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A method for rapid regulation of protein expression in *Trypanosoma cruzi*

Yan Fen Ma^a, Louis M Weiss^{a,b}, and Huan Huang^{a,*}

^aDepartment of Pathology, Albert Einstein College of Medicine Jack and Pearl Resnick Campus, 1300 Morris Park Avenue, Bronx, NY 10461 USA

^bDepartment of Medicine, Albert Einstein College of Medicine Jack and Pearl Resnick Campus, 1300 Morris Park Avenue, Bronx, NY 10461 USA

Abstract

Analysis of gene function in *Trypanosoma cruzi* is limited due to the absence of rapid, simple and reversible genetic tools to regulate gene and corresponding protein expression. We have designed a modified pTREX vector which uses an N-terminal fusion of a ligand-controlled destabilization domain (ddFKBP) to a gene/protein of interest. This vector allows rapid and reversible protein expression and efficient functional analysis of proteins in different *T. cruzi* life cycle stages.

Keywords

Trypanosoma cruzi; Chagas disease; Genetic manipulation; Destabilization domain; pTREX vector; Protein expression

Trypanosoma cruzi is a pathogenic kinetoplastid protozoan that is the etiological agent of Chagas disease (Tanowitz et al., 2009). While genetic manipulation of this organism using transfection and gene knockout technologies has been established, there are significant limitations with the currently available techniques for genetic manipulation of this organism. Although a tetracycline-inducible expression system is available (Taylor et al., 2006), the kinetics of the expression of the protein of interest is relatively slow. Utilization of the tetracycline-inducible system requires generation of a *T. cruzi* strain expressing both T7 polymerase and *tetR* genes, which entails the use of multiple selectable markers. This makes it difficult to use this system in multiple isolates of *T. cruzi*. Furthermore, this inducible system is not useful for the study of intracellular life stages since the T7 polymerase and *tetR* genes are expressed from an episome. Thus, it is difficult to utilize the tetracycline-inducible system for conditional knockouts. A new tool which is simple, rapid, reversible and versatile is needed to study the biology of this parasite.

Rapid regulation of protein levels has been demonstrated in mammalian cells, apicomplexan parasites and *Leishmania* using the expression of a target protein as a fusion protein containing the destabilization domain (ddFKBP) of the “rapamycin binding protein”. In the absence of the synthetic ligand, Shield-1, proteins bearing this domain are rapidly degraded

by the cytoplasmic proteasome. Stabilization is achieved by reversible binding of Shield-1 to ddFKBP (Banaszynski et al., 2006; Armstrong et al., 2007; Herm-Götz et al., 2007; Madeira et al., 2009).

To test whether this system could be applied in *T. cruzi*, we generated genetic fusions linking 3flag- ddFKBP N-terminally to Enhanced Yellow Fluorescent Protein (EYFP) (3flag- ddFKBP-EYFP) and C-terminally to GFP (GFP-3flag-ddFKBP) in a pTREX vector (Vazquez et al., 1999). To generate a construct N-terminally expressing 3flag-ddFKBP, we amplified the DNA encoding the 3flag-ddFKBP domain using a plasmid containing 3flag-ddFKBP domain sequences as a template by PCR (TgERK2-3flag- ddFKBP) (Huang et al., 2011) (Table 1, primer pair 1 for 3flagddFKBP) and inserted this 3flag-ddFKBP into a pTREX vector. As the restriction enzymes, *Bam*H1 and *Eco*R1, removed the HX1 region (Vazquez et al., 1999) from this pTREX vector, this expression vector is actually pRIBOTEX-3flag-ddFKBP. DNA encoding EYFP was amplified (Table 1, primer pair 2 for EYFP) using TUB-DDmycYFP-CAT plasmid as a template (gift of Dr. Kami Kim, Albert Einstein College of Medicine, USA) and EYFP was inserted into a pRIBOTEX-3flag-ddFKBP to generate a pRIBOTEX-3flag-ddFKBP-EYFP vector (Fig. 1A). To generate a pTREX- 3flag-ddFKBP-EYFP vector, the DNA encoding the 3flag-ddFKBP-EYFP was amplified using a pRIBOTEX-3flag-ddFKBP-EYFP vector as a template (Table 1, primer pair 3 for 3flagddFKBPEYFP) and this DNA was inserted into pTREX (using *Hind*III and *Xho*I restriction sites) to produce a pTREX-3flag-ddFKBP-EYFP vector (Fig. 1B). To generate a pTREX C-terminally expressing 3flag-ddFKBP, the DNA encoding the 3flag-ddFKBP was amplified using a pTREX-3flag-ddFKBP-EYFP as a template (Table 1, primer pair 4 for 3flagddFKBP) and the 3flag-ddFKBP was then inserted into a pTREX vector to create pTREX-3flag-ddFKBP with 3flag-ddFKBP at the C-terminal end of the multiple cloning sites. DNA encoding GFP was amplified using pROCKGFPNeo (Pires et al., 2008) as a template (Table 1, primer pair 5 for GFP) and this GFP fragment was ligated into a pTREX vector to generate the pTREX-GFP-3flag-ddFKBP expression vector (Fig. 1C).

