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## TEMPLATE for PROTOCOL UNIT

### Unit Title

Running Title *Trypanosoma congolense: in vitro* culture and transfection

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### Significance Statement

*Trypanosoma congolense* is a parasitic protist that, together with *T. vivax* and *T. brucei*, causes African Animal Trypanosomiasis, a livestock disease carried by bloodsucking tsetse flies in sub-Saharan Africa. This disease severely affects the health and productivity of livestock such as cattle and other ruminants in regions of Africa infested by tsetse. The ability to culture trypanosomes *in vitro* and genetically manipulate them via transfection are basic tools for the analysis and eventual control of these pathogens.

### ABSTRACT

*Trypanosoma congolense*, together with *T. vivax* and *T. brucei*, causes African Animal Trypanosomiasis (AAT) or nagana, a livestock disease carried by bloodsucking tsetse flies in sub-Saharan Africa. These parasitic protists cycle between two hosts - mammal and tsetse fly. The environment offered by each host to the trypanosome is markedly different and hence the metabolism of stages found in the mammal differs from that of insect stages. For research on new diagnostics and therapeutics, it is appropriate to use the mammalian life cycle stage, bloodstream forms. Insect stages such as procyclics are useful for studying differentiation and also serve as a convenient source of easily cultured, non-infective organisms. Here, we present protocols in current use in our lab for the *in vitro* culture of different life cycle stages of *T. congolense* - procyclics, epimastigotes and bloodstream forms – together with methods for transfection enabling the organism to be genetically modified.

**Keywords:** *Trypanosoma congolense*; transfection; transformation; tsetse

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## INTRODUCTION

*Trypanosoma congolense* is one of the causative agents of African Animal Trypanosomiasis (AAT) or nagana, a livestock disease carried by bloodsucking tsetse flies in sub-Saharan Africa (Auty, Torr, Michoel, Jayaraman, & Morrison, 2015). The disease severely affects the health and productivity of livestock in the tsetse fly infested regions, which occupy >9 million km<sup>2</sup> or one third of Africa's land mass (FAO, 2012). Hence, AAT has large negative economic consequences in terms of lost production (meat, milk, breeding stock, fertility, traction power, transport, manure) and the costs of control (drugs for treatment and/or prophylaxis; insecticides, traps and targets for tsetse control or elimination by sterile insect technique).

The life cycle of *T. congolense* is digenetic and involves mammalian hosts, including a wide range of wild and domestic animals, and an insect vector, the tsetse fly (genus *Glossina*). The developmental stage in the mammalian host is known as the bloodstream form (BSF). This stage is adapted to life at body temperatures around 37°C and a ready supply of nutrients in the serum. It has to contend with the hostile immune response of the host, which it does by changing the major glycoprotein (Variant Surface Glycoprotein, VSG) on its cell surface, a process called antigenic variation (Borst & Cross, 1982; Pays, 2005). BSF is the relevant life cycle stage for studying drug uptake, metabolism and mechanisms of action and resistance. In the past BSF were grown in experimental animals such as mice or rats, but more recently *in vitro* culture methods have been developed and are described here.

In the tsetse fly vector, *T. congolense* undergoes a complex cycle of development involving different parts of the alimentary tract and there are multiple life cycle stages. When BSF are taken up by the fly in a bloodmeal, they transform to procyclics in the midgut and proliferate to high density (~10<sup>5</sup> – 10<sup>6</sup> per midgut); VSG is lost as BSF transform to procyclics and is replaced by other surface proteins and carbohydrates (Roditi & Liniger, 2002). The bloodmeal is enclosed by the peritrophic matrix (PM), a tubular membrane that lines the midgut; the procyclics need to pass through the PM to reach the ectoperitrophic space enclosed by the midgut epithelium (Figure 1A). The trypanosomes then move forward to invade the proventriculus, the valve that separates the foregut from the midgut, pass through the PM again and then migrate to the mouthparts. Here they attach to the wall of the food canal (Figure 1B) and proliferate as epimastigotes. They then invade the

hypopharynx, the duct that carries saliva from the paired salivary glands to the tip of the proboscis, and differentiate into metacyclics, a non-dividing stage that expresses VSG and is infective to the mammalian host. The proliferative stages – procyclics and epimastigotes – can be grown in culture as described here; metacyclics are produced by mature epimastigote cultures. These life cycle stages, particularly procyclics, are easy to maintain in the lab, and are used when BSF are not essential.

