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NRCPD-OUAVM Joint Research Report

Date: June 4, 2013

Project No: 24-joint-11

1. Principal Investigator

Name Carlos E. Suarez

Position: Research Molecular Biologist

Affiliation: Animal Disease Research Unit, ARS-USDA, Pullman, Washington, USA

2. Project Title: Development of a gene knock-out/complementation system based in the WR99210/dhfr and blasticidin/bsd selection markers for functional gene characterization in *Babesia bovis*

3. Collaborating Research Members at NRCPD

Name Shin-ichiro Kawazu

Position Professor, Research Unit for Advanced Preventive Medicine

Name: Ikuo Igarashi

Position: Professor, Research Unit for Molecular Diagnosis

Name: Naoaki Yokoyama

Position: Professor, Research Unit for Molecular Diagnosis

5. Research Period, from/to (mm/dd/yyyy) and total number of years.

2012.4.1 ～2013.3.31 (one year)

6. Abstract, Results, and Research Significance:

Babesia bovis is responsible for the most severe form of bovine babesiosis. Developing improved methods of control requires a better knowledge of the molecular biology of the parasite and the parasite-host interactions. More than half of the genes identified cannot be assigned to functions based on sequence comparisons alone and no methods for functional gene characterization are available. Our main objective is to develop transfection methods for functional *B. bovis* gene characterization. Two *B. bovis* stable transfection systems using two different selectable markers were recently developed independently by our research groups in US and Japan, and targeted gene KO was achieved for the first time by Asada et al. The biologically cloned transfected parasite line TPx-1 KO *B. bovis,* with a mutation in the single copy gene 2-Cys-peroxyredoxin (*Bbtpx-1*) gene is available and will be used as a target for replacement using the proposed double-sequential transfection system (Fig. 1).

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Mutation rescue with restoration of the wild type *Bbtpx-1* gene is attempted by transfection of the mutated cell line with a plasmid construct *pBbtpx-1- ef-IG-bsd-rfp,* described in Figure 2 (Step 4). An identical control experiment will also be performed using wild type *B. bovis* as a target. The transfection plasmid construct was designed in order to target the wild type *Bbtpx-1* gene allowing expression of the *Bbtpx* and *bsd-rfp* genes (thus conferring resistance to blasticidin and expression of the red fluorescent marker RFP) for selection of the *Bbtpx-1*-restored function transfected mutant in *in vitro* cultures containing blasticidin (Figs 1 and 2).

At first, we constructed circular *ef-IG-bsd-rfp* plasmid, not aimed neither at KO nor integration (shown at step2 on Fig 2), and transfected it into gfp-expressing *B. bovis* (a parasite line which is stably transfected with circular *act-dhfr-ef-IG-gfp* plasmid) by nucleofection followed by selection with blasticidin (Fig 3) The blasticidin resistant parasites (also WR99210 resistant as well) emerged around 10 days after transfection and both green fluorescence and red fluorescence was observed on the parasites (Fig 4). This experiment demonstrated that both, WR99210/dhfr and blasticidin/bsd selection markers, can be simultaneously expressed, and that both genes fused in the the mRFP/bsd construct are functional. Therefore, the rfp-bsd fusion gene under the control of the 5'ef-1α region shown in Figure 2 can be used for the further double-sequential transfection experiments planned in the project.

We plan next to transfect both, wild type and TPx-1 KO *B. bovis* transfected parasites, with plasmid *pBbtpx-1- ef-IG-bsd-rfp* by nucleofection followed by selection with blasticidin. Emerging blasticidin-resistant parasites will be assessed by RFP production in fluorescence and western blot analysis. Integration in the desired target gene will be analyzed by Southern blot analysis. Expression of the Tpx-1 gene will be assessed by Western blot analysis. Neither wild type nor TPx-1 KO *B. bovis* parasites should be able to growth in *in vitro* cultures containing inhibitory concentrations of blasticidin. Both labs (USDA, US and Obihiro, Japan) collaborated in order to generate the transfection plasmid *pBbtpx-1- ef-IG-bsd-rfp* using the strategy and primers described in Figure 2 and based in the gene sequences shown in Figures 5, 6, and 7. Figure 8 shows the sequence at the expected mRFP-3'rap-1 junction in plasmid *pBbtpx-1- ef-IG-bsd-rfp*.