These constructs were transfected into epimastigotes using electroporation and G418 selection by the methods described in our previous publication (Bao et al., 2008). A stable line was obtained and parasites carrying the N-terminally labeled construct, pTREX-3flag-ddFKBP-EYFP, displayed regulated fusion protein expression in a Shield-1-dependent manner, e.g. in the presence of 0.5 μ M of Shield-1, the fusion protein was detectable, whereas in the absence of Shield-1 the fusion protein was not detectable. The fusion protein level increased in response to the higher concentration of Shield-1 (Fig. 2A), permitting regulated protein levels in this vector system. The time course kinetics of expression of the fusion protein was analyzed using immunoblots of parasite lysates by methods previously described (Bao et al., 2008). Following the addition of 2 μ M of Shield-1 to the transfected epimastigotes, fusion protein expression was detectable as early as 15 min, the expression level was saturated at approximately 2 h and was maintained at a steady level for several days. Using light microscopy, intense fluorescence of this regulated EYFP construct (which appeared green due to the filter set utilized) was observed at 2 h after 2 μ M of Shield-1 was added (Fig. 2B and C). All of the stably transfected parasites displayed regulated expression. To test whether the expression of fusion protein was reversible, Shield-1 was removed by washing the parasites with PBS three times and those parasites were then cultured in Shield-1-free medium. It was observed that the fusion protein gradually degraded in the absence of Shield-1 and by 120 h the fusion protein was no longer detectable. Following the addition of Shield-1 to the washed parasites, fusion protein again became detectable (Fig. 2D), indicating that this system is regulatable and reversible in *T. cruzi*. To examine whether cell density would affect this vector system, epimastigotes with pTREX-3flag- ddFKBP-EYFP were grown to a high density level (10^9 – 10^{10} /ml) and then Shield-1 was added to those cells. There was no fusion protein expression in those epimastigotes (data not shown).

This finding is consistent with a requirement of the promoter used for dividing parasites that are not in the stationary phase of the growth cycle. It is possible that the use of other promoters would allow regulated gene expression in the stationary phase. This phenomenon requires further investigation.

In contrast to the N-terminal tagged vectors, no regulation of the C-terminal GFP vector (GFP-3flag-ddFKBP) was seen in epimastigotes. We observed some fluorescent parasites 24 h after transfection in the absence of Shield-1 (data not shown), suggesting that there was insufficient degradation of the C-terminally labeled ddFKBP fusion protein. There were no stable transfectants obtained with pTREX-GFP-3flag-ddFKBP despite multiple attempts, suggesting that this fusion protein may have had a detrimental effect on the parasites. Slower degradation of C-terminal ddFKBP fusions has been observed in both mammalian cells and apicomplexan parasites (Banaszynski et al., 2006; Armstrong et al., 2007). However, this observation does not mean that C-terminal fusion of this system is useless because other fusion proteins might still be regulated by this approach. For any particular gene, therefore, one should try both N-terminal and C-terminal fusion constructs to evaluate the best construct for regulated gene expression of a particular gene.

