimerizing TMD [77, 241-245]. Furthermore, it is an accepted model TMD for the establishment of TMD-TMD interaction assays such as ToxR [77], TOXCAT [110], GALLEX [108] or BACTH [42] and is used as a reference in these assays for normalization. In addition, the TMD of the human sulfhydryl oxidase 2 (QSOX2; UniProtKB [122] accession number Q6ZRP7) was selected [246]. The S8A mutation of the QSOX2 TMD decreases the signal to GpA G83A level, which proved that serine 8 is part of the interaction interface.
In order to investigate the orientation-dependence (s. 1.3.3) of TMD-TMD interactions observed in other assays [77, 108, 121] the flexible linker between the β -lactamase fragments and the TMD was optimized to achieve a high signal-to noise signal and low orientation-dependence at the same time.

4.2.1 Quantification method

The primary experiments using soluble dimerization domains (s. 4.1.2) already indicated the low sensitivity of quantification of β -lactamase by nitrocefin turnover in the periplasmic space (Figure 20). For this reason, the β -lactamase fragments, fused to TMDs, were tested by nitrocefin turnover and ampicillin resistance.



Figure 22: Schematic illustration of the BLaTM assay (not to scale). Green: inner *E. coli* membrane. Red: C-terminal split β -lactamase fragment (C-BLa) fused to TMD (cylinder). Blue: N-terminal split β -lactamase fragment (N-BLa) fused to TMD (cylinder). Light green: cytoplasmic superfolder GFP (sfGFP). All labels count for both fusion proteins. Left side: Low affinity between the TMDs. Fusion proteins are monomeric and ampicillin or any other substrates cannot be degraded. Right side: High affinity between two TMDs. Fusion proteins form a heterodimer and the split β -lactamase fragments reconstitute to an active enzyme. Ampicillin can be degraded which leads to antibiotic resistance. PDB IDs: TEM-1 β -lactamase: 1axb, sfGFP: 4lqu.

Figure 22 illustrates the working principle of the BLaTM system. The fusion proteins N-BLa (blue) and C-BLa (red) consist of five parts (s. appendix 9.5). They differ in their large periplasmic domain, which is either the N-terminal fragment, containing the

stabilizing M182T mutation, or the C-terminal fragment of the TEM-1 β -lactamase, respectively. Both fragments are fused to a TMD of interest via a flexible linker, which enables free rotation and creates space to the membrane. The stabilizing and detectable cytoplasmic sfGFP is fused via rigid linker to each TMD.

If there is no or low affinity between the two TMDs of interest (left), both fusion proteins will be monomeric and the β -lactamase fragments cannot reconstitute. Thus, the protein is not active and ampicillin cannot be degraded. The cells die. The higher the affinity, the more proteins will form heterodimers (right). Hence, the N-terminal β -lactamase can reconstitute and form an enzymatic active enzyme which can degrade its substrate ampicillin. The more proteins form dimers, the more active enzymes are in the periplasm, the higher is the lethal ampicillin concentration.

The experiments were conducted with three different TMDs in the BLaTM 1.1_5 system (5 amino acids linker between β -lactamase fragment and TMD constructs) and it is likely that the results are similar with the other versions of the BLaTM fusion protein.





Figure 23: Initial experiments with membrane-bound split β -lactamase constructs using BLaTM 1.1₅ (5 amino acids linker between BLa fragment and TMD). A) TMD sequences **B**) Enzymatic activity of reconstituded β -lactamase after 5 h induction with 166 μ M arabinose, quantified by nitrocefin turnover after 20 min. The nitrocefin cleavage product is quantified at $\lambda = 492$ nm. n = 1. C) β -lactamase reconstition efficiency measured by mediated ampicillin resistance (LD₅₀). Induction with 1.33 mM arabinose. Means \pm SEM, n = 5.

It was expected that the GpA wt TMD containing constructs mediate a higher enzyme activity than the Leu20 TMD and the negative control GpA G83I. G83I reduces the high

affinity of the GpA wt TMD by $\sim 70 - 90\%$, depending on the assays used [42, 77, 108]. However, it was demonstrated (Figure 23 A) that the nitrocefin turnover is independent from the affinity of the measured TMD. Hence, it is not possible to use this method for the measurement and discrimination of different TMD-TMD affinities because TMDs of different known affinities cannot be distinguished. Consequently, a more sensitive method for the reliable detection of reconstituted β -lactamase was needed. The same constructs were thus tested for their ability to confer ampicillin resistance of expression *E. coli* (Figure 23 B). Indeed, different LD₅₀ values discriminated the positive control GpA from the negative control GpA G83I and from Leu20 (Figure 23 C). In conclusion the established nitrocefin based method, which is suitable to detect soluble protein-protein interactions using split β -lactamase, is not suitable for the measurement of TMD-TMD interactions [247-249]. The more sensitive ampicillin resistance is the method of choice for comparing TMD-TMD affinities.

4.2.2 **Protein expression level**

All N-BLa 1.1 and C-BLa 1.1 proteins contain a FLAG-epitope at their C-terminus [250]. Hence, full length proteins and C-terminal cleavage products can be specifically detected via Western blot. Figure 24 B shows the Western blots of *E. coli* cells expressing all eight cotransformed GpA TMD constructs and all eight QSOX2 TMD constructs in the BLaTM 1.1₁₃ version. Additionally, constructs lacking a signal peptide (Δ SP) were analyzed for insertion control. Proteins recognized by the membrane protein insertion machinery were processed by a signal peptidase which cleaves off the signal peptide (s. 1.2.2). Following, correctly inserted proteins should have the same molecular weight as the Δ SP control proteins.



Results

Figure 24: Protein expression of BLaTM_ Δ SP and BLaTM 1.1₁₃ constructs. Δ SP: N-BLa_1.1₁₃/C-BLa_1.1₁₃ with deleted signal peptide, TMD: GpA₊₁ wt. Cotransformed in *E. coli* BL21. A) LD₅₀ values of homotypic measurements in the BLaTM 1.1 system with a 13 amino acids linker B) Western blot. Marker: PageRuler Prestained Protein Ladder. Detection with monoclonal ANTI-FLAG M2 antibody and NBT/BCIP. 50 µL samples after 4 h expression, induced with 1.33 mM arabinose.

The full length β -lactamase hybrid proteins, containing the signal peptide, have a molecular mass of 54.5 kDa (N-BLa) or 45.5 kDa (C-BLa), respectively. The expression of the C-BLa proteins is marginally higher than the N-BLa proteins, probably because the copy number of the plasmid is slightly higher [130]. Besides the two β -lactamase hybrid proteins a band of about 31 kDa indicates cleaved-off GFP with the rigid linker. Surprisingly, the N-terminal fragment of the split β -lactamase fusion protein (N-BLa) is running at higher molecular mass than calculated (54.5 kDa), probably because the hydrophobic TMD influence the migration properties of the proteins in an unexpected manner [251]. After insertion into the inner membrane, the molecular weight of the split β -lactamase fusion proteins should decrease by about 2 kDa, due to cleavage of the signal peptide by a signal peptidase. On the one hand, the C-BLA 1.1 protein has a similar

molecular weight as the Δ SP_C-BLa protein. This suggests correct membrane insertion and processing of this protein. On the other hand, the signal peptide PelB of the N-BLa 1.1 protein seems not to be cleaved off, as suggested by its larger mass, which suggests insufficient protein recognition, membrane insertion and/or processing by a signal peptidase. The corresponding LD₅₀ values of all 17 constructs (Figure 24 A), show that the ampicillin resistance and the protein expression are completely independent.

4.2.3 Influence of the linker between the β-lactamase fragment and TMD

First measurements of GpA TMDs were conducted using a 5 amino acids linker between the β -lactamase fragments and the TMDs. As significant differences in the LD₅₀ values of different orientations were obtained in these experiments (Figure 25 B), it was assumed that the linker was too short to ensure free orientation of the β -lactamase fragments such that the positions of both fragments relative to each other was coupled to the relative position of the fused TMD. Therefore, the aim was the creation of a linker which is flexible enough to achieve the independent movement of the fragments of the fusion proteins and thus, enables reconstitution, on the one hand, but does not increase the background signal, on the other. Consequently, the linker was extended by one, two or four GGGS segments (Figure 25 A) to find the optimal length. To test for orientation dependence, the strongly dimerizing GpA and QSOX2 wild type TMDs and their mutants G83I (GpA) and S8A (QSOX2), respectively, were used. In order to cover all interaction interfaces all four orientations for each wild type and mutant TMD were investigated (Figure 25 B and C).



Figure 25: Effect of the linker length between the β -lactamase fragments and the TMD to the LD₅₀ in the BLaTM 1.1 assay. **A)** Amino acid sequences of the tested linkers between β -lactamase fragments and TMD. **B)** top: Amino acid sequences of all used Glycophorin A (GpA) model TMDs. Bottom: LD₅₀ of all GpA TMDs measured with a 5, 9, 13 and 25 amino acids linker between split β -lactamase fragments and TMD. Means \pm SEM, n = 3 - 4. **C)** Top: Amino acid sequences of all used Sulfhydryl oxidase 2 (QSOX2) model TMDs. Bottom: LD₅₀ of all QSOX2 TMDs measured with a 5, 13 and 25 amino acids linker between split β -lactamase fragments and TMD. Means \pm SEM, n = 3 - 4.

The homotypic affinity of the different GpA TMDs with different linkers (Figure 25 B) was characterized by a number of phenomena. First, the LD_{50} values of the negative control G83I (dashed lines) are more or less independent from the linker length for all four orientations. The smallest values were obtained with the 9 amino acids linker. The LD_{50} values of the wild-type TMDs increase up to 3-fold from the 5 amino acids linker to the 13 amino acids linker. In some cases (GpA₋₁ wt and GpA₀ wt) the resistance levels decrease with a further extension of the linker to 25 amino acids. The wild-type signals of the four orientations of the GpA wt TMDs are the closest to each other with the 13 amino acids linker.

The measurements of the different QSOX2 TMDs in the BLaTM 1.1 system with different flexible linkers (Figure 25 C) show also that some signals increase with a longer linker, nevertheless to a lesser extent than with the GpA TMDs. There is no effect on the two stronger dimerizing TMDs $QSOX2_2$ (orange) and $QSOX2_3$ (blue) – neither on the wild-

types nor on the mutants S8A. However, there is an effect on the signal intensity of the wild-type and mutant TMDs $QSOX_0$ (black) between the 5 and 13 amino acids linker, which results in more homogenous signal intensities of the four wild-type orientations and their S8A mutants of the QSOX2 TMD, respectively.

4.2.4 Orientation dependence

Figure 25 B and C lead to the assumption that the linker extension could not completely abolish orientation dependence. Orientation dependence describes the maximal difference of the LD_{50} of the four orientations of one TMD. It is the quotient of the lowest LD_{50} and the highest LD_{50} . Hence, the value is always between 0 and 1, whereas 1 means no difference and no orientation dependence. The higher is the orientation dependence. For indepth analysis, the orientation dependence was calculated for the TMDs GpA, QSOX2 and LS46 (Figure 26 and Figure 29) and averaged for each linker.



Figure 26: Orientation dependence of different TMDs and site directnisess of the interfacial amino acids. A) Absolute and averaged orientation dependence of GpA, QSOX2 and LS46 TMDs for each linker length (Figure 25 and Figure 29) in the BLaTM 1.1 system. B) Relative position of interfacial amino acids of the model TMDs GpA, QSOX2 and LS46 to the periplasmic split β -lactamase fragments. Relative propability of each amino acid position is indicated. The most important interfacial amino acids [77, 226, 252] (in bold) of the best orientations (Figure 25, Figure 29) are blotted against their position in the TMD. Each helix turn consists of 3.6 amino acids as indicated on the x-axis. The periodicity of probability is illustrated by a fitted sinus curve.

The summary of all GpA, QSOX2 and LS46 data shows (Figure 26 A) that there is a very high orientation dependence for short linkers (5 and 9) and a considerably lower one for the two longer ones. The LS46 TMD was only tested with a 13 amino acids linker. The orientation dependence might result from deleted or added amino acids located at the

termini, yet important for interaction. The orientation dependence could also systemically result from steric hindrance or necessary positioning of the β -lactamase fragments relative to the interaction interface. If the second case is correct, the interfacial amino acids of all TMDs should always be positioned at about the same relative positions in the TMD. To investigate these two hypotheses, the most strongly dimerizing orientations of the three tested TMDs GpA, QSOX2 and LS46 were chosen for analysis (Figure 26 B). The interfacial amino acids were defined according to published ToxR experiments [77, 246, 252] and blotted against their positions in the TMD. The hits are summed up to emphasize the important positions. The distribution of the interfacial amino acids follows the α -helical pattern which says that each turn exists of 3.6 amino acids [12]. Hence, the interfacial amino acids of all three TMDs are on the same side, which indicates a systematic impact of the system to the orientation dependence. Derived from this periodicity the relative probability, that a distinct amino acid is a part of the interfacion interface, can be estimated for each position in the TMD (Figure 26 B, black curve).

4.2.5 Disruption index

One important aspect in assay development is the optimization in respect to the resolution, which is defined here by the difference in LD_{50} values between positive and negative controls. This relationship can be described by the disruption index. It normalizes the impact of a mutation to the wild type signal resulting in a relative number. If the mutation has a negative impact on dimerization, the value is between 0 and 1, where 0 means no effect and 1 signifies the complete disruption of the interaction. Complete disruption would mean no growth at all. Negative values indicate a positive impact of the mutation on dimerization. As there is always a background signal due to natural ampicillin resistance of *E. coli*, a value of 1 cannot be reached. The disruption index was calculated for each GpA and QSOX2 TMD (Figure 25) and plotted against the linker length.



Figure 27: Disruption indices (([wt]-[M])/[wt]; $[wt] = LD_{50}$ of wild type, $[M] = LD_{50}$ of GpA G83I or QSOX2 S8A) of all orientations of GpA and QSOX2 BLaTM 1.1 constructs plotted against the linker length.

The analysis of the disruption indices (Figure 27) shows that they vary depend on the linker length. For the GpA TMDs (red), the disrupting impact of the mutation increases between the linkers 5 to 13, especially between linker 5 and 9. The disruption indices with the 13 amino acids and 25 amino acids linker are quite similar, while the 13 amino acids linker shows the better results, as the values are closer to each other. The shortest linker has the poorest resolution. The analysis of the QSOX2 TMD data (black) shows more similar disruption indices, especially between the 5 and 13 amino acids linkers. Here, the variations between the different orientations increase with the 25 amino acids linker, relative to the other two. This is due to two variations of the mutant signals (Figure 25 C). The wild type signals are constant for all four orientations. In sum, a linker of 13 amino acids leads to the highest resolution, overall consistent with the least orientation dependence.

4.2.6 Heterotypic interacting TMDs

After it was successfully demonstrated that the assay works for homotypic TMD-TMD interactions, it should be shown that the assay is also suitable for the measurement of heterotypic TMD-TMD interaction. As a model, the LS46 TMD was chosen. This high-affinity, homotypically interacting TMD was found by Herrmann et al. in a library screening using the ToxR/POSSYCCAT system [252, 253]. The interaction motif includes the two ionizable amino acids aspartic acid (D) and arginine (R) (Figure 28). These amino acids can be (partially) charged, where D is negative and R is positive. This results in two

D-R interaction sites. The mutation of the D on one TMD and the R on the other one to e.g. leucine (L) results in one, heterotypic D-R interaction site. These mutant TMDs should have a lower propensity for homotypic interactions than the original double charged TMDs.



Figure 28: Main interaction interface of TMD LS46 of the ionizable amino acids aspartic acid (D) and arginin (R). Solid lines: peptide backbone, dashed lines: salt-bridge like bonds. Helix wheel drew with DrawCoil 1.0 and modified (http://www.grigoryanlab.org/drawcoil/) [254].

As first experiments indicated orientation dependence (Figure 25), all four orientations of the LS46 model TMD had to be tested (Figure 29 A). The best orientation was used for the generation of the artificial heterotypic interaction interface. The orientation 3 (LS46₃) is one amino acid longer than the others. Otherwise the C-terminal GxxxG motif would be destroyed which is assumed to be essential for a strong interaction [252].



Figure 29: Homotypic interaction of the artificial model TMD LS46 in the BLaTM 1.1_{13} system. Top: Amino acid sequences of the used TMDs. Ionic amino acids and GxxxG motifs emphasized in bold. Bottom: LD₅₀ of LS46 TMDs. Means \pm SEM, n = 4 - 5.

The orientation LS46₁ generates an LD₅₀ which is significantly higher ($p \le 0.05$) than all the others (Figure 29). It is about ²/₃ of the signal of the optimal GpA wt orientation. This is much lower than in the ToxR assay where the signal is about twice that of GpA wt [252]. Nevertheless, LS46₁ was taken as a base for the generation of heterotypically interacting TMDs. Therefore, a leucine backbone was used with the two ionic amino acids and the GxxxG at the positions corresponding to the LS46₁ TMD (L19_G₁₄G₁₈_D₆R₇). This reductionism should exclude a possible influence of other amino acids in the TMD and ensure good membrane integration.

In theory, the signal should be independent from the TMD/vector combination. That means that the LD₅₀ values of the TMD pair N-BLa::TMD#1/C-BLa::TMD#2 (e.g. L19 G₁₄G₁₈ D₆ and L19 $G_{14}G_{18}$ R₇) and the LD_{50} value of the pair N-BLa::TMD#2/C-BLa::TMD#1 would be equal. Thus, it would be sufficient to measure all orientations of N-BLa::TMD#1 against all of C-BLa::TMD#2, but not necessarily in addition all N-BLa::TMD#2 against all C-BLa::TMD#1.



Figure 30: Homo- and heterotypic interactions of TMDs containing ionizable amino acids based on TMD L19_G₁₄G₁₈ in the BLaTM 1.1₁₃ system. Top: Amino acid sequences of the used model TMDs. Proposed interacting amino acids emphasized in bold. Bottom: Brown: LS46₁, Green: Positive (D₆R₇) and negative control (L19_G₁₄G₁₈). Blue: homotypic TMD pairs with one or two ionizable amino acids each. Orange: heterotypic TMD pairs with one or two ionizable amino acids to negative control. n.s. = not significant, * = p ≤ 0.05 , ** = p ≤ 0.01 , *** = p ≤ 0.001 . Means \pm SEM, n = 4.

Figure 30 shows the results of switching N-BLa for C-BLa plasmids ($D_6/R_7 \text{ vs } R_7/D_6$) and demonstrates the impact of the ionizable amino acids arginine (R) and aspartic acid (D) on the homo- (blue) and heterotypic (orange) dimerization propensity of TMDs. Moreover, the significance levels compared to the negative control L19_G14G18 (green; L19GG) are annotated. The highly dimerizing LS46₁ TMD (Figure 29) and the poly-leucine TMD containing the LS46 interaction motifs D_6R_7 and $G_{14}xxxG_{18}$ (green; positive control) are showing the same LD₅₀s of about 100 µg/mL and are about the double of the negative control. Arginines do not support homotypic dimerization. In contrast to this, one aspartic acid at position 6 significantly increases homo dimerization by about 40%, two aspartic acids at positions 6 and 7 even by more than 60%, which is still considerably less than the positive control. The ionizable amino acids of heterotypic pairs (orange bars) are in

optimal positions to each other (Figure 28) because they are derived from the orientationoptimized TMD LS46 (Figure 29). The LD₅₀ values of all four measured pairs are between 3 and 3.5 times higher than L19GG and are significant (p < 0.01). Above that, they are between 34% and 58% higher than LS46. In sum, heterotypic interactions can be measured and that the signals do not differ significantly between the two TMD/vector combinations (orange).

4.3 BLaTM 1.2

Expression analysis of the BLaTM 1.1 proteins by Western blot had shown that the signal peptide of the N-BLa proteins was not cleaved off (Figure 24). This could mean that the signal peptidase did not recognize its cleavage site. To test this issue, the signal peptidase recognition site was mutated to represent the native sequence (H21A) or the one of the C-BLa (A22L, A22L/P23L), respectively. All three modifications did not reduce the molecular weight suggesting that this strategy was not successful (data not shown). Next, the complete signal peptide was replaced by the commonly used signal peptide of Outer Membrane Protein A (OmpA, 1-22, UniProtKB [122] accession number P0A910) of E. coli, resulting in system BLaTM 1.2. As a consequence, the protein expression decreased dramatically to below the detection limit of the monoclonal ANTI-FLAG® M2 antibody in a Western blot. The ampicillin resistance strongly increased, however, as growth of neither the GpA₊₁ wt nor the GpA₊₁ G83I constructs was inhibited at ampicillin concentrations of 600 µg/mL. Thus, it was decided to use 133 µM arabinose and additionally inhibit the promoter activation with its inhibitor IPTG [255]. As a result, a tightly adjustable system which can be optimized for high and low affinity TMDs (s. 4.3.1) has been developed.

4.3.1 Adjustment to different TMD-TMD affinities

In order to tune expression using BLaTM 1.2 system, advantage was taken of the fact that the p_{BAD} promotor can be inhibited by IPTG [255]. As it was shown that inhibition with IPTG and influences the ampicillin resistance as well, several IPTG concentrations were tested to achieve the best resolution, i.e., the biggest differences between the positive (wt) and negative controls (G83I). For this purpose, the weakest (GpA₀) and the high interacting (GpA₊₁) orientation were chosen (Figure 31 B, Figure 32). The expression was induced with 133 µM arabinose and 0.1 to 0.7 mM IPTG. IPTG is a competitive inhibitor of the p_{BAD} promotor [222, 255], meaning that an increase of the IPTG concentration decreases the protein expression and thus the LD₅₀. To verify that the resistance resolution is not influenced by different TMDs, the GFP fluorescence was measured.



Figure 31: Relationship of protein expression and LD₅₀ in the BLaTM 1.2 system. Activation of p_{BAD} promotor by arabinose is repressed by the competitive inhibitor IPTG. **A)** Amino acid sequences of the used TMDs. **B)** Induction dependent LD₅₀s of GpA constructs. Induction with 133 µM arabinose and variable IPTG concentrations. Means ± SEM, n = 1 – 9. **C)** Induction dependent GFP fluorescence. Fluorecence ($\lambda_{Ex} = 485$ nm, $\lambda_{Em} = 520$ nm) measured after 4 h expression. Corrected to non-induced sample and normalized to A₅₄₄. Means ± SEM, n = 3.

Figure 31 B shows that increasing IPTG concentrations reduce the LD₅₀ for all measured GpA TMDs. At high IPTG concentrations the GpA₀ wt (black) and GpA₀ G83I (purple) constructs cannot be distinguished. The resolution between the wild type and the mutant TMDs increases with decreasing IPTG concentration. Although, the background resistance increases at lower IPTG concentrations, the wt signal is about 50% higher than the signal of the mutant at 0.1 mM IPTG. The LD₅₀ of the GpA₊₁ wt (orange) is clearly higher than the LD₅₀ of the GpA₊₁ G83I (blue) at all measured induction levels. The resolution between the two TMDs increases with lower IPTG concentrations from a factor of 2.2 at 0.7 mM IPTG to a factor of 4.2 at 0.3 mM IPTG. At higher expression levels the resistance raise to > 600 µg/mL. The LD₅₀ values of the two mutant TMDs are very similar at all IPTG concentrations. Fluorescence ($\lambda_{Ex} = 485$ nm, $\lambda_{Em} = 520$ nm) of the fused GFP (Figure 31 C) demonstrates a linear correlation between the concentration of IPTG and protein expression. At 0.7 mM IPTG, a 5-fold excess of IPTG relative to arabinose, the expression is almost completely repressed. These results (Figure 32) indicate that the concentration of

IPTG in the assay may be adjusted to the affinity of the TMD under investigation. While 0.5 mM IPTG allows the investigation of high-affinity TMD pairs, 0.3 mM IPTG is recommended for lower affinities.

4.3.2 Comparison to BLaTM 1.1

To compare the results of the BLaTM 1.1 assay with the BLaTM 1.2 assay, the same model TMD GpA was used. Again, all four orientations, each as wild type and G83I mutant, were measured. The basis for the 1.2 vectors was the BLaTM_1.1₁₃ constructs. Other linkers between the split β -lactamase fragments and the TMD were not tested again, because the β -lactamase domains should not be influenced by the signal peptide, as it ought to be cleaved off by a signal peptidase after insertion.



Figure 32: Homotypic interaction of all orientations of GpA wt (green) and GpA G83I TMDs (blue) in the BLaTM systems. A) Amino acid sequences of the used TMDs. Orientation as index. B) LD₅₀ values in the BLaTM 1.1 assay. Induction with 1.33 mM arabinose. Means \pm SEM, n = 5 - 6. C) LD₅₀ values in the BLaTM 1.2 assay. Induction with 133 μ M arabinose and 0.5 mM IPTG. Means \pm SEM, n = 5.

As already shown, the GpA wt (green) and its mutant G83I (blue) can be clearly distinguished in all four orientations (p < 0.001) in the BLaTM 1.1 system (Figure 32 B). The biggest difference of the LD₅₀ values with a factor of 10 was measured with the GpA₊₁ TMD.

The LD₅₀ measurements in the BLaTM 1.2 system of four different orientations of the GpA TMD wild type (Figure 32 B, green) and G83I mutant (blue) show, that the assay also

works with the OmpA signal peptide, as well. As GpA is a highly dimerizing TMD, an induction level of 133 μ M arabinose and 0.5 mM IPTG was chosen. The LD₅₀s of the two TMDs GpA₋₁ wt and GpA₀ wt are very low at around mutant level. Thus, the positive (wt) and negative (G83I) control can hardly be distinguished in these two cases although the difference between the GpA₋₁ wt TMD and its mutant is significant (p < 0.05). In contrast to this, the LD₅₀s of GpA₊₁ wt and GpA₊₂ wt are 3 to 4 times higher than their corresponding mutants (p < 0.001).

5 Discussion

5.1 Initial experiments

5.1.1 Optimization of protein constructs

The different parts of the BLaTM fusion proteins were optimized in respect to enzyme activity and membrane topology. The modified parts are (i) the cytoplasmic domain (EGFP or sfGFP) [227, 235] (ii) a stabilizing M182T mutation in the periplasmic β -lactamase [193] and (iii) the signal peptide.

EGFP is only active in the reducing environment of the cytoplasm but not in the oxidizing periplasm [236, 237]. sfGFP is more stable and is also fluorescent in the periplasm if it was exported via the post-translational Tat-pathway [238]. However, as the used signal peptides lack the highly conserved twin-arginine motif SRRXFLK, the proteins cannot be recognized and transported via the Tat-pathway [256]. Thus, fluorescent protein has to be cytoplasmic and functions as proof for a C_{in}-topology. As only the sfGFP containing constructs are fluorescent but not the EGFP fusion proteins (Figure 19 A), there seems to be either impropriate folding or periplasmic localization of the EGFP proteins. Both could be influenced by the different juxtamembrane regions of the two fusion proteins. Following these findings sfGFP was chosen for further assay development.

The measurement of nitrocefin turnover provides information about the folding, the activity and thus the compartment of the β -lactamase. A high turnover reflects functional, periplasmic β -lactamase [201] and thus requires signal peptide recognition and translocation of the fusion protein to the periplasm. Only the fusion proteins containing the β -lactamase stabilizing M182T mutation can degrade nitrocefin (Figure 19 B). The M182T mutation seems to be essential for the folding and the enzymatic activity of the periplasmic, membrane-bound β -lactamase [153]. The fact that EGFP and sfGFP fusion proteins can degrade nitrocefin implies correct insertion of both proteins.

Thus, the sfGFP/M182T construct was used as a template for all following experiments, as it has the desired type I topology and an enzymatic active periplasmic β -lactamase.

5.1.2 Quantification of reconstituted β-lactamase

Up to now protein complementation of split β -lactamase was only used in the cytoplasm of *E. coli* and not in its periplasm [153]. The homo-dimerizing basic leucine zipper (bZIP) was used as a soluble domain for complementation [67, 68, 240]. Only heterodimers of the N- and C-terminal fragment of the reporter protein β -lactamase, fused to the bZIP, can confer enzymatic activity as homodimers lack essential functional domains (s. 1.5.3).

Nitrocefin hydrolysis

The β -lactam nitrocefin can be used for quantification of β -lactamase activity as it changes its absorption maximum due to hydrolysis of the β -lactam ring [194]. Indeed, it is proposed that nitrocefin can solely be used in cell lysates and not in intact cells as it is not membrane permeable [257]. Harsh lysis, such as french press or sonication, of the cells is no option for the measurement of membrane protein interactions as the complete membrane and embedded membrane proteins would be fragmented and complexed by detergents. Consequently, it was attempted to quantify active β -lactamase in the periplasm which should be accessible for nitrocefin [201].

It was proven that neither the N- nor C-fragment alone can hydrolyze nitrocefin (Figure 20 A, brown). The hydrolysis rate of the cotransformed *E. coli* (orange) is only about twice of the negative control (light blue). The measurement of the full-length β -lactamase with the same features as the fragmented constructs (green) showed a high signal. Thus, the low signal of the reconstituted fragments is not due to insufficient export to the periplasm but due to low regained enzymatic activity. One reason is the unfavored construction of the fusion-proteins as both dimerization domains are attached to the C-terminus of each β -lactamase fragment. This decreases the reconstitution efficiency [153]. Next, the bZIP domains were exchanged to a TMD-sfGFP domain to measure the reconstitution of membrane bound β -lactamase conferred by TMD-TMD interaction. Although the used model TMDs GpA₀ wt, GpA₀ G83I and Leu20 have different reconstitution propensities [110, 258], they are indistinguishable in the nitrocefin assay (Figure 23). Thus, this method cannot be used for the split β -lactamase based quantification of TMD-TMD interactions in the experimental set-up.

Ampicillin resistance

Another method for quantification of the reconstitution efficiency of split β -lactamase is the detection of ampicillin resistance due to ampicillin hydrolysis. This new approach was first tested with soluble bZIP constructs (Figure 20 B).

It was successfully demonstrated that neither the N-terminal, nor the C-terminal β -lactamase fragment alone can increase the ampicillin resistance relative to the background E. coli JM83 resistance. This means, neither monomers, nor homodimers of split β -lactamase generate a survival advantage. In contrast to this, coexpressing *E. coli* survive ampicillin concentrations of up to more than 200 µg/mL. This is, however, much less than expected, as it was reported that E. coli expressing the full length protein are resistant to ampicillin concentrations of up to 1600 µg/mL on agar plates [198]. Here, it must be taken into account that in the used set-up (bZIP domain fused C-terminal to both split fragments) the proteins have to reconstitute in an unfavored conformation [153]. Additionally, it was shown that the resistance levels are very environment specific, for example between agar plates and liquid culture (data not shown). Nevertheless, the results were promising as the measured LD_{50} of the cotransformed cells is about 40 times higher than of cells with either only one of the fragments or without any plasmid. Thus, the soluble bZIP dimerization domain was exchanged to a TMD dimerizing domain (Figure 23 B). In this approach the GpA_0 wt and its negative mutant G83I could be clearly distinguished by a factor of more than two. The LD₅₀ of the Leu20 TMD is at GpA G83I level, which means very low affinity. It was expected that the Leu20 TMD forms more dimers than the GpA G83I TMD [258].

In these primary experiments it was successfully verified, that (i) the N- and C-terminal fragment of the β -lactamase alone are inactive, (ii) that the fusion of a dimerization domain to the C-terminus of both β -lactamase fragments enables reconstitution of active β -lactamase and (iii) that the dimerization affinities can be measured by an increase of ampicillin resistance. (iv) The small errors proofed reliability of the assay.

5.1.3 Data evaluation

The fitness of *E. coli* depends on the amount of ampicillin in the periplasm. This antibiotic shows only bacteriostatic effects at low concentrations but is bacteriolytic at higher concentrations [259]. If there is enough active β -lactamase to degrade the β -lactam

antibiotic faster than it enters the periplasm and inhibits the PBP (s. 1.5.1), there will be non-inhibited growth. β -Lactamase expressing, inhibited *E. coli* will decrease the ampicillin concentration in the surrounding medium until a specific, non-inhibiting concentration is reached, allowing the cells to grow and divide. It is assumed that this mechanism is the case for the used set-up. As a consequence, the calculated LD₅₀ value will increase with longer incubation times. Hence, the LD₅₀ values, which are used for data evaluation in this work, always represent the LD₅₀ after 19 h incubation. Longer or shorter incubation times will probably shift the complete curve and lead to higher or smaller calculated LD₅₀ values, respectively. Consequently, the amount of active, reconstituted split β -lactamase is proportional to the time until the ampicillin is degraded to noninhibiting concentration. An alternative output would be the minimal bacteriolytic concentration whose determination would require longer incubation times. The prolonged incubation time would however increase the probability of random natural resistance events in *E. coli* which causes false-positive results and is thus unsuitable.

The LD₅₀ data were obtained by the determination of the cell density at different ampicillin concentrations. For each measurement, 12 ampicillin concentrations were chosen, where the concentration steps should be as small as possible and the median concentration should be about the mean LD₅₀. This ensures exact LD₅₀ values based on fitting the data with the Hill equation (equation 1, chapter 3.15.4). The algorithm used for fitting needs several high cell densities and several low cell densities. The maximum A_{544} values are up to 1.8 (Figure 21) which is far higher than the linear measurement range for light scattering [260]. Hence, the maximum value is underestimated. As the LD₅₀ value is defined as the ampicillin concentration at half of the maximum absorption, the value is not correctly defined. In this special case, the effect is negligible, because the most important output is the drop of absorption, which is usually very sharp at one distinct ampicillin concentration (Figure 21).

5.2 Linker optimization

First results with a flexible 5 amino acids linker (Figure 12 and Figure 25 A) between β -lactamase fragments and the TMD indicated that there is high orientation dependence (Figure 26 A) as well as low LD₅₀ signals (Figure 25 B). The orientation dependence might be caused by a too short linker as it could sterically restrict the two β -lactamase fragments to reconstitute if the distance or flexibility between the fixed dimerization site and the

fragments is too short. Furthermore, the nearby membrane could influence the reconstitution efficacy by its properties (e.g. charges of the lipid head groups) by incorporation of parts of the β -lactamase fragments. Hence, in the next step the linker was step-wisely extended by introduction of GGGS units. These units are characterized by their flexibility due to glycines and their solubility, which is caused by the serine (Figure 25 A) [261]. The test of four different linkers (5, 9, 13 and 25 amino acids) with the two TMDs GpA and QSOX2 (Figure 25 B,C) showed only minor differences in the LD₅₀ values of the 5 and 9 or 13 and 25 amino acids linker, respectively. Thus, a linker longer than 13 amino acids does not improve the assay further. This is in line with linker analysis using a coupled soluble FRET pair [149], which shows that there is an increase of linker flexibility up to a distinct linker length and a further elongation has only minor effects. As a tendency to lower signals is visible, it is even possible that the distance between the two β -lactamase fragments becomes too large for an efficient reconstitution of the enzyme. Unspecific interactions do not seem to increase due to longer linker, as most signals of the negative control TMDs did not increase. Another feature tested using linker variations was the disruption index, representing the impact of a mutation to the dimerization signal (Figure 27). The higher the index, the better is the discrimination of different affinities. The analysis shows that the disruption indices increase for the GpA TMD with longer linkers but for the QSOX2 TMDs, they stay almost constant. The low disruption indices of the $QSOX2_0$ and $QSOX2_1$ TMD are due to low LD₅₀s of the positive controls and not due to higher negative controls. It was proven that the background signal due to unspecific reconstitution is more or less constant for all negative control TMDs and all linker lengths.

In summary, the 13 amino acids linker is preferred for further experiments due to its superior properties in (i) orientation dependence (Figure 26 A), (ii) the absolute LD_{50} values (Figure 25 B and C) and (iii) the average disruption indices (Figure 27). I note that the extension of the flexible linker could not abrogate the orientation dependence completely.

5.3 Heterotypic interaction

In measurements of TMD-TMD interactions, only half of the homotypic TMD dimers produce a signal as only the split β -lactamase heterodimers $[N \cdot C]$ generate a signal but not the homodimers $[N \cdot N]$ and $[C \cdot C]$ (Figure 33). Thus, the signal generated by a hetero interaction is influenced by potential homo-interactions of the individual TMDs. If the

homotypic affinity of one TMD is very high, the equilibrium is shifted towards this homodimer and consequently the measured signal is lower. For complete interpretation of heterotypic affinity data it is always necessary to determine the homotypic affinities, too. This is the only possibility to ensure that for example a measured negative impact of one amino acid mutation on heterodimerization is really due to decreased heterotypic affinity and not due to increased homotypic affinity of the mutated TMD. This can provide important additional data.

> $[N \cdot N] \leftrightarrow 2[N] \leftrightarrow 2[N \cdot C] \leftrightarrow 2[C] \leftrightarrow [C \cdot C]$ inactive inactive inactive inactive

Figure 33: Possible dimers, their activity and the dependence on each other. [N] N-BLa, [C] C-BLa, $[N \cdot N]$ N-BLa homodimer, $[C \cdot C]$ C-BLa homodimer, $[N \cdot C]$ N-BLa/C-BLa heterodimer.

To demonstrate that the assay is also suitable for the measurement of heterotypic interactions, a well investigated model TMD was used [252]. The used LS46 TMD was evolved from a homotypic library screening and originally contains the two ionizable amino acids aspartic acid and arginine at position six and seven. Furthermore, there is an essential GxxxG motif at the C-terminus of the TMD. The D₆ of one TMD interacts with the R₇ of another (Figure 28), which generates a heterotypic interaction motif with low homotypic affinities. Assuming that there is a D₆ on one TMD and an R₇ on the other, the counterpart in homodimers would be an inert L. To demonstrate the effect of D or R on homodimerization, the interface was extended to D₆D₇ or R₆R₇ so that there is always a complementary, possibly interacting amino acid [262]. It was expected that the heterodimer D₆D₇/R₆R₇ should be stronger than the D₆/R₇ pair, as there are two D/R interaction sites.



Figure 34: Intermoleculare interactions between the carboxyl group of aspartic acid (D) and the guanidinium group of arginine (R) in different charge states. Hashed lines: Hydrogen bonds, bold line: Salt bridge. Uncharged: Medium strong hydrogen bonds, one amino acid charged: Strong hydrogen bonds due to the negative charge of the oxygen or stronger polarization of the amino hydrogen; both amino acids charged: repulsion between amino acids of the same kind due to same charge; very strong hydrogen bonds or salt bridge for heterotypic interacting D and R.