The transfection plasmid was constructed and transfection experiments have been started. Next steps include nucleofection, selection, and analysis of the blasticidin resistant emerging transfected parasites.

An Abstract entitled "*Babesia bovis* チオレドキシンペルオキシターゼBbTPx-1 ノックアウト原虫は活性窒素種負荷に対する感受性が上昇する" authored by M. Asada, C. E.

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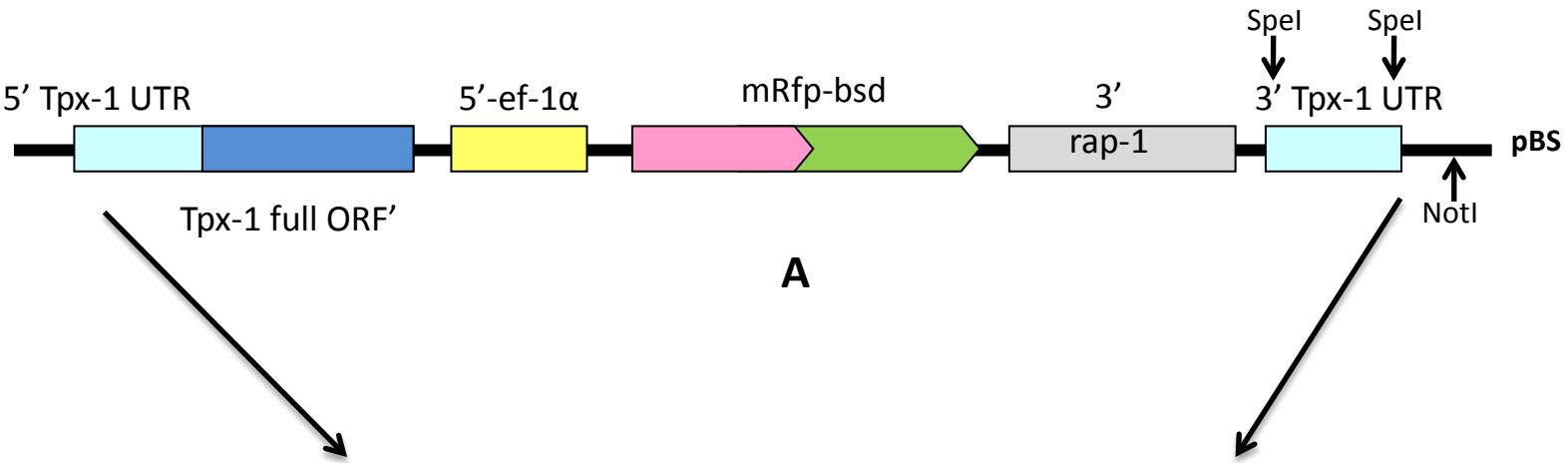
Suarez, M. Usui, Y. Goto, N. Yokoyama, N. Inoue, K. Yahata, O. Kaneko and S-I. Kawazu, describing the findings of this Project was submitted for presentation at the 156th Meeting of the Japanese Society of Veterinary Science.

7. Other (Research-related concerns, particular points of note)

It needs to be pointed out that during the developing of this research project Dr. Asada, the Japanese research partner in charge of conducting the transfection experiments switch his place of work and residence in order to pursue a new position at the Institute of Tropical Medicine at the Nagasaki University. As a result, the planned experimental work was interrupted for several months. We expect to re-assume with the pending planned experiments as soon as Dr. Asada will be able to pursue it.