Next, we tested whether *T. cruzi* genes could also be regulated using the ddFKBP system. Three *T. cruzi* mitogen activated protein kinase (TcMAPK) genes with mutations in the dual phosphorylation sites for enzyme activation were used for expression (TcMAPK1: Tc00.1047053511299.70; TcMAPK2: Tc00.1047053506007.40; TcMAPK3: Tc00.1047053509553.60. Mutations: TcMAPK1: ¹⁹⁰TEY¹⁹² to ¹⁹⁰AEF¹⁹², TcMAPK2¹⁷⁴TDY¹⁷⁶ to ¹⁷⁴ADF¹⁷⁶ and TcMAPK3 ¹⁹²TDY¹⁹⁴ to ¹⁹²ADF¹⁹⁴). These mutations have been reported to serve as dominant negative (DN) constructs (Robbins et al., 1993). These constructs would be useful tools to study TcMAPK function(s) in *T. cruzi* if these TcMAPK genes are regulated using the ddFKBP system. We amplified the TcMAPKs using *T. cruzi* genomic DNA as a template (Table 1, primer pair 6–8 for TcMAPK1–TcMAPK3). The entire open reading frames (ORFs) of TcMAPK1, TcMAPK2, or TcMAPK3 were ligated into pTREX to produce three vectors (pTREX-TcMAPK1, pTREX-TcMAPK2 and pTREX-TcMAPK3). To create mutations in the activation sites in these pTREX-TcMAPKs, we used a QuikChange® Site-Directed Mutagenesis Kit (Stratagene, USA) using the oligonucleotides described in Table 1 (primer pair 9–11 for TcMAPK1^{DN} to TcMAPK3^{DN}) to create pTREX-TcMAPK1^{DN}, pTREX-TcMAPK2^{DN} and pTREX-TcMAPK3^{DN} vectors, according to the manufacturer's instruction manual. A fragment of 3flag-ddFKBP was then generated by PCR (Table 1, primer pair 12 for 3flagddFKBP) and this 3flag-ddFKBP fragment was inserted into the pTREX-TcMAPK^{DN} vectors to generate pTREX-3flag-ddFKBP-TcMAPK1^{DN}, pTREX-3flag-ddFKBP-TcMAPK2^{DN}, and pTREX-3flag-ddFKBP-TcMAPK3^{DN} vectors (Fig. 1D) for transfection into *T. cruzi*.

These TcMAPK^{DN} vectors were transfected into epimastigotes and stable parasite lines selected. In the absence of Shield-1 TcMAPK^{DN} fusion proteins were not detectable, while the addition of Shield-1 resulted in the expression of these TcMAPK^{DN} fusion proteins (Fig. 2E), indicating that this system is suitable for regulation of the expression of endogenous genes in *T. cruzi*. We did not detect any change in endogenous TcMAPK protein levels in the presence or absence of Shield-1 (Fig. 2A).

Different *T. cruzi* expression vectors may have different results in this regulatory system. The pRIBOTEX expression vector does not contain an upstream region of the TcP2beta H1 gene (220 bp HX1 region) adjacent to the putative ribosomal promoter region (Vazquez et al., 1999; Martínez-Calvillo et al., 1997). Shield-1 failed to induce the fusion protein in epimastigotes carrying the pRIBOTEX-3flag-ddFKBP-EYFP (data not shown). In contrast, the pTREX vector that contains the 220 bp HX1 region adjacent to the putative ribosomal

promoter region, but is otherwise identical to pRIBOTEX, could regulate 3flag-ddFKBP-EYFP expression. This is probably because the HX1 region is an effective trans-splicing acceptor. It should be appreciated, however, that the pRIBOTEX expression vector has a cryptic trans-splicing site and it may be able to express other ddFKBP fusion proteins. This observation requires further investigation.