*T. congolense* is split into three genetically distinct, subgroups: savannah, forest and kilifi or Kenya coast (Gibson, 2007). Lab isolates of *T. congolense* are usually of the savannah subgroup, which is considered to be the most widely prevalent and pathogenic subgroup (Bengaly et al., 2002; Gashumba, Baker, & Godfrey, 1988; Tihon, Imamura, Dujardin, Van Den Abbeele, & Van den Broeck, 2017; Young & Godfrey, 1983). The savannah subgroup strain (IL3000) was chosen for the *T. congolense* genome project (GeneDB.org) (Jackson et al., 2012). Though the methods described here have largely been developed for *T. congolense* savannah, the protocols for procyclics, and possibly other life cycle stages, are applicable to the forest and kilifi subgroups too.

## **BASIC PROTOCOL 1**

### **CULTURING PROCYCLICS**

Procyclic forms are found in the tsetse fly midgut and are probably the easiest life cycle stage to maintain, as they grow to high density ( $\sim 10^7$  per ml) in suspension culture in various media containing 10-20% foetal calf serum (Figure 2A, Video 1). The main energy sources provided in the media are glucose and proline. Procyclics may be derived from bloodstream forms (BSF) by differentiation or from infected tsetse midguts. Established cultures need to be fed every few days and can be cryopreserved for long term storage. We routinely use an incubation temperature of 27 or 28°C, but cultures continue to grow, albeit slower, at temperatures as low as 20°C

In our lab, we use a modification of Cunningham's medium (Cunningham, 1977) (CM) routinely to culture a variety of *T. congolense* strains as procyclics and epimastigotes, as well as other trypanosome species. CM also works well for differentiating bloodstream forms of *T. congolense* or *T. brucei* to procyclics. However, there are other MEM-based media that grow *T. congolense* equally well (Brun, 1982; Coustou, Guegan, Plazolles, & Baltz, 2010; M.A. Gray, Hirumi, & Gardiner, 1987), though the derivation of robust procyclic cultures from BSF seems to be much slower (Coustou et al., 2010).

### **Materials**

Cunningham's medium, CM (see recipe)  
25 cm<sup>2</sup> flasks (T25)  
75 cm<sup>2</sup> flasks (T75)  
24-well tissue culture plates  
15 ml and 50 ml centrifuge tubes  
1.5 ml microcentrifuge tubes

Humid chamber e.g. plastic box with wet tissue paper  
Adjustable pipettes and sterile tips 0.005 - 5 ml  
Inverted microscope with phase objectives x10, x20  
28°C incubator  
Microcentrifuge  
Refrigerated centrifuge  
70% alcohol spray bottle  
Waste pot containing disinfectant solution (1% bleach or Virkon)

**NOTE:** All steps to be carried out in a biosafety cabinet (Class II hood or cabinet) using aseptic technique. Use 70% alcohol spray for disinfection of gloved hands, work surfaces and implements; use immersion in a solution of disinfectant, such as 1% bleach or Virkon, to destroy organisms in discarded medium and plasticware.

### **Protocol steps**

#### ***Starting a procyclic culture from a cryopreserved stock***

1. Aliquots of cryopreserved trypanosomes are stored in plastic, screw-top cryovials in liquid nitrogen (see Support Protocol 1 for cryopreservation).