The following results were obtained: First, the LS46₁ TMD and the L19GG_D₆R₇ TMD show the same LD₅₀ which is contrary to the published data obtained with ToxR, which is ascribed to the different methods [252]. Nevertheless, the contribution of the ionic amino acid pair D₆R₇ to the interaction becomes apparent.

Second, the D₆/D₆ pair interacts significantly better than the control, which suggests that the side chains form hydrogen bonds [263] (Figure 34). The p K_a of the β-carboxyl group is 3.90 in water [264] but it is unlikely that it is deprotonated in the apolar membrane as well. Nevertheless, it was shown in the $\zeta\zeta_{TM}$ homodimer, which is essential for its assembly with the T-cell-receptor, that the two aspartic acids of the dimerization interface share one proton, resulting in -1 net charge [265, 266]. The BLaTM assay cannot determine the charge state in the used model TMD but the net charge is probably 0 or -1 as interaction was measured (Figure 34). The D₆D₇/D₆D₇ pair dimerizes even better, as two carboxyl groups of each TMD can form hydrogen bonds.

Third, arginine containing TMDs do not mediate homotypic interactions. The pK_a , and thus the charge state, of the guanidinium group is very solvent dependent. The pK_a is 12.48 in water [267] but decreases to less than 2 in the center of cholesterol containing membranes. It depends on the position in the membrane, the saturation of the acyl chains and the lipid head groups [268]. Nevertheless, our results indicate that the side chain is protonated as

there is no interaction measured (Figure 30 and Figure 34). Probably, the lipids in the cytoplasmic membrane of *E. coli* stabilize the protonated state of the guanidinium group [4, 268-271]. Additionally, the arginine is not in the center of the TMD which could result in snorkeling effects, which stabilize the positive charge as well [272].

Forth, the heterotypic interactions of the TMD combinations D_6/R_7 and D_6D_7/R_6R_7 were more than 3 times stronger than the negative control (L19GG) and up to 1.5 times higher than the positive control (L19GG_ D_6R_7). This indicates strong heterotypic interactions, and, due to low amount of homodimers, a high content of active heterodimers (Figure 33). The charge state of the two amino acids is unclear. However it is unlikely that both amino acids are completely uncharged because in close proximity a proton will migrate from the aspartic acid to the arginine resulting in a negatively charged carboxyl group and a positively charged guanidinium group. The two unfavoured charges can stabilize each other in the apolar environment and the pK_a values are more similar to ones in aqueous solvent. These charged groups can strongly interact via two very strong hydrogen bonds or one salt-bridge. Additionally, the combination "uncharged aspartic acid/charged arginine" is possible as the hydrogens of the positively charged guanidinium group are strongly polarized which results in strong hydrogen bonds. However, the single charge in the apolar environment is unfavored and less likely. Two oppositely charged amino acids do not further increase the LD₅₀. Probably, the additional artificial R_6/D_7 interface does not have the same impact on the dimerization as the D_6/R_7 , or the heterotypic TMD-TMD affinity with one interacting pair is so strong, that the signal is already saturated.

5.4 Orientation dependence

In this work three different TMDs – GpA, QSOX2 and LS46 – were intensively investigated. In all three cases, there was orientation dependence between 0.4 and 0.6 with the 13 amino acids linker (Figure 26 A). For analysis, the interfacial amino acids in their position of the best dimerizing orientation were plotted against their position in the TMD (Figure 26 B) [77, 246, 252]. The analysis of all tested TMDs shows, that there is a periodicity of the interacting amino acids of 3 to 4 which corresponds to the 3.6 periodicity of α -helices (s. 1.3.3) [12]. Therefore, there appears to be a preferred interfacial side of the TMDs. This could be used for the prediction of probable interfacial amino acids from the signal representing homotypic interaction in the four orientations. This prediction would make the identification of interacting amino acids more efficient by reducing the number

of residues to be tested. However, more TMDs have to be analyzed for the establishment of this prediction method. Extending this approach to heterotypically interacting TMDs would require the systematic testing of four against four different orientations, resulting in 16 combinations.

5.5 Characterization of β-lactamase fusion proteins

The Western blot analysis of the BLaTM 1.1 protein expression showed a very similar protein expression for both N-BLa and C-BLa proteins of all GpA and QSOX2 constructs, (Figure 24). The N-BLa protein, including the signal peptide, has a molecular weight of 54.5 kDa, the C-BLa protein of 45.5 kDa. The migration of the proteins is not as expected, which is typical for membrane proteins [251]. SDS-PAGE is sensitive to hydrophobic amino acid stretches, such as TMDs or signal peptides. These regions bind more SDS, which alters the migration of the protein in the gel, also called "gel shifting". In addition, the unspecifically cleaved off C-terminal GFP fragment, containing the FLAG epitope and probably the rigid linker (~ 30 kDa) was detected in a very similar amount. Assuming an Nout TM topology, it was expected that the 2 kDa N-terminal signal peptide would be cleaved off by a signal peptidase (s. 1.2.2). To confirm signal peptide removal, control proteins with cut signal peptide were constructed whose molecular weight, determined by SDS-PAGE, should be equal to the molecular mass of correctly inserted proteins. This expectation was met by the C-BLa proteins but not by the N-BLa proteins, which are clearly heavier than the control proteins without signal peptide. The C-BLa protein as either not recognized by the signal peptidase (s. 1.2.2), or the molecular mass shift after signal peptide cleavage is only visible for the N-BLa proteins.

To ascertain that this problem does not simply arise from using the unusual signal peptide of pectin lyase B (PelB, UniProtKB) from *Aspergillus niger*, which is a fungus, the resistance measurements, as well as the quantification of periplasmic full-length β -lactamase by nitrocefin turnover, showed, that at least some of the proteins were exported to the periplasm or incorporated into the inner *E. coli* membrane, respectively. On this account, the signal peptide was modified by optimizing the signal peptide cleavage site, which did neither alter the Western blot, nor the ampicillin resistance values.

The complete signal peptide was exchanged to the commonly used signal peptide of pectate lyase 2 (Pel2) from *Erwinia carotovora* [273], the original β -lactamase signal

peptide (TEM-1) [274] or the signal peptide of Outer membrane protein A (OmpA) from *E. coli* [275].

According to Western blot analysis, all these signal peptides dramatically decreased the protein expression to beyond the detection limit. The Pel2 and TEM-1 signal peptide containing constructs could not generate any ampicillin resistance at all, whereas the constructs containing the OmpA signal peptide convey extremely high resistance above detection limit using the established protocol. Accordingly, the amount of protein necessary for gaining ampicillin resistance is very small and it is very likely that most of both proteins containing the PelB signal peptide is not inserted into the membrane but aggregated as inclusion bodies. However, as ampicillin is not membrane permeable these aggregates cannot generate false positive signals due to unspecific β -lactamase reconstitution. It is not clear why the expression of PelB proteins is generally higher, or why and how a signal peptide can influence protein expression.

5.6 **Tuning BLaTM for different TMD-TMD affinities**

For the OmpA signal peptide containing proteins, hereinafter called BLa 1.2 proteins, the induction strength had to be reduced to avoid unspecific reconstitution of the split β -lactamase insertion. The arabinose concentration was decreased by a factor of 10 to 133 µM, which resulted in still very high, yet sequence-specific ampicillin resistance values (GpA₊₁ wt vs GpA₊₁ G83I) and is at the border to full activation of the araBAD promotor [276]. The promotor p_{BAD} used here is assumed to have an all-or-none behavior, which means that protein expression is either on or off in a given cell. Additionally, there is a maintenance behavior, so that cells which started expression stay activated due to autoactivating themselves, by regulator protein upregulation [222, 277]. In the BLaTM 1.1 system this could be ignored, as the protein expression was induced by a high arabinose concentration, which constantly activated the promotors in all cells [277]. Expression in the BLaTM 1.2 assay was reduced by repressing the induction with isopropyl β -D-1thiogalactopyranoside (IPTG). IPTG is a competitive inhibitor of the regulator protein AraC, because it inhibits the transcription in a dose dependent manner [222, 255]. The inhibition can be circumvented by high arabinose concentrations or by distinct mutations in the AraC protein, which indicates a direct impact of IPTG on the regulator protein [255]. The impact of IPTG concentration on β -lactamase expression was proven by quantification of protein expression by GFP fluorescence and of split β-lactamase reconstitution efficiency via ampicillin resistance measurements (Figure 31). Protein expression for different GpA TMD orientations is indirectly proportional to the IPTG concentration. Orientation-dependence and the effect of the G83I mutation are preserved. Driving expression of GpA₀ wt and GpA₀ G83I TMD by decreasing IPTG concentrations allowed to distinguish the LD₅₀ values associated with them. This means that a relatively low affinity TMD-TMD interaction can be measured at higher protein concentration.

The numerical K_d values cannot be determined, because this would require determining the concentration of both β -lactamase proteins in the bacterial membrane. Further, this would require reaching saturating LD₅₀ values which has not been tested here.

This experiment showed that the induction can be adjusted to different affinities of the investigated TMDs. High affinity TMD-TMD interactions can be measured at low expression levels to get a signal as stable as possible. Less well interacting TMDs can be measured at a less inhibiting IPTG concentration, to get higher protein concentrations. High LD_{50} values, as seen for GpA₊₁ wt at 0.3 mM IPTG, go along with higher errors on the one hand. This is because the signal is more sensitive to small IPTG and arabinose concentration differences or protein expression, respectively.

Summarizing, new TMD pairs should be investigated first by intermediate induction, such as 133 μ M arabinose and 0.3 mM IPTG. For further detailed analysis, such as alanine scanning, the IPTG concentration should be adjusted to reach high signal separation with low errors. It should also be noted that although the cleaved off GFP cannot be distinguished from the full length proteins by fluorescence measurements, a link between induction and amount of periplasmic split β -lactamase can be assumed, because the ratio of the two proteins should be stable.

5.7 Comparison of BLaTM 1.1 and BLaTM 1.2

The high induction of gene transcription in the BLaTM 1.1 system starts full protein expression in all *E. coli* cells in the culture [276, 277]. This causes inclusion bodies which are an indicator of insufficient protein folding or too high protein concentrations [278]. Due to suboptimal protein recognition of the membrane proteins the proteins can accumulate in the cytoplasm and form such aggregates. Reduction of the expression strength led to non-differentiable low LD_{50} values. The insufficient membrane integration only allows the measurement of high and middle affine TMD-TMD interactions, such as

for example GpA wt, QSOX2 wt or LS46 in their optimal orientation (Figure 26 and Figure 29). Low affinity TMD-TMD pairs, such as for example GpA G83I and Leu20 (Figure 23 B) cannot be distinguished, probably because the protein concentrations in the inner membrane are below their K_d. Additionally, high affinity TMDs and their mutants can hardly be distinguished if they are measured in a low-signal orientation (Figure 26). The switch to the BLaTM 1.2 system allows, additionally to the measurement of high- and middle-affine TMDs, the quantification of low-signal interactions. It has to be noted that probably the use of the additional competitive inhibitor IPTG of the inducer arabinose in the BLaTM 1.2 systems are able to efficiently measure TMD-TMD interactions, but the BLaTM 1.2 system is more flexible and can be adjusted to TMD pairs of different affinities.

5.8 Comparison to established assays

One major difficulty in the development of BLaTM was the orientation-dependence. However, it was also reported for the ToxR and GALLEX assay [77, 108, 121] and is not a unique characteristic of the BLaTM system. There are no published results about orientation dependence, neither positive nor negative, in the AraTM, BACTH, MYTH or MaMTH systems. Beside this, BLaTM improved existing methods for the measurement of TMD-TMD interactions in different aspects. First, instead of colorimetric or fluorogenic detection methods [152, 153], BLaTM uses acquired antibiotic resistance of E. coli as a quantitative output. The LD50 directly depends on the concentration and activity of reconstituted split β -lactamase and accordingly on the affinity of both interacting TMDs fused to the β -lactamase fragments. Thus, there is a minimal number of steps from dimerization to an activation of the reporter enzyme β -lactamase. By contrast, other methods, such as GALLEX or BACTH, are based on a cascade of events [42, 108, 109]. Second, antibiotic resistance leads to survival of bacterial cells which makes the BLaTM assay perfectly suitable for screenings, where high-affinity TMD pairs can be isolated from combinational libraries. Third, the β-lactamase substrate ampicillin is not membrane permeable. Thus, only the reconstitution of the split β -lactamase fragments in the periplasm, after targeting the proteins into the inner E. coli membrane by a cleavable Nterminal signal peptide, produces ampicillin resistance. In contrast to the existing TMD-TMD interaction assays, proteins that are not membrane-integrated proteins, aggregated or

localized in the cytoplasm cannot cause false-positive results (s. 1.3.2). Forth, superfolder GFP (sfGFP) [227] is fused to the C-terminus of the TMD at its cytoplasmic end to enhance the stability, improve membrane insertion and allow direct protein quantification. It was shown that sfGFP is fluorescent in the oxidizing periplasm only if it was exported by the post-translational Tat-pathway, or by the co-translational SRP-dependent SecYEG pathway and not by the post-translational SecA-dependent pathway (s. 1.2.2) [37, 238, 279-281]. This indicates, that the sfGFP folding kinetic is too fast for the post-translational SecYEG dependent translocation, as it cannot be recognized by SecA and is thus, not transolcated through the translocon [238]. For this reason, β -lactamase fusion proteins with their C-terminal sfGFP cannot be completely transferred into the periplasm, but are forced to incorporate into the inner E. coli membrane as shown for multi-pass proteins [24]. This forced membrane integration avoids false positive signals if a TMD is too hydrophilic for membrane insertion and interacts in the periplasm (s. 1.2.3). In case of complete backward movement of the fusion protein into the cytoplasm, no ampicillin resistance would exist. In that case this would be an indicator for a too hydrophilic and thus not suitable TMD. Fifth, in contrast to other systems, there is no need for distinct features of the used E. coli strain as for example in the BACTH assay [109]. This flexibility allows the investigation of other features such as the influence of an altered lipid composition on the affinity of a TMD pair [101, 282, 283].

5.9 Conclusion and outlook

The BLaTM assay is a novel method to measure the dimerization of homo- and heterotypically interacting TMDs. It is based on the TMD-dependent reconstitution of split TEM-1 β -lactamase which mediates ampicillin resistance in its active form. Accordingly, the resistance level, represented by the LD₅₀, is directly proportional to the dimerization propensity. Thus, this method allows the very direct measurement of TMD-TMD interactions. BLaTM is an important tool to improve the understanding of intra-membranous protein-protein interactions.

On the basis of well investigated TMD pairs, it was successfully demonstrated, that the BLaTM assay can reproducibly measure TMD-TMD interactions. Nevertheless, this assay can be improved and modified to extend the areas of application or the throughput. At the moment one complete 12-well plate is needed for each data point to gain exact values. It should be possible to increase the number of samples per plate by upscaling to 24- or 48-

well plates. The shift to the 96-well format is more complicated, as the system and the protocol has to be converted to deep-well plates where, for example, the oxygen supply of *E. coli* cultures is strongly reduced compared to plates with larger wells.

One application is the creation of TMD libraries and the subsequent screening for highinteracting TMDs on ampicillin containing agar plates. As the two proteins are coded separately, the assay is especially precious for the screening of heterotypic interacting TMDs. Up to now, there is no other method capable of achieving this. Further, the BLaTM method can be used to identify specific peptide inhibitors against a TMD dimerization interface. Consequently, this method could be used to find inhibitors against distinct, pathogenic mutants of a TMD, due to the high specificity of TMD-TMD interactions. One example is the Alzheimer precursor protein (APP), which can be proteolysed by the γ -secretase as a monomer but not as a dimer [284]. Following, the formation of stable APP/peptide complexes would reduce the formation of amyloid beta and thus of amyloid plaques.

Furthermore, the designed fusion proteins can be directly purified and used for in vitro experiments, for example in order to determine the influence of different lipids to the dimerization. Here, the interaction can be quantified by nitrocefin turnover, or by anisotropy measurements using the C-terminal sfGFP. Up to now, the assay is limited to parallel TMD pairs, but can be easily adjusted to antiparallel TMD pairs, by the creation of a new, type II fusion protein. It would be the first assay to measure this kind of interactions in vivo. This could be used to find totally new interacting TMD pairs and give insights to the function of protein complexes or substrate recognition in a cellular membrane. Intramolecular, antiparallel interactions are very common in multi-pass proteins, but there are also intermolecular, antiparallel interactions, such as in the small multidrug resistance protein EmrE [285, 286]. As very little is known about this interaction mode, it would be worth to extend the assay to this application.

The cytoplasmic domain sfGFP is not necessary for the generation of the output signal, as this is generated by the periplasmic domains. Thus, it could be exchanged to another functional domain. For example it could be used to quantify the homodimerization propensity of the TMDs in the same experiment. One possibility is the combination with the AraTM system which utilizes AraC as a sensor protein. This would need another expression system such as T7 and a third plasmid containing a p_{BAD} promotor and a

reporter protein such as β -galactosidase. Another possibility would be the use of the sfGFP for homo-FRET measurements to quantify homodimers, however this would need extensive improvements regarding the protein expression and an almost complete establishment of a novel method. A fusion protein, which allows the control of membrane integration would be desirable, but is hard to implement because the sensor protein has to be on the cytoplasmic side of the membrane.

Furthermore, the IPTG-dependent adjustable protein expression is a tool to control protein expression in each cell and reduces the problem of the on/off behavior of the p_{BAD} promotor, as it inhibits the transcription start itself. In the current method, this allows the adjustment of the assay to different K_ds of the investigated TMD pairs. This method could be transferred to other protein concentration dependent systems as well to reduce unspecific signal due to protein overexpression. Moreover, it is useful for the expression of toxic proteins to reduce the selection for low or non-expressing *E. coli* in protein expression cultures.

Summing up, the current assay can be used for the reliable quantification of homo- and heterotypic TMD-TMD interactions with the possibility of fine tuning to different TMD affinities and can be adapted to a heterotypic TMD screening which is not possible with conventional methods.

6 Bibliography

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8 List of Abbreviations

ΔG_{app}	Change of Gibbs free energy for each amino acid due to membrane
	insertion
ΔG^{aa}_{app}	Change of Gibbs free energy for each amino acid at each TMD
	position due to membrane insertion
°C	Degree Celsius
μg	10 ⁻⁶ gram
μL	10 ⁻⁶ liter
μΜ	10 ⁻⁶ mol/liter
A	Alanine
Å	Ångström (10 ⁻¹⁰ meter)
A544	Absorption at 544 nm
Aa	Amino acid
Amp	Ampicillin
AP	Alkaline phosphatase
APP	Alzheimer precursor protein
APS	Ammonium persulfate
Ara	Arabinose
ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLa	β-Lactamase
bla	Gene coding for β-lactamase
bp	Base pair
bZIP	Basic leucine zipper
С	Cysteine
cAMP	Cyclic adenosine monophosphate
CAP	Catabolite activator protein
CAT	Chloramphenicol acetyltransferase
cat	Gene coding for chloramphenicol acetyltransferase
C-BLa	Fusion protein containing C-terminal fragment of β -lactamase

Cm	Chloramphenicol
Cm^R	Gene coding for chloramphenicol acetyltransferase
ColE1	ColE1/pMB1 origin of replication
ctx	Promoter of the ToxR transcription factor
Cub	C-terminal ubiquitin fragment
D	Aspartic acid
DAP12	TYRO protein tyrosine kinase-binding protein
ddNTP	Didesoxynucleotide
dH ₂ O	Distilled water
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Desoxynucleotide
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
F	Phenylalanine
FACS	fluorescence activated cell sorting
FRET	Förster resonance energy transfer
G	Glycine
gfp	Gene coding for green fluorescent protein
GFP	green fluorescent protein
Glu	Glutamic acid
GpA	Glycophorin A, UniprotKB P02724
Н	Histidine
h	Hour
HER2	Human epidermal growth factor receptor 2
HIS3	Gene coding for imidazoleglycerol-phosphate dehydratase
Ι	Isoleucine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Κ	Lysine
Kan	Kanamycin sulfate

<i>Kan^R</i>	Gene coding for aminoglycoside-3'-phosphotransferase
kb	10 ³ base pairs
KC1	Potassium chloride
K _d	Dissociation constant
kDa	10 ³ Dalton
L	Leucine
lacZ	Gene coding for β -galactosidase
LB medium	Lysogeny broth medium
LD ₅₀	Median lethal dose, ampicillin concentration at half maximum A544
	after 19 h incubation
Leu	Leucine
Lys	Lysine
Μ	Methionine
mA	10 ⁻³ ampere
MaMTH	Mammalian-membrane two-hybrid
MgCl ₂	Magnesium chloride
min	Minute
mL	10 ⁻³ liter
mM	10 ⁻³ mol/liter
MYTH	Membrane yeast two-hybrid
Ν	Asparagine
n	Number of measurements
Na ⁺	Sodium cation
Na ₃ PO ₄	Sodium phosphate
NaCl	Sodium chloride
N-BLa	Fusion protein containing N-terminal fragment of β-lactamase
NBT	Nitro blue tetrazolium
NC-BLa	Full-length β-lactamase
ng	10 ⁻⁹ gram
nm	10 ⁻⁹ meter
NubG	N-terminal ubiquitin fragment
OD ₆₀₀	Optical density at 600 nm
OmpA	Signal peptide of Outer Membrane Protein A (UniProtKB P0A910)

ONPG	Ortho-nitrophenyl-
op+/op+	Operator/promotor in homotypic GALLEX assay
op408/op+	Operator/promotor in heterotypic GALLEX assay
Р	Proline
p15a	p15a origin of replication
pbad	Promoter for AraBAD proteins
PBP	Penicillin-binding protein
PBS	Phosphate buffered saline
PCA	Protein complementation assay
PCR	Poly-chain-reaction
PDB ID	Protein data base identifier
PEG-3350	Polyethylene glycol, ~3350 g/mol
PelB	Signal peptide of Pectin lyase B, UniProtKB Q00205
PLV	protein A-LexA-VP16
PNK	T4 DNA polynucleotide kinase
QSOX2	Sulfhydryl Oxidase 2 , UniprotKB Q6ZRP7
R	Arginine
RNA	Ribonucleic acid
rop	Gene coding for rop protein
rpm	Revolutions per minute
S	Serine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis
SEM	Standard error of mean
Ser	Serine
sfGFP	Super folder green fluorescent protein
SP	Signal peptide
SRP	Signal recognition particle
Т	Threonine
TEMED	Tetramethylethylenediamine
T _M	Primer melting temperature
TMD	Transmembrane domain
TMS	Transmembransegment

TPCR	Transfer PCR
Tris	Tris(hydroxymethyl)aminomethane
U	Unit
UBP	Ubiquitin specific proteases
V	Valine
V	Volt
v/v	Volume per volume
W	Tryptophan
w/v	Weight per volume
wt	Wildtype
X/x	Any amino acid
xg	x gravity
Y	Tyrosine
YTH	Yeast two-hybrid
λ	Wave length
λ_{Em}	Emission wave length
λ_{Ex}	Excitation wave length

9.1 TMD cassettes

Name	Sequence
$L20_s$	CTAGCCTGCTCTTACTATTGCTCTTACTGCTGTTACTGTTACTGTTACTTGCTATTACTCCTGGG
$L20_{as}$	GATCCCCAGGAGTAATAGCAAGAGTAACAGTAACAGCAGTAACAGTAAGAGCAATAGTAAGAGCAGG
GpA-1_wt_s	CTAGCATTATTTTTGGCGTGATGGCGGGGGGGGGGGGGG
GpA1_wt_as	GATCCCCGCATAGCTAATCAGCAGAATGGTGCCAATCACGCCCGCC
GpA_0_wt_s	CTAGCCTGATTATTTTTGGCGTGATGGCGGGGGGGGGGG
GpA_0_wt_as	GATCCCATAGCTAATCAGCAGAATGGTGCCAATCACGCCCGCC
$GpA_+1_wt_s$	CTAGCACCCTGATTATTTTTGGCGTGATGGCGGGCGGGCG
GpA_+1_wt_as	GATCCCGCTAATCAGCAGAATGGTGCCAATCACGCCCGCC
GpA_+2_wt_s	CTAGCATTACCCTGATTATTTTGGCGTGATGGCGGGCGTGATTGGCACCATTCTGCTGATTGG
GpA_+2_wt_as	GATCCCAATCAGCAGAATGGTGCCCAATCACGCCCGCCATCACGCCCAAAAATAATCAGGGTAATG
GpA1_G831_s	CTAGCATTATTTTTGGCGTGATGGCGATTGTGATTGGCACCATTCTGCTGATTAGCTATGCGGG
GpA1_G831_as	GATCCCCGCATAGCTAATCAGCAGAATGGTGCCAATCACAATCGCCATCACGCCCAAAAATAATG
GpA_0_G831_s	CTAGCCTGATTATTTTTGGCGTGATGGCGATTGTGATTGGCACCATTCTGCTGATTAGCTATGG
GpA_0_G831_as	GATCCCATAGCTAATCAGCAGAATGGTGCCAATCACAATCGCCATCACGCCAAAAATAATCAGG
$GpA_+1_G83I_s$	CTAGCACCCTGATTATTTTTGGCGTGATGGCGATTGTGATTGGCACCATTCTGCTGATTAGCGG
GpA_+1_G831_as	GATCCCGCTAATCAGCAGAATGGTGCCAATCACAATCGCCATCACGCCAAAAATAATCAGGGTG
GpA_+2_G831_s	CTAGCATTACCCTGATTATTTTGGCGTGATGGCGATTGTGATTGGCACCATTCTGCTGATTGG
GpA_+2_G831_as	GATCCCAATCAGCAGAATGGTGCCAATCACAATCGCCATCACGCCCAAAAATAATCAGGGTAATG
QSOX2_0_wt_s	CTAGCTGCGTGGTGCTGTATGTGGCAAGCTCTCTGTTTCTGATGGTGATGTACTTCTTCTTCGCGGG
QSOX2_0_wt_as	GATCCCCGCGAAGAAGAAGTACATCACCATCAGAAACAGAGAGCTTGCCACATACAGCACCACGCAG
QSOX2_1_wt_s	CTAGCCTGTGCTGGTGCTGTATGTGGCAAGCTCTCTGTTTCTGATGGTGATGTACTTCTTCTTCGG
QSOX2_1_wt_as	GATCCCGAAGAAGAAGTACATCACCATCAGAAACAGAGAGCTTGCCACATACAGCACCACGCACAGG
QSOX2_2_wt_s	CTAGCTCTCTGTGCGTGGTGCTGTATGTGGCCAAGCTCTCTGTTTCTGATGGTGATGTACTTCTTCGG
QSOX2_2_wt_as	GATCCCGAAGAAGTACATCACCATCAGAAACAGAGAGCTTGCCACATACAGCACCACCACGCACAGAGAG

Name	Sequence
QSOX2_3_wt_s	CTAGCATGTCTCTGTGCGTGGTGCTGTATGTGGCAAGCTCTCTGTTTCTGATGGTGATGTACTTCGG
QSOX2_3_wt_as	GATCCCGGAAGTACATCACCATCAGAAACAGAGAGCTTGCCACATACAGCACCACGCACAGAGACATG
$LS46_0s$	CTAGCCTGGGCCTGCTGGATCGCCTGTATCTGCTGGTGGGCCCTGCTGGGGCGCCTGCTGGG
$LS46_0as$	GATCCCCAGCAGGCCCCAGCAGGCCCAGCAGATAGCACAGGCGATCCAGCAGGCCCAGG
$LS46_1s$	CTAGCCTGCTGGGCCTGGATCGCCTGTGCTATCTGCTGGGGGCCTGGGGGGGG
$LS46_1as$	GATCCCCAGGCCCCAGCAGCCCACCAGCAGATAGCACAGGCGATCCAGCAGGCCCAGCAGG
$LS46_2s$	CTAGCCTGCTGCTGGGCCTGCTGGATCGCCTGTGCTGCTGGTGGGCCTGCTGGGCGGGGGGGG
$LS46_2as$	GATCCCGCCCCAGCCAGCCCAGCAGCAGATAGCACAGGCGATCCAGCAGGCCCAGCAGCAGG
LS46_3_s	CTAGCCTGCTGCTGCTGGCCTGCTGGATCGCCTGTGCTATCTGCTGGTGGGCGGCGGCGGGGGGGG
$LS46_3as$	GATCCCGCCCCAGCCAGCCCACCAGCAGATAGCACAGGCGATCCAGCAGGCCCAGCAGCAGCAGCAGG
L19_G14G18_s	CTAGCCTGCTCTTACTATTGCTCTTACTGCTGCTGTTACTGGGTCTCTTGCTAGGCCTCGG
L19_G14G18_as	GATCCCGAGGCCTAGCAAGAGCCCAGTAACAGCAGTAACAGTAAGAGCAATAGTAAGAGCAGG
L19_G14G18_D6R7_s	CTAGCCTGCTCTTACTATTGGATCGCCTGCTCTTACTATTACTGGGTCTCTGCTAGGCCTCGG
L19_G14G18_D6R7_as	GATCCCGAGGCCTAGCAAGAGCCCAGTAATAGTAAGAGCAGGCGATCCAATAGTAAGAGCAGG
L19_G14G18_D6_s	CTAGCCTGCTCTTACTATTGGATTTACTGTTACTGCTGTTACTGGGTCTCTGCTAGGCCTCGG
L19_G14G18_D6_as	GATCCCGAGGCCTAGCAAGAGCCCAGTAACAGCAGTAACAGTAAATCCAATAGTAAGAGCAGG
L19_G14G18_R7_s	CTAGCCTGCTCTTACTATTGCTCCGCCTGTTACTGCTGTTACTGGGTCTCTTGCTAGGCCTCGG
L19_G14G18_R7_as	GATCCCGAGGCCTAGCAAGAGCCCAGTAACAGCAGTAACAGGCGGGGGGGAGCAATAGTAAGAGCAGG
$L19_G14G18_D6D7_s$	CTAGCCTGCTCTTACTATTGGATGATCTGTTACTGCTGTTACTGGGTCTCTTGCTAGGCCTCGG
L19_G14G18_D6D7_as	GATCCCGAGGCCTAGCAAGAGCCCAGTAACAGCAGTAACAGATCATCCAATAGTAAGAGCAGG
L19_G14G18_R6R7_s	CTAGCCTGCTCTTACTATTGCGCCGCCTGTTACTGCTGGTTCTCGGGTCTTGCTAGGCCTCGG
L19_G14G18_R6R7_as	GATCCCGAGGCCTAGCAAGAGCCCAGTAACAGCAGTAACAGGCGGCGCCAATAGTAGAGCAGG

Zame	Sequence
06ZRP7 S8A +	CCATCAGAAACAGAGGCTGCCACATACAGCACC
Q6ZRP7 S8A -	GCTGTATGTGGCAAGCGCCCTGTTTCTGATGGTG
MP_AmpR_fwd	GTTTTTTTGGGCTCTAGATAACGAGGGCAAAAAATGAGTATTCAACATTTCCGTGTC
MP_AmpR_rev	CCAGAATGATCACGCTAGCTCGATTAGCAGAACCGCTGCCACTAGTCCAATGCTTAATCAGTGAGGCAC
BLa_M182T_s	CGACGAGCGTGACGCCTGTAGCAATGGCAACAAC
BLa_M182T_as	GTTGTTGCCATTGCTACAGGCGTCGTGGTGTCACGCTCGTCG
BLa_PelB_s	GATAACGAGGGCAAAAAATGCATTATAAACTGCTGTGTTTGCGGGCCGCGGGCGG
BLa_PelB_as	AGCGTTTCTGGGTGAGCGCTCACCGCGCCCGGGCCGGGC
GCN4_fwd_15x_spel	ATCTGCAGTTACTTGTCGTCGTCGTCGTTGTAGTCGCGTTCGCCAACTAATTTCTTTAATCTG
GCN4_rev_ft_PstI	GACTAGTGGCGGTGGCGGCGGTGGCGGCGGCGGCGGCGGCGGCGG
BLa_194G_rev	GCCAGTTAATAGTTTGCGCAAC
BLa_spel-15x_fwd	ACTAGTGGCGGTGGCGGC
BLa_PelB+H_rev	GTGAGCGCTCACCGCGC
BLa_196L_fwd	CTACTTACTTCTAGCTTCCCGG
pToxRV_fwd	GCATTCTGTAACAAAGCGGG
pToxRV_rev_XhoI	AACTCGAGGAGGCTTATGGACTGACTTG
BLa_Spacer_fwd	GGTTCTGCTAATCGAGCTAGC
N-BLa_Spacer_GGGS_rev	GCTACCGCCACCGCTGCCAGTTA
C-BLa_Spacer_GGGS_rev	GCTACCGCCACCGCTGCCACTGC
C-BLa_2xGGGS_rev	GCTGCCGCCGCCGCCGCCGCCGCCGCCACTAGTCCAATGC
BLa_delta_SP_fwd	CCAGAAACGCTGGTGAAAGTA
OmpA_rev	CGCCGCCTGCGCCACGGTCGCCAAGCCCCGCCAGCGCCAATCGCGGGTCTTTTTCATTTTGCCCTCGTTATCTAG

9.2 PCR primers

Name	Sequence	Plasmids	Platform
GATC-pBAD-FP	ATGCCATAGCATTTTTATCC	N-BLa and C-BLa	GATC SupremeRun
BFP_Y66H_rev	GGGTCAGGGTGGTAACCAGG	N-BLa and C-BLa	GATC LightRun
N-BLa_fwd_IRD700	CACGACGCCTGTAGCAATG	N-BLa	In-house
C-BLa_fwd_IRD800	CGAAATAGACAGATCGCTG	C-BLa	In-house

9.3 Sequencing primers

9.4 Bacteria strains

<i>E. coli</i> strain	Genotype	References
BL21	$F^- e14^- (McrA^-) hsdR (rK^- mK^-) glnV44 thr-1 leuB6 thi-1 lacY1 fhuA21 mcrB hflA150::Tn10 (TetR)$	[230, 231]
JM83	F ⁻ ara Δ (lac-proAB) rpsL (Str ^R)[φ 80 dlac Δ (lacZ)M15] thi	[232]
XL1-blue	F' :: $Tn10 \ proA^+B^+ \ lacI^q \ \Delta(lacZ)M15/ \ recA1 \ endA1 \ gyrA96$ (Nal ^R) thi hsdR17 ($rK^- mK^+$) glnV44 relA1 lac	[287]

9.5 Protein sequences

А	BLaTM 1.1:	PelB MHYKLLFAAAAASLASAVSAH
	BLaTM 1.2:	OmpA MKKTAIAIAVALAGFATVAQAA

В

23 BLa_N-term
APETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMS
IF KVLLCGAVLSKVDAGGEGLGRRI HISGNDLVEISPVIEKHLIDGMI
BLa N-term
VRELCSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWE
BLa_N-term M1821 # 194 Spacer
PELNEATPNDERDITTPVAMATTLRKLLTGTSGSGGGSGGGSGSGSANRA
Int a5 GP Spacer
Spacer sfGFP
AAAKAASKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLT
of CEP
I KE LUTTOKI DVDWDTI VTTI TVOVOCESDVDDHMKOHDEEKSAMDEG
sfGFP
YVQERTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGH
(255)
STGFP
KLEYNFNSHNVYTTADKQKNGTKANFKTRHNVEDGSVQLADHYQQNTP
sfGFP
IGDGPVI I PDNHYL STOSVI SKOPNEKROHMVI I EEVTAAG I THGMDE
sfGFP FLAG-tag
LYKGDYKDHDG*

С

#196 BLa	_C-term		
LLTLASF	ROQLIDWMEADKVAGP	LLRSALPAGWFI	ADKSGAGERG
BLa_C-term			
SRGIIAALGPE	OGKPSRIVVIYTTGSQ.	ATMDERNRQIAE	GASLIKHWT
Spacer	Int a5 GP		Spacer
SGSGGGSGGGS	SGSANRASVIILAVGP	GLLLLGIHKLAVN	VQLPAEAAA
Spacer	sfGFI	Р	
KEAAAKEAAA	KEAAAKEAAAKAASKG	EELFTGVVPILVE	LDGDVNGHK
sfGFP			
FSVRGEGEGD/	ATNGKLTLKFICTTGK	LPVPWPTLVTTL	TYGVQCFSRY
sfGFP			
PDHMKQHDFF	SAMPEGYVQERTISF	KDDGTYKTRAEV	KFEGDTLVNR
sfGFP			
IELKGIDFKED	OGNILGHKLEYNFNSH	NVYITADKQKNG	KANFKIRHN
sfGFP			
VEDGSVQLAD	HYQQNTPIGDGPVLLP	DNHYLSTQSVLSH	KDPNEKRDHM
efGEP	EL AG-ta	a	
VLLEFVTAAG	THGMDELYKGDYKDH	DG*	

Figure 35: Amino acid sequences (single letter code) of the BLaTM proteins. A) Signal peptides used in BLaTM 1.1 (PelB) and BLaTM 1.2 (OmpA). B) N-BLa protein with a 13 residue spacer and the integrin α 5 GP TMD. Numbers at the start and end of the sequence denote residues of TEM-1 β -lactamase (UniProtKB annotation number P62593). C) C-BLa protein with a 13 residue spacer and the integrin α 5 GP TMD. Numbers at the start of the sequences denote residue of TEM-1 β -lactamase (UniProtKB annotation number P62593).