The ability to regulate a protein of interest or to express an inhibitor to interdict a signal transduction pathway in the intracellular form of *T. cruzi* (amastigotes) is of importance because there is no reported regulatable genetic system which currently permits regulated protein/gene expression in this life stage. It has been reported that pTREX vectors are integrated into the *T. cruzi* nuclear genome within the first 5 h following transfection and that any non-integrated vectors (i.e. episomal copies) are rapidly degraded (Lorenzi et al., 2003). We therefore examined whether the pTREX-3flag-ddFKBP-EYFP was integrated into the parasitic genome. Southern blot analysis was performed using digoxigenin-dUTP labeled 3flag-ddFKBP as a probe. The result revealed that 3flag-ddFKBP-EYFP had integrated into the genome in epimastigotes (Fig. 3A).

To generate amastigotes carrying pTREX-3flag-ddFKBP-EYFP, we infected human fibroblast cells with transfected epimastigotes containing metacyclic trypomastigotes, which resulted in an infection of human fibroblast cells and the development of intracellular amastigotes. Without Shield-1, the intracellular amastigotes did not show any fluorescence. Within 1 h after addition of 2 μ M of Shield-1 we observed that all of the amastigotes appeared fluorescent (Fig. 3B) and that these parasites maintained fluorescence (i.e. cells were still fluorescent 8 weeks after the initial observation as long as Shield-1 was present in the media). Trypomastigotes released from these Shield-1 treated amastigotes were fluorescent; however, trypomastigotes released from amastigotes without Shield-1 did not turn fluorescent when incubated with 2 μ M Shield-1 (tested at 2, 4, 8, 12, 24 h after Shield-1 treatment) (data not shown). This may be due to the decrease of *polII* and *polIII* and associated transcription in the trypomastigote life stage (Schenkman et al., 2011) making it likely that in this non-replicating life cycle stage the expression of this fusion protein is very low. Next, we tested pTREX-3flag-ddFKBP-TcMAPK3^{DN} in tissue culture amastigotes and found that this *T. cruzi* gene was also clearly regulated by Shield-1 in amastigotes (Fig. 3C).

We previously found that cAMP-Protein Kinase A (PKA) signaling is essential for *T. cruzi* in epimastigotes and that over-expression of Protein Kinase A inhibitor (PKI) has a lethal effect in epimastigotes (Bao et al., 2008). To explore the possibility of specifically blocking PKA activity in amastigotes, we constructed a pTREX-3flag-ddFKBP-PKI. To generate a pTREX vector expressing 3flag-ddFKBP-PKI, a 3flag-ddFKBP fragment was amplified (Table 1, primer pair 13 for 3flagddFKBP) and the DNA fragment introduced into a pTREX vector. Then, DNA encoding a mouse PKI (GenBank accession number **BC026550**) was amplified using a pTREX-PKI-HA construct (Bao et al., 2008). (Table 1, primer pair 14 for PKI) and the PKI gene was ligated into the vector to make the pTREX-3flag-ddFKBP-PKI construct (Fig. 1E).

The pTREX-3flag ddFKBP-PKI construct was transfected into epimastigotes and tissue culture amastigotes carrying this construct were generated by the same method mentioned above. The effect of Shield-1 on both forms of *T. cruzi* was tested. The fusion protein level of 3flag-ddFKBP-PKI in epimastigotes was confirmed by immunoblot using monoclonal antibody (mAb) anti-3flag (Fig. 3D) while expression of 3flag-ddFKBP-PKI in amastigotes was verified by IFA using mAb anti-3flag (Fig. 3E). To examine the effect of 3flag-ddFKBP-PKI on intracellular amastigotes, we measured the growth of amastigotes by ³H-Uracil incorporation. Addition of Shield-1 resulted in growth inhibition; at 96 h post addition of Shield-1 there was an 80% reduction in growth (Fig. 3F). Shield-1 alone or

expression of 3flag-ddFKBP-EYFP did not inhibit the growth of amastigotes (data not shown).