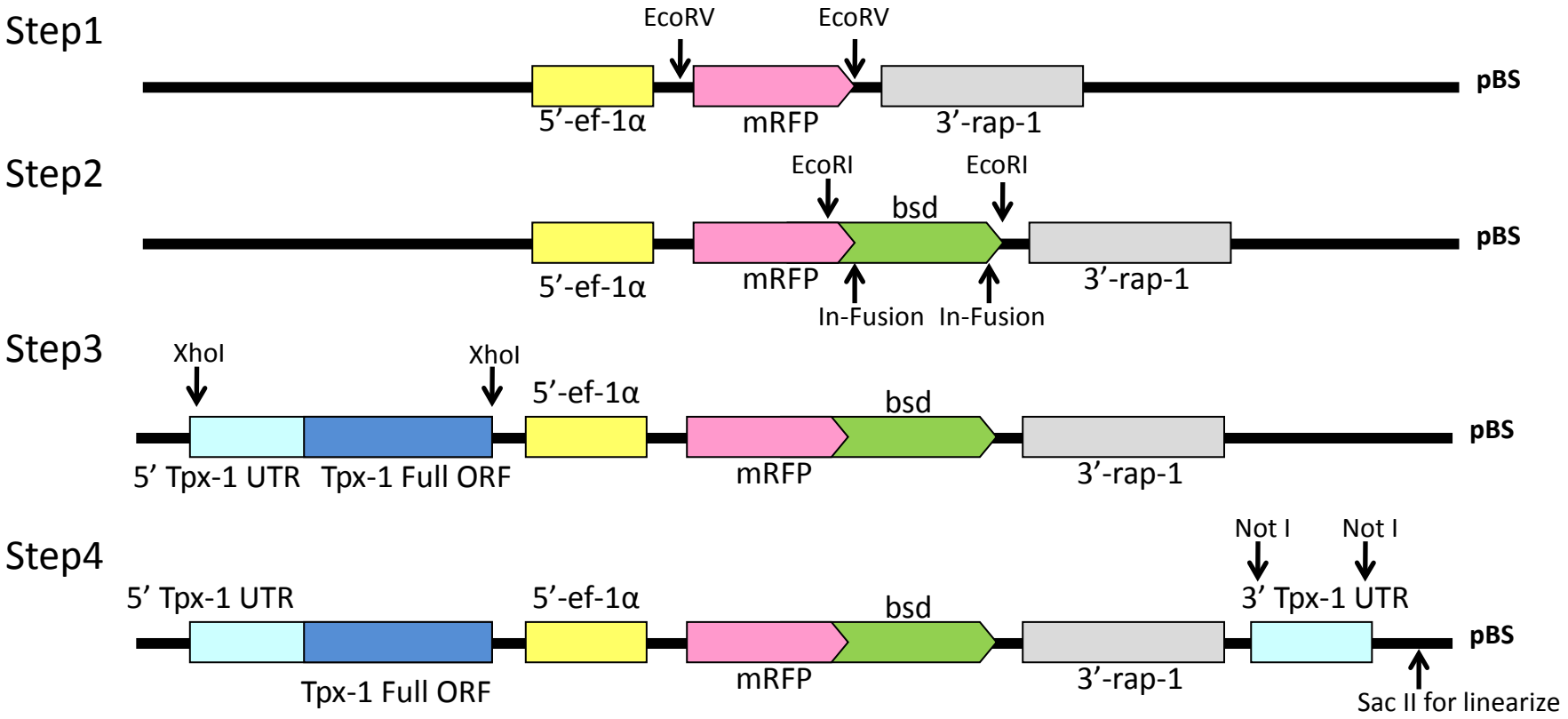
※Please attach any reference material as necessary: Please find attached the document: ”All Figures Final report” and the meeting articlet and registration receipt.



A: rescue of function plasmid ***pBbtpx-1- ef-IG-bsd-rfp***

B: targeted disrupted Tpx-1 locus in mutated Tpx-1 KO *B. bovis* line

Figure 1

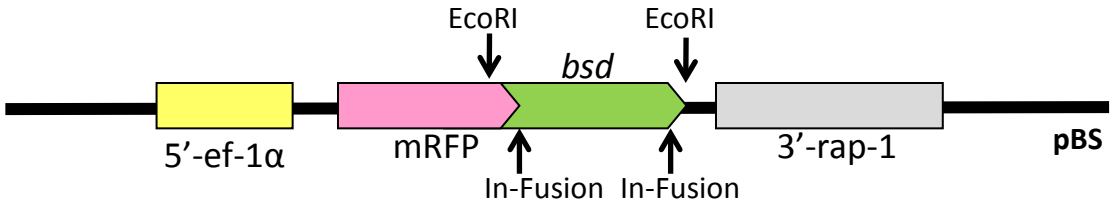


Primers	Sequence
mRFP-F-EcoRV	AATTGATATCATGGCCTCCTCCGAGGAC
mRFP-R-EcoRV	AATTGATATCGGCGCCGGTGGAGTGGCG
Bsd-F-infusion	CGCCGATATCGAATTCCAGGCCAAGCCTTTGTCT
Bsd-R-infusion	CATCCTGCAGGAATTTTATAAACGCATCTCATC
5Bbtpx1-F-Infusion	CGGGCCCCCCCTCGAATTGCAAGGCAACCAATTTAC
5Bbtpx1-R-Infusion	TACCGTCGACCTCGATCGCATTTCGCATTAATTCAC
3Bbtpx1-F-Infusion	AGTTCTAGAGCGGCCTTCGTTAATGCCTTCAACTCG
3Bbtpx1-R-Infusion	ACCGCGGTGGCGGCCGAATCTGCAGCAACCATTAGC