9.6 Plasmid sequences

9.6.1 N-BLa 1.1

AGTCAGCCCC	Ĩ		40		60 I	arabinose_O2_op	erator_region 00		1
AGICAGCCCC	атасдатата	AGTTGTAATT	CTCATGTTTG	ACAGCTTATC	ААТСБАТААĠ	AAACCAATTG	TCCATATTGC	ATCAGACATT	GCCGTCACTG
									Mlul
	120		140		160		180		200
CGTCTTTTAC	төөстсттст	CGCTAACCAA	ACCGGTAACC	CCGCTTATTA	AAAGCATTCT	GTAACAAAGC	GGGACCAAAG	CCATGACAAA	AACGCGTAAC
	arabin	ose O1 operator	region 240		CA	P binding site	280		- 300
AAAAGTGTCT	ATAATCACGG	CAGAAAAGTC	CACATTGATT	ATTTGCACGG	CGTCACACT	TGCTATGCCA	TAGCATTTTT	АТССАТААДА	TTAGCGGATG
arabinose_i	minimal_promoter		340 I		360		Pe	B signal peptide	400
CTACCTGACG	CTTTTTATCG	CAACTCTCTA	CIGTITCICC	ATACCCGTTT	TTTTGGGCTC	TAGATAACGA	GGGCAAAAA	TGCATTATAA	ACTGCTGTTT
PelB signal peptide	9 420		440 I	N-BLa	460 		480		500
GCGGCCGCGG	CCGCGAGCCT	GGCGAGCGCG	GTGAGCGCTC	ACCCAGAAAC	GCTGGTGAAA	GTAAAAGATG	CTGAAGATCA	GTTGGGTGCA	CGAGTGGGTT
N-BLa	520		540		560		580		600
ACATCGAACT	GGATCTCAAC	AGCGGTAAGA	TCCTTGAGAG	TTTTCGCCCC	GAAGAACGTT	TTCCAATGAT	GAGCACTTTT	AAAGTTCTGC	TATGTGGCGC
	620		640		660		690		700
	COTOTOOO	CCCCCCAACA	Ĩ	CCCCCCATAC		CAATCACTTC	GTICACTACT	CACCAGTCAC	
GGTATTATCC	CGIGIIGACG	CCGGGCAAGA	GCAACTEGGT	CGCCGCATAC	ACTATICICA	GAATGACTTG	GIIGAGIACI	CACCAGICAC	AGAAAAGCAT
N-BLa	720 I		740 I		760 I		780 I		800 I
CTTACGGATG	GCATGACAGT	AAGAGAATTA	TGCAGTGCTG	CCATAACCAT	GAGTGATAAC	ACTGCGGCCA	ACTTACTTCT	GACAACGATC	GGAGGACCGA
N-BLa	820		840		860		880		900
AGGAGCTAAC	CGCTTTTTTG	CACAACATGG	GGGATCATGT	AACTCGCCTT	GATCGTTGGG	AACCGGAGCT	GAATGAAGCC	ATACCAAACG	ACGAGCGTGA
					Spel				
N-BLa	920		940		flexible li	nker	980		JM 1.000
CACCACGACG	CCTGTAGCAA	TGGCAACAAC	GTTGCGCAAA	CTATTAACTG	GCACTAGTGG	CAGCGGCGGC	GGCAGCGGCG	GCGGCAGCGG	TTCTGCTAAT
Nhel			Apal		BamHI				v
JM alp	haVmut-TMD 1.020		1.040	JM	1.060		1.080	rigid linker	1.100
CGAGCTAGCG	TGATCATTCT	GGCGGTGGGG	сссосстос	тестссте	GATCCACAAA	CTGGCAGTTA	ATGTTCAGCT	GCCTGCCGAA	GCAGCAGCGA
		-		v					1.000
rigid linker	1.120 I		1.140 I		1.160	sfGl	FP 1.180		1.200 I
AAGAAGCCGC	TGCCAAAGAA	GCGGCAGCGA	AAGAGGCTGC	CGCGAAAGAG	GCAGCAGCTA	AAGCCGCAAG	CAAAGGTGAA	GAACTGTTTA	CCGGTGTTGT
sfGFP	1.220		1.240	1	1.260		1.280)	1.300
TCCGATTCTG	GTTGAACTGG	ATGGTGATGT	TAATGGCCAC	AAATTCTCAG	TTCGTGGTGA	AGGCGAAGGT	GATGCAACCA	ATGGTAAACT	GACCCTGAAA
sfGFP	1.320		1.340		1.360		1.380)	1.400
TTTATCTGTA	CCACCGGCAA	ACTGCCGGTT	CCGTGGCCGA	CCCTGGTTAC	CACCCTGACC	TATGGTGTTC	AGTGTTTCAG	CCGTTATCCG	GATCATATGA
sfGFP	1.420 I		1.440 I)	1.460 I		1.480 I)	1.500 I
AACAGCACGA	TTTCTTCAAA	TCTGCAATGC	CGGAAGGTTA	TGTTCAAGAA	CGTACCATCT	CCTTTAAAGA	TGATGGCACC	TATAAGACCC	GTGCCGAAGT
sfGFP	1.520		1.540)	1.560		1.580)	1.600
TAAATTTGAA	GGTGATACCC	TGGTGAATCG	CATTGAACTG	AAAGGCATCG	ATTTCAAAGA	AGATGGTAAT	ATCCTGGGCC	ATAAACTGGA	ATATAATTTC
sfGFP	1.620						1.68()	1.700
AACAGCCACA	1		1.640)	1.660				GTTCAGCTGG
	ACGTGTATAT	CACCGCAGAT	1.640 I AAACAGAAGA	ATGGCATCAA	1.660 I AGCCAACTTT	AAGATCCGCC	ATAATGTTGA	AGATGGCAGC	1.000
10.55	ACGTGTATAT	CACCGCAGAT	1.640 I AAACAGAAGA	ATGGCATCAA	1.660 I AGCCAACTTT	AAGATCCGCC	ATAATGTTGA	AGATGGCAGC	
sfGFP	ACGTGTATAT	CACCGCAGAT	1.640 I AAACAGAAGA 1.740 I	ATGGCATCAA	1.660 AGCCAACTTT 1.760 	AAGATCCGCC	ATAATGTTGA 1.780	AGATGGCAGC	1.800
SIGFP CAGATCATTA	ACGTGTATAT 1.720 I TCAGCAGAAT	CACCGCAGAT	1.640 I AAACAGAAGA 1.740 I GTGATGGTCC	ATGGCATCAA GGTTCTGCTG	1.660 I AGCCAACTTT 1.760 I CCGGATAATC	AAGATCCGCC	ATAATGTTGA 1.780 CACCCAGAGC	AGATGGCAGC	AAGATCCGAA
sfGFP CAGATCATTA sfGFP	ACGTGTATAT 1.720 I TCAGCAGAAT 1.820 I	CACCGCAGAT	1.640 I AAACAGAAGA 1.740 GTGATGGTCC 1.840 I 1.840 I	ATGGCATCAA GGTTCTGCTG	1.660 I AGCCAACTTT 1.760 I CCGGATAATC 1.860 I CCGGATAATC	AAGATCCGCC	ATAATGTTGA 1.780 CACCCAGAGC	AGATGGCAGC GTTCTGAGCA	AAGATCCGAA
SfGFP CAGATCATTA SfGFP TGAGAAACGT	ACGTGTATAT 1.720 I TCAGCAGAAT 1.820 GATCACATGG	CACCGCAGAT ACACCGATTG TGCTGCTGGA	1.640 I AAACAGAAGA I GTGATGGTCC I .1.840 I ATTTGTTACC	ATGGCATCAA GGTTCTGCTG GCAGCAGGTA	AGCCAACTTT 1.760 CCGGATAATC 1.8600 I TTACCCATGG	AAGATCCGCC ATTATCTGAG TATGGATGAA	ATAATGTTGA 1.780 CACCCAGAGC 1 FL CTGTATAAAG	AGATGGCAGC GTTCTGAGCA AG-tag GCGATTATAA	AAGATCCGAA 1.900 AGATCACGAC
sfGFP CAGATCATTA sfGFP TGAGAAACGT <i>Xhol</i>	ACGTGTATAT 1.720 I TCAGCAGAAT 1.820 I GATCACATGG	CACCGCAGAT ACACCGATTG TGCTGCTGGA	1.64 AAACAGAAGA 1.740 I GTGATGGTCC 1.840 I ATTTGTTACC	ATGGCATCAA GGTTCTGCTG GCAGCAGGTA	1860 AGCCAACTTT 1.760 CCGGATAATC 1.860 I TTACCCATGG	AAGATCCGCC ATTATCTGAG TATGGATGAA	ATAATGTTGA 1.786 CACCCAGAGC 1 FL CTGTATAAAG	AGATGGCAGC GTTCTGAGCA AG-tag GCGATTATAA	AAGATCCGAA 1.900 AGATCACGAC
sfGFP CAGATCATTA sfGFP TGAGAAACGT FLAG-tag	ACGTGTATAT 1.720 I TCAGCAGAAT 1.820 GATCACATGG 1.920 1.920	CACCGCAGAT ACACCGATTG TGCTGCTGGA	1.64C AAACAGAAGA 1.74C I GTGATGGTCC 1.64C I ATTTGTTACC	ATGGCATCAA GGTTCTGCTG GCAGCAGGTA	1 feed agccaacttt 1.760 CCGGATAATC 1.860 1.740 1.740 1.960	AAGATCCGCC ATTATCTGAG TATGGATGAA	ATAATGTTGA 1788 CACCCAGAGC CTGTATAAAG	AGATGGCAGC GTTCTGAGCA AG-tag GCGATTATAA	AAGATCCGAA 1.900 AGATCACGAC 2.000
sfGFP CAGATCATTA sfGFP TGAGAAACGT FLAG-tag GGCTAACTCG	ACGTGTATAT 1.720 I TCAGCAGAAT 1.820 I GATCACATGG 1.920 I AGCACCACCA	CACCGCAGAT ACACCGATTG TGCTGCTGGA CCACCACCAC	1.540 I AAACAGAAGA 1.740 I GTGATGGTCC 1.540 I ATTTGTTACC 1.940 TGAGATCCGG	ATGGCATCAA GGTTCTGCTG GCAGCAGGTA CTGCTAACAA	AGCCAACTTT AGCCAACTTT 1.760 1.760 1.760 1.860 1.860 1.960 AGCCCGAAAG	AAGATCCGCC ATTATCTGAG TATGGATGAA GAAGCTGAGT	ATAATGTTGA 17.8% CACCCAGAGC 1FL CTGTATAAAG 1.8% 1.9%	AGATGGCAGC GTTCTGAGCA AG-tag GCGATTATAA CACCGCTGAG	1.800 AAGATCCGAA 1.900 AGATCACGAC CAATAACTAG
sfGFP CAGATCATTA sfGFP TGAGAAACGT FLAG-tag GGCTAACTCG	ACGTGTATAT 1.721 TCAGCAGAAT 1.822 GATCACATGG AGCACCACCA 2.020	CACCGCAGAT ACACCGATTG TGCTGCTGGA CCACCACCAC	1.540 I AAACAGAAGA 1.740 I GTGATGGTCC I ATTTGTTACC I I GGGATCCGG 2.040	ATGGCATCAA GGTTCTGCTG GCAGCAGGTA CTGCTAACAA	11660 AGCCAACTTT 1.760 1.76	AAGATCCGCC ATTATCTGAG TATGGATGAA GAAGCTGAGT	ATAATGTTGA 1.78 CACCCAGAGC CTGTATAAAG 1.98 TGGCTGCTGC 2.08(AGATGGCAGC GTTCTGAGCA AG-tag GCGATTATAA CACCGCTGAG	1.800 AAGATCCGAA 1.900 AGATCACGAC 2.000 CAATAACTAG 2.100
sfGFP CAGATCATTA sfGFP TGAGAAACGT FLAG-tag GGCTAACTCG T7_terminator CATAACCCCT	ACGTGTATAT 1.720 TCAGCAGAAT 1.820 I GATCACATGG AGCACCATGG 1.920 I I	CACCGCAGAT ACACCGATTG TGCTGCTGGA CCACCACCAC	1.560 I AAACAGAAGA 1.740 I GTGATGGTCC I 1.560 I 1.560	ATGGCATCAA GGTTCTGCTG GCAGCAGGTA CTGCTAACAA	AGCCAACTTT Interim and a state of the state	AAGATCCGCC ATTATCTGAG TATGGATGAA GAAGCTGAGT GCCTTCAACC	ATAATGTTGA 1.784 CACCCAGAGC CTGTATAAAG 1.984 1 TGGCTGCTGC CAGTCAGCTC	AGATGGCAGC GTTCTGAGCA AG-tag GCGATTATAA CACCGCTGAG	1.800 AAGATCCGAA 1.900 4 AGATCACGAC 2.000 1 CAATAACTAG 2.100 1 GCGCGGGGGC
sfGFP CAGATCATTA sfGFP TGAGAAACGT FLAG-tag GGCTAACTCG T7_terminator CATAACCCCT	ACGTGTATAT 1720 1 TCAGCAGAAT 1820 GATCACATGG 1.9200 1.9200 1.920	CACCGCAGAT ACACCGATTG TGCTGCTGGA CCACCACCAC AAACGGGTCT	1 1846 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	ATGGCATCAA GGTTCTGCTG GCAGCAGGTA CTGCTAACAA TTTGACCGAT	AGCCAACTTT AGCCAACTTT 17800 1 CCGGATAATC 1880 1 TTACCCATGG 1980 4 AGCCCGAAAG 2.060 GCCCTTGAGA	AAGATCCGCC ATTATCTGAG TATGGATGAA GAAGCTGAGT GCCTTCAACC	ATAATGTTGA 1.784 CACCCAGAGC CTGTATAAAG TGGCTGCTGC CAGTCAGCTC	AGATGGCAGC GTTCTGAGCA AG-tag GCGATTATAA CACCGCTGAG CTTCCGGTGG	AAGATCCGAA 1.900 J AGATCACGAC 2.000 J CAATAACTAG C GCGCGGGGCA J GCGCGGGGGCA
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			0100001400	10010010AA	GIIGOCOCC	ACAGAGAGIG	GARCOARCOG	OTOXIACOAC	GATACTATOA
CTGAGAGTCA		COCCTOATT		AGTTACAACA		CIGICCOGIA		GTGGGGGGGG	
CIGAGAGICA	ACGCCATGAG	CGGCCTCATT	TCTTATICIG	AGTTACAACA	GILLGLALLG	CIGICCGGIA	GUICUITUUG	GIGGCGCGCGG	GGCATGACTA
	3.220 I		3.240 I		3.260 I		3.280		3.300
TCGTCGCCGC	ACTTATGACT	GTCTTCTTTA	TCATGCAACT	CGTAGGACAG	GTGCCGGCAG	CGCCCAACAG	TCCCCCGGCC	ACGGGGCCTG	CCACCATACC
	3.320 I)	3.340 I		3.360 I)	3.380 I		3.400 I
CACGCCGAAA	CAAGCGCCCT	GCACCATTAT	GTTCCGGATC	TGCATCGCAG	GATGCTGCTG	GCTACCCTGT	GGAACACCTA	CATCTGTATT	AACGAAGCGC
	3.420 I)	3.440 I		3.460 I)	3.480 I		3.500 I
TAACCGTTTT	TATCAGGCTC	TGGGAGGCAG	AATAAATGAT	CATATCGTCA	ATTATTACCT	CCACGGGGAG	AGCCTGAGCA	AACTGGCCTC	AGGCATTTGA
	3.520)	3.540		3.560)	3.580		3.600
GAAGCACACG	GTCACACTGC	TTCCGGTAGT	CAATAAACCG	GTAAACCAGC	AATAGACATA	AGCGGCTATT	TAACGACCCT	GCCCTGAACC	GACGACCGGG
	3.620)	3.640		3.660)	3.680		3.700
TCGAATTTGC	TTTCGAATTT	CTGCCATTCA	TCCGCTTATT	ATCACTTATT	CAGGCGTAGC	ACCAGGCGTT	TAAGGGCACC	AATAACTGCC	ттааааааат
	3.720)	3.740		3.760)	3.780		CAT
TACGCCCCGC	CCTGCCACTC	ATCGCAGTAC	TGTTGTAATT	CATTAAGCAT	TCTGCCGACA	TGGAAGCCAT	CACAGACGGC	ATGATGAACC	TGAATCGCCA
	3.820	1	3.840		3.860	1	3.880		CAT
GCGGCATCAG	CACCTTGTCG	CCTTGCGTAT	AATATTTGCC	CATGGTGAAA	ACGGGGGCGA	AGAAGTTGTC	CATATTGGCC	ACGTTTAAAT	CAAAACTGGT
	3 920	,	3 940		3 060		3 980		CAT
GAAACTCACC	CAGGGATTGG	CTGAGACGAA		TCAATAAACC		ATAGGCCAGG	TTTTCACCGT	AACACGCCAC	ATCTTGCGAA
TATATOTOTA	4.020	CAAATCOTCO		TOCACACCOA		TCACTITCCT	4.080	COTOTAACAA	CAT
TATATGIGIA	GAAACIGUUG	GAAATCGTCG	IGGIATICAC	TCCAGAGCGA	IGAAAACGII	TCAGITIGCT	CATGGAAAAC	GGIGIAACAA	GGGTGAACAC
	4.120 I)	4.140 I		4.160 I		4.180 I		CAT
TATCCCATAT	CACCAGCTCA	CCGTCTTTCA	TTGCCATACG	GAATTCCGGA	TGAGCATTCA	TCAGGCGGGC	AAGAATGTGA	ATAAAGGCCG	GATAAAACTT
	4.220 I)	4.240		4.260	2	4.280		CAT
GTGCTTATTT	4.220 I TTCTTTACGG	ТСТТТААААА	4.240 I GGCCGTAATA	TCCAGCTGAA	4.260 I CGGTCTGGTT	ATAGGTACAT	4.280 I TGAGCAACTG	ACTGAAATGC	CTCAAAATGT
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GTGCTTATTT	4.20 I TTCTTTACGG 4.320 I GCCATTGGGA 4.420	TCTTTAAAAA TATATCAACG	4.240 I GGCCGTAATA 4.340 I GTGGTATATC 4.440	TCCAGCTGAA CAGTGATTTT	4.26 CGGTCTGGTT CAT 360 1 TTTCTCCAT 4.460	ATAGGTACAT TTAGCTTCCT	4.280 TGAGCAACTG 4.380 1 TAGCTCCTGA 4.480	ACTGAAATGC AAATCTCGAT	CTCAAAATGT 4.400 AACTCAAAAA 4.500
GTGCTTATTT TCTTTACGAT ATACGCCCGG	4.220 TTCTTTACGG 4.320 I GCCATTGGGA 4.420 I TAGTGATCTT	TCTTTAAAAA TATATCAACG ATTTCATTAT	4.240 I GGCCGTAATA 4.340 I GTGGTATATC 4.440 I GGTGAAAGTT	TCCAGCTGAA CAGTGATTTT GGAACCTCTT	4.26 CGGTCTGGTT CAT 36C TTTCTCCAT T ACGTGCCGAT	ATAGGTACAT TTAGCTTCCT CAACGTCTCA	4,280 TGAGCAACTG 4,380 TAGCTCCTGA 4,480 TTTTCGCCAA	ACTGAAATGC AAATCTCGAT AAGTTGGCCC	CTCAAAATGT 4400 AACTCAAAAA 4500 I AGGGCTTCCC
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GTGCTTATTT TCTTTACGAT ATACGCCCGG GGTATCAACA	4 220 I TTCTTTACGG 4 220 I GCCATTGGGA 4.420 TAGTGATCTT 4.520 I GGGACACCAG 4.620	TCTTTAAAAA TATATCAACG ATTTCATTAT GATTTATTTA	4 240 1 GGCCGTAATA 4 340 1 GTGGTATATC 4,440 GGTGAAAGTT 4,540 1 TTCTGCGAAG 4,640	TCCAGCTGAA CAGTGATTTT GGAACCTCTT TGATCTTCCG	4.866 CGGTCTGGTT CAT TTTCTCCAT ACGTGCCGAT 4.466 1 CCACGGCGAT	ATAGGTACAT TTAGCTTCCT CAACGTCTCA TTATTCGGCG	4 280 1 TGAGCAACTG 4380 1 TAGCTCCTGA 4480 TTTTCGCCAA 4580 1 CAAAGTGCGT 4680	ACTGAAATGC AAATCTCGAT AAGTTGGCCC CGGGTGATGC	CTCAAAATGT 4400 AACTCAAAAA 4500 AGGGCTTCCC 4600 TGCCAACTTA
GTGCTTATTT TCTTTACGAT ATACGCCCGG GGTATCAACA CTGATTTAGT	4 220 I TTCTTTACGG 4 320 I GCCATTGGGA 4 420 I TAGTGATCTT 4 520 I GGGACACCAG 4 620 I GTATGATGGT	TCTTTAAAAA TATATCAACG ATTTCATTAT GATTTATTTA GTTTTTGAGG	4 240 I GGCCGTAATA 4 340 I GTGGTATATC 4 440 GGTGAAAGTT I TTCTGCGAAG I TGCTCCAGTG	TCCAGCTGAA CAGTGATTTT GGAACCTCTT TGATCTTCCG GCTTCTGTTT	4.866 CGGTCTGGTT TTTCTCCAT ACGTGCCGAT 4.466 1 CCACAGGTAT 4.566 1 CCACAGGTAT	ATAGGTACAT TTAGCTTCCT CAACGTCTCA TTATTCGGCG	4.280 I TGAGCAACTG 4.380 I TAGCTCCTGA 4.480 I TTTTCGCCAA I CAAAGTGCGT CAAAGTGCGT I TCAGCTACTG	ACTGAAATGC AAATCTCGAT AAGTTGGCCC CGGGTGATGC ACGGGGTGGT	CTCAAAATGT 4400 AACTCAAAAA 4500 AGGGCTTCCC 4400 I TGCCAACTTA 4700 GCGTAACGGC
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GTGCTTATTT TCTTTACGAT ATACGCCCGG GGTATCAACA CTGATTTAGT AAAAGCACCG AAAGCTGCAC CGCCCCCTG TCGTGCGCTC CAGTTCGCTC TGCAAAAGCA TGACTGCGCT	4 220 1 TTCTTTACGG 4.320 1 GCCATTGGAA 4.420 1 GGGACACCAG GTATGATGGT 4.520 1 CCGGACATCA 4.520 1 CCGGACGTCA 4.520 1 CCGGACGTCA 5.120 1 CCACTGGCAC 5.120 1 CCACTGGCAC	TCTTTAAAAA TATATCAACG ATTTCATTAT GATTTATTTA GATTTATTTA GTTTTTGAGG GCGCTGGCGG GCAGAATATG GGAGATTTCC CGAAATCTGA GCCTTTCGGT TGTATGCACG AGTTACCTCG	4 240 GGCCGTAATA 4.340 GTGGTATATC 4.440 TTCTGCGAAG TTCTGCGAAG 4.640 TGCTCCAGTG 4.640 TGCTCCAGTG 4.640 TGCTCCAGTG 4.640 TGGTATACT 4.640 TGGTACAGGA 5.040 TGGTCAAAGTG 5.440 TTACCGGTGT AACCCCCCGT TAATTGATTT 5.440 GTTCAAAGAG	TCCAGCTGAA CAGTGATTTT GGAACCTCTT TGATCTTCCG GCTTCTGTTT GGCTTACTAT TATATTCCGC CAGGAAGATA AGTGGTGGCG CATTCCGCTG TCAGTCCGAC AGAGGAGTTA TTGGTAGCTC	4.866 CGGTCTGGTT CAT 4.466 1 CCACAGGTAT 4.667 CTATCAGGTAT CTATCAGGTAT GTTGGCACTG 4.967 4.967 1 TTCCTCGCTC 4.967 1 CTTAACAGGG 4.967 1 TTCCTCGCTC 4.967 1 CTTAACAGGG 5.967 1 CTTAACAGGGC 5.967 1 CTTAGGCCGC 1 CTTAGGCCGC 5.967 1 CTTGGCACT 5.967 1 CTTGGCACT 5.967 1 CTTGGCACT 5.967 1 CTTGGCACT 5.967 1 CTTGGCACT 5.967 1 CTTGGCGCC 1 CTTGGCGCCC 1 CTTGGCGCCC 1 CTTGGCGCCCC 5.967 1 CTTGGCGCCCC 1 CTTGGCGCCCCC 5.967 1 CTTGGCGCCCCC 5.967 1 CTTGGCGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	ATAGGTACAT TTAGCTTCCT CAACGTCTCA TTATTCGGCG TCCCTCCTGT ATGAGGGTGT ACTGACTCGC GGACTATAAA GTTTGTCTCA TATCCGGTAA CATGCGCCGG CGAAAAACCG	TGAGCAACTG A 380 TAGCTCCTGA A 480 TTTTCGCCAA CAAAGTGCGT CAAAGTGCGT TCAGCTACTG A 480 TCAGCTCGGT A 480 TACGCTCGGT A 480 CAGTGAAGTG GCCGCGGCAA A 480 TTCCACGCT CAGTGAAGTG CAGTGAAGTG CGCTGCGGCAA CCTGCAAGGC CAAGGCTAA	ACTGAAATGC AAATCTCGAT AAGTTGGCCC CGGGTGATGC ACGGGGTGGT CTTCATGTGG CGTTCGACTG Q150 O AGCCGTTTTT GTTTCCCCCT GACACTCAGT GAGTCCAACC ACTGAAAGGA CGGTTTTTTC	CAT CTCAAAAATGT AACTCAAAAA 4.500 1 AGGGCTTCCC 4.600 1 TGCCAACTTA 4.700 CGGCAACGGC P15a_OR CGGCGAGCGG CATAGGCTC CGGCGAGCGG CCATAGGCTC CGGCGAGCGG CCATAGGCTC CGGCAAGACA 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.100 1 CGGCGGCTCCC 5.000 1 CGGCGCCCC CCC CGGCGCCCC CCC CCC
GTGCTTATTT TCTTTACGAT ATACGCCCGG GGTATCAACA CTGATTTAGT AAAAGCACCG AAAGCTGCACC CAGCTCCCCTG TCGTGCGCTC CAGTTCGCTC TGCAAAAGCA TGACTGCGCT	4 220 TTCTTTACGG 4 320 GCCATTGGAA 4 420 TAGTGATCTT GGGACACCAG GTATGATGGT 4 520 GTATGATGGT 4 520 CGGACAGCGC 4 420 CGGACATCA 4 520 CGGACAGCGC 5 120 CCACTGGCAC 5 120 CCACTGGCAC 5 220 CCACTGGCAC 5 220 CCACTGGCAC 5 220 CCACTGGCAC 5 220 CCACTGGCAC 5 220 CCACTGGCAC 5 220 CCACTGGCAC	TCTTTAAAAA TATATCAACG ATTTCATTAT GATTTATTTA GATTTATTTA GTTTTTGAGG GCGCTGGCGG GCAGAATATG GGAGATTTCC CGAAATCTGA GCCTTTCGGT TGTATGCACG AGTTACCTCG	4 240 GGCCGTAATA 4.340 GTGGTATATC 4.440 TCTGCGAAG TTCTGCGAAG 4.640 TGCTCCAGTG 4.640 TGCTCCAGTG 4.640 TGGTATACT 4.640 TGGAAGATGC 5.040 TGGCTCAAATC 5.040 TTACCGGTGT AACCCCCCGT TAATTGATTT 5.440 GTTCAAAGAG	TCCAGCTGAA CAGTGATTTT GGAACCTCTT TGATCTTCCG GCTTCTGTTT GGCTTACTAT TATATTCCGC CAGGAAGATA AGTGGTGGCG CATTCCGCTG TCAGTCCGAC AGAGGAGTTA TTGGTAGCTC ATCTTATTAA	4.866 CGGTCTGGTT CAT 4.66 1 CCACAGGTAT 4.66 1 CCACAGGTAT 4.867 1 CTATCAGCTG GTTGGCACTG 4.967 1 TTCCTCGCTC 4.967 1 CTTAACAGGG 4.967 1 CTTAACAGGG 5.967 1 CTTAACAGGG 5.967 1 CTTAGGCCGC 1 CTTAGGCCGC 5.967 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 5.967 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACTG 1 CTTGGCACCTG 1 CTTGGCACCTG 1 CTTGGCACCTG 1 CTTGGCACCTG 1 CTTGGCACTG 1 CTTGGCACCTG 1 CTTGGCACCTG 1 CTTGGCACCTG 1 CTTGGCACCTG 1 CTTGGCACCTG 1 CTTGGCACCTG 1 CTTGGCACCTG 1 CTTGGCACCTG 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGC 1 CTTGGCACTGCACTGCACTGCACTGCACTGCACTGCACT	ATAGGTACAT TTAGCTTCCT CAACGTCTCA TTATTCGGCG TCCCTCCTGT ATGAGGGTGT ACTGACTCGC AAGTGAGAGAGG GGACTATAAA GTTTGTCTCA TATCCGGTAA CATGCGCCGG	TGAGCAACTG A 380 TAGCTCCTGA A 480 TTTTCGCCAA CAAAGTGCGT CAAAGTGCGT TCAGCTACTG A 480 TCAGCTCGGT A 480 TACGCTCGGT A 480 CAGTGAAGTG GCCGCGGCAA A 480 TTCCACGCT CAGTGAAGTG CAGTGAAGTG A 480 CCCTGCAGGC A 480 C 4	ACTGAAATGC AAATCTCGAT AAGTTGGCCC CGGGTGATGC ACGGGGTGGT CTTCATGTGG CGTTCGACTG Q150 Q AGCCGTTTTT GTTTCCCCCT GACACTCAGT GAGTCCAACC ACTGAAAGGA CGGTTTTTTC	CAT CTCAAAATGT 4.40 AACTCAAAAA A AGGGCTTCCC 4.60 TGCCAACTTA GCGTAACGGC P15a.0R CAGGAGAAAAA P15a.0R CAGGGGAGCGG CCATAGGCTC CCATAGGCTC CCAGGGGAGCAG CCATAGGCTC CCACATTTGG CGGAAAGACA CAAGTTTTGG GTTTCAGAG S500 CCAAGTTTGAG S500 CCAAGTTTGG S500 CCAAGTTTGAG S500 CCAAGTTCAGGC S500 CCAAGTTCAGGC S500 CCAAGTCAC
GTGCTTATTT TCTTTACGAT ATACGCCCGG GGTATCAACA CTGATTTAGT AAAAGCACCG AAGGCTGCAC AAATGGCTTA CGCCCCCCTG TCGTGCGCTC CAGTTCGCTC TGCAAAAGCA TGACTGCGCT CAAGAGATTA	4 220 TTCTTTACGG 4 320 GCCATTGGAA 4 420 TAGTGATCTT GGGACACCAG GTATGATGGT 4 520 GTATGATGGT 4 520 GTATGATGGT 4 520 CGGACAGCGGC 5 120 TCCTGTTCCT CAAGCTGGAC 5 120 CCACTGGCAG 5 120 CCACTGGCAG 5 220 CCACTGGCAG 5 220 CCCCCAAGCC 5 220 CCCCCAAGCC 5 220 CCCCCCACCCAGCC	TCTTTAAAAA TATATCAACG ATTTCATTAT GATTTATTTA GATTTATTTA GTTTTTGAGG GCGCTGGCGG GCAGAATATG GGAGATTTCC CGAAATCTGA GCCTTTCGGT TGTATGCACG CAGCCACTGG AGTTACCTCG	4 240 GGCCGTAATA 4.340 GTGGTATATC 4.440 TTCTGCGAAG TTCTGCGAAG 4.640 TGCTCCAGTG 4.640 TGCTCCGTG 4.640 TGCTCCGCG 4.640 TGCTCCGG 4.640 TGCTCCGGTG 4.640 TGCTCCGGTG 4.640 TGCTCCGGTG 4.640 TGCTCCGGTG 4.640 TGCTCCGGTG 4.640 TGCTCCGGTG 4.640 TGCTCCGGCG 4.640 TGCTCCGGCG 4.640 TGCTCCGGCG 4.640 TGCTCCGGCG 4.640 TGCTCCGGCG 4.640 TGCTCCGGCG 4.640 TGCTCCGGCG 5.400 TGCTCCGGTG 5.400 TGCTCCGGTG 5.400 TGCTCCGGTG 5.400 TGCTCCGGTG 5.400 TGCTCCGGTG 5.400 TGCTCCGGTG 5.400 TGCTCCGGTG 5.400 TGCTCCGGTG 5.400 TGCTCCGGTG 5.400 TGCTCCGGTG 5.400 TGCTCGGCGCC 5.400 TGCTCGGCGC 5.400 TGCTCGGCG 5.400 TGCTCGGCG 5.400 TGCTCGGGG 5.400 TGCTCGGCG 5.400 TGCTCGGCG 5.400 TGCTCGGCG 5.400 TGCTCGGCGC 5.400 TGCTCGGCGC 5.400 TGCTCGGCGC 5.400 TGCTCGGCG 5.400 TGCTCGGCG 5.400 TGCTCGGCGC 5.400 TGCTCGCGCG 5.400 TGCTCGCGCG 5.400 TGCTCGCGCG 5.400 TGCTCGCGCG 5.400 TGCTCGCGCG 5.400 TGCTCGCGC 5.400 TGCTCGCGCG 5.400 TGCTCGCCCCCG 5.400 TGCTCGCCCCCG 5.400 TGCTCGCCCCCG 5.400 TGCTCGCCCCCCG 5.400 TGCTCGCCCCCCG 5.400 TGCTCGCCCCCCGCG 5.400 TGCTCGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	TCCAGCTGAA CAGTGATTTT GGAACCTCTT TGATCTTCCG GCTTCTGTTT GGCTTACTAT TATATTCCGC CAGGAAGATA AGTGGTGGCG CATTCCGCTG TCAGTCCGAC AGAGGAGTTA TTGGTAGCTC ATCTTATTAA	4.866 CGGTCTGGTT CAT ACGTGCCGAT CACAGGTAT CACAGGTAT CTATCAGGTAT GTTGGCACTG GTTGGCACTG A.866 A.766 GTTGGCACTG CTTAACAGGG CTTAACAGGG CTTAACAGGG CTTAACAGGG CTTAACAGGG CTTAACAGGG CTTAACAGGG CTTAACAGGC CT CTTAACAGGC CT CTTAACAGGC CT CTTAACAGGC CT CTTAACAGGC CT CTTAACAGGC CT CTTAACAGGC CT CTTAACAGCC CT CTTAACAGCCGACA CT CTTAACAGCCG CT CTTAACAGCCG CT CTTAACAGCCG CT CTTAACAGCCG CT CT CTTAACAGCCG CT CT CTTAACAGCCG CT CT CTTAACAGCCG CT CT CTTAACAGCCG CT CT CTTAACAGCCG CT CT CTTAACAGCCG CT CT CTTAACAGCCG CT CT CTTAACAGCCG CT CT CTTAACAGCCG CT CT CTTAACAGCCG CT CT CTTAACAGCCG CT CT CT CTTAACAGCCG CT CT CT CT CT CT CT CT CT CT CT CT CT	ATAGGTACAT TTAGCTTCCT CAACGTCTCA TTATTCGGCG TCCCTCCTGT ATGAGGGTGT ACTGACTCGC GGACTATAAA GTTTGTCTCA TATCCGGTAA CATGCGCCGG CGAAAAACCG	TGAGCAACTG 4.380 TAGCTCCTGA 4.480 TTTTCGCCAA 4.680 CAAAGTGCGT 4.780 CAGTGAAGTG CAGTGAAGTG 4.780 CAGTGAAGTG 4.880 TACGCTCGGT 4.880 GCCGCGGCAA 5.180 TTCCACGCCT 5.280 CTATCGTCTT 5.380 TTAAGGCTAA 5.480 CCCTGCAAGGC 5.480	ACTGAAATGC AAATCTCGAT AAGTTGGCCC CGGGTGATGC ACGGGGTGGTG CTTCATGTGG CGTTCGACTG GACCCTCAGT GACACTCAGT GAGTCCAACC ACTGAAAGGA CGGTTTTTTC ATTTATCTCT	CAT CTCAAAATGT 4.400 AACTCAAAATGT 4.400 AACTCAAAATGT 4.800 TGCCAACTTCC 4.800 TGCCAACTTC 4.800 TGCCAACTTC 4.800 TGCCAACTTC 4.800 TGCCAACTTC 4.800 TGCCAACTTC 4.800 TGCCAACTTC 5.100 CCATAGGCT CCATAGGCT CCATAGGCT CCACGGCAGCAGG 5.200 TCCGGGAAAGACA 5.400 CAAGTTTTCAGAG GTTTTCAGAGG TCAAATGTAG
GTGCTTATTT TCTTTACGAT ATACGCCCGG GGTATCAACA CTGATTTAGT AAAAGCACCG AAAGCTGCAC AAATGGCTTA CGCCCCCCTG TCGTGCGCTC CAGTTCGCTC TGCAAAAGCA TGACTGCGCT	4 220 TTCTTTACGG 4 320 GCCATTGGGA 4 420 TAGTGATCTT GGGACACCAG GGAACACCAG GTATGATGGT 4 720 CCGGACATCA 4 820 CGGTGCGTCA 4 820 CGGACGGGC 5 100 CCACTGGCAG 5 100 CCACTGGCAG 5 100 CCCCCAAGCC 5 100 CCCCCCAAGCC 5 100 CCCCCCAAGCC 5 100 CCCCCCAAGCC 5 100 CCCCCCCCCCCCCC 5 100 CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	TCTTTAAAAA TATATCAACG ATTTCATTAT GATTTATTTA GATTTATTTA GTTTTTGAGG GCGCTGGCGG GCAGAATATG GGAGATTTCC CGAAATCTGA GCCTTTCGGT TGTATGCACG CAGCCACTGG AAAACGATCT	4 240 GGCCGTAATA 4.340 GTGGTATATC 4.440 TTCTGCGAAG TTCTGCGAAG 4.640 TGCTCCAGTG 4.740 AGTGTATACT 4.840 TGGATACAGGA 1 TGGATACAGGA 1 TGGATACAGGA 5.440 TGCTCCAGTG 4.640 TGGTCAAGGA 5.540 GTTCAAAGAG 5.540 CAAGAAGATC	TCCAGCTGAA CAGTGATTTT GGAACCTCTT TGATCTTCCG GCTTCTGTTT GGCTTACTAT TATATTCCGC CAGGAAGATA AGTGGTGGCG CATTCCGCTG TCAGTCCGAC AGAGGAGTTA TTGGTAGCTC ATCTTATTAA	4.866 CGGTCTGGTT CAT ACGTGCCGAT CATACAGGTAT CCACAGGTAT CCACAGGTAT CTATCAGCTG GTTGGCACTG GTTGGCACTG CTTAACAGGG CTTAACAGGG CTTAACAGGG CTTAACAGGG CGCTGCGCCC CGCTGCGCCC GTCTTGAAGT AGAGAACCTT S.566 CCCCGCCCC CCCCGCCCC CCCCGCCCCC CCCCGCCCCC CCCCGCCCCCC	ATAGGTACAT TTAGCTTCCT CAACGTCTCA TTATTCGGCG TCCCTCCTGT ATGAGGGTGT ACTGACTCGC GGACTATAAA GTTTGTCTCA TATCCGGTAA CATGCGCCGG CGAAAAACCG TATTTCTAGA	TGAGCAACTG 4.380 TAGCTCCTGA 4.480 TTTTCGCCAA 4.480 CAAAGTGCGT CAGTGAAGTG CAGTGAAGTG CAGTGAAGTG GCCCCCGGCAA 5.180 TTCCACGCCT CTATCGTCTT 5.380 CTATCGTCTT 5.180 TTAAGGCTAAGG CTATCGTCTT 5.180 TTAAGGCTAAGG 5.180 TTCAGGCCAAGGC 5.180 TTCAGGCCAAGGC 5.180 TTCAGGCCAAGGC	ACTGAAATGC AAATCTCGAT AAGTTGGCCC CGGGTGATGC ACGGGGTGGT CTTCATGTGG CGTTCGACTG GAGCCGTTTTT GACACTCAGT GAGTCCAACC ACTGAAAGGA CGGTTTTTTC ATTTATCTCT	CAA CTCAAAAATGT 4400 4400 4400 4400 4400 4400 4500 1400 14