*There are specific risks (e.g. asphyxiation, cold burns) associated with handling liquid nitrogen and training is required. There is a very small risk that vials may explode by rapid expansion of trapped nitrogen. This can result in contamination of a wide area with shards of plastic and potentially infective material, with associated risks of injury and infection to those nearby. Minimize these hazards by containment (thaw vials in a large robust container, e.g. empty plastic chemical pot with screw lid) and appropriate protective wear (labcoat, gloves, visor). Make sure that any other workers in the vicinity are aware of the risks and are protected.*

2. Remove vial from liquid nitrogen using insulated gloves and forceps if needed. Transfer immediately to the screw top container to thaw. In event of the vial exploding inside the container, disinfect the contents with 1% bleach or Virkon and discard.

3. Transfer the container to the Class II hood. Remove vial, slightly open the cap to release any pressure and thaw rapidly. Transfer contents into culture medium using sterile tips and an adjustable pipette; typically a 1:5 dilution is sufficient to reduce the concentration of glycerol, i.e. 1 ml from the vial into 4 ml of medium in a T25 flask; a 24-well plate is suitable for smaller volumes (with same dilution factor). Discard vial and tips into discard pot with disinfectant.

4. Check culture for viability under an inverted microscope and incubate at 28°C. Caps on T25 flasks should be tightly closed to prevent evaporation, whereas 24-well plates should be enclosed in a humid chamber (e.g. plastic box with wet tissue).

#### ***Maintaining and harvesting a procyclic culture***

5. Check for growth daily by microscopy and subculture when dense ( $\sim 10^7$  per ml) by replacing 2 - 4 ml of medium; well-adapted lines tolerate 1:5 or 1:10 dilution every few days,

whereas less robust cultures may need to be kept at higher density by regular 1:1 dilution. The trypanosomes should be actively swimming in the medium (Video 1); dead and dying trypanosomes accumulate at the bottom of the flask. Start by focussing on the bottom of the flask and then roll up through the depth of the medium to estimate density.

*The colour and turbidity of the medium are also good guides to growth; trypanosome metabolism produces waste products such as acetate and succinate, which will acidify the medium and change the colour from red through orange to yellow (Figure 2B). T. congolense procyclics do not tolerate acidic conditions well, so it is advisable to subculture before the medium becomes yellow.*

6. To bulk up culture to harvest large numbers of cells, double the volume every day or every few days using T25 then T75 flasks until the desired volume is reached.

7. Cells are harvested by centrifugation at 3000g for 5-10 minutes at 4°C in 15 ml or 50 ml tubes; pour off clear supernatant into a discard pot and recover pellet. For smaller volumes (~1 ml), use a microcentrifuge (5000g for 3 minutes at room temperature); carefully remove supernatant without disturbing pellet using a pipette.

#### **Appearance of procyclic cultures**

*Location: Trypanosomes are found dispersed throughout the depth of the medium and are not attached to the plastic. However, they tend to accumulate at the bottom by gravity unless dispersed by shaking.*

*Activity/movement: Cells are very active and occasionally swim rapidly in a straight line (Video 1); the anterior of the cell shows the greatest movement, while the posterior is relatively stiff.*

*Cell division: Cells in the last stage of division remain joined by the posterior (Figure 2A; Video 1; (Wheeler, Scheumann, Wickstead, Gull, & Vaughan, 2013), and are easily found in healthy, log phase cultures.*

*Morphology: The procyclic cell is a slender trypomastigote with the kinetoplast posterior to the nucleus and no free flagellum. The kinetoplast is subterminal and the nucleus is found at about one third cell length from the posterior. The mean length of non-dividing cells is 15.3 +/- 0.15 µm (late log phase culture of T. congolense IL3000).*

### **ALTERNATE PROTOCOL 1**

#### **DIFFERENTIATION OF BLOODSTREAM FORM TO PROCYCLIC TRYPANOSOMES**

Procyclics may be derived by differentiation *in vitro* of bloodstream forms grown in rodents or culture (see Basic Protocol 3). The triggers appear to be lower temperature and the presence of tricarboxylic acid (TCA) cycle intermediates (Brun & Schonberger, 1981); these may be supplemented into the medium but are already present in Cunningham's medium.