In short, this system has many merits for studies of either the cellular or molecular biology of this pathogenic organism. The regulation of protein expression is tightly controlled, fast and reversible. Quickly over-expressing a protein from a gene of interest or a lethal gene is feasible in this system and this can be done in multiple *T. cruzi* isolates using the same vector construct without any need to genetically modify the isolates. Finally, since this system is simple and requires only one drug for selection, it can be used for conditional gene knockout in this organism (i.e. after transfection with the ddFKBP system one can use Shield-1 treatment to maintain the expression of the regulated gene while knocking out the endogenous gene locus).

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Fig. 1.

Schematic diagrams of vector constructs used for transfection of *Trypanosoma cruzi*. Two expression vectors, pRIBOTEX and pTREX, were used in producing these constructs. DD represents the 3flag ligand-controlled destabilization domain (ddFKBP); EYFP represents Enhanced Yellow Fluorescent Protein; GFP represents Green Fluorescent Protein; TcMAPK^{DN} represents the *T. cruzi* mitogen activated protein kinase dominant negative mutants; PKI represents the Protein Kinase A Inhibitor. The detailed strategies for construction of these vectors are described in the text.

Fig. 2.

Regulation of proteins fused to ligand-controlled destabilization domain (ddFKBP) by Shield-1 in *Trypanosoma cruzi* epimastigotes. Epimastigotes (Tulahuen strain) were grown at 26°C in a liver digest-neutralized, tryptose medium (LDNT) supplemented with 10% FCS (Gibco Life Technologies, Gaithersburg, MD, USA). (A) To examine Shield-1-dependent regulation, protein lysates (150 μ g/lane) from epimastigotes carrying the N terminal 3flag-ddFKBP-Enhanced Yellow Fluorescent Protein vector (pTREX-3flag- ddFKBP-EYFP) treated with different concentration of Shield-1 for 12 h were examined by immunoblot analysis using rabbit polyclonal antibody to GFP (Abcam, catalog number: ab6556, USA) which is suitable for detection of EYFP. The fusion protein level increased in response to the higher concentration of Shield-1. The same lysates (Loading) were stained with Coomassie Brilliant Blue in an independent SDS Page to verify the loading equivalency and the bands around 100 kDa were shown. (B) Immunoblot analysis demonstrates that Shield-1 (2 μ M) induction is rapid. Within 15 min, the Enhanced Yellow Fluorescent Protein containing the ligand-controlled destabilization domain (DD-EYFP) can be detected. DD-EYFP expression peaks at 2 h following the addition of Shield-1 and then maintains a steady level. The same lysates (Loading) were stained with Coomassie Brilliant Blue in an independent SDS Page to verify equal protein loading of the examined samples. (C) Fluorescence detected by light microscopy after addition of 2 μ M of Shield-1 in epimastigotes with pTREX-3flag-ddFKBP-EYFP. Without Shield-1, no fluorescence was detected. (D) Induced epimastigotes expressing pTREX-3flag- ddFKBP-EYFP (2 μ M of Shield-1, 12 h) were washed and cultured in medium without Shield-1. Immunoblot analysis demonstrated that Enhanced Yellow Fluorescent Protein containing the ligand-controlled

destabilization domain (EYFP) gradually degraded following removal of Shield-1. The addition of Shield-1 (2 μ M of Shield-1, 12 h) to washed parasites that had been without Shield-1 for 14 days resulted in the expression of EYFP, indicating that this system is reversible and that the vectors persist in the parasite. The same lysates (Loading) were stained with Coomassie Brilliant Blue in an independent SDS Page to verify equal protein loading of the examined samples. (E) Three *T. cruzi* mitogen activated protein kinase genes with mutations in activation sites (TcMAPK1^{DN}, TcMAPK2^{DN} and TcMAPK3^{DN}) were N-terminally fused with 3flag-ddFKBP and transfected into epimastigotes. Immunoblot analysis using a mAb anti-flag (Sigma, USA) demonstrated that without Shield-1 (0 μ M), there was no protein expression while addition of 2 μ M of Shield-1 for 12 h resulted in detectable levels of these TcMAPK^{DN} fusion proteins. The same lysates (Loading) were stained with Coomassie Brilliant Blue in an independent SDS Page to verify equal protein loading of the examined samples.