alphaVmut-TMD = TMD Int_a5GP

JM = Juxtamembrane region

CAT = Chloramphenicol acetyltransferase

9.6.2 C-BLa 1.1

	20 I		40 I		60 I		80 I		AraC
GACAACIIG	ACGGCIACAI	CATICACITI	140	CCGGCACGGA	ACTOGCTOGG	GCIGGCCCCG	GIGCAIIII 180	TAAATACCCG	CGAGAAATAG
AGTTGATCGT	CAAAACCAAC	ATTGCGACCG	ACGGTGGCGA	TAGGCATCCG	GGTGGTGCTC	AAAAGCAGCT	TCGCCTGGCT	GATACGTTGG	TCCTCGCGCC
	220		240		260		280		AraC
AGCTTAAGAC	GCTAATCCCT	AACTGCTGGC	GGAAAAGATG	TGACAGACGC	GACGGCGACA	AGCAAACATG	CTGTGCGACG	CTGGCGATAT	CAAAATTGCT
	320 I		340 I		360 I		380 I		AraC
GTCTGCCAGG	TGATCGCTGA	TGTACTGACA	AGCCTCGCGT	ACCCGATTAT	CCATCGGTGG	ATGGAGCGAC	TCGTTAATCG	CTTCCATGCG	CCGCAGTAAC
AATTGCTCAA	GCAGATTTAT	CGCCAGCAGC	TCCGAATAGC	GCCCTTCCCC	TTGCCCGGCG	TTAATGATTT	GCCCAAACAG	GTCGCTGAAA	TGCGGCTGGT
	520		540		560		580		AraC
GCGCTTCATC	CGGGCGAAAG	AACCCCGTAT	TGGCAAATAT	TGACGGCCAG	TTAAGCCATT	CATGCCAGTA	GGCGCGCGGA	CGAAAGTAAA	CCCACTGGTG
	620 I		640 I		660 I		680 I		AraC
ATACCATTCG	CGAGCCTCCG	GATGACGACC	GTAGTGATGA	ATCTCTCCTG	GCGGGAACAG	CAAAATATCA	CCCGGTCGGC	AAACAAATTC	TCGTCCCTGA
TTTTCACCA		GCGAATGGTG		TATAACCTTT		GGTCGGTCGA		GAGATAACCG	AraC
1111104004	820	COCATOOTO	840		860		880		AraC
TCGGCGTTAA	ACCCGCCACC	AGATGGGCAT	TAAACGAGTA	TCCCGGCAGC	AGGGGATCAT	TTTGCGCTTC	AGCCATACTT	TTCATACTCC	CGCCATTCAG
arabinos	e_O2_operator_regi	ion							
		AraC	940 I		960 I		980 I		1.000 I
AGAAGAAACC	AATTGTCCAT	ATTGCATCAG		CACTGCGTCT	TTTACTGGCT	CTTCTCGCTA	ACCAAACCGG	TAACCCCGCT	TATTAAAAGC
	1.020	3	1.040		1.060	arabinose_01_	operator_region 080		1.100
ATTCTGTAAC	AAAGCGGGAC	CAAAGCCATG	ACAAAAACGC	GTAACAAAAG	тдтстатаат	CACGGCAGAA	AAGTCCACAT	TGATTATTTG	CACGGCGTCA
CAP bindi	ng site)	1.140 I	ar	abinose_minimal_p	romoter	1.180 I		1.200 I
CACTTTGCTA	TGCCATAGCA	TTTTTATCCA	TAAGATTAGC	GGATGCTACC	TGACGCTTTT	TATCGCAACT	CTCTACTGTT	TCTCCATACC	CGTTTTTTG
Xbal	1.220	PelB Signa	peptide 1.240		1.260)	1.280	C-BLa	1.300
GGCTCTAGAT	AACGAGGGCA	AAAAATGCAT	TATAAACTGC	TGTTTGCGGC	CGCGGCCGCG	AGCCTGGCGA	GCGCGGTGAG	CGCTCACCTA	CTTACTCTAG
C-BLa	1.320)	1.340		1.360	l.	1.380		1.400
CTTCCCGGCA	ACAATTAATA	GACTGGATGG	AGGCGGATAA	AGTTGCAGGA	CCACTTCTGC	GCTCGGCCCT	TCCGGCTGGC	TGGTTTATTG	CTGATAAATC
C-BLa	1.420		1.440		1.460		1.480 I		1.500 I
C-BLa TGGAGCCGGT	1.420 GAGCGTGGGT	CTCGCGGTAT	1.440 I CATTGCAGCA	CTGGGGCCAG	1.460 I ATGGTAAGCC	CTCCCGTATC	1.480 I GTAGTTATCT	ACACGACGGG	1.500 I GAGTCAGGCA
C-BLa TGGAGCCGGT C-BLa	1.420 GAGCGTGGGT 1.520	CTCGCGGTAT	1.440 CATTGCAGCA 1.540	CTGGGGCCAG	1.460 ATGGTAAGCC 1.560	CTCCCGTATC Spel flexible linke	1.480 J GTAGTTATCT r 1.580	ACACGACGGG	1.500 I GAGTCAGGCA 1.600
C-BLa TGGAGCCGGT C-BLa ACTATGGATG	1.420 GAGCGTGGGT 1.520 I AACGAAATAG	CTCGCGGTAT	1.440 I CATTGCAGCA I GAGATAGGTG	CTGGGGCCAG	1.460 ATGGTAAGCC 1.560 TAAGCATTGG	CTCCCGTATC Spel flexible linke	1.480 J GTAGTTATCT r 1.580 GCGGCGGCGG	ACACGACGGG CAGCGGCGGC	1.500 I GAGTCAGGCA 1.600 I GGCAGCGGTT
C-BLa TGGAGCCGGT C-BLa ACTATGGATG	1.420 GAGCGTGGGT 1.520 AACGAAATAG <u>Nhel</u>	CTCGCGGTAT	1.440 CATTGCAGCA 1.540 I GAGATAGGTG	CTGGGGCCAG CCTCACTGAT Apai	1.460 I ATGGTAAGCC 1.560 I TAAGCATTGG Bam	CTCCCGTATC Spel flexible linke ACTAGTGGCA	1.480 GTAGTTATCT - GCGGCGGCGG 1.680	ACACGACGGG CAGCGGCGGC	1.500 J GAGTCAGGCA 1.600 J GGCAGCGGTT
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM: linker CTGCTAATCG	AACGAAATAG	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG	1.440 I CATTGCAGCA 1.540 I GAGATAGGTG GGGTGG <u>GGCC</u>	CTGGGGCCAG CCTCACTGAT Apai CGGCCTGCTG	1.460 ATGGTAAGCC 1.560 1 TAAGCATTGG Bam JM 1.660 1 CTCCTGGGA	CTCCCGTATC Spel flexible linke ACTAGTGGCA HI	1.480 GTAGTTATCT I GCGGCGGGGGG GGCAGCGGCGG I GGCAGTTAAT	ACACGACGGG CAGCGGCGGC GTTCAGCTGC	1.500 GAGTCAGGCA 1.600 GGCAGCGGTT rigid linker CTGCCGAAGC
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM: linker CTGCTAATCG rigid linker	AACGAAATAG	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG	1.440 CATTGCAGCA 1.540 GAGATAGGTG 1.640 1.640 1.640 1.740 1.740	CTGGGGCCAG CCTCACTGAT Apal CGGCCTGCTG	1.46C 1 ATGGTAAGCC 1.56C 1.56C 1.56C 1.56C 1.56C 3 JM 1.66C 3 JM 1.66C 3 JM 1.66C 3 JM 1.66C 3 JM 1.66C 3 JM 1.66C 3 JM 1.66C 3 JM 1.66C 3 JM 3 JM 3 JM 3 JM 3 JM 3 JM 3 JM 3 J	CTCCCGTATC Spel flexible linke ACTAGTGGCA HI TCCACAAACT	GTAGTTATCT T 1.580 GCGGCGGCGG GGCAGTTAAT SfGFP	ACACGACGGG CAGCGGCGGC GTTCAGCTGC	1.500 J GAGTCAGGCA 1.600 GGCAGCGGTT rigid linker 700 C T GCCGAAGC 1.800
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM3 linker CTGCTAATCG rigid linker AGCAGCGAAA	1.422 GAGCGTGGGT 1.522 AACGAAATAG Nhei AGCTAGCGTG GAAGCCGCTG	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC	1.440 CATTGCAGCA 1.541 GAGATAGGTG 1.6444 1.6444 1.6444 1.6444 1.	CTGGGGCCAG CCTCACTGAT Apal CGGCCTGCTG GAGGCTGCCG	1466 1 ATGGTAAGCC 1588 TAAGCATTGG JM 1666 JM 1666 CGGAAGGGGC	CTCCCGTATC Spel flexible linke ACTAGTGGCA HI TCCACAAACT	1.480 GTAGTTATCT I 1.580 GCGGCGGGCGG I GGCAGTTAAT STGFP3 GCCGCAAGCA	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA	1.500 J GAGTCAGGCA 1.600 I GGCAGCGGTT rigid linker CTGCCGAAGC 1.800 I ACTGTTTACC
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM: linker CTGCTAATCG rigid linker AGCAGCGAAA s(GFP	1.422 GAGCGTGGGT 1.527 AACGAAATAG Mhei alpha AGCTAGCGTG GAAGCCGCTG	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC	1.440 CATTGCAGCA 1.540 1.64	CTCGCGCCAG CCTCACTGAT Apai CCGCCCTGCTG GACGCTGCCG	1.46C ATGGTAAGCC 1561 TAAGCATTGG JM 160 CTCCTCGGGAA 1760 CGAAAGAGGC 1000 1	CTCCCGTATC Spel flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA	1.480 GTAGTTATCT I 1.580 GCGGCGGCGG GGCAGCGGCGG GGCAGTTAAT GGCCGCAAGCA 1.080 I 1.000 I	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA	1.500 J GAGTCAGGCA 1600 1 GGCAGCGGTT rigid linker 70 CTGCCGAAGC 1800 1 ACTGTTTACC 1.900 1 0 1 1 1 1 1 1 1 1 1 1 1 1 1
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM: linker CTGCTAATCG rigid linker AGCAGCGAAA sfGFP GGTGTTGTTC	AACGAAATAG AACGAAATAG AACGAAATAG AACGAACGTG AAGCTAGCGTG GAAGCCGCTG 1.022 CGATTCTGGT	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC TGAACTGGAT	1.440 CATTGCAGCA 1.540 GAGATAGGTG 1.640	CTGGGGCCAG CCTCACTGAT Apai CGGCCTGCTG GAGGCTGCCG ATGGCCACAA	1.46C ATGGTAAGCC 1.56C TAAGCATTGG JM 16C CTCCTGGGGAA 1.76C 1.6CCC 1.6CC 1.6CC 1.6CC 1.6CCC 1.6CCC 1.6CCC 1.6CCC 1.6CCC 1.6CCC 1.6CCC 1.6CCC 1.6CCC 1.6CCC 1.6CCCC 1.6CCCC 1.6CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	CTCCCGTATC Spel flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA	GGCGCGCGGCGG GGCGGCGGCGG GGCGGCGGCGG GGCGGC	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT	1.500 J GAGTCAGGCA I GGCAGCGGTT rigid linker 700 C TGCCGAAGC 1.800 J ACTGTTTACC GGTAAACTG C
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM: linker CTGCTAATCG rigid linker AGCAGCGAAA siGFP GGTGTTGTTC siGFP CCCTGAAATT	AACGAAATAG AACGAAATAG AACGAAATAG AACGAAATAG AACTAGCTG 1.722 GAAGCCGCTG 1.222 1	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC TGAACTGGAT	1.440 CATTGCAGCA 1.540 1.54	CTGGGGCCAG CCTCACTGAT Apal GAGGCTGCCG ATGGCCACAA GTGGCCGACC	1.46C ATGGTAAGCC 1.56C 1.56C 3.000 1.000 1.000 1.76C 1.76	CTCCCGTATC Spel flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG	1.480 GTAGTTATCT I 1.580 GCGGCGGCGG GGCAGTTAAT SGGCAAGCA 1.680 SGGCAAGCA 1.680 I 1.680 I 1.680	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC	1.500 GAGTCAGGCA I GGCAGCGGTT rigid linker CTGCCGAAGC ACTGTTTAC GGTAAACTGA 22000 GTTAACCGA
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM3 linker CTGCTAATCG rigid linker AGCAGCGAAA srGFP GGTGTTGTTC srGFP CCCTGAAATT srGFP	1.422 GAGCGTGGGT 1.522 AACGAAATAG AGCTAGCGTG 1.722 GAAGCCGCTG 1.822 1.822 1.824 1.8	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC TGAACTGGAT	1.440 CATTGCAGCA 1.540 GAGATAGGTG GGGGGGGGCC 1.740 1	CTGGGGCCAG CCTCACTGAT Apal CGGCCTGCTG GAGGCTGCCG ATGGCCACAA GTGGCCGACC	1.466 ATGGTAAGCC 1.566 JM 1.666 JM JM JM JM JM JM JM JM JM JM	CTCCCGTATC Spel flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA	1.480 GTAGTTATCT GCGGCGGCGGG GGCGGCGGCGG GGCGCGAGGTAAT SGGPGO GCCGCAAGGCA 1.800 GCGAAGGTGA 1.900 I 1.900 I 1.900 I 1.900 I 1.900 I 2.000	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC	1.500 J GAGTCAGGCA 1860 GGCAGCGGTT rigid linker700 CTGCCGAAGC 1800 1 GGTAAACTGA 2000 J GTTATCCGGA 2.100
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM3 linker CTGCTAATCG rigid linker AGCAGCGAAA srGFP GGTGTTGTTC sfGFP TCATATGAAA	1.422 GAGCGTGGGT 1.522 AACGAAATAG Nhei AGCTAGCGTG GAAGCCGCTG 1.22 GAAGCCGCTG 1.22 GAAGCCGCTG 1.22 CGATTCTGGT 1.22 CGATTCTGTACC 2.02 CAGCACGATT	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC GCAAAGAAGC ACCGGCAAAC	1.440 CATTGCAGCA 1.541 GAGATAGGTG 1.6444 1.6444 1.6444 1.6444 1.	CTGGGGCCAG CCTCACTGAT Apal CGGCCTGCTG GAGGCTGCCG ATGGCCACAA GTGGCCGACC GAAGGTTATG	1.466 1 ATGGTAAGCC 1.560 JM 1660 JM 16	CTCCCGTATC Speil flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA	1.480 GTAGTTATCT GCGGCGGCGG 1.680 1 GGCGGCGGCGG 1 GGCGGCGGCGG 1 GGCGCAAGCA 1.690 1 GCGGAAGGTGA 1.800 1 GCGGAAGGTGA 1.800 1 1.800 1 1 1 1 1 1 1 1 1 1 1 1 1	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA	1.500 J GAGTCAGGCA 1600 1 GGCAGCGGTT rigid linker CTGCCGAAGC 1800 1 GGTAAACTGA 2000 1 GGTAAACTGA 2.100 1 TAAGACCCGI
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM3 linker CTGCTAATCG rigid linker AGCAGCGAAA siGFP GGTGTTGTTC sfGFP CCCTGAAATT sfGFP TCATATGAAA siGFP	1.422 GAGCGTGGGT 1.522 AACGAAATAG Nhei alpha AGCTAGCGTG 1.222 1 GAAGCCGCTG 1.22 1 GAAGCCGCTG 1.22 1 CGATTCTGGT 1.22 1 CGATTCTGGT 1.22 1 CGATTCTGGT 1.22 1 CGATTCTGGT 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCTGGT 1.22 1 CGATCCGCTGGT 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCTG 1.22 1 CGATCCGCGCTG 1.22 1 CGATCCGGCTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGCGCTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGGTG 1.22 1 CGATCCGCGCGC 1 CGATCCGCGCGCTG 1.22 1 CGCCGCCGCTG 1.22 1 CGCCGCCGCTG 2.22 1 CGCCGCGCGCTG 2.22 1 CGCCGCGCGCGCTG 2.22 1 CGCCGCGCGGTG 2.22 1 CGCCGCGCGCGCTG 2.22 1 CGCCGCGCGCGCTG 2.22 1 CGCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGC	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC GCAAAGAAGC TGAACTGGAT ACCGGCAAAC	1.440 CATTGCAGCA 1.541 GAGATAGGTG 1.641	CTGGGGCCAG CCTCACTGAT Apal CGGCCTGCTG GAGGCTGCCG ATGGCCACAA GTGGCCGACC GAAGGTTATG	1466 1 ATGGTAAGCC 1581 TAAGCATTGG JM 1666 JM 1666 CTCCTGGGGA 1767 CTCCTGGGGA 1776 1 CTGGTTACCA 2060 TTCCAGGACG 2.160 2.160 2.160	CTCCCGTATC Speil flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA	1.480 GTAGTTATCT 1 1580 GCGGCGGCGG GGCAGCGA GGCAGTTAAT 3 GGCAAGGTAA 1.680 1 GGCGAAGGTAA 1 GGCGAAGGTGA 1 GGGGGTGTTCAG 2.000 TTTAAAGATG 2.180	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA	1.500 1 GAGTCAGGCA 1600 1 GGCAGCGGTT rigid linker 1 CTGCCGAAGC 1.800 1 CTGCCGAAGC 1.800 1 GGTAAACTGA 2.000 1 GGTAAACTGA 2.100 1 GGTAAACTGA 2.200 1 TAAGACCCGT
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM: linker CTGCTAATCG rigid linker AGCAGCGAAA sfGFP GGTGTTGTTC sfGFP CCCTGAAATT sfGFP GCCGAAGTTA	1.422 GAGCGTGGGT 1.522 AACGAAATAG I.522 alpha adpha adpha adpha adpha adpha adpha 1.722 1 GAAGCCGCTG 1.222 1 CGATTCTGGT 1.222 1 CGATCCGCTG 1.222 1 CGATCCGCGCTG 1.222 1 CGATCCGCTG 1.222 1 CGATCCGCGCTG 1.222 1 CGATCCGCGCTG 1.222 1 CGATCCGCGCGCTG 1.222 1 CGATCCGCGCGCTG 1.222 1 CGATCCGCGCGCTG 1.222 1 CGATCCGCGCGCTG 1.222 1 CGATCCGCGCGCGCTG 1.222 1 CGATCCGCGCGCGCTG 1 CGCCGCCGCGCTG 1 CGCCGCCGCGCTG 1 CCCCCCGCGCGCGCTG 1 CCCCCCGCGCGCGCGCGCTG 1 CCCCCCGCGCGCGCGCGCGCTG 1 CCCCCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGC	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC GCCAAAGAAGC TGAACTGGAT ACCGGCAAAC TCTTCAAATC	1.440 CATTGCAGCA 1.540 1.64	CTGGGGCCAG CCTCACTGAT Apai CGGCCTGCTG GAGGCTGCCG ATGGCCACAA GTGGCCGACC GAAGGTTATG TTGAACTGAA	1.466 1 ATGGTAAGCC 1 1 1 TAAGCATTGG 3 3 3 3 1 1 5 CTCCTGGGGA 1 1 7 CGGAAAGAGGC 1 0 CGAAAGAGGC 1 0 CGGAAGAGGC 1 0 CTGGTTACCAGT 1 1 0 C CGGAAGAGGC 1 0 C CGGAAGAGGC 1 0 C CGGAAGAGGC 1 0 C CGGAAGAGGC 1 0 C CGGAAGAGGC 1 0 C CGGAAGAGGC 1 0 C C CGAAAGAGCC 1 0 C C CGAAAGAGCC 1 0 C C C C C C C C C C C C C C C C C	CTCCCGTATC Speil flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA TACCATCTCC	1.480 GTAGTTATCT 1 580 GCGGCGGCGG 1.680 1 GGCAGTTAAT 1.680 1 GGCAGTTAAT 1.680 1 GGCGAAGCA 1.680 1 GGCGAAGCA 1.680 1 GGCGAAGCA 1 1 GGCGAAGCA 1 1 1 1 1 1 1 1 1 1 1 1 1	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA CCTGGGCCAT	1.500 1 GAGTCAGGCA GGCAGCGGTT rigid linker rigid linker CTGCCGAAGC 1.800 1 GGTAACTGA 2.000 1 GTTATCCGAA 2.100 1 TAAGACCCGT 2.000 1 TAAGACCCGT
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM: linker CTGCTAATCG rigid linker AGCAGCGAAA siGFP GGTGTTGTTC siGFP CCCTGAAATT siGFP TCATATGAAA siGFP	AACGAAATAG AACGAAATAG AACGAAATAG AACGAAATAG AACGAACGTG 1.72 GAAGCCGCTG 1.82 CGATTCTGGT 1.92 CGATTCTGGT 1.92 CAGCACGATT 1.92 CAGCACGATT 1.92 CAGCACGATT 1.92 CAGCACGATT 1.92 CAGCACGATT 1.92 CAGCACGAT 1.92 CAGCACGAT 1.92 CAGCACGAT 1.92 CAGCACGAT 1.92 CAGCACGAT 1.92 CAGCACACACA 1.92 CAGCACACACACACACACACACACACACACACACACACA	CCAAAGAAGC CCAAAGAAGC CCAAAGAAGC CCAAAGAAGC CCAAAGAAGC CCAAAGAAGC CCAAAGAAGC CCAAAGAAGC	1.440 CATTGCAGCA 1.541 GAGATAGGTG 1.641	CTGGGGCCAG CCTCACTGAT Apai CGGCCTGCTG GAGGCTGCCG ATGGCCGACC GAAGGTTATG TTGAACTGAA	1.46C 1 ATGGTAAGCC 1 SE 1 AAGCATTGG 1 AAGCATTGG 1 M 1 M 1 M 1 CTCCTGGG 1 CTCCTGGG 1 CGAAAGAGGC 1 M 1 CTGGTTACCA 1 C	CTCCCGTATC Spei flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA TACCATCTCC	1.480 GTAGTTATCT 1.580 GCGGCGGCGG GGCAGCTAAT 1.680 1 GGCAGTTAAT 1.680 1 GGCAGTTAAT 1.680 1 GGCAGTTAAT 1.680 1 GGCAGTTAAT 1.680 1 GGCAGTTAAT 1.680 1 1 1 1 1 1 1 1 1 1 1 1 1	ACACGACGGG GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA CCTGGGCCAT	1.500 GGGAGCGGGTT rigid linker rigid linker CTGCCGAAGC 1.800 1.800 ACTGTTTACC 1.900 GGTAAACTGA 2.000 1 GTTATCCGGA 2.100 1 AAACTGGAAT 2.000 1 AAACTGGAAT 2.000 1 AAACTGGAAT
C-BLa TGGAGCCGGT ACTATGGATG flexJM) linker CTGCTAATCG rigid linker AGCAGCGAAA srGFP GGTGTTGTTC srGFP TCATATGAAA SrGFP TCATATGAAA SrGFP ATAATTTCAA	AACGAAATAG 1152 AACGAAATAG 1152 AACGAAATAG 1172 alphai AGCTAGCGTG 1172 GAAGCCGCTG 1182 1172 CGATTCTGGT 1182	CCAAAGAAGC CCAAAGAACC CCAAACC CCAAAGAACC CCACC CCAAAGAACC CCAAAGAACC CCAAAGAACC CCACC CCAAAGAACC CCACC CCAAAGAACC CCACC CCAAAGAACC	1.440 CATTGCAGCA 1.540 GAGATAGGTG GGGGGGGGCC 1.740 GGCGGGGGGGAAA 1.940 1.740 1.940 1.740 1.940	CTGGGGCCAG CCTCACTGAT Apai CGGGCCTGCTG GAGGCTGCCG GTGGCCGACC GAAGGTTATG TTGAACTGAA ACAGAAGAAT	1.46C 1 ATGGTAAGCC 1 1 1 1 1 1 1 1 1 1 1 1 1	CTCCCGTATC Spel flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA TACCATCTCC TTCAAAGAAG CCAACTTTAA	1.480 GTAGTTATCT GCGGCGGCGG GGCGCGGCGG GGCAGTTAAT SGGCGCAAGGA 1.880 GCGCAAGGAA 1.880 GCGAAGGTGA 1.880 GCGAAGGTGA 1.880 1.800 1.8	ACACGACGGG GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA CCTGGGCCAT AATGTTGAAG	1.500 GAGTCAGGCA 1600 1 GGCAGCGGTT rigid linker 700 1 CTGCCGAAGC 1.800 1 GGTAACTGA 2000 1 GGTAACCGG 2100 1 TAAGACCCGT 2200 1 AAACTGGAAT 2200 1 AAACTGGAAT
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM) linker CTGCTAATCG rigid linker AGCAGCGAAA srGFP GGTGTTGTTC srGFP TCATATGAAA srGFP GCCCGAAGTTA srGFP ATAATTTCAA srGFP	AACGAAATAG AACGAAATAG AACGAAATAG AACGAAATAG AACGAAATAG AACTAGCGTG 1.722 GAAGCCGCTG 1.724 1.724 GAAGCCGCGTG 1.7244 1.724 1.724 1.724 1.724 1.	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGAGC GCGAAAGAAGC TGAACTGGAT ACCGGCAAAC TGTTCAAATC GTGTATATCA AGCAGAAATAC	1.440 CATTGCAGCA 1.540 1.64	CTGGGGCCAG CCTCACTGAT Apar CGGGCCTGCTG GAGGCTGCCG ATGGCCACAA GTGGCCGACC GAAGGTTATG TTGAACTGAA ACAGAAGAAT	1.46C ATGGTAAGCC 1.56C JM 160C JM 160C 160C 170C CGGAAAGAGGGC 100C 170C 190C	CTCCCGTATC Spel flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA TACCATCTCC TTCAAAGAAG CCAACTTTAA	1.480 GTAGTTATCT GCGGCGGCGG GGCGCGGCGG GGCAGTTAAT SGGPA GGCGCAAGGTA 1980 GCGCAAGGTGA 1980 GCGAAGGTGA 1980 GCGAAGGTGA 1980 GCGAAGGTGA 2.980 TTTAAAGATG ATGGTAATAT 2.280 GATCCGCCAT 2.280 GATCCGCCAT	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA CCTGGGCCAT AATGTTGAAG CCCAGAGCGT	1.500 GAGTCAGGCA 1.600 1 GGCAGCGGTT rigid linker 700 1 CTGCCGAAGC 1.800 1 GGTAAACTGG CTGTTTACCGGA 2.100 1 GGTAAACTGGAT 2.200 2.100 1 AAACTGGAT 2.200 1 AAACTGGAT 2.200 1 AAACTGGAC 1 AAACTGGAC 1 2.200 1 AAACTGGAC 1 2.200 1 AAACTGGAC 1 2.200 1 AAACTGGAC 1 2.200 1 3 3 4 4 4 4 4 4 4 4 4 4 4 4 4
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM: linker CTGCTAATCG rigid linker AGCAGCGAAA siGFP GGTGTTGTTC siGFP CCCTGAAATT SiGFP GCCGAAGTTA siGFP ATAATTTCAA siGFP TCAGCTGGCA siGFP	1.422 GAGCCGGGGG 1.527 AACGAAATAG I.727 AGCTAGCGTG 1.727 GAAGCCGCTG 1.277 GAAGCCGCTG 1.277 GAAGCCGCTG 1.277 GAAGCCGCTG 1.277 GAAGCCGCTG 1.277 1.277 GAAGCCGCTG 1.2777 1.2777 1.2777 1.2777 1.	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC GCAAAGAAGC TGAACTGGAT ACCGGCAAAC TCTTCAAATC GTGTATATCA GTGTATATCA	1.440 CATTGCAGCA 1.640 1.64	CTGGGGCCAG CCTCACTGAT Apal CGGCCTGCTG GAGGCTGCCG ATGGCCACAA GTGGCCGACC GAAGGTTATG TTGAACTGAA ACAGAAGAAT GATGGTCCGG	1466 1 ATGGTAAGCC 1580 1580 1580 1580 1580 1580 1580 1580	CTCCCGTATC Speil flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA TACCATCTCC TTCAAAGAAG CCCAACTTTAA	1.480 GTAGTTATCT GCGCGCGGCGG 1.880 1.880 GGCGCGCGGCGG 1.880 1.890 1.8000 1.8000 1.8000 1.8000 1.8000	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA CCTGGGCCAT AATGTTGAAG CCCAGAGCGT	1.500 GAGTCAGGCA 1600 1 GGCAGCGGTT rigid linker CTGCCGAAGC 1800 CTGCCGAAGC 1800 1 GGTAAACTGA 2000 1 GGTAACCGGA 2100 1 ACTGTTACC 3 2001 1 AAACTGGAAT 2300 1 ATGGCAGCGT 2400 1 TCTGAGCAAA Tag 2.500
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM: linker CTGCTAATCG rigid linker AGCAGCGAAA sfGFP GGTGTTGTTC sfGFP TCATATGAAA sfGFP GCCGAAGTTA sfGFP ATAATTTCAA sfGFP TCAGCTGGCA sfGFP	1.422 GAGCGTGGGT AACGAAATAG IST AACGAAATAG INHE AGCTAGCGTG 1.222 I GAAGCCGCTG 1.222 I GAAGCCGCTG 1.222 I CAGCACGATT 2.222 I CAGCCACGACA 2.222 I CAGCCACGACA 2.222 I GATCATTATC 2.222 I GATCATTATC 2.222 I GATCATTATC	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGAGC GCGAAAGAAGC ACCGGCAAAC TGTTCAAATC GTGTATATCA AGCAGGAATAC	1.440 CATTGCAGCA 1.540 GAGATAGGTG 1.640	CTGGGGCCAG CCTCACTGAT Apai CGGCCTGCTG GAGGCTGCCG ATGGCCGACC GAAGGTTATG TTGAACTGAA ACAGAAGAAT GATGGTCCGG TTGTTACCGC	1.46C 1.475 1.	CTCCCGTATC Speil flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA TACCATCTCC TTCAAAGAAG CCCAACTTTAA GGATAATCAT	1.480 GTAGTTATCT GCGGCGGCGG GGCGGCGGCGG 1.680 1 GGCAGTTAAT GGCGGCAAGCA 1.680 1 GGCGCAAGCA 1.680 1 GGCGCAAGCA 1.680 1 GGCGCAAGCA 1.680 1 GGCGCAAGCA 1.680 1 GGCGCAAGCA 1 1 GGCGAAGGTGA 1 2.800 1 GGTCGCCAA 2.480 TGGATGAACT	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA CCTGGGCCAT AATGTTGAAG CCCAGAGCGT FLAG GTATAAAGGC	1.500 1 GAGTCAGGCAT GGCAGCGGTT rigid linker CTGCCGAAGC 1.800 1 CTGCCGAAGC 1.800 1 GGTAACTGA 2.000 1 GGTAACCGGA 2.100 1 TAAGACCCGT 2.000 1 AACTGGAAA 2.000 1 TAAGACCGGAT 2.000 1 TAAGACCGGAAG 1 1 CTGGCAAGCGAAG 1 2 2 1 3 1 2 1 3 2 1 3 2 1 3 2 5 1 3 1 3 1 3 1 1 1 1 1 1 1 1 1 1 1 1 1
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM: linker CTGCTAATCG rigid linker AGCAGCGAAA sfGFP GGTGTTGTTC sfGFP TCATATGAAA sfGFP GCCGAAGTTA sfGFP TCAGCTGGCA sfGFP TCAGCTGGCA	1.422 GAGCGTGGGT AACGAAATAG IST AACGAAATAG INDE AGCTACGTG 1.722 GAAGCCGCTG 1.722 1 GAAGCCGCTG 1.722 1 CGATTCTGGT CGATTCTGACG 2 2 1 CAGCCACGATT 2.12 CAGCCACGACCACAC 2.12 CAGCCACGACCACAC 2.12 CAGCCACGACCACAC 2.12 CAGCCACGACCACAC 2.12 CAGCCACGACCACACAC 2.12 CAGCCACGACCACACACACACACACACACACACACACAC	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC CCAAAGAAGC TGAACTGGAT ACCGGCAAAC TGTTCAAATC GTGTATATCA AGCAGAATAC	1.440 CATTGCAGCA 1.640 1.64	CTGGGGCCAG CCTCACTGAT Apai CGGGCCTGCTG GAGGCTGCCG ATGGCCACAA GTGGCCGACC GAAGGTTATG TTGAACTGAA ACAGAAGAAT GATGGTCCGG TTGTTACCGC	1466 ATGGTAAGCC 156 TAAGCATTGG Bam JM 166 CTCCTGGGAA 1776 CGAAAGAGGC 107 CGAAAGAGGC 107 CGAAAGAGGC 107 107 107 107 107 107 107 107	CTCCCGTATC Spei flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA TACCATCTCC TTCAAAGAAG CCAACTTTAA GGATAATCAT ACCCATGGTA	1,480 GTAGTTATCT GCGGCGGCGG GGCGCGGGGGGG 1,680 1 GGCAGTTAAT GGCAGTTAAT 1,600 1 GGCGCAAGCA 1,600 1 GGCGCAAGCA 1,600 1 GGCGCAAGCA 1,600 1 1 GGCGCAAGCA 1,600 1 1 GGCGCAAGCA 1,600 1 1 1 1 1 1 1 1 1 1 1 1 1	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA CCTGGGCCAT AATGTTGAAG CCCAGAGCGT FLAG GTATAAAGGC	1.500 GGGTAGGGGTT rigid linker 700 1 GGCAGCGGAGG 1.800 1 C GCCGAAGC 1.800 ACTGTTTACC 1.900 1 GGTAACTGA 2.000 1 TAAGACCCGT 2.000 1 AACTGGAGCAAA 2.000 1 TAGGCAGCGT 2.000 1 TGTGAGCAAA 1 3 2.000 1 3 3 3 4 3 4 3 4 3 4 3 4 3 4 4 4 4 4 4 4 4 4 4 4 4 4
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM: linker CTGCTAATCG rigid linker AGCAGCGAAA sfGFP CCCTGAATT SfGFP TCATATGAAA sfGFP ATAATTCAA sfGFP TCAGCTGCA SfGFP GATCCGAATG FLAG-Tag ATCAGACGG	AACGAAATAG AACGAAATAG AACGAAATAG AACGAAATAG AACTACGTG 1.722 GAAGCCGCTG 1.822 CGATTCTGGT CGATTCTGGT CAGCACGATT AATTTGAAGG CAGCCACAACCTGA CAGCACGACACACC 2.282 CAGCACGTGA CAGCACGTGA	CTCGCCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC CCAAAGAAGC TGAACTGGAT ACCGGCAAAC TCTTCAAATC GTGTATATCA AGCAGAATAC TCACATGGTG	1.440 CATTGCAGCA 1.540 GAGATAGGTG GGGGGGGGCC 1.6400 1.6400 1.640	CTTGGGGCCAG CCTCACTGAT Apai CGGGCCTGCTG GAGGCTGCCG ATGGCCGACC GAAGGTTATG TTGAACTGAA ACAGAAGAAT GATGGTCCGG TTGTTACCGC accord	1.46C 1 ATGGTAAGCC 1 AAGCATTGG 1 AAGCATTGG 1 AAGCATTGG 1 AGCAAGAGGC 1 OF 1 CTCCTGGGGA 1 OF 1 CTGGTTACCA 1 OF 1 CTGGTTACCA 2 OF 1 OF 1 CTGGTTACCA 2 OF 1 OF 1 CTGGTTACCA 2 OF 1	CTCCCGTATC Spei flexible linke ACTAGTGGCA ff TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA TACCATCTCC TTCAAAGAAG CCAACTTTAA GGATAATCAT ACCCATGGTA	1,480 GTAGTTATCT GCGGCGGCGG GGCGCGGCGGG GGCAGTTAAT SGGPA GGCGCAAGCA 1,680 SGGPA GGCGCAAGCA 1,680 1 GGCGCAAGCTGA 1,680 1 GGCGCAAGCT 1,680 1 GGCGCAAGCT 1,680 1 GGCGCAAGCTGA 2,280 1 TTTCAGCCTG 2,280 1 TTTCAGCCTG	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA CCTGGGCCAT AATGTTGAAG CCCAGAGCGT FLAG GTATAAAGGC	1.500 GAGTCAGGCAT rigid linker 700 1 CTGCCGAAGC 1.800 1 CTGCCGAAGC 1.800 1 CTGCCGAAGC 1.800 2.000 CTTATCCGGA CTTATCCGGA CTGCCGACGT 2.000 1 AAACTGGAACTGA 2.000 1 CTGGCGCGAAGC 2.100 1 CTGGCAGCGAAGC 1 CTGGCAGCAA 2.000 1 CTGGCCGAAGCGT 2.000 1 CTGGCCGAAGCGT 2.000 1 CTGGCCGAAGCGT 2.000 1 CTGGCGCGAAGCGT 2.000 1 CTGGCGCGAAGCGT 2.000 1 CTGGCGCGAAGCGT 1 CTGGCGCGAAGCGT 1 CTGGCGCGAAGCGT 1 CTGGCGCGAAGCGT 1 CTGGCGCGAAGCGT 1 CTGGCGCGAAGCGT 1 CTGGCGCGAAGCGT 1 CTGGCGCGAAGCGT 1 CTGGCGCGAAGCGGT 1 CTGGCGCGAAGCGGT 2.000 1 CTGGCGCGAAGCGGT 2.000 1 CTGGCGCGAAGCGGT 2.000 1 CTGGCGCGAAGCGGT 2.000 1 CTGGCGCGAAGCGGT 2.000 1 CTGGCGCGAAGCGGT 2.000 1 CTGGCGCGAAGCGGT 2.000 1 CTGGCGCGAAGCGGT 2.000 1 CTGGCGCGAAGCGGT 2.000 1 CTGGCGCGAAGCGGT 2.000 1 CTGGCGCGAAGCGGT 2.000 1 CTGGCGCGCGGAAGCGGT 2.000 1 CTGGCGCGCGAAGCGGT 2.000 1 CTGGCGCGCGAAGCGGT 2.000 1 CTGGCGCGCGGAAGCGGT 2.000 1 CTGGGCGCGCGAAGCGGT 2.000 1 CTGGCGCGCGCGCGGAAGCGGT 2.000 1 CTGGCGCGCGCGCGAAGCGGT 2.000 1 CTGGCGCGCGCGCGGCGGCGGCGGCGGG 2.000 1 CTGGCGCGCGCGGCGGCGGCGGCGGGCGGGCGGGCGGGGGG
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM) linker CTGCTAATCG rigid linker AGCAGCGAAA srGFP GGTGTTGTTC srGFP TCATATGAAA srGFP ATAATTTCAA srGFP CCCCGGAGTTA srGFP CCAGCTGGCA srGFP GATCCGAATG FLAG-Tag ATCACGACGG	AACGAAAATAG AACGAAATAG AACGAAATAG AACGAAATAG AACGAAATAG AACTACCTTG GAAGCCGCTG CGATTCTGGT CGATTCTGGT CAGCACGATT CAGCACGATT CAGCACGACACAC CAGCCACAAC CAGCCACCACACAC CAGCCACCACACAC CAGCCACCACACAC	ACAGATCGCT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC CCAAAGAAGC GCGAACTGGAT ACCGGCAAAC TCTTCAAATC GTGTATATCA AGCAGAATAC TCACATGGTG GTCGACC <u>TGC</u>	1.440 CATTGCAGGA GAGATAGGTG GAGATAGGTG CGGTGG <u>GGGC</u> 1.740 GGCAGCGAAA 1.940 1.740 GGCAGCGAAA 1.940 1.740 GGCGGTGCGAAA 1.940 1.7	CTGGGGCCAG CCTCACTGAT Apail CGGGCCTGCTG GAGGCTGCCG ATGGCCACAA GTGGCCGACC GAAGGTTATG TTGAACTGAA ACAGAAGAAT GATGGTCCGG TTGTTACCGC <u>GCT</u> TGGCTGT nation	1.46C ATGGTAAGCC 1.46	CTCCCGTATC Spel flexible linke ACTAGTGGCA ff TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA TACCATCTCC TTCAAAGAAG CCAACTTTAA GGATAATCAT ACCCATGGTA	1,480 GTAGTTATCT GCGGCGGCGGG GGCGCGGCGGG GGCAGTTAAT SGGCGCAAGCA 1,880 GCGCAAGGTGA 1,880 GCGAAGGTGA 1,880 GCGAAGGTGA 1,880 TGGTGTTCAGCA 2,180 ATGGTATTA C2,180 ATGGTAATAT C2,180 TTTCAGCCA CA CCCCCAAGCA 2,180 TTTCAGCCA CA CA CA CA CA CA CA CA CA CA CA CA	ACACGACGGG GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA CCTGGGCCAT AATGTTGAAG CCCAGAGCGT FLAG GTATAAAGGC ATACAGATTA	1.500 GAGTCAGGCA IL600 I GGCAGCGGTT I Igid linker 700 CTGCCGAAGC IL800 GGTAACTGA GGTAACTGGA GGTAACTGGA C100 GGTAACCGGT 22000 I AAACTGGAACT 2300 I AAGCCGCAAG CT CTGAGCAGCA CT GGTAACTGGAA C C C C C C C C C C C C C C C C C C
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJMD linker CTGCTAATCG Tigid linker AGCAGCGAAA STGFP GGTGTTGTTC STGFP TCATATGAAA STGFP ATAATTTCAA STGFP GATCCGAATG FLAG-TAG ATCACGACGG CAGAAGCGGT	AACGAAACGTGGT AACGAAATAG AACGAAATAG AACGAAATAG AACGAAATAG AACTAGCGTG 1.722 GAAGCCGCTG 1.724 GAAGCCGCTG 1.724 GAAGCCGCTG 1.724 CAGCACGATT 2.124 CAGCACCACAC 2.222 CAGCACCACAC 2.224 CAGCACCACAC CAGCACCACAC 2.224 CAGCACCACAC CAGCACCACAC CAGCACCACAC CAGCACCACAC CAGCACCACAC CAGCACCACAC CAGCACCACAC CAGCACCACAC CAGCACCACAC CAGCACCACAC CAGCACCACAC CAGCACCACAC CAGCACACACACAC	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC GCCAAAGAAGC TGAACTGGAT ACCGGCAAAC TCTTCAAATC GTGTATATCA AGCAGAATAC TCACATGGTG GTCGACC <u>TGC</u> rmB_tr AGAATTTGCC	1.440 CATTGCAGCA 1.540 GAGATAGGTG GGGGGGGGGG 1.740 1.640 1.640 1.640 1.740 1.640 1.640 1.740 1.640 1.640 1.740 1.640 1.740 1.640 1.740 1.640 1.740 1.640 1.740 1.640 1.740 1.640 1.740 1.640 1.740 1.740 1.640 1.740 1.740 1.640 1.740 1	CTGGGGCCAG CCTCACTGAT Apail CGGGCCTGCTG GAGGCTGCCG ATGGCCACAA GTGGCCGACC GAAGGTTATG TTGAACTGAA ACAGAAGAAT GATGGTCCGG TTGTTACCGC MIM AGCGCGGTGG	1.46C 1 AAGGTAAGCC 1 AAGCATTGG 1 AAGCATTGG 1 Sec 1 Se	CTCCCGTATC Speil frexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA TACCATCTCC TTCAAAGAAG CCCACTTTAA GGATAATCAT ACCCATGGTA	1.480 GTAGTTATCT GCGGCGGCGGG GGCGCGGCGGG GGCAGTTAAT SGGP30 GCCGCAAGCAA 1900 GCGAAGGTGA 1900 GCGAAGGTGA 1900 GCGAAGGTGA 1900 GCGAAGGTGA 1900 GGCGCAAGCA 1900 GGCGCAAGCA 1900 GGCGCAAGCA 1900 GCGAAGGTGA 1900 GCGAAGGTGA 1900 TTTAAAGATG 2,180 GATCCGCCAT 2,280 TTTCAGCCTG C TTTCAGCCTG 2,280 C	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA CCTGGGCCAT AATGTTGAAG CCCAGAGCGT GTATAAAGGC ATACAGATTA TGAAACGCCG	1.500 GAGTCAGGCA 11600 I GGCAGCGGTT I Igid linker 700 C GGCAGCGAT ACTGTTTACC I GGTAAACTGGA C C C GTTATCCGA C C C C C C C C C C C C C C C C C C
C-BLa TGGAGCCGGT C-BLa ACTATGGATG flexJM) linker CTGCTAATCG flexJM) linker CTGCTAATCG GTGTTGTTC sfGFP GCCGGAAGTTA sfGFP TCATATGAAA sfGFP TCAGTATGAAA sfGFP TCAGCTGGCA sfGFP GATCCGAATG FLAG-Tag ATCACGACGG CAGAAGCGGT rmB_transcription	AACGAAATAG AACGAAATAG AACGAAATAG AACGAAATAG AGCTAGCGTG GAAGCCGCTG GAAGCCGCTG 122 GAAGCCGCTG 132 CGATTCTGGT CAGCACGAT CAGCCACGAT 222 CAGCCACGAT 222 GATCATTATC CAGCCACGAC 222 GATCATTATC CAGCCACGAC 222 GATCATTATC CAGCCACGAC 222 CAGCCACGACAC 222 CAGCCACGAC 222 CAGCCACGAC 222 CAGCCACGAC 222 CAGCCACGACAC 222 CAGCCACGAC 222 CAGCCACGAC 222 CAGCCACGAC 222 CAGCCACGAC CAGC 222 CAGCCACGAC CAGC 222 CAGCCACGAC CAGC 222 CAGCCACGAC CAGC 222 CAGCCACGAC CAGC 222 CAGCCACGAC CAGC 222 CAGCCACACAC CAGC 222 CAGCCACACAC CAGC 222 CAGCCACACAC CAGCACGACGAC CAGCACCACGAC CAGCACCACAC CAGCACCACACCAC CAGCACCACGAC CAGCACCACACCAC CAGCACCACACCAC CAGCACCACACCAC CAGCACCACACCACAC CAGCACCACACCACAC CAGCACCACACCACAC CAGCACCACACCACAC CAGCACCACACCACAC CAGCACCACACACA	CTCGCGGTAT ACAGATCGCT Vmut-TMD ATCATTCTGG CCAAAGAAGC CCAAAGAAGC GCGAACTGGAT ACCGGCAAAC TGATACCCTG GTGTATATCA AGCAGAATAC CCGCCCTGC GTCGACCTGC GTCGACCTGC	1.440 CATTGCAGCA 1.640 1.64	CTGGGGCCAG CCTCACTGAT Apai CGGCCTGCTG GAGGCTGCCG ATGGCCACAA GTGGCCGACC GAAGGTTATG TTGAACTGAA ACAGAAGAAT GATGGTCCGG TTGTTACCGC dilli <u>GCT</u> TGGCTGT nation AGCGCGGTGG	1466 1 ATGGTAAGCC 1560 1 TAAGCATTGG JM 1660 1 CTCCTGGGGA 1770 CGGAAAGAGGC 1 ATTCTCAGTT 2 CGGAAAGAGGC 2 1 TTCAAGAACG 2 2 CTGGTTACCA 2 2 CTGGTTACCA 2 2 CTGGTTACCA 2 2 CTGGTTACCA 2 2 CTGGTTACCA 2 2 CTGGTTACCA 2 2 CTGGTTACCA 2 CTGTTACCA	CTCCCGTATC Speil flexible linke ACTAGTGGCA HI TCCACAAACT AGCAGCTAAA CGTGGTGAAG CCCTGACCTA TACCATCTCC TTCAAAGAAGA CCAACTTTAA GGATAATCAT ACCCATGGTA GAGAGAAGAT	1.480 GTAGTTATCT GCGGCGGCGG GGCGCGGCGG GGCGCGCGGCGG GGCGCGCGGCGG GGCGCGCAGGCA 1.680 1 GGCGAGGTGA 1 GGCGAAGGTGA 1 1 GGGTGTTCAG 2.480 ATGGTAGAAGA C 2.480 ATGGATGAACA C C C C C C C C C C C C C	ACACGACGGG CAGCGGCGGC GTTCAGCTGC AAGGTGAAGA TGCAACCAAT TGTTTCAGCC ATGGCACCTA CCTGGGCCAT AATGTTGAAG CCCAGAGCGT GTATAAAGGC ATACAGATTA TGAAACGCCG	1.500 GAGTCAGGCA IGCCAGCGGTT rigid linker 700 CTGCCGAAGC 1.800 I ACTGTTTACC GGTAAACTGA 2.100 I GGTAAACTGAA 2.100 I AAACTGGAAT 2.200 I AAACTGGAAT 2.300 AAACTGGAAT 1.300 I GTTATCCGGAAGCGT 2.400 I CTGAGCAAGC 2.100 I AAACTGGAACAC 2.100 I AAACTGGAAC 2.000 I AAACTGAACGAAC 2.000 I AAACTGAAAC 2.000 I AAACTGAAAC 2.000 I AAACTGAAAC 2.000 I AAACTGAAAC 2.000 I AAACTGAAAC 2.000 I AAACTGAAAC 2.000 I AAACTGAAAC 2.000 I AAACTGAAAC 2.000 I AAACTGAAAC 2.000 I I AAACTGAAAC 2.000 I AAACTGAAAC 2.000 I AAACTGAAAC I I I I I I I I I I I I I