### ***Additional materials***

Blood from rodents infected with *Trypanosoma congolense*

### **Protocol steps**

1. Use blood from a rodent with a parasitaemia of at least  $10^8$  per ml. Infected blood should be obtained aseptically, e.g. by heart puncture of a terminally anaesthetized animal.

*Alternatively, use an equivalent number of culture-derived bloodstream forms – see Basic Protocol 3, Support protocol 2).*

2. Pipette 1 ml of CM into each of two wells of a 24-well plate and then add ~50  $\mu$ l of infected blood to each well; higher parasitaemias work best and duplicate wells increase the chance of obtaining a healthy culture without contamination.

3. Place the plate in a humid chamber to incubate at 28°C; propping the plate at a slight angle allows the red blood cells to accumulate by gravity on the lower side of the well, leaving the trypanosomes in clear medium.

4. Inspect growth after about 24 hours. By this time the bloodstream trypanosomes should have differentiated into procyclics and started to proliferate. If sufficiently dense, transfer 0.5 ml of medium from each well to a clean well, trying not to carry over any red blood cells, and add 0.5 ml fresh medium to all four wells; leave a further 24 hours if not very dense. Incubate at 28°C as before.

5. Inspect growth after a further 24 hours. Growth in the new wells should now be vigorous; if not, try making a further subculture from the original wells as in step 4.

6. After a few days the subcultured wells should be dense and ready to be moved on to a larger volume in a T25 flask. It is also advisable to cryopreserve the culture at this stage (see Support Protocol 1).

### ***ALTERNATE PROTOCOL 2***

#### **ISOLATION OF PROCYCLIC TRYPANOSOMES FROM TSETSE MIDGUT**

Procyclics grow to high density in the tsetse midgut and  $\sim 10^5$  to  $10^6$  trypanosomes are found in a single midgut. However, bacterial and/or fungal contamination can prove problematic, so we routinely set up replicate cultures from individual tsetse midguts and select the least contaminated.

### ***Additional materials***

Tsetse flies infected with *Trypanosoma congolense*

96-well tissue culture plates with flat bottom wells  
Anti-contamination cocktail (100x ACC; see recipe)  
For tsetse dissection:  
Phosphate buffered saline (PBS)  
Fine forceps  
Dissecting microscope

### **Protocol steps**

1. Add 5x anti-contamination cocktail (ACC (Maser, Grether-Buhler, Kaminsky, & Brun, 2002)) to CM and set up a 96-well plate with as many 100  $\mu$ l wells of this medium as required for the number of flies to be dissected.
2. Cold anaesthetize tsetse flies and hold on ice until dissection; dissect flies using sterile PBS and forceps wiped with 70% alcohol. Before dissection, remove the head of the fly with a scalpel and pull off the legs and wings.
3. For each fly, place it on its back in a drop of PBS and open the abdomen using forceps. Tease out the internal organs into the PBS without rupturing the gut. Sever the midgut at either end with forceps or a needle and, using forceps, quickly transfer the midgut to one well of CM in the 96-well plate; keep the plate covered with its lid as much as possible.
4. When all flies have been dissected, view the plate under an inverted microscope and mark the wells that contain infected midguts. Incubate the plate overnight at 28°C in a humid box to minimize evaporation.
5. Examine the plate the following day and check for growth of trypanosomes. Selecting wells with least contamination and highest trypanosome density, transfer 50  $\mu$ l from the original to a new well containing 50  $\mu$ l of fresh medium. Replace the 50  $\mu$ l of medium in the original well, as often growth remains vigorous.
6. Continue to monitor growth in subcultured wells. When trypanosomes become dense, try subculturing to a 24-well plate to reduce the amount of fly debris and obtain a pure trypanosome culture, which can then be expanded and cryopreserved.