Fig. 3.

Regulation of proteins fused to ligand-controlled destabilization domain (ddFKBP) by Shield-1 in *Trypanosoma cruzi* amastigotes and functional analysis of the cAMP-Protein Kinase A (cAMP-PKA) pathway using the ddFKBP system. (A) Southern blot analysis performed on wild type (WT) and N terminal 3flag- ddFKBP-Enhanced Yellow Fluorescent Protein vector (DD-EYFP) transfected *T. cruzi* using digoxigenin-dUTP labeled 3flag- ddFKBP as a probe. The result indicated that N terminal 3flag- ddFKBP-Enhanced Yellow Fluorescent Protein vector (pTREX-3flag- ddFKBP-EYFP) was integrated into the parasitic genome. Genomic DNA was isolated from epimastigotes with and without pTREX- 3flag- ddFKBP-EYFP using a Wizard Genomic DNA Purification kit (Promega, USA). DNA (10 μ g) was digested with 20 units of the indicated restriction enzymes (*HindIII* or *XhoI*) at 37°C overnight. The restriction fragments were separated by electrophoresis on 0.7% agarose gels and transferred to nylon membranes. Probe labeling, hybridization conditions and detection of signals were performed using the PCR DIG Probe Synthesis Kit, DIG Easy Hyb Granules and DIG Wash and Block Buffer Set systems according to manufacturer's instructions (Roche, USA). kb represents kilobase in the DNA standard. (B) Within 1 h after addition of 2 μ M of Shield-1, fluorescence microscopy revealed that amastigotes with pTREX-3flag-ddFKBP-EYFP expressed EYFP and were fluorescent. Tissue culture

trypomastigotes were generated by incubating transfected stationary epimastigotes containing metacyclic trypomastigotes with human foreskin fibroblasts (HFF) maintained in DMEM supplemented with 10% FCS. The trypomastigotes released by this method were used for experiments to infect HFF to achieve intracellular amastigotes for this experiment. (C) Immunoblot analysis with a monoclonal antibody (mAb) anti-flag demonstrated that the mutated *T. cruzi* gene, TcMAPK3^{DN} N-terminally fused with 3flag-ddFKBP (DD-TcMAPK3), was regulated by 2 μ M of Shield-1 (12 h) in amastigotes. The same lysates (Loading) were stained with Coomassie Brilliant Blue in an independent SDS Page to verify equal protein loading of the examined samples. (D) A PKA inhibitor gene (PKI) N-terminally fused with 3flag-ddFKBP (DD-PKI), was induced by 2 μ M of Shield-1 (12 h) in epimastigotes. Immunoblot analysis with a mAb anti-flag detected a fusion protein band in Shield-1-induced parasite lysate. (E) IFA using a mAb anti-flag indicated that 3flag-ddFKBP-PKI was expressed in amastigotes following the addition of 2 μ M of Shield-1 (12 h). Fibroblasts were cultured on cover slips to 80% confluence and then infected with tissue culture-derived trypomastigotes ($3.1 \times 10^6/\text{cm}^2$ surface area of culture plates) for 24 h. The fibroblast cultures were fixed with 2% formaldehyde (30 min), permeabilized with 0.2% Triton $\times 100$ (20 min) and blocked with 1% BSA in PBS overnight at 4°C in a moist chamber. A mAb to flag (Sigma, USA) was diluted to 1:200 in 1% BSA, incubated with the cover slips at 37°C for 90 min and washed with PBS. Secondary antibody (Alexa 488, goat anti-mouse IgG, 1:5,000) was incubated with the cover slips at 37°C for 90 min and washed with PBS. 1,4-diazabicyclo[2.2.2]octane (DABCO) was added to the slides (to minimize fluorescent bleaching), cover slips applied and the slides were observed under a fluorescent microscope. (F) A ³H-Uracil incorporation test indicated that the expression of 3flag-ddFKBP-PKI in intracellular amastigotes inhibited their growth. Twelve-well tissue culture plates containing confluent fibroblast monolayers were inoculated with trypomastigotes derived from pTREX-3flag-ddFKBP-PKI transfected epimastigotes ($3\text{--}4 \times 10^6/\text{well}$) with 2 μ M of Shield-1 to induce the fusion protein, 3flag-ddFKBP-PKI, for 24, 48, 72 and 96 h, and one set of the same culture was used as a negative control by adding the same amount of vehicle solution (2 μ l of 100% ethanol per well). Mean values of three independent experiments $\pm$ S.D. are shown. Asterisk indicates a significant difference in ³H-Uracil incorporation ($P < 0.01$, two-tailed Student's *t*-test). DPM indicates disintegrations per minute.