Figure 2

Combination of *hDHFR*/WR99210 system and *bsd*/blastcidin system

mRFP-bsd construct (shown at step2 on Fig. 2)



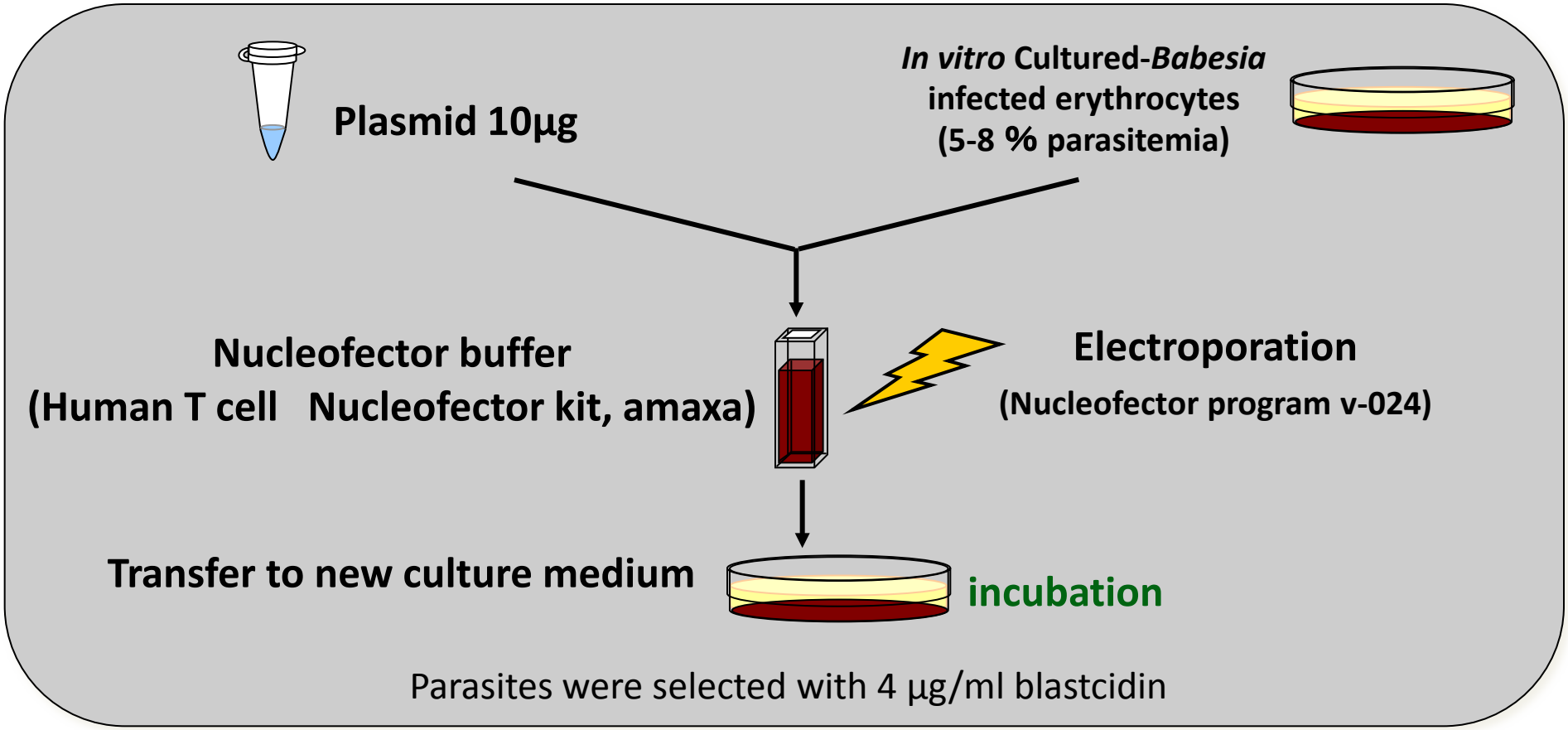
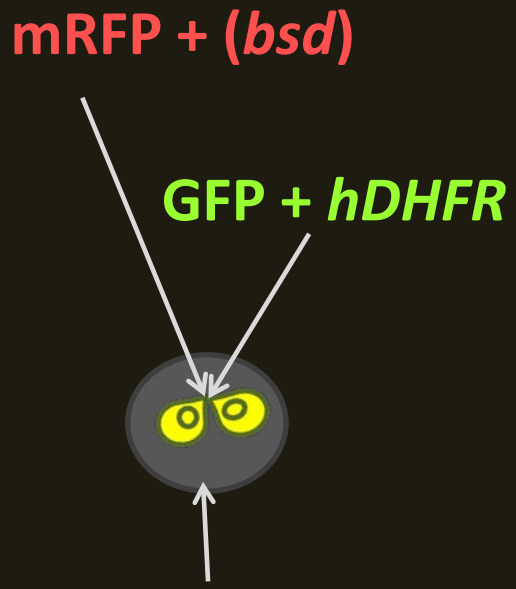
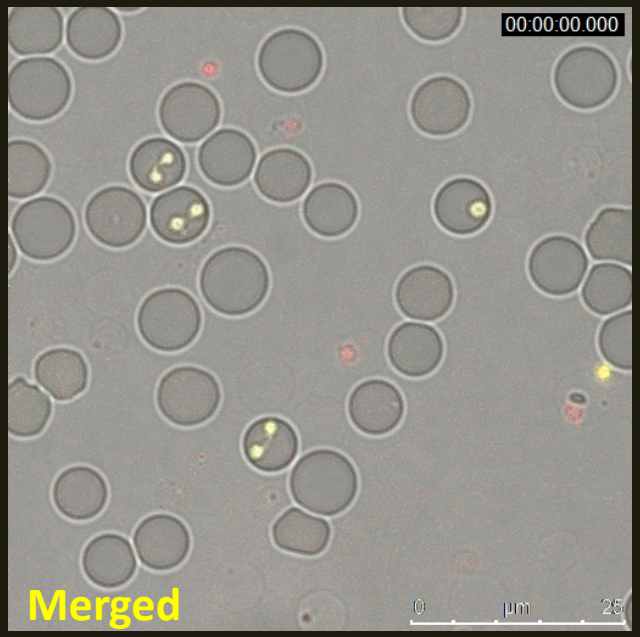