### ***SUPPORT PROTOCOL 1***

#### **CRYOPRESERVATION**

Cryopreservation is a way of preserving trypanosome strains in the lab without the need for continuous culture and also provides a safeguard against loss through contamination with other microorganisms or other disaster. We routinely cryopreserve using glycerol as cryoprotectant, but DMSO and trehalose may also be used (Wen et al., 2016).

#### ***Additional materials***



1 ml cryovials with screw-tops  
Indelible pen  
Glycerol  
0.2 µm filters  
10 or 20 ml syringes  
Slow cooler e.g. Mr Frosty (Nalgene)  
-70 or -80 °C freezer  
Liquid nitrogen storage dewar

### **Protocol steps**

1. For best results, cultures should be dense and nearing the end of logarithmic growth when cryopreserved.
2. Prepare a stock freezing medium of 20% glycerol in CM (v/v) and sterilize by passing through a 0.2 µm filter. This can be stored at 4°C for a few weeks.

*Glycerol is viscous, so warming it briefly in a 37°C waterbath makes it easier to pipette.*

3. Mix an equal volume of freezing medium with dense culture and dispense 1 ml aliquots into cryovials labelled with an indelible pen.
4. Slow cool the vials in a Mr Frosty freezing container (Nalgene) or well-insulated box (e.g. small polystyrene box) to about -70 or -80°C (usually overnight), before transferring to longterm storage at -196°C in a liquid nitrogen storage dewar.

## **SUPPORT PROTOCOL 2**

### **CLONING**

Cloning single cells is a way of obtaining a genetically homogeneous population. Cultures of procyclic trypanosomes generally do not tolerate dilutions of more than 1:10, possibly because the cells produce metabolic products that condition the medium. Hence cloning presents a challenge. For *T. brucei*, cloning is facilitated by incubation of the dilute cultures in a 5% CO<sub>2</sub> atmosphere, but we found that this did not work for *T. congolense* as it does not tolerate acidic pH well. Instead we found that the omission of glutamine from the growth medium facilitates cloning.

Cloning can be achieved in two ways: by limiting dilution, where cells are diluted to a density of <1 cell per culture well, or by visual inspection of a small drop of the culture inoculum by microscopy. We routinely use limiting dilution as a simple way of obtaining multiple procyclic clones as described below.

### **Additional materials**

CM without glutamine (see recipe)

## Protocol steps

1. Dilute a mid-log phase culture to a density of <10 trypanosomes per ml, so that on average 100  $\mu$ l will contain a single trypanosome. This is best achieved by sequential 1:100 or 1:1000 dilutions.

2. Plate out 100  $\mu$ l aliquots of the dilution into rows of a 96-well plate. A repeating pipette such as a Finn pipette Stepper makes this less laborious. Also add a few wells of the higher density dilutions to the plate – if these fail to grow, you cannot expect any clones.

*Alternatively, dilute to a density of <1 trypanosome per ml and inoculate a 24-well plate with 1ml of medium per well. The larger volume helps mitigate the loss of water through evaporation when plates are incubated for more than a week.*

3. Incubate the plate for 1-2 weeks at 28°C in a humid box to minimize evaporation.

*Starting from a single trypanosome, the population in each 100  $\mu$ l well will need to double 19 times to increase to  $10^6$  trypanosomes, i.e. a density of  $10^7$  trypanosomes per ml. If the population doubles twice each day, that is 9 or 10 days.*

4. After 1-2 weeks, first check that the high density wells have grown, then systematically check the cloning wells, marking any wells that contain trypanosomes as you go.

*You should expect at least half the wells to be negative, as a cloning dilution of <10 trypanosomes per ml will mostly give 100  $\mu$ l aliquots with 0, 1 or 2 trypanosomes. Be suspicious if you are too successful – it means that your original cloning dilution was higher than 10 trypanosomes per ml.*

5. If any wells are dense, i.e.  $>10^6$  trypanosomes per ml, transfer 50  $\mu$ l from the original to a new well containing 50  $\mu$ l of fresh medium, and add fresh medium to the original well too. If not, monitor growth again in 1-2 days.