Table 1

Primers used for construction of the vectors and cloning of genes from *Trypanosoma cruzi*.

PRIMERS	FORWARD	REVERSE
1. 3flgddFKBP	BamH1 5'-CGGGATCCATGGACTACAAAGACCATGAC-3'	EcoR1 5'-CCGGAATTCTTCCGGTTTTAGAAGCTC-3'
2. EYFP	EcoR1 5'-CCGGAATTCGTGAGCAAGGGCGAGGAG-3'	Sal1 5'-ACGCGTCGACTTATCGAAGCTTGAGCTCGAG-3'
3.3flgddFKBPEYFP	Hind III 5'-CCCAAGCTTATGGACTACAAAGACCATGAC-3'	Xho1 5'-CCGCTCGAGTTATCGAAGCTTGAGCTCGA-3'
4. 3flgddFKBP	Sal1 5'-ACGCGTCGACGACTACAAAGACCATGAC-3'	Xho1 5'-CCGCTCGAGTTATTCGGTTTTAGAAGCT-3'
5. GFP	EcoR1 5'-CGGAATTCATGAGTAAAGGAGAAGAACT-3'	Sal1 5'-ACGCGTCGACTTTGTATAGTTCATCCATG-3'
6. TcMAPK1	EcoR1 5'-CCGGAATTCATGGATAAACCCAGGAAG-3'	Sal1 5'-CCGGTCGACCTACGGCTCAGGTGAGT-3'
7. TcMAPK2	EcoR1 5'-CCGGAATTCATGTCATCTGAAATAGAGCC-3'	Xho1 5'-CCGCTCGAGCTACTTTTGCATGGTTCGT-3'
8. TcMAPK3	EcoR1 5'-CCGGAATTCATGCACGATAGCAACCAGG-3'	Xho1 5'-CCGCTCGAGCTAAGGAGAAGCAGGGGGC-3'
9. TcMAPK1 ^{DN}	5'-GTCTCAGATGCCCTCCGCGGAATTCGTTGTACGCGATG-3'	5'-CATCGCGTGACAACGAATTCGCGAGGGCATCTGAGAC-3'
10. TcMAPK2 ^{DN}	5'-CGCCCCGCCCTCGCGGATTCATCATGACCCG-3'	5'-CGGGTCATGATGAAATCCGCGAGGGCGGGGCG-3'
11. TcMAPK3 ^{DN}	5'-GCCGTACTATGAGCTGGCGGATTTTGTATCACACGTGG-3'	5'-CCAGCGTGTGATGACAAAATCCGCCAGCTCATAGTACGGC-3'
12. 3flgddFKBP	EcoR1 5'-GGAATTCATGGACTACAAAGACCATGACG-3'	EcoR1 5'-GGAATTCTTCCGGTTTTAGAAGCTCCAC-3'
13. 3flgddFKBP	EcoR1 5'-CCGGAATTCATGGACTACAAAGACCATGAC-3'	Hind III 5'-CCGGAATTCATGGACTACAAAGACCATGAC-3'
14. PKI	Hind III 5'-CCCAAGCTTATGACTGATGTGAAACTAC-3'	Xho1 5'-CCGCTCGAGTTAGCTTTCAGACTTGGCTG-3'