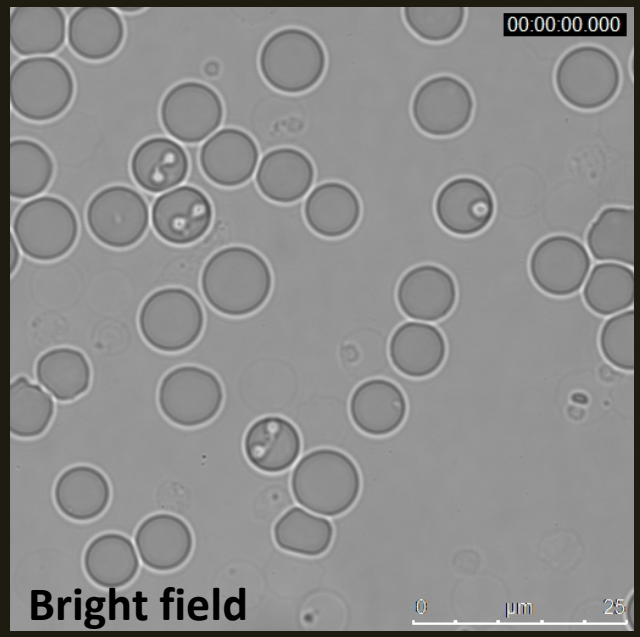
Figure 3

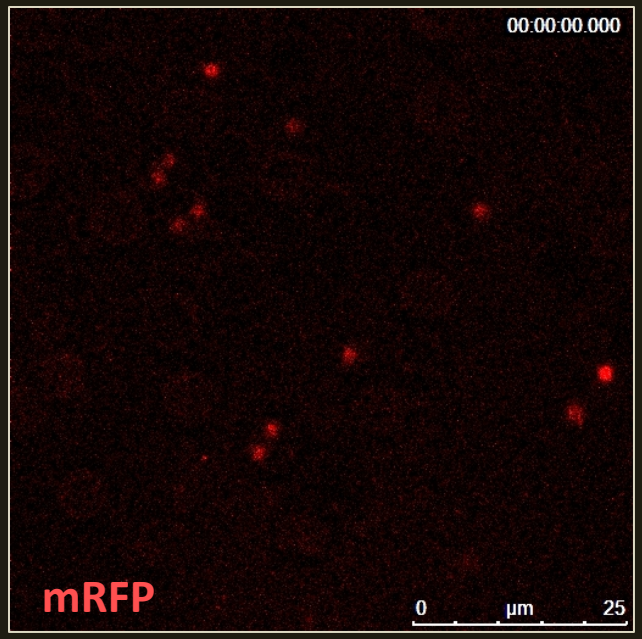
bsd/blastcidin system was established



Cultured with
4 μg/ml blastcidin







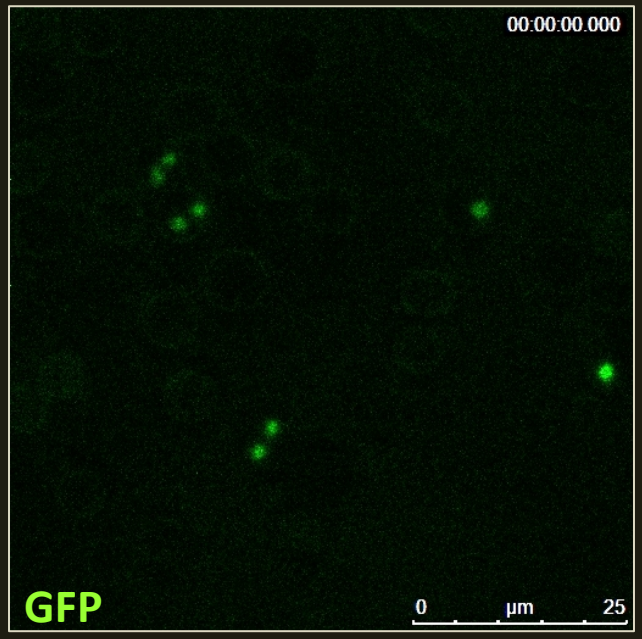


Figure 4

mRFP

atggcctcctccgaggacgtcatcaaggagttcatgcgcttcaaggtgcgcatggagg
gctccgtgaacggccacgagttcgagatcgagggcgagggcgagggccgcccctacga
gggcacccagaccgccaagctgaaggtgaccaagggcggccccctgcccttcgcctgg
gacatcctgtcccctcagttccagtacggctccaaggcctacgtgaagcaccccgccg
acatccccgactacttgaagctgtccttccccgagggcttcaagtgggagcgcgtgat
gaacttcgaggacggcggcgtggtgaccgtgacccaggactcctccctgcaggacggc
gagttcatctacaaggtgaagctgcgcggcaccaacttcccctccgacggccccgtaa
tgcagaagaagaccatgggctgggaggcctccaccgagcggatgtaccccgaggacgg
cgccctgaagggcgagatcaagatgaggctgaagctgaaggacggcggccactacgac
gccgaggtcaagaccacctacatggccaagaagcccgtgcagctgcccggcgcctaca
agaccgacatcaagctggacatcacctcccacaacgaggactacaccatcgtggaaca
gtacgagcgcgccgagggccgccactccaccggcgcctaa

bsd

caggccaagcctttgtctcaagaagaatccaccctcattgaaagagcaacggctacaa
tcaacagcatccccatctctgaagactacagcgtcgccagcgcagctctctctagcga
cggccgcatcttcactggtgtcaatgtatatcattttactgggggaccttgtgcagaa
ctcgtggtgctgggcactgctgctgctgcggcagctggcaacctgacttgtatcgtcg
cgatcggaaatgagaacaggggcatcttgagcccctgcggacggtgccgacaggtgct
tctcgatctgcatcctgggatcaaagccatagtgaaggacagtgatggacagccggcg
gcagttgggattcgtgaattgctgccctctggttatgtgtgggagggcgatatcgaat
tcctgcaggatgagatgcgtttataa