6. Expand each clonal culture to at least 1 ml before cryopreservation (Support Protocol 1).

## **BASIC PROTOCOL 2**

### **CULTURING EPIMASTIGOTES**

Epimastigotes of *T. congolense* readily attach to tissue culture plastic or glass coverslips and grow as dense layers of dividing trypanosomes at the bottom of the culture flask or well, covered with a layer of medium (M. A. Gray, Cunningham, Gardiner, Taylor, & Luckins, 1981; M.A. Gray, Ross, Taylor, & Luckins, 1984). We routinely use Cunningham's medium (CM), but MEM-based media are widely used (e.g. (Coustou et al., 2010)). The main energy sources provided in CM are glucose and proline.

The exact conditions for triggering transformation of procyclics to epimastigotes are not well defined, but it is observed that clusters of attached epimastigotes arise spontaneously

in procyclic cultures that have been maintained for several weeks at high density. Temporary removal of serum from the medium may help (Coustou et al., 2010), but in our hands, this caused adhesion of procyclics to the bottom of the flask, not transformation to epimastigotes.

Typically metacyclics are found above the epimastigote layer in the supernatant after a few weeks, e.g. (Coustou et al., 2010; M.A. Gray et al., 1984; Hendry & Vickerman, 1988). However, this is not our experience; although we have observed cells with trypomastigote morphology in the supernatant, these have not proved to be infective to mice. The exact conditions for triggering metacyclogenesis are not well defined, although it has been reported that the addition of 4 mM glutamine and/or 4 mM proline to MEM medium enhanced differentiation of epimastigotes to metacyclics (Ross, 1987).

### **Materials**

As for culturing procyclics (see Basic Protocol 1)  
Sterile glass coverslips 10-13 mm diameter  
Forceps

### **Protocol steps**

1. Follow Basic Protocol 1 steps 1-4 to initiate a culture from a cryopreserved aliquot of epimastigotes. Cultures are maintained at 28°C in CM.

*The epimastigotes adhere to either glass or plastic (Figure 3), so cultures may be grown directly in T25 flasks or 24-well plates, or on glass coverslips placed in 24-well plates. The advantage of using coverslips is that these can be removed from the well with forceps and observed under the microscope as live or fixed and stained preparations.*

2. Check for growth daily by microscopy and subculture when dense, i.e. when there is a dense covering of attached cells and/or the medium has started to turn yellow. As the cells are firmly attached, the medium can be completely removed with a 5 ml pipette and replaced with fresh medium, typically every 2-3 days. The medium removed contains both epimastigotes and trypomastigotes and can either be discarded or used to initiate new cultures.

*Cultures are initially seeded at low density of singly attached epimastigotes and subsequently expand to large rosettes of attached cells that completely cover the surface (Figure 3B).*

3. Epimastigotes are firmly attached and are damaged by removal with a cell scraper. More gentle release is achieved by vigorous shaking or vortexing of the flask for several minutes. To harvest only the attached epimastigotes, before vortexing, first remove the culture supernatant and wash the cell layer briefly with sterile PBS, before replacing with fresh medium. The free epimastigotes can be transferred to new culture vessels, harvested by centrifugation as for procyclics (Basic Protocol 1, step 7) or cryopreserved.

*Epimastigotes may also arise spontaneously in long term procyclic cultures. In this case, the epimastigote population will be selected by removal of spent medium containing unattached cells, and the epimastigotes grown as described in steps 1-3.*

### **Cryopreserving an epimastigote culture**

4. Free attached cells as described in step 3 and then proceed as described for freezing procyclics (Basic Protocol 1, Support Protocol 1).