	2.820		2.840		2.860		2.880		2.900
TTGTCGGTGA	ACGCTCTCCT	GAGTAGGACA	AATCCGCCGG	GAGCGGATTT	GAACGTTGCG	AAGCAACGGC	CCGGAGGGTG	GCGGGCAGGA	CGCCCGCCAT
	2.020		2.040		2.000		2.000		2.000
	2.920	rrnB_T2_tr	anscrip_term 2.940		2.960		2.980		3.000 I
AAACTGCCAG	GCATCAAATT	AAGCAGAAGG	CCATCCTGAC	GGATGGCCTT	TTTGCGTTTC	TACAAACTCT	TTTGTTTATT	тттстааата	CATTCAAATA
	3.020		3.040 I		3.060		3.080		3.100 I
TGTATCCGCT	CATGGAGCTC	AAAGCCACGT	TGTGTCTCAA	AATCTCTGAT	GTTACATTGC	ACAAGATAAA	AATATATCAT	CATGAACAAT	AAAACTGTCT
	3.120		KanR 3.140		3.160		3.180		3.200
GCTTACATAA	ACAGTAATAC	AAGGGGTGTT	ATGAGCCATA	TTCAACGGGA	AACGTCTTGC	TCCAGGCCGC	GATTAAATTC	CAACATGGAT	GCTGATTTAT
KanR	3.220 I		3.240 I		3.260 I		3.280 I		3.300 I
ATGGGTATAA	ATGGGCTCGC	GATAATGTCG	GGCAATCAGG	TGCGACAATC	TATCGTTTGT	ATGGGAAGCC	CGATGCGCCA	GAGTTGTTTC	TGAAACATGG
KanR	3.320		3.340		3.360		3.380		3.400
CAAAGGTAGC	GTTGCCAATG	ATGTTACAGA	TGAGATGGTC	AGACTAAACT	GGCTGACGGA	ATTTATGCCT	CTTCCGACCA	TCAAGCATTT	TATCCGGACT
KanP	3.420		3.440		3.460		3.480		3 500
COTCATCAAC	CATCOTACT	CACCACTOCC		AAACACCATT	CCACCTATTA	CAACAATATC		TCAAAATATT	
CCTGATGAAG	CATGGTTACT	CACCACIGCO	ATCCCCGGCA	AAACAGCATT	CCAGGIAITA	GAAGAATATC	CIGATICAGG	IGAAAATATT	GIIGAIGCGC
KanR	3.520		3.540 I		3.560 I		3.580 I		3.600 I
TGGCAGTGTT	CCTGCGCCGG	TTGCATTCGA	TTCCTGTTTG	TAATTGTCCT	TTTAACAGCG	ATCGCGTATT	TCGTCTCGCT	CAGGCGCAAT	CACGAATGAA
KanR	3.620		3.640		3.660		3.680		3.700
TAACGGTTTG	GTTGATGCGA	GTGATTTTGA	TGACGAGCGT	AATGGCTGGC	CTGTTGAACA	AGTCTGGAAA	GAAATGCACA	AACTTTTGCC	ATTCTCACCG
KanR	3 720		3 740		3 760		3 780		3 800
GATTCAGTCG	TCACTCATGG	TGATTTCTCA	CTTGATAACC	TTATTTTGA	CGAGGGGAAA	TTAATAGGTT	GTATTGATGT	TGGACGAGTC	GGAATCGCAG
KanR	3.820		3.840		3.860		3.880		3.900
ACCGATACCA	GGATCTTGCC	ATCCTATGGA	ACTGCCTCGG	TGAGTTTTCT	CCTTCATTAC	AGAAACGGCT	TTTTCAAAAA	TATGGTATTG	ATAATCCTGA
KanR	3.920		3.940		3.960		3.980		4.000
TATGAATAAA	TTGCAGTTTC	ATTGATOCT	CGATGAGTT	TTCTAAGAGC	TCTCCATGAC	CAAAATCCCT	TAACGTGAGT	TTTCGTTCCA	CTGAGCGTCA
TATOAATAAA	TICOACTITO	ATTICATOOT	U GATUAGITI	TICIAACAGO	TOTOGRIGAG		TARGOTOAGT	111001100A	
	4.020		4.040 I		4.060 I		4.080 I		4.100 I
GACCCCGTAG	AAAAGATCAA	AGGATCTTCT	TGAGATCCTT	TTTTTCTGCG	CGTAATCTGC	TGCTTGCAAA	CAAAAAACC	ACCGCTACCA	GCGGTGGTTT
	4.120		4.140		4.160		4.180		4.200
GTTTGCCGGA	TCAAGAGCTA	CCAACTCTTT	TTCCGAAGGT	AACTGGCTTC	AGCAGAGCGC	AGATACCAAA	TACTGTCCTT	CTAGTGTAGC	CGTAGTTAGG
	4.000		4.040		4.000		4 200		4.000
	4.220		4.240		4.260		4.280 I		4.300
CCACCACTTC	AAGAACTCTG	TAGCACCGCC	TACATACCTC	GCTCTGCTAA	TCCTGTTACC	AGTGGCTGCT	GCCAGTGGCG	ATAAGTCGTG	TCTTACCGGG
	4.320		4.340		4.360		4.380		4.400
TTGGACTCAA	GACGATAGTT	ACCGGATAAG	GCGCAGCGGT	CGGGCTGAAC	GGGGGGTTCG	TGCACACAGC	CCAGCTTGGA	GCGAACGACC	TACACCGAAC
	4.420		4.440		4.460		4.480		4.500
TGAGATACCT	ACAGCGTGAG	CTATGAGAAA	GCGCCACGCT	TCCCGAAGGG		ACAGGTATCC	GGTAAGCGGC	AGGGTCGGAA	CAGGAGAGCG
ICACATACOT	ACAGOGICAC	OTATOAGAAA	COCCACCO	TUTUTA		1010011100	00111100000	AUGUIUUUAA	UNUUNUNUU
	4.520		4.540 I		4.560 I		4.580 I		4.600 I
CACGAGGGAG	CTTCCAGGGG	GAAACGCCTG	GTATCTTTAT	AGTCCTGTCG	GGTTTCGCCA	CCTCTGACTT	GAGCGTCGAT	TTTTGTGATG	CTCGTCAGGG
	4.620		4.640		4.660		4.680		4.700
GGGCGGAGCC	TATGGAAAAA	CGCCAGCAAC	GCGGCCTTTT	TACGGTTCCT	GGCCTTTTGC	төөссттттө	CTCACATGTT	стттсствсв	TTATCCCCTG
	4 700		4.740		4.700		4 700		4.000
	4.720		4.740		4.700		4.760		4.000
ATTCTGTGGA	TAACCGTATT	ACCGCCTTTG	AGIGAGCIGA	TACCGCTCGC	CGCAGCCGAA	CGACCGAGCG	CAGCGAGICA	GIGAGCGAGG	AAGCGGAAGA
	4.820		4.840		4.860		4.880		4.900
GCGCCTGATG	CGGTATTTTC	TCCTTACGCA	TCTGTGCGGT	ATTTCACACC	GCATATGGTG	CACTCTCAGT	ACAATCTGCT	CTGATGCCGC	ATAGTTAAGC
	4.920		4.940		4.960		4.980		5.000
CAGTATACAC	TCCGCTATCG	CTACGTGACT	GGGTCATGGC	TGCGCCCCGA	CACCCGCCAA	CACCCGCTGA	CGCGCCCTGA	COOCTTOTC	TGCTCCCGGC
CAGIAIACAG	1000014100	CIACCICACI	COULTRAILOGO	ICCCCCCCC	CAUCUUUUU	CACCOUCTER	000000104	0000011010	
	5.020		5.040 I		5.060 I		5.080 I		ROP_ORF
ATCCGCTTAC	AGACAAGCTG	TGACCGTCTC	CGGGAGCTGC	ATGTGTCAGA	GGTTTTCACC	GTCATCACCG	AAACGCGCGA	GGCAGCTGCG	GTAAAGCTCA
	5.120		5.140		5.160		5.180		ROP_ORF
TCAGCGTGGT	CGTGAAGCGA	TTCACAGATG	TCTGCCTGTT	CATCCGCGTC	CAGCTCGTTG	AGTTTCTCCA	GAAGCGTTAA	TGTCTGGCTT	CTGATAAAGC
	5.220 I		ROP_ORF 5.240		5.260 I		5.280 I		5.300 I
GGGCCATGTT	AAGGGCGGTT	TTTTCCTGTT	TGGTCACTGA	тесстссете	TAAGGGGGAT	TTCTGTTCAT	GGGGGTAATG	ATACCGATGA	AACGAGAGAG
	5.320		5.340		5.360		5.380		5.400
GATGCTCACG				TTACTGGAAC	GTTGTGAGGG	ТАААСААСТС	GCGGTATGGA	TGCGGCGGGA	CCAGAGAAAA
	ATACGGGTTA	CIGAIGAIGA	ACATGCCCGG						
	ATACGGGTTA	CIGAIGAIGA	ACATGCCCGG		E 480		5 490		5 500
ATCACTO 400	ATACGGGTTA 5.420	CIGAIGAIGA	5.440	GTCAAA TOO T	5.460 I		5.480 I	CTCCCTC++C	5.500
ATCACTCAGG	ATACGGGTTA 5.420 I GTCAATGCCA	GCGCGATGCA	ACATGCCCGG 5.440 I TAATGTGCCT	GTCAAATGGA	5.460 I CGAAGCAGGG	ATTCTGCAAA	5.480 I CCCTATGCTA	CTCCGTCAAG	5.500 I CCGTCAATTG
ATCACTCAGG	ATACGGGTTA 5.420 I GTCAATGCCA	GCGCGATGCA	5.440 TAATGTGCCT	GTCAAATGGA	5.460 I CGAAGCAGGG	ATTCTGCAAA	5.480 I CCCTATGCTA	CTCCGTCAAG	5.500 I CCGTCAATTG

alphaVmut-TMD = TMD Int_a5GP

JM = Juxtamembrane region

KanR = Aminoglycoside O-phosphotransferase

9.6.3 N-BLa 1.2

AGTCAGCCCC	ATACGATATA	AGTTGTAATT	CTCATGTTTG	ACAGCTTATC	AATCGATAAG	arabinose_O2_op AAACCAATTG	erator_region 80 I TCCATATTGC	ATCAGACATT	GCCGTCACTG
CGTCTTTTAC	TGGCTCTTCT	CGCTAACCAA	ACCGGTAACC	CCGCTTATTA	AAAGCATTCT	GTAACAAAGC	GGGACCAAAG	CCATGACAAA	
AAAAGTGTCT	arabir A T A A T C A C G G	nose_O1_operator_ CAGAAAAGTC	region 240 I CACATTGATT	ATTTGCACGG	CGTCACACT	AP_binding_site	TAGCATTTTT	ATCCATAAGA	300 I TTAGCGGATG
arabinose_ CTACCTGACG	minimal_promoter	CAACTCTCTA	340 I CTGTTTCTCC	ATACCCGTTT	360 I TTTTGGGCTC	TAGATAACGA	GGGCAAAAAA	npA signal peptide TGAAAAAGAC	400 I CGCGATTGCG
OmpA signal pept	ide 420 I CGCTGGCGGG	CTTTGCGACC	440 I GTGGCGCAGG	N-BLa CGGCGCCAGA	460 I AACGCTGGTG	AAAGTAAAAG	480 I ATGCTGAAGA	TCAGTTGGGT	500 I GCACGAGTGG
N-BLa GTTACATCGA	ACTGGATCTC	AACAGCGGTA	540 I AGATCCTTGA	GAGTTTTCGC	560 I CCCGAAGAAC	GTTTTCCAAT	580 I GATGAGCACT	TTTAAAGTTC	600 I TGCTATGTGG
N-BLa CGCGGTATTA	620 I TCCCGTGTTG	ACGCCGGGCA	640 I AGAGCAACTC	GGTCGCCGCA	660 I TACACTATTC	TCAGAATGAC	680 I TTGGTTGAGT	ACTCACCAGT	700 I CACAGAAAAG
N-BLa CATCTTACGG	720 I ATGGCATGAC	AGTAAGAGAA	740 I TTATGCAGTG	CTGCCATAAC	760 I CATGAGTGAT	AACACTGCGG	780 I CCAACTTACT	TCTGACAACG	800 I ATCGGAGGAC
N-BLa CGAAGGAGCT	AACCGCTTTT	TTGCACAACA	840 I TGGGGGATCA	TGTAACTCGC	860 I CTTGATCGTT	GGGAACCGGA	880 I GCTGAATGAA	GCCATACCAA	900 I ACGACGAGCG
N-BLa	920 I		940 I		Spel flexi	ble linker	980 I		JM ⁰⁰
TGACACCACG	ACGCCTGTAG	CAATGGCAAC	AACGTTGCGC	AAACTATTAA	CTGGCACTAG	TGGCAGCGGC	GGCGGCAGCG	GCGGCGGCAG	CGGTTCTGCT
Nhel		_	Apal		BamHI				
JM	alphaVmut-TM	D	1.040	TOCTOCTOCT		AAAGTGGGAG	1.080		inker 1.100
AATCGAGCTA	A	TETEGEGGTG		IGUIGUICUI	GGGGATCCAC	AAACIGGCAG	TTAATGTTCA	GUIGUUIGUU	GAAGCAGCAG
rigid linker	1.120		1.140		1.160		sfGFP 1.180		1.200 I
CGAAAGAAGC	CGCIGCCAAA	GAAGCGGCAG	CGAAAGAGGC	IGCCGCGAAA	GAGGCAGCAG	CTAAAGCCGC	AAGCAAAGGI	GAAGAACIGI	TTACCGGTGT
sfGFP	1.220)	1.240 I		1.260 I)	1.280 I		1.300 I
TGTTCCGATT	CTGGTTGAAC	TGGATGGTGA	TGTTAATGGC	CACAAATTCT	CAGTTCGTGG	TGAAGGCGAA	GGTGATGCAA	CCAATGGTAA	ACTGACCCTG
sfGFP	1.320 I)	1.340 I)	1.360 I)	1.380 I		1.400 I
AAATTTATCT	GTACCACCGG	CAAACTGCCG	GTTCCGTGGC	CGACCCTGGT	TACCACCCTG	ACCTATGGTG	TTCAGTGTTT	CAGCCGTTAT	CCGGATCATA
SIGFP TGAAACAGCA	1.420 CGATTTCTTC	AAATCTGCAA	1.440 I TGCCGGAAGG	TTATGTTCAA	1.460 GAACGTACCA	ТСТССТТТАА	1.480 I AGATGATGGC	ACCTATAAGA	1.500 I CCCGTGCCGA
sfGFP AGTTAAATTT	1.520 J GAAGGTGATA	CCCTGGTGAA	1.540 I TCGCATTGAA	CTGAAAGGCA	1.560 I TCGATTTCAA	AGAAGATGGT	1.580 I AATATCCTGG	GCCATAAACT	1.600 I GGAATATAAT
sfGFP	1.620	0	1.640		1.660	0	1.680		1.700
TTCAACAGCC	ACAACGTGTA	TATCACCGCA	GATAAACAGA	AGAATGGCAT	CAAAGCCAAC	TTTAAGATCC	GCCATAATGT	TGAAGATGGC	AGCGTTCAGC
SIGFP TGGCAGATCA	1.720 I TTATCAGCAG	AATACACCGA	1.740 I TTGGTGATGG	TCCGGTTCTG	1.760 I CTGCCGGATA	ATCATTATCT	1.780 I GAGCACCCAG	AGCGTTCTGA	1.800 I GCAAAGATCC
sfGFP	1.820	5	1.840	1	1.860)	1.880	FLAG-tag	1.900
GAATGAGAAA	CGTGATCACA	TGGTGCTGCT	GGAATTTGTT	ACCGCAGCAG	GTATTACCCA	TGGTATGGAT	GAACTGTATA	AAGGCGATTA	TAAAGATCAC
	Xhol								
FLAG-tag	1.920 7)	1.940 I		1.960)	1.980 I		2.000 I
GACGGCTAAC	TCGAGCACCA	CCACCACCAC	CACTGAGATC	CGGCTGCTAA	CAAAGCCCGA	AAGGAAGCTG	AGTTGGCTGC	TGCCACCGCT	GAGCAATAAC
T7_termina	100 2.020)	2.040 I		2.060 I)	2.080 I		2.100 I
TAGCATAACC	CCTTGGGGCC	TCTAAACGGG	TCTTGAGGGG	TTTTTGACC	GATGCCCTTG	AGAGCCTTCA	ACCCAGTCAG	CTCCTTCCGG	TGGGCGCGGG
GCATGACTAT	CGTCGCCGCA	CTTATGACTG	2.140 I TCTTCTTTAT	CATGCAACTC	2.160 I GTAGGACAGG	TGCCGGCAGC	GCTCTGGGTC	ATTTTCGGCG	2.200 I AGGACCGCTT
TCGCTGGAGC	2.220	тсоосстотс	GCTTGCGGTA	TTCGGAATCT	TGCACGCCCT	CGCTCAAGCC	2280 I TTCGTCACTG	GTCCCGCCAC	2.300 I CAAACGTTTC
	2.320	0	2.340	1	2.360)	2.380		2.400
GGCGAGAAGC	AGGCCATTAT	CGCCGGCATG	GCGGCCGACG	CGCTGGGCTA	сстсттсстс	GCGTTCGCGA	CGCGAGGCTG	GATGGCCTTC	CCCATTATGA
TTCTTCTCGC	2.420 I TTCCGGCGGC	ATCGGGATGC	2.440 I CCGCGTTGCA	GGCCATGCTG	2.460 I TCCAGGCAGG	TAGATGACGA	2480 I CCATCAGGGA	CAGCTTCAAG	2.500 I GATCGCTCGC
	2.520)	2.540	1	2.560)	2.580		2.600
GGCTCTTACC	AGCCTAACTT	CGATCACTGG	ACCGCTGATC	GTCACGGCGA	TTTATGCCGC	CTCGGCGAGC	ACATGGAACG	GGTTGGCATG	GATTGTAGGC
	2.620)	2.640	1	2.660)	2.680		2.700
GCCGCCCTAT	ACCTTGTCTG	сстссссссс	TTGCGTCGCG	GTGCATGGAG	CCGGGCCACC	TCGACCTGAA	TGGAAGCCGG	CGGCACCTCG	CTAACGGATT
CACCACTCCA	AGAATTGGAG	CCAATCAATT	CTTGCGGAGA	ACTGTGAATG	CGCAAACCAA	CCCTTGGCAG	AACATATCCA	тсссстсссс	CATCTCCAGC