Figure 5

Babesia bovis elongation factor 1 alpha-B 5′ intergenic region

CACGTAATAAATGAGATAAATAAGTATATGTCATGTATAAATTTGGGGTATAATTTTAATTGTAGAAGGTGGAGATAGTACGGT
AAAAACTGAGATGATATATAATATATGATGAATATATAAACTTGACAGGTCAAGATTACTTGGTCAGATTTAATTAGTTGTGAT
AAAGCGGAAATTCCCATATATGTCATCCCGTTAAAGGTATTAATAGCATTTTAAGAATATATTTCACAAATATGATATACCATA
CATCTGATAGAATTATGCATCGATATTCGTCGTTATAGGCTTAGTTGCGCACAACGAAAAGGTATGTGTATATACTTAATGATA
TTTCAATCAAACGATGGGTGACAAATGTGTGGCGTGGAGCAAAGCAAAACAGCACCACAACCTTTACAAAGGGATCTTCATTAG
TCCTTTGCAGTGTCTTTATAACTTAATAAAGTAATTCCACGCAAGAATAGTGATTATATCGCCAACAACGCACTTTTGTGATGT
AAGTCAGGCCCCTGTCATTTTGCTTTGTGGGTGGTGTTATTTCATGAAGCAGGCGACTACTTCTTGTCGAGCTGCTGTGTGTAT
ATCCTACTTTTGAGGCTATTATATACACTGATGTGTTTGGACATTTGTGTTATTTAATGCTTGATTTAACGTTTTTTCAATTGT
GTTTGCCGACACTAATGTTGTCTCAGAGATAATTTATTGCAACTTTACGAAA (724bp)

Babesia bovis rap-1 3′ intergenic region

GATGAGATGCGTTTATAATGGCACAAACTCAACAAATGATGTATCGTCATCTGATCCATCGGTTTTCAATATTGTATTGGATGC
AATATCTGAATGCATATGATGCGACAGTTTCCATCATCGGGTGCCGAATCGTAACTCTCATAACACCATTTTAAGTTATGTAAA
CTAGTATCTATGTTTATGGTTTACGTAAAATGAATGTTTGAGTCTACAGGAAGGAACACGTGTCTAGAAGTATTTATTTCGTGA
ATGTACGTCATATATTTTTATGTATACGTGAACCTCACTAAAGGAAATTTTTGATGTTTTACGTATGCATTTATTAAACAATAC
TCTGTTAAATTATTCCATACGCTAAGATATGATTCCTTTCTCGCAAATCTCGGTTATTTTATAACAATAAAGTATACAAATAAG
CATAATATTCCCATGAAGTGATCTATAATGCAGTTATATTCTCATCAATAATAAAACAACAAATTAAACGTATTTGTTATAAAA
ATGAAACATAACATATATGATGTTCATTCAAGATGTATTATATATATATATATACACAAACATCTTTTTTTAAAAATTATTTGC
TTATAAATGTAAACATATCATGCAATTGTGCACAAACACATCGCATGATTATGAAGAATTAAAATTAACATTACACAATGAAAT
TTTATATTAAGTGGACGAATTAGGTTTATGTTTACATATAAGGTGGTGATATGTTTGGTCTTATGATGTTGTTATACACATTCA
TGGCACGACATTTTGATAGTTTAGTAATGAAGTCTAGTTAATGTTGCCAATGCTAGCCAATTAAAATAGGCTAAATTATATTTT
TATGAATAATTTCTTTTTTTTCCAATCCACGATGGCAGACTTGGCAACAGCAATTGCTGAATTTGATTCTCTTAACGAATTTTC
AAAACCTGATGCTAAATTATTATCCACAATACTACTATCGGATGGCGTAAAATTATCACAACAACTTAAGGCGCTTTACTTCTG
TCGTGATTTATCTTCTAGTGAATGTGCTACTATCCTTAAAAAGGCACTTGAAGTACACTACGATACATTCTTGAGGTAGGTAAT
AGCACGTAATGTTATTATGTTACCTTATTCATTCTTCATCTTCTTATAGACATGAAATTGCTTATGTAATTGGACAAGCAGAAT
GCGAGGAAGCAGCTGACGTATTGGTTCGCCTTTTGGAGGATACTAATGAAGACCCTATGGTCAGACATGAGGTATGTCGTTTAA
CTCTTTGACATATCGTTCGTAGG
(1283 bp)

Figure 6

BbTpx-1 ORF, 5’-,3’- UTR

BBOV_II004970
(BbTpx-1)

Figure 7