*In our experience, epimastigotes do not recover as well as procyclics after cryopreservation.*

### **Cloning an epimastigote culture**

5. Harvest attached epimastigotes as described in step 3 and then proceed as described for cloning procyclics (Basic Protocol 1, Support Protocol 2). Since each clonal culture starts from a single attached cell, it is easy to verify which wells are true clones by visual inspection: there should be only a single rosette of proliferating epimastigotes. Clones take 2 – 3 weeks to emerge.

### **Appearance of epimastigote cultures**

*Location: Epimastigotes adhere tightly to cell culture plastic and glass; free-swimming cells, both epimastigotes and trypomastigotes, are found in the medium above the adherent cell layer.*

*Activity/movement: Epimastigotes are firmly attached by the anterior and gently pivot about this point (Video 2); the cell body shows little undulatory movement.*

*Cell division: When single cells divide, the daughter cells often attach nearby, eventually forming large rosettes (Video 2).*

*Morphology: Epimastigote cultures typically consist of very short (~6  $\mu\text{m}$ ), stout attached cells of uniform appearance, but cell length becomes more variable in dense or starved cultures and longer, needle-like cells appear; cultures revert to the short cell type after subpassage. The arrangement of kinetoplast and nucleus is more varied than in other life cycle stages, so individual colonies might contain cells in trypomastigote (kinetoplast posterior to the nucleus), epimastigote (kinetoplast anterior to the nucleus) or juxta-nuclear configuration; there is no visible free flagellum.*

## **ALTERNATE PROTOCOL 2**

### **DERIVATION OF EPIMASTIGOTE TRYPANOSOME CULTURE FROM TSETSE PROVENTRICULUS**

The tsetse proventriculus contains a large population of proventricular forms –  $3 \times 10^3$  to  $7 \times 10^4$  per proventriculus - which can be used to derive epimastigotes *in vitro* (Peacock, Kay, Bailey, & Gibson, 2018). This provides a straightforward route for deriving an epimastigote culture rather than waiting for spontaneous transformation to occur in longterm cultures. However, it requires access to tsetse flies infected with *T. congolense*.

### **Additional materials**

Tsetse flies infected with *Trypanosoma congolense*

Anti-contamination cocktail (100x ACC; see recipe)

For tsetse dissection:

Phosphate buffered saline (PBS)

Fine forceps

Hypodermic needles

Dissecting microscope

### **Protocol steps**

1. Pool the proventriculi of about 10 infected flies as follows. Pipette 100  $\mu$ l of CM containing 1x anti-contamination cocktail (ACC) into a microcentrifuge tube.
2. Cold anaesthetize tsetse flies and hold on ice until dissection; dissect flies using sterile PBS and forceps wiped with 70% alcohol. Before dissection, remove the head of the fly with a scalpel and pull off the legs and wings.
3. For each fly, place it on its back in a drop of PBS and open the abdomen using forceps. Gently pull on the anterior midgut to retrieve the proventriculus; it doesn't matter if the foregut breaks. Sever the connection to the midgut as close as possible to the proventriculus with a needle or forceps, and also free the proventriculus from the foregut if still connected. With forceps, transfer the proventriculus to the CM in the microcentrifuge tube. Do this as quickly as possible to minimize loss of trypanosomes from the proventriculus.
4. When all flies have been dissected, tap the tube gently to mix and leave on the bench for 10 minutes or so while preparing the culture plate.
5. Set up a 24-well plate as follows. Place a sterile round (10–13 mm diameter) glass coverslip in each of 6 wells so the coverslip sits flat at the bottom, and add 1 ml of CM plus 1x ACC.
6. Retrieve the tube of pooled proventriculi, tap to mix and pipette a 15  $\mu$ l aliquot of the suspension into each well. Inspect each well using an inverted microscope and focussing on the upper surface of the coverslip. The seeding density will depend on how many flies had an infected proventriculus and the average number of trypanosomes per proventriculus. Several attached trypanosomes should be visible in each field.
7. Incubate the plate at 28°C overnight in a humid box to minimize evaporation.