	2.820		2.840		2.860		2.880		2.900
AGCCGCACGC	GGCGCATCTC	GGGCAGCGTT	GGGTCCTGGC	CACGGGTGCG	CATGATCGTĠ	стсстбтсбт	TGAGGACCCG	GCTAGGCTGG	CGGGGTTGCC
	2.920 I		2.940 I		2.960 I		2.980 I		3.000 I
TTACTGGTTA	GCAGAATGAA	TCACCGATAC	GCGAGCGAAC	GTGAAGCGAC	TGCTGCTGCA	AAACGTCTGC	GACCTGAGCA	ACAACATGAA	TGGTCTTCGG
TTTCCGTGTT		TGGAAACGCG		ACGIGCIGCI		GCAACAGAGA		CCGGTGATAC	
1110001011	3 120	IGGAAACGCG	3 140	ACGIGCIGCI	3 160	GCAACAGAGA	3180	CCGGIGATAC	3200
TGACTGAGAG	TCAACGCCAT	GAGCGGCCTC	ATTTCTTATT	CTGAGTTACA	ACAGTCCGCA	ссостотссо	GTAGCTCCTT	CCGGTGGGCG	CGGGGCATGA
	3.220		3.240		3.260		3.280		3.300
CTATCGTCGC	CGCACTTATG	ACTGTCTTCT	TTATCATGCA	ACTCGTAGGA	CAGGTGCCGG	CAGCGCCCAA	CAGTCCCCCG	GCCACGGGGC	CTGCCACCAT
	3.320 I		3.340 I		3.360 I		3.380 I		3.400 I
ACCCACGCCG	AAACAAGCGC	CCTGCACCAT	TATGTTCCGG	ATCTGCATCG	CAGGATGCTG	CTGGCTACCC	TGTGGAACAC	CTACATCTGT	ATTAACGAAG
	3.420 		3.440 I		3.460 		3.480 I		3.500 I
CGUIAACCGI		CICIGGGAGG	CAGAATAAAT	GAICATAICG		CUICCALGGG	GAGAGECTGA	GUAAAUIGGU	CICAGGCATT
TGAGAAGCAC	ACGGTCACAC	TGCTTCCGGT	AGTCAATAAA	CCGGTAAACC	AGCAATAGAC	ATAAGCGGCT	ATTTAACGAC	CCTGCCCTGA	ACCGACGACC
	3.620		3.640		3.660		3.680		3.700
GGGTCGAATT	TGCTTTCGAA	TTTCTGCCAT	TCATCCGCTT	АТТАТСАСТТ	ATTCAGGCGT	AGCACCAGGC	GTTTAAGGGC	АССААТААСТ	GCCTTAAAAA
	3.720		3.740		3.760		3.780		CAT
AATTACGCCC	CGCCCTGCCA	CTCATCGCAG	TACTGTTGTA	ATTCATTAAG	CATTCTGCCG	ACATGGAAGC	CATCACAGAC	GGCATGATGA	ACCTGAATCG
	3.820 I		3.840 I		3.860 I		3.880 I		CAT
CCAGCGGCAT	CAGCACCTTG	TCGCCTTGCG	ΤΑΤΑΑΤΑΤΤΤ	GCCCATGGTG	AAAACGGGGGG	CGAAGAAGTT	GTCCATATTG	GCCACGTTTA	AATCAAAACT
COTCANACTO	3.920	TCCCTCACAC	3.940 I	TTOTOAATAA	3.960	CAAATACCCC	3.980	COTAACACCC	CACATOTICS
GGIGAAACIC	ACCCAGGGAT	TOGCTGAGAC	GAAAAACATA	TICICAATAA	ACCCITIAGG	GAAATAGGCC	AGGITTTCAC	CGTAACACGC	CACATCITIGC
GAATATATGT	GTAGAAACTG	CCGGAAATCG	TCGTGGTATT	CACTCCAGAG	CGATGAAAAC	GTTTCAGTTT	GCTCATGGAA	AACGGTGTAA	CAAGGGTGAA
	4.120		4.140		4.160		4.180		CAT
CACTATCCCA	TATCACCAGC	TCACCGTCTT	TCATTGCCAT	ACGGAATTCC	GGATGAGCAT	TCATCAGGCG	GGCAAGAATG	TGAATAAAGG	CCGGATAAAA
	4 000								
	4.220		4.240		4.260		4.280		CAI
CTTGTGCTTA	TTTTTCTTTA	CGGTCTTTAA	4.240 I AAAGGCCGTA	ATATCCAGCT	4.260	GTTATAGGTA	CATTGAGCAA	CTGACTGAAA	TGCCTCAAAA
CTTGTGCTTA	4.220 I TTTTTCTTTA 4.320 I	CGGTCTTTAA	4.240 I AAAGGCCGTA 4.340 I	ATATCCAGCT	4.260 1 GAACGGTCTG 4.36	GTTATAGGTA CAT	4.280 I CATTGAGCAA 4.380 I	CTGACTGAAA	TGCCTCAAAA
CTTGTGCTTA TGTTCTTTAC	4.220 I TTTTTCTTTA 4.320 I GATGCCATTG	CGGTCTTTAA GGATATATCA	4.240 I AAAGGCCGTA 4.340 I ACGGTGGTAT	ATATCCAGCT	4.260 I GAACGGTCTG 4.36 I TTTTTTCTCC	GTTATAGGTA CAT ATTTTAGCTT	LACTTGAGCAA 4.380 I CCTTAGCTCC	СТGАСТGААА ТGAAAATCTC	TGCCTCAAAA 4.400 1 GATAACTCAA
CTTGTGCTTA TGTTCTTTAC	4.20 TTTTTCTTTA 4.320 I GATGCCATTG 4.420 I 4.420 I 4.420 I 4.20 I I I I I I I I I I I I I	CGGTCTTTAA	4.240 1 AAAGGCCGTA 4.340 1 ACGGTGGTAT 4.440 1	ATATCCAGCT	4.280 I GAACGGTCTG 4.38 I TTTTTTCTCC 4.460 I	GTTATAGGTA CAT ATTTTAGCTT	4,280 CATTGAGCAA 4,3800 CCTTAGCTCC 4,4800 1	CTGACTGAAA TGAAAATCTC	CA1 TGCCTCAAAA 4.400 J GATAACTCAA 4.500
CTTGTGCTTA TGTTCTTTAC AAAATACGCC	4.20 TTTTTCTTTA 4.320 GATGCCATTG GATGCCATTG CGGTAGTGAT	CGGTCTTTAA GGATATATCA CTTATTTCAT	4.240 AAAGGCCGTA 4.340 ACGGTGGTAT 4.440 TATGGTGAAA	ATATCCAGCT ATCCAGTGAT GTTGGAACCT	4.260 I GAACGGTCTG 4.36 TTTTTTTCTCC 4.460 I CTTACGTGCC	GTTATAGGTA CAT ATTTTAGCTT GATCAACGTC	4,280 CATTGAGCAA 4,380 I CCTTAGCTCC 4,480 I TCATTTTCGC	CTGACTGAAA TGAAAATCTC CAAAAGTTGG	GATAACTCAA GATAACTCAA 4.500 CCCAGGGCTT
CTTGTGCTTA TGTTCTTTAC AAAATACGCC	4 220 TTTTTCTTTA 4 220 CGGTAGTGGT 4 420 4 420 CGGTAGTGGT 4 420 4 420 CGGTAGTGGT 4 400 CGGT 4 400 CGGT C	GGATATATCA CTTATTTCAT	4 240 1 AAAGGCCGTA 4.340 1 ACGGTGGTAT TATGGTGAAA 4.540 TTATTCTGCG	ATATCCAGCT ATCCAGTGAT GTTGGAACCT	4 260 I GAACGGTCTG 4 360 TTTTTTCTCC CTTACGTGCC 4 460 CCGTCACGGGC 4 560 CCGTCACGGG	GTTATAGGTA ATTTTAGCTT GATCAACGTC	4 280 1 CATTGAGCAA 4380 1 CCTTAGCTCC 4480 1 TCATTTTCGC 4580 1 GCGCAAAGTG	CTGACTGAAA TGAAAATCTC CAAAAGTTGG	GATAACTCAA 4.400 GATAACTCAA 4.500 CCCAGGGCTT 4.600 1GCTGCCAAC
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA	4 220 TTTTTCTTTA 4.220 GATGCCATTG 4.420 CGGTAGTGAT 4.520 1 ACAGGGACAC 4.520 1 ACAGGGACAC 4.520 1 4.500 1 4.500	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT	4.440 1 AAAGGCCGTA 4.340 1 ACGGTGGTAT TATGGTGAAA 4.540 1 TTATTCTGCG 4.640	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT	4 280 I GAACGGTCTG 4 380 I TTTTTTCTCC 4 460 CTTACGTGCC 4 560 I CCGTCACAGG 4 660	GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG	4 280 T CATTGAGCAA CCTTAGCTCC 4480 TCATTTTCGC TCATTTTCGC GCGCAAAGTG 4680	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA	GATAACTCAAA GATAACTCAA CCCAGGGCTT 4.600 1 TGCTGCCAAC 4.700
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT	4 200 T TTTTTCTTTA 4.200 GATGCCATTG 4.200 CGGTAGTGAT 4.500 ACAGGGACAC 4.500 ACAGGGACAC 4.500 ACAGGGACAC	GGATATATCA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG	4.240 1 AAAGGCCGTA 4.340 1 ACGGTGGTAT 4.540 TTATTCTGCG 4.640 AGGTGCTCCA	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG	4 260 I GAACGGTCTG 4 360 I TTTTTTCCCC 4 460 I CCGTCACAGG 4 660 I TTTCTATCAG G	GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC	4 280 T CATTGAGCAA 4 380 TCATTGACTCC 4 480 TCATTTTCGC 4 450 TGGTCAGCTA	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA CTGACGGGGT	CAI TGCCTCAAAA 4400 GATAACTCAA 4500 CCCAGGGCTT 4600 TGCTGCCAAC 4700 J GGTGCGTAAC
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT	4 200 TTTTTCTTTA 4.200 GATGCCATTG CGGTAGTGAT ACAGGGACAC AGTGTATGAT 4.200 AGTGTATGAT 4.200 AGTGTATGAT	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG	4 240 1 AAAGGCCGTA 4.340 1 ACGGTGGTAT 4.440 TATGGTGAAA 4.540 TTATTCTGCG 4.640 AGGTGCTCCA 4.740	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG	4 260 GAACGGTCTG 4.360 CTTACGTGCC 4.660 CCGTCACAGG 4.660 CCGTCACAGG 4.660 TTTCTATCAG 4.660 A 760	GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC	4 280 T CATTGAGCAA 4 380 T CCTTAGCTCC 4 480 T GCGCAAAGTG GCGCAAAGTG 4680 TGTTCAGCTA 4680 4680 TGTTCAGCTA	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA CTGACGGGGT	CAL TGCCTCAAAA 4.400 GATAACTCAA 4.500 CCCAGGGCTT 4.600 TGCTGCCAAC 4.700 GGTGCGTAAC 915a_ORI
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCA	4.20 TTTTTCTTTA 4.20 GATGCCATTG CGGTAGTGAT ACAGGGACAC AGTGTATGAT 4.20 1 ACAGGGACAC 4.20 1 CGGCCGGACA	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG	4.240 1 AAAGGCCGTA 4.340 1 ACGGTGGTAT 4.440 1 TATGGTGAAA 4.540 1 TATTCTGCG 4.640 1 AGGTGCTCCA 4.640 1 CGGGAGTGTAT	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC	4 280 I GAACGGTCTG 4.860 CTTACGTGCC CCGTCACAGG CCGTCACAGG TTTCTATCAG 4.660 TTTCTATCAG 4.660 TTTCTATCAG	GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG	44200 TCATTGAGCAA 43800 CCTTAGCTCC 44800 TCATTTTCGC GCGCAAAGTG GCGCAAAGTG TGTTCAGCTA 47800 TGTCAGTGAA	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGGTGA CTGACGGGGT GTGCTTCATG	GATAACTCAAAA 4400 1 GATAACTCAA 4500 1 CCCCAGGGCT TGCCGCCAAC 4700 1 GGTGCGTAAC 4700 1 GGTGCGTAAC
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCA	4 420 TTTTTCTTTA 4.320 GATGCCATTG 4.420 T CGGTAGTGAT 4.520 T ACAGGGACAC 4.620 T AGTGTATGAT 4.520 T CGCCCGGACA 4.20 T CCGCCGGACA 4.20 T CCGCCGGACA	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG	4.240 AAAGGCCGTA 4.340 ACGGTGGTAT 4.40 1 TATGGTGAAA 4.540 1 TTATTCTGCG 4.640 1 AGGTGCTCCA 4.640 1 CGGAGTGTAT 4.640 1 4.640 1 4.640 1 4.640 1 4.640 1 4.640 1 4.640 1 4.640 1 4.640 1 4.640 1 4.640 1 1 4.640 1 1 4.640 1 1 4.640 1 1 4.640 1 1 1 1 1 1 1 1 1 1 1 1 1	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC	4 280 4 280 1 GAACGGTCTG 4.860 4.860 4.860 4.860 1 1 1 1 1 1 1 1 1 1 1 1 1	GTTATAGGTA GAT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG	4 280 T CATTGAGCAA 4380 T CCTTAGCTCC 4480 T CCTTAGCTCA T GCGCAAAGTG 4680 T GCGCAAAGTG 4680 T GTTCAGCTA 4780 T GTCAGTGAA 4780 T GTCAGTGAA	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA CTGACGGGGT GTGCTTCATG	GATAACTCAAAA 4.400 GATAACTCAA CCCAGGGCTT TGCTGCCAAC 4.600 TGCTGCCAAC 4.700 TGCTGCCAAC 915a_ORT TGGCAGGAGA p15a_ORT
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCA AAAAAGGCTG	4 420 TTTTTCTTTA 4.320 GATGCCATTG CGGTAGTGAT 4.220 ACAGGGACAC 4.220 AGTGTATGAT 4.220 CCGCCGGACA 4.220 CCGCCGGACA 4.220 CCGCCGGACA 4.220 CCGCCGGACA	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAT	4.240 AAAGGCCGTA 4.340 ACGGTGGTAT 4.40 1 TATGGTGAAA 4.540 1 TTATTCTGCG 4.640 1 AGGTGCTCCA 4.640 1 CGGAGTGTTC 4.740 1 AGGTGCTCCA 4.740 1 AGGTGCTCCA 4.740 1 1 4.740 1 1 1 1 1 1 1 1 1 1 1 1 1	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC GGATATATTC	4 280 1 GAACGGTCTG 4.360 1 TTTTTTCCC 4.560 1 CCGTCACAGG 4.560 1 CCGTCACAGG 4.560 1 CCGTCACAGG 4.760 1 TTTCTATCAG 4.760 1 CCGTCACAGG 4.760 1 CCGTCACAGG 4.760 1 1 1 1 1 1 1 1 1 1 1 1 1	GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT	CATTGAGCAA 4380 CCTTAGCTCC 1 CCTTAGCTCC 1 TCATTTTCGC 4480 1 CCCCAAAGTG 4680 1 TGTTCAGCTA 4780 1 TGTCAGCTAA 4780 1 CGCCACGCTC	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA CTGACGGGGT GTGCTTCATG GGTCGTTCGA	GATAACTCAAAA 4.400 4.500 CCCAGGGCT TGCTGCCAAC 4.600 1 TGCTGCCAAC 4.700 1 GGTGCGTAAC 915a_OR TGGCGGGGAGA
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCA AAAAAGGCTG	4.20 TTTTTCTTTA 4.30 GATGCCATTG CGGTAGTGAT 4.420 ACAGGGACAC ACAGGGACAC 4.20 I AGTGTATGAT 4.70 I CCGCCGGACA 4.20 I CCGCCGGACA 4.20 I CCGCCGGACA 4.20 I CCGCCGGACA 4.20 I CCGCCGGACA	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAT	4 240 4 240 AAAGGCCGTA 4 340 1 ACGGTGGTAT 4 440 1 TATGGTGAAA 4 540 1 TTATTCTGCG 4 540 1 AGGTGCTCCA 4 740 1 CGGAGTGTAT 4 840 1 AGGTGATAC 4 340 1 1 1 1 1 1 1 1 1 1 1 1 1	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC GGATATATTC	4 280 1 GAACGGTCTG 4.36 1 TTTTTTCCC 4.460 1 CTTACGTGCC 4.500 1 CCGTCACAGG 4.600 1 TTTCTATCAGG 4.600 1 CGCTCCCACAGG 4.600 1 CGCTCCCACAGG 4.600 1 CGCTCCCACAGG 4.600 1 CGCTCCCACAGG 4.600 1 CGCTCCCACAGG 4.600 1 CGCTCCCACAGG 4.600 1 CGCCACAGGTCCC 4.600 1 CGCCACAGGTCCC 4.600 1 CGCCACAGGTCCC 4.600 1 CGCCACAGGCCACAGG 4.600 1 CGCCACAGGCCACAGG 4.600 1 CGCCACAGGCCACAGG 4.600 1 CGCCACAGGCCACAGG 4.600 1 CGCCACAGGCCACAGG 4.600 1 CGCCACAGGCCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGGCACAGGCACAGG 4.600 1 CGCCACAGGCACAGG 4.600 1 CGCCACAGGCACAGGCACAGGCACAGGCACAGG 4.600 1 CGCCACAGGCAGGCAGGCAGGCAGGCAGGCAGGCAGGCA	GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT	CATTGAGCAA 4380 CCTTAGCTCC 1 CCTTAGCTCC 1 TCATTTTCGC 4480 1 CGCCAAAGTG 4680 1 TGTTCAGCTA 4780 1 TGTCAGTGAA 4800 1 CGCTACGCTC 49800 1	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGGTGA CTGACGGGGT GTGCTTCATG GGTCGTTCGA	CCCAGGGCTAAAA 4.400 4.400 4.500 CCCAGGGCTT 4.600 TGCTGCCAAC 4.700 GGTGCGTAAC p15a_ORI CTGCGGCGAG CTGCGGCGAG
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCA AAAAAGGCTG CGGAAATGGC	4 200 TTTTTCTTTA 4 320 GATGCCATTG 4 420 CGGTAGTGAT 4 420 ACAGGGACAC 4 420 AGTGTATGAT 4 720 CCGCCGGACA 4 420 CCGCCGGACA 4 420 CACCGGTGCG 1 CACCGGTGCG 4 420 CACCGGTGCG	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAT GGCGGAGATT	4 240 4 240 4 340 4 340 1 ACGGTGGTAT 4 440 1 TATGGTGAAA 4 540 1 TTATTCTGCG 4 640 1 AGGTGCTCCA 4 740 CGGAGTGTAT 4 840 1 AGGTGATACA 4 340 1 1 1 1 1 1 1 1 1 1 1 1 1	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC GGATATATTC TGCCAGGAAG	4 280 GAACGGTCTG 4 480 1 TTTTTTTCTCC 4 460 1 CTTACGTGCC 4 560 1 CCGTCACAGG 4 660 1 TTTCTATCAG 4 760 1 TATGTTGGCA 4 760 1 CGCTTCCTCG 4 60 1 1 1 1 1 1 1 1 1 1 1 1 1	GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT GGGAAGTGAG	CATTGAGCAA 4380 CCTTAGCTGC 1 CCTTAGCTGC 4480 TCATTTTCGC GCGCAAAGTG 4580 TGTTCAGCTA 4780 TGTCAGTGAA 4880 CGCTACGCTC 4880 1 CGCTACGCTC 4880 1 CGCTACGCTC	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGGTGA CTGACGGGGT GTGCTTCATG GGTCGTTCGA P ¹ CAAAGCCGTT	CCCAGGGCT GATAACTCAAA 4.500 CCCAGGGCT CCCAGGGCT TGCTGCCAAC 4.700 GGTGCGTAAC p15a_OR CTGCGGCGAG p15a_OR CTGCGGCGAG
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCA AAAAAGGCTG CGGAAATGGC CTCCGCCCCC	4 200 TTTTTCTTTA 4.200 GATGCCATTG 4.200 TCGGTAGTGAT 4.200 AGTGTATGAT 4.200 AGTGTATGAT 4.200 AGTGTATGAT 4.200 CCGCCGGACA 4.200 TTACGAACGG 1 TTACGAACGG	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAT GGCGGAGATT	4 240 1 AAAGGCCGTA 4.340 1 ACGGTGGTAT 4.440 1 TTATTCTGCG 4.640 AGGTGCTCCA 4.640 CGGAGTGTAT 4.840 ATGTGATACA 4.840 1 ATGTGATACA 4.840 CGGAGGCTCAA	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC GGATATATTC TGCCAGGAAG ATCAGTGGTG	4 280 1 GAACGGTCTG 4.360 CTTACGTGCC 4.600 CCGTCACAGG 4.600 1 TTTCTATCAG 4.600 1 TTCCAC 4.600 1 TTCCAC 4.600 1 TTCCAC 4.600 1 TTCCAC 4.600 1 TTCCAC 4.600 1 TTCCAC 4.600 1 TTCCAC 4.600 1 TTCCAC 4.600 1 TTCCAC 4.600 1 TTCCAC 4.600 1 TTCCACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT GGGAAGTGAG	4 280 T CATTGAGCAA 4 380 T CCTTAGCTCC 4 480 T GCGCAAAGTG 4 480 TGTTCAGCTA 4 480 TGTTCAGCTA 4 480 TGTCAGTGAA 4 680 TGTCAGCGCG 4 680 CGCTACGCTC	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGGTGA CTGACGGGGT GTGCTTCATG GGTCGTTCGA P1 CAAAGCCGTT GGCGTTTCCC	CCAGGGGGGG CCCAGGGCTT CCCCAGGGCTT CCCCAGGGCTT CCCCAGGGCTT CCCCAGGGCT CCCGGCGAGC P15a_ORI CCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCA AAAAAGGCTG CGGAAATGGC CTCCGCCCCC	4 200 TTTTTCTTTA 4.200 GATGCCATTG 4.200 T CGGTAGTGAT ACAGGGACAC 4.200 CGGCCGGACA 4.200 CCGCCGGACA 4.200 CCGCCGGACA 4.200 TTACGAACGG C CCGCCGGACA	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAT GGCCGGAGATT TCACGAAATC	4 240 1 AAAGGCCGTA 4.340 1 ACGGTGGTAT 4.440 1 TATGGTGAAA 4.540 4.640 AGGTGCTCCA 4.640 CGGAGTGTAT 4.640 CGGAGTGTA	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC GGATATATTC TGCCAGGAAG ATCAGTGGTG	4 280 GAACGGTCTG 4.360 CTTACGTGCC 4.600 CCGTCACAGG TTTCTATCAG 4.600 TTTCTATCAG 4.600 TTTCTATCAG 4.600 TTTCTATCAG 4.600 CGCTTCCTCG 4.600 1 CGCTTCCTCG 4.600 1 CGCTTCCTCG 4.600 1 CGCTTCCTCG 4.600 1 CGCTTCCTCG 4.600 1 CGCTCCCCC 4.600 1 CGCTCCCCC 4.600 1 CGCTCCCCC 4.600 1 CGCTCCCCCC 4.600 1 CGCTCCCCCC 4.600 1 CGCTCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT GGGAAGTGAG ACAGGACTAT	4 280 T CATTGAGCAA 4 380 T CCTTAGCTCC 4 480 T CCTTAGCTA GCGCAAAGTG 4 680 TGTTCAGCTA 4 780 TGTCAGTGAA 4 780 TGTCAGTGAA 4 480 TGTCAGCTGA 1 CGCTACGCTC 4 480 TGTCAGCGCGCG 4 480 CGCTACGCTC 1 AGGGCCGCGCG AAAGATACCA	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGGTGA CTGACGGGGT GTGCTTCATG GGTCGTTCGA P1 CAAAGCCGTT GGCGTTTCCC	CALL TGCCTCAAAA 4400 TGCTAACTCAA 4500 TGCCAGGGCTT 4600 TGCCGCCAAC 4700 GGTGCGTAAC 4700 GGTGCGTAAC 4700 GGTGCGTAAC 4700 TGCCGGCGAAC 4700 TGCCGGCGAAC 15a_0R1 TTGCCACAGGAGA 15a_0R1 TTTCCATAGG 5.100 CCTGGCGGCGT
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCTG CGGAAATGGC CTCCGCCCCC CCCTCGTGCG	4 200 TTTTTCTTTA 4.200 I GATGCCATTG 4.200 I CGGTAGTGAT ACAGGGACAC 4.200 I CGGCCGGACA 4.200 I CCGCCGGACA 4.200 I CCGCCGGACA 4.200 I CCGCCGGACA I CACCGGTGCG I CTCCCGCGACA	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAT GGCGGAGATT TCACGAAATC CCTGCCTTTC	4 240 1 AAAGGCCGTA 4.340 1 ACGGTGGTAT 4.440 1 TATGGTGAAA 4.540 CGGAGTGTAT 4.640 CGGAGTGTAT 4.640 CGGAGTGTACA 4.640 CGGAAGA 4.540 TCCTGGAAGA 5.140 GGTTTACCGG	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG GGATATATTC TGCCAGGAAG ATCAGTGGTG TGTCATTCCG	4 280 4 280 1 GAACGGTCTG 4.860 CTTACGTGCC 4.600 CCGTCACAGG 4.600 TTTCTATCAG 4.600 TTTCTATCAG 4.600 TATGTTGGCA 4.600 TATGTTGGCA 4.600 CGCTTCCTCG 4.600 CGCTTCCTCG 4.600 5.000 CGCGAAACCCG 5.100 CTGTTATGCC	GTTATAGGTA GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT GGGAAGTGAG ACAGGACTAT	4 420 CATTGAGCAA 4 380 CCTTAGCTCC 4 480 1 CCTTAGCTA 4 580 1 CCTTAGCTA 4 580 1 CCTTAGCTA 4 680 1 CGCTACGCTC 4 680 1 CGCTACGCTC 1 1 1 1 1 1 1 1 1 1 1 1 1	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGGTGA CTGACGGGGT GTGCTTCATG GGTCGTTCGA P ¹ CAAAGCCGTT GGCGTTTCCC CCTGACACTC	CALL TGCCTCAAAA 4400 GATAACTCAAA CCCCAGGGCTT TGCTGCCAAC 4500 TGCTGCCAAC 4700 GGTGCGTAAC 4700 GGTGCGTAAC 4700 TGCCGGCGAG TGCGGGGGAG TTGCCGGGGAG TTTCCATAGG 5100 CCTGGCGGCGAG
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGGCTG CGGAAATGGC CTCCGCCCCC CCCTCGTGCG	4 200 TTTTTCTTTA 4.320 I GATGCCATTG 4.220 I CGGTAGTGAT ACAGGGACAC AGTGTATGAT ACAGGGACAC ACCCGGACA 4.20 I CCGCCGGACA 4.20 I CCGCCGGACA 4.20 I CCGCCGGACA 4.20 I CCGCCGGACA CCGCCGGACA 4.20 I CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCGCCGGACA CCCCCGGACA CCCCGGACA CCCCGGACA CCCCGGACA CCCCGGACA CCCCGGACA CCCCGCGACA CCCCGCGGACA CCCCGGACA CCCCGGACA CCCCGGACA CCCCGGACA CCCCGGACA CCCCGGACA CCCCGGACA CCCCGGACA CCCCGCGGACA CCCCGGACA CCCGCCGGACA CCCGCCGGACA CCCCGCGGACA CCCGCCGGACA CCCGCCGGACA CCCGCCGGACA CCCGCCGGACA CCCGCCGGACA CCCGCCGGACA CCCGCCGGACA CCCGCCGGACA CCCCGCCGGACA CCCGCCGGACA CCCGCCGGACA CCCGCCGGACA CCCGCCGGACA CCCCGCCGGACA CCCCCGCGGACA CCCCGCCGGACA CCCCGCCGGACA CCCCCGCGGACA CCCCCGCGGACA CCCCCGCGGACA CCCCCGCCGGACA CCCCCCGCCGGACA CCCCCCGCCGGACA CCCCCGCCGGACA CCCCCCCGCCGGACA	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAT GGCCGGAGATT TCACGAAATC CCTGCCTTTC	4 240 4 240 1 AAAGGCCGTA 4.340 1 ACGGTGGTAT 4.440 1 TATGGTGAAA 4.540 1 TTATTCTGCG 4.640 1 AGGTGCTCCA 4.740 1 CGGAGTGTAT 4.840 1 CGGAGTGTACA 4.940 TCCTGGAAGA 5.140 GGTTTACCGG 5.240	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG GGATATATTC GGGATATATTC TGCCAGGAAG ATCAGTGGTG TGTCATTCCG	4 280 GAACGGTCTG 4.360 CTTACGTGCC CTTACGTGCC CCGTCACAGG TTTCTATCAG 4.600 TTTCTATCAG 4.600 TATGTTGGCA 4.600 TATGTTGGCA 4.600 TATGTTGGCA 4.600 TATGTTGGCA 4.600 TATGTTGGCA 4.600 TATGTTGGCA 4.600 TATGTTGGCA 4.600 TATGTTGGCA 4.600 TATGTTGGCA 4.600 TATGTTGGCA 4.600 TATGTTGGCA 4.600 TATGTTCTCC CGCTCCCCG ATACTTAACA	GTTATAGGTA GATTATAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG GCGAAGTGAGG ACAGGACTAT CGCGTTTGTC	CATTGAGCAA 4380 CCTTAGCTCC 4480 TCATTTTCGC GCGCAAAGTG GCGCAAAGTG TGTTCAGCTA 4680 1 GGCTACGCTC 4680 1 GGCTACGCTC 4680 1 AGGGCCGCGG AAAGATACCA 5180 TCATTCCACG	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA CTGACGGGGT GTGCTTCATG GGTCGTTCGA P ¹ CAAAGCCGTT GGCGTTTCCC CCTGACACTC	CALL TGCCTCAAAA 4.400 GATAACTCAA CCCAGGGCTT TGCTGCCAAC 4.700 GGTGCGTAAC P15a_ORI TGGCGGGGAG 5.000 TTTCCATAGG 5.100 CCTGGCGGCT 5.200 AGTTCCGGGT 5.200 CCTGCGGCGAC
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGGCTG CGGAAATGGC CTCCGCCCCC CCCTCGTGCG AGGCAGTTCG	4 420 TTTTTCTTTA 4.320 GATGCCATTG 4.220 CGGTAGTGAT 4.220 ACAGGGACAC 4.200 1 CGCCCGGACA 4.200 1 CCGCCGGACA 5.200 CCGCCGGCGACA	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAT GGCCGGAGATT TCACGAAATC CCTGCCTTTC GACTGTATGC	4 240 4 240 AAAGGCCGTA 4.340 TATGGTGAAA 4.540 TTATTCTGCG 4.640 1 TTATTCTGCG 4.640 1 AGGTGCTCCA 4.740 1 CGGAGTGTAT 4.840 1 CGGAGTGTAT 4.840 1 CGGAGTGTACA 4.940 1 CGGAGTGTACA 4.940 1 CGGAGTGTCCA 4.940 1 CGGAGTGTACA 4.940 1 CGGAGTGTCCA 4.940 1 CGGAGTGCTCA 4.940 1 CGGAGTGCTCA 4.940 1 CGGAGTGCTCCA 4.940 1 CGGAGGCTCCA 4.940 1 CGGAGGCCCCA 4.940 1 CGGAGGCCCCA 4.940 1 CGGAGCCCCA 5.140 CGGCCCCCA 5.140 CGGAGCCCCCC	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC GGATATATTC TGCCAGGAAG ATCAGTGGTG TGTCATTCCG CGTTCAGTCC	4 280 GAACGGTCTG 4.360 TTTTTTCCC 4.760 CTTACGTGCC 4.500 CCGTCACAGG TTTCTATCAG 4.760 TTTCTATCAG 4.760 TTTCTATCAG 4.760 CGCTTCCTCG 4.500 CGCTTCCCC 4.500 CGCTCCCCG 5.600 CGCTCCCCG 5.500 CGCTCCCCG 5.500 CGCTCCCCG	GTTATAGGTA GATTATAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT GGGAAGTGAG ACAGGACTAT CGCGTTTGTC CCTTATCCGG	CATTGAGCAA 4390 CCTTAGCTCC 4480 TCATTTTCGC 4580 1 CCTTAGCTGC 4580 1 GCGCAAAGTG 4580 1 GCGCAAGCTA 4780 1 GCGCACGCTG 4680 1 GCGCACGCTG 4680 1 GCGCACGCGG 1 AGGGCCGCGGG 1 AGGGCCGCGGG 1 AGGGCCCGCGG 1 AGGGCCGCGG 1 AGGGCCCGCGG 1 AGGGCCCGCGG 1 AGGGCCCGCGG 1 AGGGCCCGCGG 1 AGGGCCCGCGG 1 AGGGCCGCGG 1 AGGGCCGCGG 1 AGGGCCGCGGG 1 AGGGCCGCGGG 1 AGGGCCGCGGG 1 AGGGCCGCGGG 1 AGGGCCGCGGG 1 AGGGCCGCGGG 1 AGGGCCGCGGG 1 AGGGCCGCGGG 1 AGGGCCGCGGG 1 AGGGCCGCGGG 1 AGGGCCGCGG 1 AGGGCCGCGG 1 AGGGCCGCGG 1 AGGGCCGCGG 1 AGGGCCGCGGG 1 AGGGCCGCGG 1 AGGGCCGCGG 1 AGGGCCGCGG 1 AGGGCCGCGG 1 AGGGCCGCGG 1 AGGGCCGCGCGG 1 AGGGCCGCGCGG 1 AGGGCCGCGCGG 1 AGGGCCGCGCG 1 AGGGCCGCGCG 1 AGGGCCGCGCG 1 AGGGCCGCGCGCG 1 AGGGCCGCGCGCGCG 1 AGGGCCGCGCGCGCGCGCG 1 AGGCCGCGCGCGCGCGCGCG 1 AGGCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 1 AGGCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGC	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA CTGACGGGGT GTGCTTCATG GGTCGTTCGA GGCGTTTCCC CCTGACACTC CTTGAGTCCA	CALL TGCCTCAAAA 44.00 GATAACTCAA CCCAGGGCT TGCTGCCAAC 4.00 TGCTGCCAAC 915a_ORI TGGCAGGAGA 915a_ORI CTGCGGCGAG 5.00 TTTCCATAGG CCTGCCGCGCT 5.00 CCTGCCGGCT AGTTCCGGGT
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCA AAAAAGGCTG CGGAAATGGC CTCCGCCCCC CCCTCGTGCG AGGCAGTTCG	4 420 TTTTTCTTTA 4.320 GATGCCATTG 4.420 CGGTAGTGAT 4.420 1 ACAGGGACAC 4.420 1 ACAGGGACAC 4.420 1 CGCCCGGACA 4.720 1 CCGCCGGCGACA 4.720 1 CCGCCGGACA 5.700 1 CCGCCGCGACA 1 CCGCCGGACA 1 CCGCCGCGACA 1 CCGCCGCGACA 1 CCGCCGGACA 1 CCGCCGCGACA 1 CCGCCGCGACA 1 CCGCCGCGACA 1 CCGCCGCGCGCGCA 1 CCGCCGCGCA 1 CCGCCGCGCA 1 CCGCCGCGCA 1 CCGCCGCGCA 1 CCGCCGCA 1 CCGCCGCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCA 1 CCGCCGCGCA 1 CCGCCGCACA 1 CCGCCG	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAT GGCCGGAGATT TCACGAAATC CCTGCCTTTC GACTGTATGC	4 240 4 340 AAAGGCCGTA 4 340 1 ACGGTGGTAT 4 450 1 TATGGTGAAA 4 540 1 TTATTCTGCG 4 640 1 AGGTGCTCCA 4 740 1 CGGAGTGTAT 4 640 1 AGGTGCTCCA 4 740 1 AGGTGCTCCA 4 740 1 AGGTGCCCCCA 4 740 1 AGGTGCTCCA 4 740 1 AGGTGCCCCCA 4 740 1 AGGCGCCCCCA 4 740 1 AGGCGCCCCCA 4 740 1 AGGCGCCCCCA 4 740 1 AGGCGCCCCCCA 4 740 1 AGGCGCCCCCCA 4 740 1 AGGCGCCCCCCC 4 740 1 AGGCGCCCCCCC 4 740 1 AGGCGCCCCCCC 4 740 1 AGGCCCCCCCC 4 740 1 AGGCCCCCCCC 4 740 1 AGGCCCCCCCC 4 740 1 AGGCCCCCCCC 4 740 1 AGGCCCCCCCCC 4 740 1 AGGCCCCCCCCC 4 740 1 AGGCCCCCCCCC 4 740 1 AGGCCCCCCCCCC 4 740 1 AGGCCCCCCCCC 4 740 1 AGGCCCCCCCCC 4 740 1 AGGCCCCCCCCC 4 740 1 AGGCCCCCCCCCC 4 740 1 AGGCCCCCCCCCCC 4 740 1 AGGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC GGATATATTC TGCCAGGAAG ATCAGTGGTG TGTCATTCCG CGTTCAGTCC	4 280 GAACGGTCTG 4.360 TTTTTTCCCC 4.560 CTTACGTGCC 4.560 TTTCTACGTGCC 4.560 TTTCTATCAG CGGTCACAGG 4.660 TTTCTATCAG 4.760 1 TATGTTGCCA 4.760 1 CGCTTCCTCG 4.660 1 CGCTTCCCCG 4.560 1 CGCTACCGCG 4.560 1 CGCTACCCG 4.560 1 CGCTCCCCGC 5.560 CGCTCCCCG 5.560 CGCTCCCCG 5.560 CGCTCCCCG 5.560 CGCCGCCGCGCG 5.560 CGCCGCGCGCG 5.560 CGCCGCCGCG 5.560 CGCCGCCGCG 5.560 CGCCGCCGCG 5.560 CGCCGCCGCG 5.560 CGCCGCCGCG 5.560 CGCCGCCGCG 5.560 CGCCGCCGCG 5.560 CGCCGCCGCG 5.560 CGCCGCCGCG 5.560 CGCCGCCGCG 5.560 CGCCGCCGCG 5.560 CGCCGCCGCGCG 5.560 CGCCGCCGCGCGCGCG 5.560 CGCCGCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG	GTTATAGGTA GTTATAGGTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT GGGGAAGTGAG ACAGGACTAT CGCGTTTGTC	CATTGAGCAA 4380 CCTTAGCTCC 4480 TCATTTTCGC 4580 GCGCAAAGTG 4680 1 GCGCAAAGTG 4680 1 GCGCAAAGTG 4780 1 GGCTACGCTA 480 1 GGCCACGCCG 4800 1 GGCCCCGCGG 4800 1 GGCCCCGCGG 4800 1 GGCCCCCGCGG 1 AAAGATACCA 5100 1 CCTTAGCTCCCCG 1 1 1 1 1 1 1 1 1 1 1 1 1	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA CTGACGGGGT GTGCTTCATG GGCGTTCCGA CCTGACACTC CTTGAGTCCA	CALL TGCCTCAAAA 44.00 GATAACTCAAA CCCCAGGGCT TGCTGCCAAC 47.00 GGTGCGTAAC p15a_OR CTGCGGCGAG 51.00 CTGCGGCGAG 51.00 CTGCGGCGAG 53.00 CTGCCGGCGAC 53.00 AGTTCCGGGT
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCA AAAAAGGCTG CGGAAATGGC CTCCGCCCCC CCCTCGTGCG AGGCAGTTCG ACATGCAAAA	4 add TTTTTCTTTA 4.320 GATGCCATTG 4.420 CGGTAGTGAT ACAGGGACAC 4.520 AGTGTATGAT 4.720 1 CGCCCGGACA 4.720 1 CCGCCGGACA 5.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCG 5.70 1 CCGCCGGCGGCG 4.70 1 CCGCCGGCGGCGG 5.70 1 CCGCCGGCGGCGG 5.70 1 CCGCCGGCGGCG 5.70 1 CCGCCGGCGGCG 5.70 1 CCGCCGGCGGCG 5.70 1 CCGCCGGCGGCG 5.70 1 CCGCCGGCGGCG 5.70 1 CCGCCGGCGGCG 5.70 1 CCGCCGGCGGCG 5.70 1 CCGCCGGCGGCGG 5.70 1 CCGCCGGCGGCG 5.70 1 CCGCCGGCGGCG 1 CCGCCGGCGGCG 1 CCGCCGGCGGCGGCG 1 CCGCCGGCGGCG 1 CCGCCGGCGGCGGCG 1 CCGCCGGCGGCGGCG 1 CCGCCGGCGGCGGCGCG 1 CCGCCGGCGGCGCGCGCGCGCGCGCGCGCGCGCGCGCG	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAATT CCTGCCTTTC GACTGTATGC CAGCAGCCAC	4 240 4 340 AAAGGCCGTA 4 440 TATGGTGAAA 4 540 TTATTCTGCG 4 640 AGGTGCTCCA 4 640 AGGTGCTCCA 4 740 CGGAGTGTAT 4 640 10 10 10 10 10 10 10 10 10 1	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC GGATATATTC TGCCAGGAAG ATCAGTGGTG TGTCATTCCG CGTTCAGTCC TTTAGAGGAG	4 280 GAACGGTCTG 4.360 1 TTTTTTCCC 4.560 1 CTTACGTGCC 4.560 1 CCGTCACAGG 4.660 1 TTTCTATCAG 4.660 1 TTTCTTCCG 4.660 1 TTTCTTCCG 4.660 1 TTTCTTCCG 4.660 1 TTTCTTCCG 5.600 1 TTTCTTCGG 5.100 1 TTTGTTGGC 5.100 1 TTTGTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTTGGC 5.100 1 TTTGTTGGC 5.100 1 TTTGTTGGC 5.100 1 TTTGTTGGC 5.100 1 TTTGTTGGC 5.100 1 TTTGTTGGC 5.100 1 TTTGTTGGC 5.100 1 1 TTTGTTGGC 5.100 1 1 1 1 1 1 1 1 1 1 1 1 1	GTTATAGGTA GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT GGGGAAGTGAG ACAGGACTAT CGCGTTTGTC CCTTATCCGG AGTCATGCGC	CATTGAGCAA A380 CCTTAGCTGC CTTAGCTGC CTTAGCTGC CCTTAGCTG CCTTAGCTG CGCGCAAGTG A480 TGTTCAGCTA CGCTACGCTC A480 TGTCAGTGAA CGCTACGCC A480 AGGGCCGCGG AAAGATACCA CA CGCTACGCC CGCTACGCC CGCTACGCC C CGGTTAAGCC	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGGTGA CTGACGGGGT GTGCTTCATG GGCCGTTCCA CCTGACACTC CTTGAGTCCA TAAACTGAAA	CALL TGCCTCAAAA 44.00 GATAACTCAA CCCCAGGGCTT TGCTGCCAAC 4.00 TGCTGCCAAC 915a_OR CTGCGGCGAG 15a_OR CTGCGGCGAG 15a_OR CTGCGGGCGAG CCTGGCGGCG CCTGCGGCGAG CCTGGCGGCT AGTTCCGGGT AGTTCCGGGT
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCA AAAAAGGCTG CGGAAATGGC CTCCGCCCCC CCCTCGTGCG AGGCAGTTCG ACATGCAAAA	A 200 TTTTTCTTTA 4.320 GATGCCATTG 4.420 CGGTAGTGAT ACAGGGACAC 4.520 AGTGTATGAT 4.520 AGTGTATGAT 4.720 4.520 CGCCCGGACA 4.620 1 CCGCCGGACA 4.620 1 CCGCCGGACA 4.620 1 CCGCCGGACA 4.620 1 CCGCCGGACA 5.720 1 CTCCCAGCTG 5.720 1 CTCCCAGCTG 5.720 1 CCCCCCGCGACA 5.720 1 CCCCCCGCGACA 5.720 1 CCCCCCGACCA 5.720 1 CCCCCCGACCA 5.720 1 CCCCCCGACCA 5.720 1 CCCCCCGACCA 5.720 1 CCCCCCGACCA 5.720 1 CCCCCCGACCA 5.720 1 CCCCCCGCGACA 5.720 1 CCCCCCGCGACA 5.720 1 CCCCCCGCGACA 5.720 1 CCCCCCGCGACA 5.720 1 CCCCCCGCGACA 5.720 1 CCCCCCGCGACA 5.720 1 CCCCCCGCGACA 5.720 1 CCCCCCGCGCGACA 5.720 1 CCCCCCGCGCGACA 5.720 1 CCCCCCGCGCGCGC 1 CCCCCCGCGCGCGCGC 1 CCCCCCGCGCGCGCGCGC 1 CCCCCCGCGCGCGCGCGCCGC 1 CCCCCCGCGCGCGCGCGCGCCGCCGCCGCCGCCGCGCCGCCGCG	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAATT GGCCGGAGATT TCACGAAATC CCTGCCTTTC GACTGTATGC CAGCAGCCAC	4 240 4 240 AAAGGCCGTA 4 340 TATGGTGGAAA 4 540 TTATTCTGCG 4 640 1 TTATTCTGCG 4 640 1 GGGAGTGTAT 4 840 1 CGGAGTGTAT 4 840 1 CGGAGTGTAT 4 840 1 CGGAGTGTACA 4 940 1 CGGAGGTCCAA 5 940 1 GGTTTACCGG 6 94 1 CGGAACCCCC 5 340 1 CGGAACCCCC 5 1 1 CGGAACCCCC 5 1 1 CGGAACCCCCC 5 1 1 CGGAACCCCCC 5 1 1 CGGAACCCCC 5 1 1 CGGAACCCCCC 5 1 1 CGGAACCCCCCC 5 1 1 CGGAACCCCCC 5 1 1 CGGAACCCCCC 5 1 1 CGGAACCCCCC 5 1 1 CGGAACCCCCC 5 1 1 CGGAACCCCCC 1 CGGAACCCCCC 1 1 CGGAACCCCCC 1 1 1 1 1 1 1 1 1 1 1 1 1	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC GGATATATTC TGCCAGGAAG ATCAGTGGTG TGTCATTCCG CGTTCAGTCC TTTAGAGGAG	4 280 4 280 4 280 4 360 1 TTTTTTTCCC 4 460 1 CTTACGTGCC 4 580 1 CCGTCACAGG 4 680 1 TTTCTATCAG 4 780 1 TTTCTATCAG 4 780 1 TTTCTATCAG 4 780 1 CGCTCCCC 4 580 1 CGCAAACCCG 5 800 1 CGCCGCTGCG 5 800 1 CGCCCCCC 5 800 1 CGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	GTTATAGGTA GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT GGGAAGTGAG ACAGGACTAT CGCGTTTGTC CCTTATCCGG AGTCATGCGC	CATTGAGCAA 4380 CCTTAGCTGC CCTTAGCTGC CCTTAGCTGC CCTTAGCTGC GCGCAAAGTG GCGCAAAGTG A4980 TGTTCAGCTA 4580 TGTTCAGCTA 4580 CGCTACGCTC A4980 CGCTACGCTC A4980 CGCTACGCCG A4980 CGCCAAAGTG A4980 CGCCAAAGTG A4980 CGCCAAAGTG A4980 CGCCAAAGTG A4980 CGCCAAAGTG A4980 CGCCAAAGTG A4980 CGCCAAAGTG A4980 CGCCAAAGTG A4980 CGCCAAAGTG A4980 CGCCACGCGG A4980 CGCCACGCGG A4980 CGCCACGCCG A4980 CGCCACGCCG A4980 CGCCACGCCG A4980 CGCCACGCCGCG A4980 CGCCACGCCGCG A4980 CGCCACGCCGCG A4980 CGCCACGCCG A4980 CGCCACGCCG A4980 CGCCACGCCG A4980 CGCCACGCCG A4980 CGCCACGCGCG A4980 CGCCACGCGCG A4980 CGCCACGCCG A4980 CGCCACGCCGCG A4980 CGCCACGCCGCG A4980 CGCCACGCGCG A4980 CGCCACGCGCG A4980 CGCCACGCGCG A4980 CGCCACGCGCG A4980 CGCCACGCGCGG A4980 CGCCACGCGCGCG A4980 CGCCACGCCGCGG CGCCACGCGCG CGC CGCCACGCGCG CGCCACGCGCG CGCCACGCGCGCG	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA CTGACGGGGT GTGCTTCATG GGCGTTTCCC CCTGACACTC CTTGAGTCCA TAAACTGAAA	CAA GGCTCCAAAA 4.400 4.500 CCCAGGGCTT CCCCAGGGCTT TGCTGCCAAC 4.700 GGTGCGTAAC P15a_OR CTGCGGCGAA P15a_OR CTGCGGCGAC 15a OR 5.100 CCTGGCGGCT AGTTCCGGGT AGTTCCGGGT AGTTCCGGGT AGTCCGGAAAG GGACAAGTTT 5.000 1
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCCGGTATCA TTACTGATTT GGCAAAAGCA AAAAAGGCTG CGGAAATGGC CTCCGCCCCC CCCTCGTGCG AGGCAGTTCG ACATGCAAAA TGGTGACTGC	TTTTTCTTTA GATGCCATTG GATGCCATTG CGGTAGTGAT ACAGGGACAC ACAGGGACAC AGTGTATGAT 4.520 AGTGTATGAT 4.720 CCGCCGGACA 4.620 CACCGGTGGG CACCGGTGGG CTGACAAGCA 5.120 CTCCCAAGCTG CTCCCAAGCTG GCACCACTGG GCTCCTCCAA	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAATC CCTGCCTTTC GACTGTATGC CAGCAGCCAC GCCAGTTACC	4 240 4 240 1 AAAGGCCGTA 4 340 1 TATGGTGAAA 4 540 1 TATTCTGCG 4 640 1 AGGTGCTCCA 4 740 CGGAGTGTAT 4 840 CGGAGTGTAT 4 840 1 CGGAGTGTCCA 4 840 1 CGGAGTGTCCA 5 140 1 GGTTTACCGG 5 240 ACGAACCCCC 5 340 1 CGGTTCAAA	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTAC GGATATATTC TGCCAGGAAG ATCAGTGGTG TGTCATTCCG CGTTCAGTCC TTTAGAGGAG GAGTTGGTAG	4 280 GAACGGTCTG 4.360 CTTACGTGCC 4.460 CTTACGTGCC 4.600 TTTCTATCAG 4.600 TTTCTATCAG 4.600 TTTCTATCAG 4.600 TATGTTGGCA 4.600 CGCTTCCTCG 4.600 CGCTTCCTCG 4.600 CGCTTCCTCG 4.600 5.000 CGCGAAACCCG 5.300 TTAGTCTGA 5.460 CCCGCCGCG 5.300 CCCGCGCGCG 5.300 CCCGCGCGCG 5.300 CCCGCGCGCGCG 5.300 CCCGCGCGCGCG 5.300 CCCGCGCGCGCG 5.300 CCCGCGCGCGCG 5.300 CCCGCGCGCGCG 5.300 CCCGCGCGCGCG 5.300 CCCGCGCGCGCGCG 5.300 CCCGCGCGCGCGCG 5.300 CCCGCGCGCGCGCG 5.300 CCCGCGCGCGCGCG 5.300 CCCGCGCGCGCGCG 5.300 CCCGCCGCGCGCGCG 5.300 CCCGCGCGCGCGCGCG CCCGCCGCGCGCGCGCGCG CCCGCCGCGCGCG	GTTATAGGTA ATTITAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT GGGAAGTGAG ACAGGACTAT CGCGTTTGTC CCTTATCCGG AGTCATGCGC	CATTGAGCAA 4380 CCTTAGCTGC 4480 TCATTTTCGC GCGCCAAAGTG GGCGCAAAGTG TGTTCAGCTA 4580 TGTTCAGCTA 4580 TGTTCAGCTA 4580 CGCTACGCTC 4580 AGGGCCGCGG AAAGATACCA 5180 TCATTCCACG TAACTATCGT 520 CGGTTAAGGC CGGTTAAGGC	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA CTGACGGGGT GTGCTTCATG GGCCGTTCCC CCTGACACTC CCTGACACTC CTTGAGTCCA TAAACTGAAA AGGCGGTTTT	CAAAA TGCCTCAAAAA 4.400 4.500 CCCAGGGCTT CCCAGGGCTT TGCTGCCAACA 4.600 1 TGCTGCCAAC 4.700 1 GGTGCGTAAC 0 15a_ORI 1 TGCGGGGGGA 0 1 1 CCCGGGGGGCA 1 CCCGGGGGGCA 1 CCCGGGGGGC 5.100 CCCGGGGGT 5.100 CCCGGGGGGT 5.100 CCCGGGGGT 5.100 CCCGGGGGT 5.100 CCCGGGGGT 5.100 CCCGGGGGT 5.100 CCCGGGGGT 5.100 CCCGGGGGT 5.100 CCCGGGGGT 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGGAAAG 5.100 CCCGGAAAG 5.100 CCCGGAAAG CCCGGGAAAG CCCGGGAAAAG CCCGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGGAAAAG CCCGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGGAAAAG CCCGGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAAG CCCGGAAAG CCCGGAAAAG CCCGAAAAAAAAAA
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCCGGTATCA TTACTGATTT GGCAAAAGCA AAAAAGGCTG CGGAAATGGC CTCCGCCCCC CCCTCGTGCG AGGCAGTTCG ACATGCAAAA TGGTGACTGC	4 400 TTTTTCTTTA 4.200 GATGCCATTG 4.200 CGGTAGTGAT 4.500 ACAGGGACAC 4.500 AGTGTATGAT 4.720 CCGCCGGACA 4.200 CTGACAAGCA 5.120 CTCCCAAGCTG 5.200 GCACCACTGG 5.200 GCACCACTGA	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAATC CCTGCCTTTC GACTGTATGC CAGCAGCCAC GCCAGTTACC	4 240 1 AAAGGCCGTA 4.340 1 ACGGTGGTAT 4.440 1 TATGGTGAAA 4.540 1 AGGTGCTCCA 4.640 AGGTGCTCCA 4.640 CGGAGTGTAT 4.840 TCCTGGAAGA 5.440 GGTTTACCGG ACGAACCCCC 5.340 TGGTAATTGA 5.540 TCGGTTCAAAA 5.540 CGGTTCAAAA	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG GGATATATTC GGGATATATTC GGCCAGGAAG ATCAGTGGTG GAGTTGGTAG ATCATCTTA	4 280 GAACGGTCTG 4.360 CTTACGTGCC 4.460 CTTACGTGCC 4.600 TTTCTATCAG 4.600 TTTCTATCAG 4.600 TTTCTATCAG 4.600 TATGTTGGCA 4.600 CGCTTCCTCG 4.600 CGCTTCCTCG 4.600 CGCTTCCTCG 4.600 CGCTTCCTCG 4.600 CGCTTCCTCG 4.600 CGCTTCCTCG 4.600 CGCTCCCCG 5.000 CGCTCCCCG 5.000 CGCTCCCCG 5.000 CGCTCCCCG 5.000 CGCTCCCCG 5.000 CGCTCCCCG 5.000 CGCTCCCCG 5.000 CGCTCCCCG 5.000 CGCTCCCCGC 5.000 CGCTCCCCGC 5.000 CGCTCCCCGC 5.000 CGCCCCCCCCG 5.000 CGCCCCCCCCG 5.000 CGCCCCCCCCG 5.000 CGCCCCCCCCG 5.000 CGCCCCCCCCG 5.000 CCCCCCCCCCCCG 5.000 CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	GTTATAGGTA GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT GGGAAGTGAG ACAGGACTAT CCCTTATCCGG AGTCATGCGC CTTCGAAAAA	CATTGAGCAA 4380 CCTTAGCTGC TCATTTTCGC GCGCAAAGTG GCGCAAAGTG TGTTCAGCTA 4780 TGTCAGTGAA 4780 TGTCAGTGAA 4880 CGCTACGCTC AGGGCCGCGG AAAGATACCA 5180 TCATTCCACG CGGTTAAGGC CGGCTTAGGC CGGCTTAGGC CGGCCTGCA 5580 CCGCCCTGCA	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA CTGACGGGGT GTGCTTCATG GGCGTTCCC CCTGACACTC CTTGAGTCCA TAAACTGAAA AGGCGGTTTT GCAATTATC	CAAAAA TGCCTCAAAAA 4400 4500 CCCAGGGCTT CCCAGGGCTT TGCTGCCAACA 4500 1 TGCTGCCAAC 4500 1 TGCGCGCAAC 4700 1 GGTGCGTAAC 4700 1 GGTGCGTAAC 4700 1 GGGCGGGGAA 1 CCTGGCGGCGA 5000 1 CCTGGCGGCG 5000 1 CCTGGCGGCA 5000 1 CCTGGCGGCA 5000 1 CCTGGCGGCA 5000 1 CCTGGCGGCAA 5000 1 CCTGGCGGCAA 5000 1 CCTGGCGGCAA 5000 1 CCTGGCGGCAA 5000 1 CCTGGCGGCAA 5000 1 CCTGGCGGCAA 5000 1 CCTGGCGGCAA 5000 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGCGGCGAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCGAA 1 CCTGGCGGCAA 1 CCTGGCGGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGAA 1 CCTGGCGGCGAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCCAA 1 CCTGGCGGCCAA 1 CCTGGCGGCCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCCAA 1 CCTGGCGGCGCAA 1 CCTGGCGGCCAA 1 CCTGGCGGCAAA 1 CCTGGCGGCAAA 1 CCTGGCGGCAAA 1 CCTGGCGGCAAA 1 CCTGGCGGCAAAA 1 CCTGGCGGCAAAA 1 CCTGGCGGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
CTTGTGCTTA TGTTCTTTAC AAAATACGCC CCCGGTATCA TTACTGATTT GGCAAAAGCA AAAAAGGCTG CGGAAATGGC CTCCGCCCCC CCCTCGTGCG AGGCAGTTCG ACATGCAAAA TGGTGACTGC GAGCAAGAGA	4 200 TTTTTCTTTA 4.320 GATGCCATTG 4.220 CGGTAGTGAT 4.220 CGGTAGTGAT ACAGGGACAC 4.200 I ACAGGGACAC 4.200 I CCGCCGGACA I CCGCCGGACA 5.200 I CCGCCGGCACA 5.200 I CCCCCGCACAGCA I CCCCCGCCGACA 5.200 I CCCCCGGACA 5.200 I CCCCCGGACA 5.200 I CCCCCGGACA I CCCCCGGACA 5.200 I CCCCCGGACA I CCCCCGGACA 5.200 I CCCCCGGACA I CCCCCGGACA 5.200 I CCCCCGGACA I CCCCCGGACA I CCCCCGGACA I CCCCCGGACA I CCCCCGGACA I CCCCCGGACA I CCCCCCGACA I CCCCCCGACA I CCCCCCCCAAGCA I CCCCCCCCAA I CCCCCCCCAA I CCCCCCCCAA I CCCCCCCCAA I CCCCCCCCAA I CCCCCCCCAA I CCCCCCCCAA I CCCCCCCCAA I CCCCCCCCAA I CCCCCCCCAA I CCCCCCCCAA I I CCCCCCCCAA I I CCCCCCCCAA	CGGTCTTTAA GGATATATCA CTTATTTCAT CAGGATTTAT GGTGTTTTTG TCAGCGCTGG TCAGCAGAAATC CCTGCCTTTC GACTGTATGC CAGCAGCCAC GCCAGTTACC ACCAAAACGA	4 240 4 240 AAAGGCCGTA 4 340 1 ACGGTGGTAT 4 440 1 TATGGTGAAA 4 540 1 TTATTCTGCG 4 640 4 640 1 AGGTGCTCCA 4 770 1 CGGAGTGTAAA 4 80 1 TCCTGGAAGA 5 400 1 GGTTTACCGG 5 540 1 TCGGTTCAAGAAG	ATATCCAGCT ATCCAGTGAT GTTGGAACCT AAGTGATCTT GTGGCTTCTG ACTGGCTTCTG GGATATATTC GGATATATTC TGCCAGGAAG ATCAGTGGTG GAGTTGGTAG ATCATCTTAT	4 280 GAACGGTCTG 4.360 CTTACGTGCC CTTACGTGCC CCGTCACAGG TTTCTATCAG CTTACGTGCC 4.600 TTTCTATCAG CGCTCCCCG AAGO CGCTTCCTCG ATACTTAACA CGCGAAACCCG CGCAAACCCG CGCGAAACCCG CGCGCTGCGC CGC CCGCTGCGCGCG CGCCGCTGCG CGCCCCCG CCGCTCCCCG CCGCTCCCCG CCGCTCCCCG CCGCTCCCCG CCGCTCCCCG CCGCTCCCCG CCGCTCCCCG CCGCTCCCCG CCGCCCCCGC CCGCCCCCGC CCGCCCCCG CCGCCCCCG CCGCCCCCGC CCGCCCCCC	GTTATAGGTA GTTATAGGTA ATTTTAGCTT GATCAACGTC TATTTATTCG CTGTCCCTCC CTGATGAGGG CTCACTGACT GGGAAGTGAG ACAGGACTAT CGCGTTTGTC CCTTATCCGG AGTCATGCGC CTTCGAAAAA AAATATTTCT	CATTGAGCAA 4380 CCTTAGCTCC 4480 TCATTTTCGC GCGCAAAGTG GCGCAAAGTG TGTTCAGCTA 4580 TGTTCAGCTA GCGCAAGTG A480 TGTCAGTGAA 4880 AAAGATACCA 5180 TCATTCCAGG AAAGATACCA 5180 TCATTCCAGG CGGTTAAGCC CGGCTACGCTG CGGTTAAGCC CGGTTAAGCC AGATTTCAGT	CTGACTGAAA TGAAAATCTC CAAAAGTTGG CGTCGGGTGA CTGACGGGGT GTGCTTCATG GGTCGTTCATG GGCGTTTCCC CCTGACACTC CTTGAGTCCA TAAACTGAAA AGGCGGTTTT GCAATTTATC	CALL TGCCTCAAAA 44.00 GATAACTCAA CCCAGGGCTT TGCTGCCAAC 915a_ORI TGCGGGGGGAA 071 CTGCGGCGAG 5100 CTGCGGCGAG 5200 TTTCCATAGG CCTGGCGGCAG CCTGGCGGCT 5200 AGTTCCGGGT AGTTCCGGGT AGTTCCGGGT CCTGGCGAAAG CCTGGCGAAAG CCTGGCGAAAG CCTGGCGAAAG CCTGCGGAAAG CCTGCGGAAAG CCTGCGGAAAG CCTGCGGAAAG CCTGCGGAAAG CCTGCGGAAAG CCTGCGGAAAG CCTGCGGAAAG

alphaVmut-TMD = TMD Int_a5GP

JM = Juxtamembrane region

CAT = Chloramphenicol acetyltransferase

9.6.4 C-BLa 1.2

	20 1	CATTOACTT	40 I		60 I		80 I	*******	AraC
GACAACTIG	120	CATTCACTTT	140	CUGGCAUGGA	160		180	TAAATACCCG	AraC
AGTTGATCGT	CAAAACCAAC	ATTGCGACCG	ACGGTGGCGA	TAGGCATCCG	GGTGGTGCTC	AAAAGCAGCT	TCGCCTGGCT	GATACGTTGG	TCCTCGCGCC
	220 1		240 I		260 I		280 I		AraC
AGCTTAAGAC	GCTAATCCCT	AACTGCTGGC	GGAAAAGATG	TGACAGACGC	GACGGCGACA	AGCAAACATG	CTGTGCGACG	CTGGCGATAT	CAAAATTGCT
GTCTGCCAGG	TGATCGCTGA	TGTACTGACA	AGCCTCGCGT	ACCCGATTAT	CCATCGGTGG	ATGGAGCGAC	TCGTTAATCG	CTTCCATGCG	AraC CCGCAGTAAC
	420		440		460		480		AraC
AATTGCTCAA	GCAGATTTAT	CGCCAGCAGC	TCCGAATAGC	GCCCTTCCCC	ттосссоосо	TTAATGATTT	GCCCAAACAG	GTCGCTGAAA	TGCGGCTGGT
CCCCTTCATC	520 1	*****	540 I	TOACCOCAC	560	CATCCCACTA	580		AraC
GUGUIIUAIU	620	AACCCCGTAT	1GGCAAATAT 640	IGALGGULAG	11AAGCCATT 660	CATGULAGIA	GGCGCGCGGGA	CGAAAGTAAA	AraC
ATACCATTCG	CGAGCCTCCG	GATGACGACC	GTAGTGATGA	ATCTCTCCTG	GCGGGAACAG	CAAAATATCA	CCCGGTCGGC	AAACAAATTC	TCGTCCCTGA
	720		740		760		780 I		AraC
TTTTTCACCA	CCCCCTGACC	GCGAATGGTG	AGATTGAGAA	TATAACCTTT	CATTCCCAGC	GGTCGGTCGA	TAAAAAAATC	GAGATAACCG	TTGGCCTCAA
TCGGCGTTAA		AGATGGGCAT		TCCCGGCAGC		TTTGCGCTTC		TTCATACTCC	
arabinose	O2 operator regi	on	TARACOACTA		AUCCONTONT		ACCONTACT !		
		AraC	940 I		960 I		960 I		1.000
AGAAGAAACC	AATTGTCCAT	ATTGCATCAG	ACATTGCCGT	CACTGCGTCT	TTTACTGGCT	CTTCTCGCTA	ACCAAACCGG	TAACCCCGCT	TATTAAAAGC
	1.020)	1.040		1.060	arabinose O1	operator region 080		1.100
ATTCTGTAAC	AAAGCGGGAC	CAAAGCCATG	ACAAAAACGC	GTAACAAAAG	TGTCTATAAT	CACGGCAGAA	AAGTCCACAT	TGATTATTTG	CACGGCGTCA
CAP bindin	g site 1.120)	1.140 I	ar	abinose_minimal_p	romoter	1.180 I		1.200
CACTTTGCTA	TGCCATAGCA	TTTTTATCCA	TAAGATTAGC	GGATGCTACC	TGACGCTTTT	TATCGCAACT	CTCTACTGTT	TCTCCATACC	CGTTTTTTG
xbal	1.220	OmpA Sign	al peptide 1.240		1.260		1.280		C-BLa 1.300
GGCTCTAGAT	AACGAGGGCA	AAAAATGAAA	AAGACCGCGA	TTGCGATTGC	GGTGGCGCTG	GCGGGCTTTG	CGACCGTGGC	GCAGGCGGCG	CTACTTACTC
C-BLa	1.320)	1.340 I		1.360 I		1.380 I		1.400 I
TAGCTTCCCG	GCAACAATTA	ATAGACTGGA	TGGAGGCGGA	TAAAGTTGCA	GGACCACTTC	TGCGCTCGGC	CCTTCCGGCT	GGCTGGTTTA	TTGCTGATAA
C-BLa		COLCICOCCO	1.440 I	CCACTCCCC	1.460 I	CCCCTCCCCT		TCTACACGAC	1.500 I
C-BLa ATCTGGAGCC	1.420 I GGTGAGCGTG	GGTCTCGCGG	1.440 I TATCATTGCA	GCACTGGGGC	1.460 I CAGATGGTAA	GCCCTCCCGT	1.480 I ATCGTAGTTA	TCTACACGAC	1.500 I GGGGAGTCAG
C-BLa ATCTGGAGCC C-BLa	1.420 I GGTGAGCGTG 1.520 I	GGTCTCGCGG	1.440 I TATCATTGCA 1.540 I	GCACTGGGGC	1.460 I CAGATGGTAA 1.560 I	GCCCTCCCGT	1.480 I ATCGTAGTTA linker 1.580 I	TCTACACGAC	1.500 I GGGGAGTCAG 1.600 I
C-BLa ATCTGGAGCC C-BLa GCAACTATGG	1.420 I GGTGAGCGTG 1.520 I ATGAACGAAA	GGTCTCGCGG	1.440 I TATCATTGCA 1.540 I GCTGAGATAG	GCACTGGGGC	1.460 I CAGATGGTAA 1.560 I GATTAAGCAT	GCCCTCCCGT	1.480 I ATCGTAGTTA I linker I GCAGCGGCGG	TCTACACGAC	1.500 J GGGGAGTCAG 1.600 J GGCGGCAGCG
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible I JMar	1.422 GGTGAGCGTG 1.522 ATGAACGAAA Nhel 1.622	GGTCTCGCGG TAGACAGATC	1.440 1 TATCATTGCA 1.540 1 GCTGAGATAG 1.640	GCACTGGGGC GTGCCTCACT Apal	1.460 I CAGATGGTAA 1.560 I GATTAAGCAT	GCCCTCCCGT Spel flexible TGGACTAGTG BamHI	1.480 ATCGTAGTTA linker 1.580 GCAGCGGCGG 1.680	TCTACACGAC	1.500 I GGGGAGTCAG 1.600 I GGCGGCAGCG rigid linker
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible I JMer GTTCTGCTAA	1.422 GGTGAGCGTG 1.522 ATGAACGAAA Nhel 1.622 TCGAGCTAGC	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC	1.440 TATCATTGCA 1.540 I GCTGAGATAG 1.640 I TGGCGGTGGG	GCACTGGGGC GTGCCTCACT Apal GCCCGGCCTG	1.460 I CAGATGGTAA 1.560 I GATTAAGCAT CTGCTCCTGG	GCCCTCCCGT	1.480 I ATCGTAGTTA Iinker 1.580 GCAGCGGCGG I ACTGGCAGTT	TCTACACGAC CGGCAGCGGC AATGTTCAGC	1.500 GGGGAGTCAG 1.600 J GGCCGCCAGCG rigid linker TGCCTGCCGA
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible I JMbr GTTCTGCTAA rigid linker	1420 GGTGAGCGTG 1522 ATGAACGAAA Whel TCGAGCTAGC 1622 1721	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC	1.440 I TATCATTGCA 1.540 GCTGAGATAG I TGGCGGTGGG 1.640 I 1.640 I 1.640 I 1.640 I 1.640 I 1.640 I I 1.640 I I I I I I I I I I I I I I I I I I I	GCACTGGGGC GTGCCTCACT Apal GCCCGGCCTG	1.460 I CAGATGGTAA I.500 GATTAAGCAT CTGCTCCTGG I 1.7600 I	GCCCTCCCGT	1.480 I ATCGTAGTTA GCAGCGGGGGG ACTGGCAGGT 1.680 I ACTGGCAGTT	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP	1.500 GGGGAGTCAG 1600 GGCGGCAGCG rigid linker TGCCTGCCGA 1800 1
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible I JMbr GTTCTGCTAA rigid linker AGCAGCAGCG	1422 GGTGAGCGTG ATGAACGAAA (Whei) 1.822 TCGAGCTAGC 1.722 AAAGAAGCCG	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC CTGCCAAAGA	1.440 TATCATTGCA 1.540 GCTGAGATAG 1.640 1.640 TGGCGGTGGG 1.740 1.740 1.740 1.740 1.740 1.740 1.740 1.740 1.740 1.640 1.740 1.640 1.640 1.640 1.640 1.740 1.640 1.7	GCACTGGGGC GTGCCTCACT Apal GCCCGGCCTG AAAGAGGCTG	1.460 CAGATGGTAA 1.560 GATTAAGCAT 1.560 I GATTAAGCAT 1.760 CCGCCGAAAGA	GCCCTCCCGT	1.480 I ATCGTAGTTA GCAGCGGCGG ACTGGCAGGT AAAGCCGCAA	TCTACACGAC CGGCAGCGGC AATGTTCAGC GCAAAGGTGA	1.500 GGGGAGTCAG GGCGGCAGCG rigid linker TGCCTGCCG AGAACTGTTT
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible IJMer GTTCTGCTAA rigid linker AGCAGCAGCG srGFP ACCGGTGTTG	1.422 GGTGAGCGTG 1.522 ATGAACGAAA TGGAACGAAA 1.822 1.722 AAAGAAGCCG 1.722 AAAGAAGCCG	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC CTGCCAAAGA GGTTGAACTG	1.440 H TATCATTGCA 1.540 GCTGAGATAG TGGCGGTGGG 1.740 AGCGGCAGCG 1.740 GATGGTGATGG 1.840 CATGGTGATG	GCACTGGGGC GTGCCTCACT Apai GCCCGGCCTG AAAGAGGCTG TTAATGGCCA	1.460 CAGATGGTAA 1.560 I GATTAAGCAT CTGCTCCT CCGCCGAAAGA 1.760 1	GCCCTCCCGT Spal flexible TGGACTAGTG BamHI GGATCCACAA GGCAGCAGCT GTTCGTGGTG	1.480 I ATCGTAGTTA I GCAGCGGCGG ACTGGCAGGT ACTGGCAGTT I AAAGCCGCAAG I BAGGCGAAGG	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC	1.500 I GGGGAGTCAG 1.600 I GGCCGCCAGCG rigid linker TGCCTGCCGA 1.800 I AGAACTGTTT 1.900 AATGGTAAACAG
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible IJMar GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG sfGFP	1.422 GGTGAGCGTG 1.522 ATGAACGAAA TCGAGCTAGC 1.722 AAAGAAGCCG 1.822 TTCCGATTCT 1.927	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC CTGCCAAAGA GGTTGAACTG	1.440 I TATCATTGCA 1.540 I GCTGAGATAG I GGCGGGGGGGGGGG I GATGGGGAGGG 1.840 GATGGTGATG 1.940	GCACTGGGGC GTGCCTCACT Apai GCCCGGCCTG AAAGAGGCTG TTAATGGCCA	1.460 I CAGATGGTAA GATTAAGCAT CTGCTCCTGG 1.760 I.760 I.760 CCGCGAAAGA 1.860 CAAATTCTCA	GCCCTCCCGT	1.480 I ATCGTAGTTA Inker 1.680 I GCAGCGGGGGG I ACTGGCAGTT AAAGCCGCAA 1.880 AAGGCGAAGG 1.980	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC	1.500 I GGGGAGTCAG GGCGGCAGCGG Figid linker TGCCTGCCGA 1800 AGAACTGTTT 1.900 AATGGTAAAC 2.000
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible I JMyr GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG sfGFP TGACCCTGAA	1420 GGTGAGCGTG ATGAACGAAA Vinel 1622 1721 AAAGAAGCCG 1722 AAAGAAGCCG 1822 1721 AAAGAAGCCG 1822 1722 AAAGAAGCCG	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA	1.440 TATCATTGCA 1.540 1 GCTGAGATAG 1 TGGCGGTGGGG 1.640 1 TGGCGGCGGGGG 1.640 1 4 GATGGTGATG 1.400 1 1 4 1 4 1 1 4 1 1 1 1 1 1 1 1 1 1 1 1 1	GCACTGGGGC GTGCCTCACT Apal GCCCGGGCCTG AAAGAGGCTG TTAATGGCCA TCCGTGGCCG	1.460 CAGATGGTAA 1.500 GATTAAGCAT CTGCTCCTGG 1.700 1.000 1.600 1	GCCCTCCCGT	1.480 IIIIKER 1.580 CAGCGGGGGG ACTGGCAGTT AAAGCCGCAA AAGGCGAAGG 1.880 AAGGCGAAGG 1.890 CTATGGTGTT	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA	1.500 1 GGGGGAGTCAG 1600 GGCGGCAGCG rigid linker TGCCTGCCGA 1800 1 AGAACTGTTT 1.900 1 AATGGTAAAC 2000 GCCGTTATCC
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible I JMbr GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG sfGFP TGACCCTGAA sfGFP	1420 GGTGAGCGTG ATGAACGAAA Vinel TCGAGCTAGC 1.622 1.62 1.6	GGTCTCGCGG TAGACAGATC alphaVmutTMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA	1.440 TATCATTGCA 1.540 GCTGAGATAG 1.640 1 1 1.640 1.640 1.64	GCACTGGGGC GTGCCTCACT Apa GCCCGGGCCTG AAAGAGGGCTG TTAATGGCCA TCCGTGGCCG	1.460 CAGATGGTAA 1.500 1.50	GCCCTCCCGT	1.480 I ATCGTAGTTA GCAGCGGGGGG ACTGGCAGGT AACTGGCAGTT 1.680 I AAGGCCGAAGG 1.800 I AAGGCGAAGG 1.900 I CTATGGTGT 1.200 I CTATGGTGT 1.200 I	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA	1.500 1 GGGGGAGTCAG 1600 1 GGCGGCAGCG rigid linker TGCCTGCCGA 1800 1 AGAACTGTTT 1.900 1 AATGGTAAAC 2.000 1 GCCGTTATCC 2.100 1
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible I JMir GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG sfGFP TGACCCTGAA sfGFP GGATCATATG	1.422 GGTGAGCGTG ATGAACGAAA TCGAGCTAG 1.822 TCGAGCTAG 1.822 1.72 AAAGAAGCCG 1.822 TTCCGATTCT 1.922 ATTTATCTG ATTTATCTG 1.922 AAACAGCACG	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA	1.440 TATCATTGCA 1.540 1.540 1.540 1.64	GCACTGGGGC GTGCCTCACT Apal GCCCGGGCCTG AAAGAGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT	1.460 CAGATGGTAA 1.500 1.50	GCCCTCCCGT Spei TGGACTAGTG BamHil GGATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC	1.480 I ATCGTAGTTA GCAGCGGGGGG ACTGGCAGCG AACTGGCAGT AAAGCCGCAA 1.880 I AAGGCGAAGG CTATGGTGT I CTATGGTGT I CCTTTAAAG	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC	1.500 1 GGGGGAGTCAG 1600 1 GGCGGCAGCG rigid linker 1000 1 AGAACTGTTT 1.000 1 AATGGTAAAC 2.000 1 GCCGTTATCC 2.100 1 CTATAAGACC
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible IJMar GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG sfGFP TGACCCTGAA sfGFP GGATCATATG sfGFP	1.422 GGTGAGCGTG ATGAACGAAA TCGAGCTAGC 1.522 ATGAACGAAA TCGAGCTAGC 1.722 AAAGGAAGCCG 1.822 TTCCGATTCT 1.922 AAACAGCACG 2.022 AAACAGCACG 2.122	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA	1.440 TATCATTGCA 1.540 1.540 1 GCTGAGATAG 1.540 1.5	GCACTGGGGC GTGCCTCACT Apai GCCCGGGCCTG AAAGAGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT GCATTGAACT	1.460 1.560 1.560 1.560 1.560 1.560 1.560 1.560 1.560 1.560 1.560 1.760 1.	GCCCTCCCGT Spei flexible TGGACTAGTG BamHil GGATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC GATTTCAAAG	1.480 Inker 1.580 GCAGCGGGGGG I ACTGGCAGTT AAAGCCGCAA 1.880 I AAGGCGAAGG I CTATGGTGTT I CTATGGTGT I CCTTTAAAG I CCTTTAAAG	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC TATCCTGGGC	1.500 1 GGGGGAGTCAG 1 GGCGGCAGCGA 1 1 GGCCTGCCGA 1 1 0 0 1 0 0 1 0 0 1 0 0 1 0 0 1 0 0 1 0 0 1 0 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible I JM2r GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG SfGFP GGATCATATG sfGFP CGTGCCGAAG sfGFP	1422 GGTGAGCGTG 1522 ATGAACGAAA ICGAGCTAGC 1622 TCGAGCTAGC 1722 AAAAGAAGCCG 1722 AAAAGAAGCCG 1822 1724 AAACAGCACG 2.022 AAAACAGCACG 2.122 TTAAATTTGA 2.222	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA AGGTGATACC	1.440 TATCATTGCA 1.540 1.54	GCACTGGGGC GTGCCTCACT Apal GCCCGGCCTG AAAGAGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT GCATTGAACT	1.460 CAGATGGTAA 1.560 CTGCTCCTGG 1.700 CTGCTCCTGG 1.700 CCGCGAAAGA 1.8000 1.800 1.800 1.800 1.800 1.8000 1.8000	GCCCTCCCGT Spel flexible TGGACTAGTG BamHil GGATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC GATTTCAAAG	1.480 ATCGTAGTTA IInker 1.580 1.680 1.680 1.680 1.680 1.680 1.680 1.680 1.680 1.680 1.680 1.680 1.680 1.800 AAGGCGAAGG 1.800 AAGGCGAAGG 1.800 AAGGCGAAGG 1.8000 1.8000 1.800 1.8000 1.8000	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC TATCCTGGGC	1.500 1 GGGGGAGTCAG 1600 GGCGGCAGCG rigid linker TGCCTGCCGA 1800 1 AGAACTGTTT 1900 AATGGTAAAC 2000 1 GCCGTTATCC 2.100 1 CTATAAGACC 2.200 1 CTATAAGACC 2.200 2.300
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible I JMyr GTTCTGCTAA rigid linker AGCAGCAGCG srGFP ACCGGTGTTG SrGFP GGATCATATG SrGFP CGTGCCGAAG SrGFP	1422 GGTGAGCGTG 1522 ATGAACGAAA IS2 ATGAACGAAA IS2 1522 1522 1522 AAAGAAGCCG 1522 1722 AAAGAAGCCG 1522 1723 1724 17	GGTCTCGCGG TAGACAGATC alphaVmuLTMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA AGGTGATACC	1.440 TATCATTGCA 1.540 1.54	GCACTGGGGC GTGCCTCACT Apal GCCCGGGCCTG AAAGAGGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT GCATTGAACT TAAACAGAAG	1.460 1 CAGATGGTAA GATTAAGCAT GATTAAGCAT CTGCTCCTGG 1.500	GCCCTCCCGT Spel flexible TGGACTAGTG BamHI GGATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC GATTTCAAAG AAGCCAACTT	1,480 IIIIKER 1,580 IGCAGCGGGGG ACTGGCAGTT AAAGCCGCAAA 1,680 I AAGGCGAAGT AAGGCGAAGG I CTATGGTGTT 2,600 I CCTTTAAAG 2,180 I AAGATGGTAA 2,280 I TAAGATCCGC	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC TATCCTGGGC CATAATGTTG	1.500 1 GGGGGAGTCAG 1600 GGCGGCAGCG rigid linker TGCCTGCCGA 1800 1 AGAACTGTTT 1900 1 AATGGTAAAC 2000 1 GCCGTTATCC 2.100 1 CTATAAGACC 2.200 1 CATAAACTGG 2.200 2.200 1 CATAAACTGG 2.200 1 CATAAACTGG
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible IJMbr GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG sfGFP GGATCATATG sfGFP CGTGCCGAAG sfGFP AATATAATTT sfGFP	1420 GGTGAGCGTG 1520 ATGAACGAAA IS200 IS20 IS200	GGTCTCGCGG TAGACAGATC alphaVmutTMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA AGGTGATACC AACGTGTATA	1.440 TATCATTGCA 1.540 GCTGAGATAG 1.640 1 1 1.640 1.640	GCACTGGGGC GTGCCTCACT Apa GCCCGGGCCTG AAAGAGGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT GCATTGAACT TAAACAGAAG	1.460 1.60	GCCCTCCCGT Spei flexible TGGACTAGTG BamHI GGATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC GATTTCAAAG AAGCCAACTT	1,480 I ATCGTAGTTA GCAGCGGGGGG ACTGGCAGGT AACTGGCAGTT AAAGCCGGAAG 1,880 1 AAGGCGAAGG 1 AAGGCGAAGG 1 1 CTATGGTGT 2,800 1 AAGGTGGTAA 2,200 1 TAAGATCCGC 2,290	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC TATCCTGGGC CATAATGTTG	1.500 1 GGGGGAGTCAG 1 GGCGGCAGCG rigid linker TGCCTGCCGA 1800 1 AGAACTGTTT 1.900 1 AATGGTAAAC 2.000 1 GCCGTTATCC 2.100 1 CTATAAGACC 2.200 1 CATAAACTGG 2.2
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible IJMbr GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG sfGFP GGATCATATG sfGFP CGTGCCGAAG sfGFP AATGFP CGTTCAGCTG	1422 GGTGAGCGTG ATGAACGAAA IS22 ATGAACGAAA IS22 ICGAGCTAG IS22 IS23 IS23 IS23 IS23 IS23 IS23 IS23	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA AGGTGATACC AACGTGTATA ATCAGCAGAA	1.440 TATCATTGCA 1.540 1.64	GCACTGGGGC GTGCCTCACT Apal GCCCGGGCCTG AAAGAGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT GCATTGAACT TAAACAGAAG GGTGATGGTC	1.460 1.460 1.500 1.	GCCCTCCCGT Fexible TGGACTAGTG BamHH GGATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC GATTTCAAAG AAGCCAACTT GCCGGATAAT	1,480 I ATCGTAGTTA GCAGCGGGGG ACTGGCAGGGGG ACTGGCAGGTA AAAGCCGGAA 1,680 I AAAGCCGGAA A CTATGGTGT CTATGGTGT CTATGGTGT AAGATGGTAA I AAGATGGTAA I AAGATGGTAA I CATTATCTGA	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC TATCCTGGGC CATAATGTTG GCACCCAGAG	1.500 I GGGGGAGTCAG I GGCGGCAGCG rigid linker TGCCTGCCGA 1.800 I AGAACTGTTT 1.900 I AATGGTAAAC 2.000 I CTATAAGACC 2.100 I CTATAAGACC 2.100 I CTATAAGACC 2.100 I CTATAAGACC 2.100 I CTATAAGACC 2.100 I CTATAAGACCAG I CTATAAGACCAG I CATAAACTGGC I CATAAACTGG I I CATAAACTGG I I CATAAACTGG I I I I I I I I I I I I I
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible IJMar GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG SfGFP GGATCATATG sfGFP CGTGCCGAAG sfGFP AATATAATTT SfGFP CGTTCAGCTG	1422 GGTGAGCGTG 1522 1522 1522 1522 1522 1522 1522 152	GGTCTCGCGG TAGACAGATC alphaVmutTMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA AGGTGATACC AACGTGTATA ATCAGCAGAA	1.440 TATCATTGCA 1.540 1.540 1.540 1.540 1.540 1.540 1.540 1.540 1.540 1.540 1.540 1.540 1.74	GCACTGGGGC GTGCCTCACT Apai GCCCGGCCTG AAAGAGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT GCATTGAACT TAAACAGAAG GGTGATGGTC	1.460 1.460 1.560 1.	GCCCTCCCGT Spel flexible TGGACTAGTG GGATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC GATTTCAAAG AAGCCAACTT GCCGGATAAT	1.480 ATCGTAGTTA GCAGCGGGGG ACTGGCAGTT AAAGCCGCAGTT AAAGCCGCAA 1.880 AAGGCGAAGG CTATGGTGTT AAGATGGTAA 2.180 AAGATGGTAA 2.180 CTATGGTGTT AAGATGGTAA 2.280 CATTATCCGC 2.380 CATTATCCGC	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC TATCCTGGGC CATAATGTTG GCACCCAGAG ACTGTATAA	1.500 1 GGGGGAGTCAG 16000 1600 1600 1600 1600 1600 1600 1600
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible IJMyr GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG SfGFP GGATCATATG sfGFP CGTGCCGAAG sfGFP AATATAATTT sfGFP CGTTCAGCTG sfGFP	1422 GGTGAGCGTG 1522 ATGAACGAAA TCGAGCTAGC 1102 1102 1102 1102 1102 1102 1102 110	GGTCTCGCGG alphaVmut-TMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA AGGTGATACC AACGTGTATA ATCAGCAGAA	1.440 TATCATTGCA 1.540 1.54	GCACTGGGGC GTGCCTCACT Apa GCCCGGGCCTG AAAGAGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT GCATTGAACT TAAACAGAAG GGTGATGGTC AATTTGTTAC HindIII	1.460 AGATGGTAA GATTAAGCAT GATTAAGCAT CTGCTCCTGG 1.500 1.50	GCCCTCCCGT Spel flexible TGGACTAGTG BamHil GGATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC GATTTCAAAG AAGCCAACTT GCCGGATAAT ATTACCCATG	1,480 ATCGTAGTTA IINKET 1,580 1,680 1,680 ACTGGCAGT AAGGCGGAGG AAGGCGGAAGG 1,880 AAGGCGGAAGG 1,880 AAGGCGGAAGG 1,880 1,880 AAGGCGGAAGG 2,180 AAGGTGGTTA 2,280 1,70	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC TATCCTGGGC CATAATGTTG GCACCCAGAG ACTGTATAAÀ	1.500 1 GGGGGAGTCAG Figid linker TGCCTGCCGA 1.6000 1.6000 1.600
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible I JMir GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG SfGFP GGATCATATG SfGFP CGTGCCGAAG sfGFP AATATAATTT SfGFP CGTTCAGCTG SfGFP AATATAATTT	1422 GGTGAGCGTG 1522 ATGAACGAAA ICCGAGCTAGC 1622 1622 1622 1722 1722 AAAGAAGCCG 1722 AAAGAAGCCG 1722 AAAGAAGCCG 2122 TTAAATTTGA 2222 CAACAGCCAC 2.323 CAACAGCCAC 2.323 CAACAGCCAC 2.323 CAACAGCCAC 2.323 CAACAGCCAC 2.323 CAACAGCCAC 2.323 CAACAGCCAC 2.323 CAACAGCCAC 2.323 CAACAGCCAC	GGTCTCGCGG TAGACAGATC alphaVmuLTMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA AGGTGATACC AACGTGTATA ATCAGCAGAA	1.440 TATCATTGCA GCTGAGATAG GCTGAGATAG 1.540 1 TGGCGGTGAGG 1.640 1 TGGCGGCAGCG 1.640 1 TGGCGGCAGCG 1.640 1 1 AGCGGCAGCG 1.640 1 1 AGCGGCAGCG 1.640 1 1 AGCGGCAGCG 1.640 1 1 AGCGGCAGCG 1.640 1 1 AGCGGCAGCG 1.640 1 1 AGCGGCAGCG 1.640 1 1 AGCGGCAGCGG 1.640 1 1 AGCGGCAGCGG 1.640 1 1 AGCGGCAGCG 1.640 1 1 AGCGGCAGCGG 1.640 1 1 AGCGGCAGCGG 1.640 1 1 AGCGGCAGCGG 1.640 1 1 AGCGGCAGCGG 1.640 1 1 AGCGGCAGCGG 1.640 1 1 1 1 1 1 1 1 1 1 1 1 1	GCACTGGGGC GTGCCTCACT Apa GCCCGGGCCTG AAAGAGGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT GCATTGAACT TAAACAGAAG GGTGATGGTC AATTTGTTAC	1.460 1 CAGATGGTAA 1.500 GATTAAGCAT CTGCTCCTGG 1.700 1 CCGCGAAAGA 1.600 1 1 CCGCGAAAGA 1.600 1 1 CCGCGAAAGA 1.600 1 1 1 CCGCGAAAGA 1.600 1 1 1 CCGCGAAAGA 1.600 1 1 1 1 1 1 1 1 1 1 1 1 1	GCCCTCCCGT Spel TGCACTAGTG BamHI GCATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC GATTTCAAAG AAGCCAACTT GCCGGATAAT ATTACCCATG	1,480 ATCGTAGTTA GCAGCGGGGG ACTGGCAGTG ACTGGCAGTT AAAGCCGCAAA 1,680 1 AAAGCCGCAAT AAGGCGAAGG 1980 1 CTATGGTGTT 2,800 1 AAGATCCGC 2,380 CATTATCTGA 2,480 1 GTATGGATGA 2,480 1 GTATGGATGA	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC CATAATGTTG GCACCCAGAG ACTGTATAAA	1.500 1 GGGGGAGTCAG 1600 GGCGGCAGCG rigid linker TGCCTGCCGA 1800 1 AGAACTGTTT AATGGTAAAC 2.000 CGTTATAGACC 2.100 CATAAACTGG 2.200 CATAAACTGC 2.200 CATAAACTGC 2.200 CATAAACTGC 2.200 CATAAACTGC 2.200 CATAAACTGC CATAAAACTGC CATAAACTGC CATAAACTGC CATAAACTGC CATAAACTGC
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible IJMbr GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP ACCGGTGTTG sfGFP GGATCATATG sfGFP CGTGCCGAAG sfGFP CGTGCCGAAG sfGFP AATATAATTT sfGFP CGTTCAGCTG sfGFP AATATAATTT	1420 GGTGAGCGTG 1520 ATGAACGAAA IS200 IS20 IS20 IS20 IS20 IS20 IS200 IS20 IS20 IS20 IS20 IS20 IS20 IS20 IS20	GGTCTCGCGG alphaVmutTMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA AGGTGATACC AACGTGTATA ATCAGCAGAA TGATCACATG	1.440 TATCATTGCA GCTGAGATAG GCTGAGATAG 1.540 1	GCACTGGGGC GTGCCTCACT Apal GCCCGGGCCTG AAAGAGGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT GCATTGAACT TAAACAGAAG GGTGATGGTC AATTTGTTAC Hindlil CA <u>AGCT</u> TGGC	1.460 1.60	GCCCTCCCGT Fexible TGGACTAGTG BamHH GGATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC GATTCAAAG AAGCCAACTT GCCGGATAAT ATTACCCATG GATGAGAGAA	1,480 ATCGTAGTTA GCAGCGGGGGG ACTGGCAGGT ACTGGCAGTT 1,680 1 ACTGGCAGTT 1,680 1 AAGGCGCAAGT 1,680 1 AAGGCGAAGG 1,980 1 CTATGGTGTT 2,080 1 TAAGATCCGC 2,380 1 TAAGATCCGC 2,380 1 CATTATCTGA 2,480 GTATGGATGA	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC CATAATGTTG GCACCCAGAG ACTGTATAAA CTGATACAGA	1.500 1 GGGGGAGTCAG 1 GGCGGCAGCG rigid linker TGCCTGCCGA 1000 1 AGAACTGTTT 1.900 1 AATGGTAAAC 2.000 1 GCCGTTATCCG 2.300 1 AAGAACTGGCA 2.400 1 GGGGATTATA GGCGATTATA GGCGATTATA
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible IJMbr GTTCTGCTAA rigid linker AGCAGCAGCAGCG sfGFP ACCGGTGTTG sfGFP GGATCATATG sfGFP CGTGCCGAAG sfGFP CGTGCCGAAG sfGFP AATATAATTT sfGFP CGTTCAGCTG sfGFP AAAGATCCGA	1422 GGTGAGCGTG ATGAACGAAA IS22 ATGAACGAAA IS22 ICGAGCTAG 1.622 ICGAGCTAG I.622 ICGAGAAGCCG I.622 ICGAGAAGCCG I.622 ICGAGAAGCCG I.622 ICGAGACGAC I.622 ICGAGAAGCCG I.622 ICGAGAACGCAC I.622 ICGAGAACAGCCAC ICGACAACAGCAACAGCAC ICGACAACAACAGCAC ICGACAACAGCACAACAGCAC ICG	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA AGGTGATACC AACGTGTATA ATCAGCAGAA GGTCGACCG GAGGTCGACC	1.440 TATCATTGCA 1.540 1.54	GCACTGGGGC GTGCCTCACT Apal GCCCGGCCTG AAAGAGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT GCATTGAACT TAAACAGAAG GGTGATGGTC AATTTGTTAC HindIII CAAGCTTGGC	1.460 1.460 1.60	GCCCTCCCGT GGACTAGTG GGATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC GATTTCAAAG AAGCCAACTT GCCGGATAAT ATTACCCATG GATGAGAGAA	1,480 ATCGTAGTTA GCAGCGGGGGG ACTGGCAGGGGGG ACTGGCAGGT AAAGCCGGAA 1,680 1 AAAGCCGGAA 1,680 1 AAAGCCGGAA 1,680 1 AAAGCCGGAA 1,680 1 CTATGGCGAA 2,880 1 CATTATCTGA CATTATCTGA CATTATCTGA CATAGGTGATGA	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC CATAATGTTG GCACCCAGAG ACTGTATAAA CTGATACAGA	1.500 GGGGGAGTCAG 1600 GGCGGCAGCG rigid linker TGCCTGCCGA 1800 1 AGAACTGTTT 1.900 1 AATGGTAAACC 2.100 1 CTATAAGACC 2.100 1 CTATAAGACC 2.100 1 GGCGTTCTGAGC FLAG-Tag 2.500 1 GGCGATTATA 2.600 1 GGCGATTATA
C-BLa ATCTGGAGCC C-BLa GCAACTATGG flexible IJMir GTTCTGCTAA rigid linker AGCAGCAGCG sfGFP TGACCCTGAA sfGFP GGATCATATG sfGFP CGTGCCGAAG sfGFP AATATAATTT sfGFP CGTTCAGCTG sfGFP AATATAATTT sfGFP CGTTCAGCTG sfGFP AAAGATCCGA FLAG-Tag AAGATCACGA	1420 GGTGAGCGTG 1522 ATGAACGAAA 1.822 TCGAGCTAC 1.822 TCGAGCTAC 1.822	GGTCTCGCGG TAGACAGATC alphaVmut-TMD GTGATCATTC CTGCCAAAGA GGTTGAACTG ACCACCGGCA ATTTCTTCAA AGGTGATACC AACGTGTATA ATCAGCAGAA TGATCACATG GAGGTCGACC	1.440 TATCATTGCA 1.540 1.54	GCACTGGGGC GTGCCTCACT Apal GCCCGGGCCTG AAAGAGGCTG TTAATGGCCA TCCGTGGCCG CCGGAAGGTT GCATTGAACT TAAACAGAAG GGTGATGGTC AATTTGTTAC HindIII CAAGCTTGGC	1.460 1.460 1.500 1.	GCCCTCCCGT TGCACTAGTG GCATCCACAA GGCAGCAGCT GTTCGTGGTG CCACCCTGAC ACGTACCATC GATTTCAAAG AAGCCAACTT GCCGGATAAT ATTACCCATG GATGAGAGAA TGACCCCATG	1,480 ATCGTAGTTA GCAGCGGCGG ACTGGCAGCG ACTGGCAGTT 1,680 ACTGGCAGTT 1,680	TCTACACGAC CGGCAGCGGC AATGTTCAGC SFP GCAAAGGTGA TGATGCAACC CAGTGTTTCA ATGATGGCAC CATAATGTTG GCACCCAGAG ACTGTATAAA AGTGAAACG	1.500 I GGGGGAGTCAG GGCGGCAGCG rigid linker TGCCTGCCGA AGAACTGTTT AATGGTAAAC GCCGTTATCC CTATAAGACC CTATAAGACC CTATAAGACC CGTTCTGAGC FLAGTag 2.000 I GGCGATTATA CGTACCAG CCGTAGCGCC CCGTAGCGCC 2.000 CCGTAGCGCC CCC CCC CCCC CCC CCCCC CCCCC CCCCC CCCC CCCCC CCCCC CCCCC CCCCCC

	2.820		2.840		2.860	0	2.880		2.900
TGTTTGTCGG	TGAACGCTCT	CCTGAGTAGG	ACAAATCCGC	CGGGAGCGGA	TTTGAACGTT	GCGAAGCAAC	GGCCCGGAGG	GTGGCGGGCA	GGACGCCCGC
	2.920	rrnB_	T2_transcrip_term		2.960		2.980		3.000
CATAAACTGC	CAGGCATCAA	ATTAAGCAGA	AGGCCATCCT	GACGGATGGC	CTTTTTGCGT	TTCTACAAAC	тсттттбттт	АТТТТТСТАА	АТАСАТТСАА
	3.020		3.040		3.060	L. C.	3.080		3.100
ATATGTATCC	GCTCATGGAG	CTCAAAGCCA	сөттөтөтст	СААААТСТСТ	GATGTTACAT	TGCACAAGAT	ΑΑΑΑΑΤΑΤΑΤ	CATCATGAAC	аатаааастб
	3.120		KanR 3.140		3.160		3.180		3.200
TCTGCTTACA	тааасадтаа	TACAAGGGGT	GTTATGAGCC	ATATTCAACG	GGAAACGTCT	TGCTCCAGGC	CGCGATTAAA	TTCCAACATG	GATGCTGATT
KanR	3.220		3.240		3.260		3.280		3.300
TATATGGGTA	TAAATGGGCT	CGCGATAATG	TCGGGCAATC	AGGTGCGACA	ATCTATCGTT	TGTATGGGAA	GCCCGATGCG	CCAGAGTTGT	TTCTGAAACA
KanR	3.320		3.340		3.360		3.380		3.400
TGGCAAAGGT	AGCGTTGCCA	ATGATGTTAC	AGATGAGATG	GTCAGACTAA	ACTGGCTGAC	GGAATTTATG	CCTCTTCCGA	CCATCAAGCA	TTTTATCCGG
KanR	3.420		3.440		3.460		3.480		3.500
ACTCCTGATG	AAGCATGGTT	ACTCACCACT	GCGATCCCCG	GCAAAACAGC	ATTCCAGGTA	TTAGAAGAAT	ATCCTGATTC	AGGTGAAAAT	ATTGTTGATG
KanR	3.520		3.540		3.560		3.580		3.600
CGCTGGCAGT	GTTCCTGCGC	CGGTTGCATT	CGATTCCTGT	TTGTAATTGT	CCTTTTAACA	GCGATCGCGT	ATTTCGTCTC	GCTCAGGCGC	AATCACGAAT
KanR	3.620		3.640		3.660		3.680		3.700
GAATAACGGT	TTGGTTGATG	CGAGTGATTT	TGATGACGAG	CGTAATGGCT	GGCCTGTTGA	ACAAGTCTGG	AAAGAAATGC	ACAAACTTTT	GCCATTCTCA
KanR	3.720		3.740		3.760		3.780		3.800
CCGGATTCAG	TCGTCACTCA	TGGTGATTTC	TCACTTGATA	ACCTTATTTT	TGACGAGGGG	AAATTAATAG	GTTGTATTGA	TGTTGGACGA	GTCGGAATCG
KanR	3.820		3.840		3.860		3.880		3.900
CAGACCGATA	CCAGGATCTT	GCCATCCTAT	GGAACTGCCT	CGGTGAGTTT	TCTCCTTCAT	TACAGAAACG	GCTTTTTCAA	AAATATGGTA	TTGATAATCC
KanP	3.920		3.940		3 960		3 980		4 000
TGATATGAAT	AAATTGCAGT	TTCATTTGAT	GCTCGATGAG	TTTTTCTAAG	AGCTCTCCAT	GACCAAAATC	CCTTAACGTG	AGTTTTCGTT	CCACTGAGCG
	4.020		4.040	· · · · · · · · · · · · · · · · · · ·	4.060		4 090		4 100
TCAGACCCCG	TAGAAAAGAT	CAAAGGATCT	TCTTGAGATC	стттттттст	GCGCGTAATC	тестесттес		ACCACCGCTA	CCAGCGGTGG
	4100		1011040410		4400		4.490	AUGAUUUUIA	4000
	4.120		4.140	GGTAACTGGC	4.160	CCCACATACC	4.100	CTICIACICI	
					TTCAGCAGAG				
	GGATCAAGAG	CIACCAACIC	TTTTTCCGAA	GGTAACTGGC	TTCAGCAGAG	COCAGATACC	AAATACIGIC	CITCIAGIGI	AGCCGTAGTT
	4.220	CTACCAACIC	4.240	CICCCICICC	4.260	ACCAGIGGET	4.280 4.280	CCCATAGET	4.300
AGGCCACCAC	4.220 I TTCAAGAACT	CTGTAGCACC	4.240 GCCTACATAC	CTCGCTCTGC	4.260 I TAATCCTGTT	ACCAGTGGCT	4.280 GCTGCCAGTG	GCGATAAGTC	4.300 GTGTCTTACC
AGGCCACCAC	4.220 1 TTCAAGAACT 4.320	CTGTAGCACC	4.240 GCCTACATAC 4.340	CTCGCTCTGC	4.260 1 TAATCCTGTT 4.360	ACCAGTGGCT	4.280 GCTGCCAGTG 4.380	GCGATAAGTC	4.300 GTGTCTTACC 4.400
AGGCCACCAC	4,220 1 TTCAAGAACT 4,320 1 CAAGACGATA	CTGTAGCACCC	4.240 J GCCTACATAC 4.340 I AAGGCGCAGC	CTCGCTCTGC	4.260 I TAATCCTGTT 4.360 I AACGGGGGGGT	ACCAGTGGCT	4,280 I GCTGCCAGTG 4,380 I AGCCCAGCTT	GCGATAAGTC	4.300 I GTGTCTTACC 4.400 I ACCTACACCG
AGGCCACCAC	4,220 1 TTCAAGAACT CAAGACGATA 4,320 1 CAAGACGATA	CTGTAGCACCC	4.240 GCCTACATAC 4.340 AAGGCGCAGC 4.440	CTCGCTCTGC	4.260 TAATCCTGTT AACGGGGGGGT 4.460 AACGGGGGGGT	ACCAGTGGCT	4,280 GCTGCCAGTG AGCCCAGTG AGCCCAGCTT 4,4800 I	GCGATAAGTC	4.300 GTGTCTTACC 4.400 I ACCTACACCG 4.500 1
AGGCCACCAC GGGTTGGACT AACTGAGATA	4220 I TTCAAGAACT CAAGACGATA CCTACAGCGT	CTGTAGCACCC GTTACCGGAT GAGCTATGAG	4240 I GCCTACATAC AAGGCGCAGC AAAGCGCCAC	CTCGCTCTGC GGTCGGGCTG GCTTCCCGAA	4.260 TAATCCTGTT AACGGGGGGGT GGGAGAAAGG	ACCAGTGGCT TCGTGCACAC CGGACAGGTA	42800 I GCTGCCAGTG AGCCCAGCTT 4,480 TCCGGTAAGC	GCGATAAGTC GGAGCGAACG GGCAGGGTCG	4.300 I GTGTCTTACC 4.400 I ACCTACACCG 4.500 GAACAGGAGA
AGGCCACCAC GGGTTGGACT AACTGAGATA	4220 I TTCAAGAACT 4320 I CAAGACGATA 4420 I CCTACAGCGT 4520 I	CTACCAACTC CTGTAGCACC GTTACCGGAT GAGCTATGAG	4240 I GCCTACATAC 4.340 AAGGCGCAGC AAAGCGCCAC 4.540 AAAGCGCCAC	CTCGCTCTGC GGTCGGGCTG GCTTCCCGAA	4 4800 TAATCCTGTT AACGGGGGGGT 4,380 J AACGGGGGGGG 4,580 GGGAGAAAGG 4,580 J	ACCAGTGGCT TCGTGCACAC CGGACAGGTA	42800 I GCTGCCAGTG AGCCCAGCTT AGCCCAGCTT 4.4800 TCCCGGTAAGC 4.5800 I	GCGATAAGTC GGAGCGAACG GGCAGGGTCG	4.000 GTGTCTTACC 4.400 1 ACCTACACCG 4.500 1 GAACAGGAGA 4.600 1 GAACAGGAGA 4.600 1 1 1 1 1 1 1 1 1 1 1 1 1
AGGCCACCAC GGGTTGGACT AACTGAGATA GCGCACGAGG	4220 1 TTCAAGAACT 4320 CAAGACGATA 4420 1 CCTACAGCGT 4520 1 GAGCTTCCAG	CTGTAGCACC GTTACCGGAT GAGCTATGAG GGGGAAACGC	4.240 I GCCTACATAC AAGGCGCAGC AAAGCGCCAGC AAAGCGCCAC CTGGTATCTT	CTCGCTCTGC GGTCGGGCTG GCTTCCCGAA TATAGTCCTG	44260 TAATCCTGTT AACGGGGGGGT GGGAGAAAGG TCGGGTTTCG	ACCAGTGGCT TCGTGCACAC CGGACAGGTA CCACCTCTGA	4280 I GCTGCCAGTG AGCCCAGCTT AGCCCAGCTT TCCGGTAAGC CTTGAGCGTC	GCGATAAGTC GGAGCGAACG GGCAGGGTCG GATTTTTGTG	4.300 GTGTCTTACC 4.400 J GAACAGCAGA GAACAGGAGA 4.500 J GAACAGGAGA A 4.500 J GAACAGGAGA
AGGCCACCAC GGGTTGGACT AACTGAGATA GCGCACGAGG	4220 1 TTCAAGAACT 4320 CAAGACGATA 4420 1 CCTACAGCGT GAGCTTCCAG 4620 4620 1 4620 1 4620 1 4620 1 4620 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	CTGTAGCACC GTTACCGGAT GAGCTATGAG GGGGAAACGC	4.240 I GCCTACATAC AAGGCGCAGC AAAGCGCCAGC AAAGCGCCAC CTGGTATCTT 4.640 I CTGGTATCTT	CTCGCTCTGC GGTCGGGCTG GCTTCCCGAA TATAGTCCTG	44260 TAATCCTGTT AACGGGGGGGT GGGAGAAAGG TCGGGTTTCG 46600 1 GGGAGAAAGG	ACCAGTGGCT TCGTGCACAC CGGACAGGTA CCACCTCTGA	42800 I GCTGCCAGTG AGCCCAGCTT AGCCCAGCTT TCCCGGTAAGC CTTGAGCGTC 46800 I CTTGAGCGTC	GCGATAAGTC GGAGCGAACG GGCAGGGTCG GATTTTTGTG	4.300 GTGTCTTACC 4.400 1 ACCTACACCG GAACAGGAGA 4.500 1 GAACAGGAGA 4.500 1 ATGCTCGTCA 4.700 1 4.000 1 4.400 1
AGGCCACCAC GGGTTGGACT AACTGAGATA GCGCACGAGG GGGGGGCGGA	4220 1 TTCAAGAACT 4320 CAAGACGATA 4420 1 CCTACAGCGT 4520 1 GAGCTTCCAG 4620 1 GCCTATGGAA	CTGTAGCACC GTTACCGGAT GAGCTATGAG GGGGAAACGC AAACGCCAGC	4.240 GCCTACATAC 4.340 AAGGCGCAGC 4.440 I AAAGCGCCAC 4.540 I AAAGCGCCAC 4.540 I AAAGCCCCAC	CTCGCTCTGC GGTCGGGCTG GCTTCCCGAA TATAGTCCTG TTTTACGGTT	44260 1 TAATCCTGTT AACGGGGGGGT GGGAGAAAGG TCGGGTTTCG 4660 4660 4660 4660 4660 4660	ACCAGTGGCT TCGTGCACAC CGGACAGGTA CCACCTCTGA	4280 GCTGCCAGTG 4380 AGCCCAGCTT 4480 1 TCCGGTAAGC 4 1 TCCGGTAAGC 1 TTGGTCACAT	GCGATAAGTC GGAGCGAACG GGCAGGGTCG GATTTTTGTG GTTCTTTCCT	4.300 GTGTCTTACC 4.400 1 ACCTACACCG GAACAGGAGA 4.500 1 GAACAGGAGA 4.500 1 GGGTTATCCC
AGGCCACCAC GGGTTGGACT AACTGAGATA GCGCACGAGG GGGGGGCGGA	4220 1 TTCAAGAACT 4320 1 CAAGACGATA 4420 1 CCTACAGCGT 4520 1 GAGCTTCCAG 4620 1 GAGCTTCCAG 4620 1 GCCTATGGAA 4720 1 CAAGACGATA	CTACCAACTC CTGTAGCACC GTTACCGGAT GAGCTATGAG GGGGAAACGC AAACGCCAGC	4.240 GCCTACATAC 4.340 AAGGCGCAGC 4.440 I AAAGCGCCAC 4.540 CTGGTATCTT 4.540 I AAAGCGCCCT 4.540 I AAAGCCCCAC 4.540 I AAAGCCCCAC 4.540 I AAAGCCCCAC 4.540 I AAAGCCCCAC 4.540 I AAAGCCCCAC 4.540 I AAAGCCCCAC 4.540 I AAAGCCCCAC 4.540 I AAAGCCCCAC 4.540 I AAAGCCCCAC 4.540 I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAAGCCCCAC I AAGCCCCCAC I AAAGCCCCCCAC	CTCGCTCTGC GGTCGGGCTG GCTTCCCGAA TATAGTCCTG TTTTACGGTT	4 2600 TAATCCTGTT 4 3600 AACGGGGGGGT GGGAGAAAGG TCGGGTTTCG 4 6600 CCTGGCCTTT 4 7600	ACCAGTGGCT TCGTGCACAC CGGACAGGTA CCACCTCTGA	4280 GCTGCCAGTG 4380 AGCCCAGCTT 44800 1 TCCGGTAAGC 4 1 TCCGGTAAGC 4 1 TTGGTCACAT 4 4 1 1 1 1 1 1 1 1 1 1 1 1 1	GCGATAAGTC GGAGCGAACG GGCAGGGTCG GATTTTTGTG GTTCTTTCCT	4.300 GTGTCTTACC 4.400 1 ACCTACACCG GAACAGGAGA 4.500 1 GGAACAGGAGA 4.500 1 GGGTTATCCC 4.800 1 GCGTTATCCC 4.800 1 4.900 1 1 1 1 1 1 1 1 1 1 1 1 1
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AGGCCACCAC GGGTTGGACT AACTGAGATA GCGCACGAGG GGGGGGGCGGA CTGATTCTGT AGAGCGCCTG AGCCAGTATA GGCATCCGCT TCATCAGCGT AGCGGGCCAT GAGGATGCTC AAAATCACTC	4420 TTCAAGAACT 4320 CAAGACGATA 4420 CCTACAGCGT GAGCTTCCAG GGCTATGGAA 4520 GGCTATGGAA 4520 GGATAACCGT 4520 CACTCCGCTA 4520 CACTCCGCTA 4520 CACTCCGCTA 4520 CACTCCGCTA 5120 CACTCCGCAAG 5120 CACTCCGCAAG 5120 CACGACAAG 5120 CACGACAAG 5120 CACGACAAG 5120 5120 CACGACAACG 5120 5	CTACCAACTC CTGTAGCAACC GTTACCGGAT GAGCTATGAG GGGGAAACGC AAACGCCAGC ATTACCGCCAGC TTCTCCTTAC TCGCTACGTG CTGTGACCGT CGATTCACAG GTTTTTCCT TTACTGATGA CCAGCGCGAT	4.240 GCCTACATAC 4.340 AAGGCGCAGC 4.440 CTGGTATCTT AAAGCGCCAC 4.540 CTGGTATCTT AACGCGGCCT 4.740 TTGAGTGAGC 4.840 GCATCTGTGC 4.840 1 CTCCGGGAGC 5.140 CTCCGGGAGC 5.140 CTCGGTCAC	CTCGCTCTGC GGTCGGGCTG GCTTCCCGAA TATAGTCCTG TTTTACGGTT TGATACCGCT GGTATTTCAC GGCTGCGCCC TGCATGTGTC GTTCATCCGC TGATGCCTCC CGGTTACTGG CCTGTCAAAT	TTCAGCAGAGA 4.260 TAATCCTGTT AACGGGGGGGT GGGAGAAAAGG TCGGGTTTCG 4.600 CCTGGCCTTT 4.600 CCTGGCCTGC 4.600 CCTGGCCTGC 4.600 CCTGGCCTGC 4.600 CCTGGCCTGC 5.000 CCGCCGCAGCC 5.000 CCGCGCAGCC 5.000 CCGCGCAGCC 5.000 CCGCGCAGCC 5.000 CCGCGCAGCC 5.000 CCGCGCAGCC 5.000 CCGCGCAGCTGC 5.000 CCGCGCAGCC CCGCGCAGCC CCGCGCAGCC CCGCGCC CCGCGCAGCC CCGCGCAGCC CCGCGCAGCC CCGCGCAGCC CCGCGCAGCC CCGCGCAGCC CCGCGCAGCC CCGCGCAGCC CCGCGCAGCC CCGCGCAGCC CCGCGCAGCC CCGCGCCAGCC CCGCGCCAGCC CCGCGCAGCC CCGCGCCCAGCC CCGCGCCC CCGCGCCAGCC CCGCGCCCCGCAGCC CCGCGCCCCCGCC CCGCGCCCCC CCGCCCCCCCC	ACCAGTGGCT TCGTGCACAC CGGACAGGTA CCACCTCTGA TGCTGGCCTT GAACGACCGA GTGCACTCTC CAACACCCGC ACCGTCATCA TTGAGTTTCT GATTTCTGTT GGGTAAACAA GGGATTCTGC	4440 1910 GCTGCCAGTG 44300 AGCCCAGCTT 4400 TCCCGGTAAGC 4500 CTTGAGCGTC 4600 TTGCTCACAT 4600 TGCCCAGCGAG 4800 AGTACAATCT 4900 TGACGCGCCC 5000 CCGAAACGCG 5180 CCGGAAACGCG 5180 CCGGAAACGCG 5180 CCGGAAACGCG 5180 CCGGAAACGCG 5180 CCGGAAACGCG 5180 CCGGAAACGCG 5180 CCGGAAACGCG 5180 CCGGCGGTA 5380 CTGGCGGGTA 5480 AAACCCTATG	GCGATAAGTC GGAGCGAACG GGCAGGGTCG GATTTTTGTG GTTCTTTCCT TCAGTGAGCG GCTCTGATGC TGACGGGCTT CGAGGCAGCT TAATGTCTGG ATGATACCGA GGATGCGGCG CTACTCCGTC	AGCCAGAGAG 4.00 1 GTGTCTTACC 4.400 1 GAACAGGAGA 4.00 1 GAACAGGAGA 4.00 1 GGGTTATCCC 4.00 1 GCGTTACCCC 4.00 1 GCGTTACCCC 4.00 1 GCGTTACCCC 4.00 1 GCGTTACCCC 4.00 1 GCGTACAGGAGCGGA 4.00 1 GCGCTACACGAGAC 4.00 1 GCGCTACACGAGAC 4.00 1 GCGCTACACGAGAC 4.00 1 GCGCCACAGACCAGAC 4.00 1 GCGCCACAGACCAGACCAGAC
AGGCCACCAC GGGTTGGACT AACTGAGATA GCGCACGAGG GGGGGGGCGGA CTGATTCTGT AGAGCGCCTG AGCCAGTATA GGCATCCGCT TCATCAGCGT AGCGGGCCAT GAGGATGCTC AAAATCACTC	4420 TTCAAGAACT 4320 CAAGACGATA 4420 CCTACAGCGT GAGCTTCCAG GGCTATGGAA 4520 GGATAACCGT 4520 GGATAACCGT 4520 CACTCCGCTA 4520 CACTCCGCTA 4520 CACTCCGCTA 5020 TACAGACAAG 5120 GGTCATGGAAG 5120 GGTCATGGAAG 5120 CACTCCGCG 5120 CACGATACGGG 5120 5120 CACGATACGGG 5120 512	CTACCAACTC CTGTAGCACC GTTACCGGAT GAGCTATGAG GGGGAAACGC AAACGCCAGC ATTACCGCCT TTCTCCTTAC TCGCTACGTG CTGTGACCGT CGATTCACAG GTTTTTTCCT TTACTGATGA CCAGCGCGAT	4.240 GCCTACATAC 4.340 AAGGCGCAGC 4.440 CTGGTATCTT AAAGCGCCAC 4.540 CTGGTATCTT AACGCGGCCT 4.740 TTGAGTGAGC GCATCTGTGC 4.840 GCATCTGTGC 4.840 1 CTCCGGGAGC 5.440 CTCCGGGAGC 5.440 GCATAATGTG GCATAATGTG	CTCGCTCTGC GGTCGGGCTG GCTTCCCGAA TATAGTCCTG TTTTACGGTT TGATACCGCT GGTATTTCAC GGCTGCGCCC TGCATGTGTC GTTCATCCGC TGATGCCTCC CGGTTACTGG CCTGTCAAAT	TTCAGCAGAG 4280 TAATCCTGTT 4480 GGGAGAAAAGG TCGGGTTTCG CCTGGCCTTT 4780 CCTGGCCTTT 4780 CCTGGCCTTT 4780 CGCCGCAGCC 4800 ACCGCAAGCC 4800 ACCGCAAGCC 5000 1 GGCCAGCCGC 5100 1 GTCCAGCTGG 3500 1 GTCCAGCTGG 3500 1 GGCCGAGCC	ACCAGTGGCT TCGTGCACAC CGGACAGGTA CCACCTCTGA TGCTGGCCTT GAACGACCGA GTGCACTCTC CAACACCCGC ACCGTCATCA TTGAGTTTCT GATTTCTGTT GGGTAAACAA GGGATTCTGC	AAATACTOTE 4280 GCTGCCAGCT AGCCCAGCT AGCCCAGCT TCCCGGTAAGC 4480 CTTGAGCGGC GCGCAGCGAG AGTACAATCT 4580 TGGCGCAGCGAG 4800 AGTACAATCT 4980 TGACGCGCCC 5080 CCGAAACGCG 5180 CCGGAAACGCG 5180 CCGGAAACGCG 5180 CCGGAAACGCG 5180 CCGGAAACGCG 5180 CCGGAAACGCG 5180 CCGGAAACGCG 5180 5280 CCGGCGGTAT CAGGGGGTAT	GCGATAAGTC GGAGCGAACG GGCAGGGTCG GATTTTTGTG GTTCTTTCCT TCAGTGAGCG GCTCTGATGC TGACGGGCTT CGAGGCAGCT TAATGTCTGG ATGATACCGA GGATGCGGCG CTACTCCGTC	AGCCGTAGTA 4.300 GTGTCTTACC 4.400 J GAACAGGAGA 4.500 GAACAGGAGA 4.600 J GGGTTATCCC 4.600 1 GCGTTATCCC 4.600 1 GCGCTAGTCA 4.700 1 GCGCTAGTCA 4.700 1 GCGCTAGTCA 4.700 1 GCGCTAGTCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 GCGCCAGAGCA 4.900 1 4.900 1 4.

alphaVmut-TMD = TMD Int_a5GP

JM = Juxtamembrane region

KanR = Aminoglycoside O-phosphotransferase

Publications

Schanzenbach C, Schmidt FC, Breckner P, Teese MG, Langosch D. Identifying ionic interactions within a membrane using BLaTM, a genetic tool to measure homo- and heterotypic transmembrane helix-helix interactions. *Sci. Rep.* **7**, 43476; (2017).

Schanzenbach C, Ilver D, Blomgren J, Jonasson C, Johansson C, Krozer A, Ye L, Rustas BO. Preparation and characterisation of a sensing system for wireless pH measurements in vivo, in a rumen of a cow. Sensors and Actuators, B: Chemical. 2017 Apr 1;242:637-644.

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