8. The following day, remove most of the CM (about 900 µl) and replace with PBS containing 20% v/v FCS.

*In preliminary trials, we found that trypanosomes ceased proliferating and died after a few days if left in CM, whereas diluting the medium tenfold with PBS enhanced survival and the chance of establishing a stable epimastigote culture. Replacing the medium after 24 hours also serves to reduce microbial contamination, though this is generally very low in these preps anyway, so that 1x ACC can be omitted from the medium after the first 24 hours.*

9. Continue to monitor growth daily and replace the medium every few days.

*Seeded proventricular trypanosomes form a firm attachment to the glass coverslip after about 30 minutes; they become shorter and stouter over the next 24 hours and start to divide; rosettes of dividing epimastigotes appear in a few days (Peacock et al., 2018).*

### **BASIC PROTOCOL 3**

#### **CULTURING BLOODSTREAM FORMS**

Bloodstream form (BSF) trypanosomes are found in the mammalian host and are the appropriate life cycle stage for testing drugs or studying antigenic variation. *T. congolense* BSF are more demanding to grow than procyclics or epimastigotes and relatively few strains have been adapted to BSF culture, e.g. (Coustou et al., 2010; Hirumi & Hirumi, 1991). The genome strain, IL3000, is well-adapted to BSF culture and relatively easy to maintain.

Initial culture methods for *Trypanosoma brucei* and *T. congolense* BSF relied on the presence of mammalian or insect cells in a feeder layer (Brun, Jenni, Schonenberger, & Schell, 1981; Hirumi & Hirumi, 1984; Kaminsky, Beaudoin, & Cunningham, 1987), but it was subsequently found that reducing agents such as mercaptoethanol and cysteine removed the need for a feeder layer (Baltz, Baltz, Giroud, & Crockett, 1985; Duszenko, Ferguson, Lamont, Rifkin, & Cross, 1985) and further improvements, notably the addition of the chelating agent bathocuproine sulphate to maintain cysteine in the reduced form (Yabu, Takayanagi, & Sato, 1989), resulted in media suitable for long term culture of *T. brucei* – HMI-9 (Hirumi & Hirumi, 1989) and *T. congolense* – HMI-93 (Hirumi & Hirumi, 1991). The main energy source provided in HMI-93 is glucose.

#### **Materials**

HMI-93 (see recipe)

Vented 25 cm<sup>2</sup> (T25) or 75 cm<sup>2</sup> flasks (T75)

5% CO<sub>2</sub> incubator set at 34°C with humidity tray

Other materials as for Basic Protocol 1

## Protocol steps

### ***Starting a BSF culture from a cryopreserved stock***

1. Follow steps 1-4 of Basic Protocol 1 to retrieve a cryopreserved aliquot of BSF culture and set up a BSF culture using HMI-93 medium pre-warmed to 34°C.

*Addition of antibiotics such as Gentamycin to the medium may inhibit growth. Substitution of goat serum with foetal calf serum (FCS) is possible but significantly diminishes peak density (<30% of peak density); varying the concentration of goat serum 15-25% (v/v) does not greatly change maximum density.*

2. Check culture for viability under an inverted microscope and incubate at 34°C in a 5% CO<sub>2</sub> incubator.

*HMI-93 relies on bicarbonate buffering and therefore incubation in a 5% CO<sub>2</sub> atmosphere is essential. Flasks should have vented caps to allow gas exchange; alternatively, leave non-vented caps slightly loose; unsealed 24-well plates allow gas exchange. Make sure the humidity tray is filled with distilled water to maintain a humid atmosphere in the incubator; alternatively, incubate cultures in a humid box (e.g. plastic box with wet tissue).*

### ***Maintaining and harvesting a BSF culture***

3. Check for growth daily by microscopy and subculture when dense (~5 x 10<sup>6</sup> to 10<sup>7</sup> per ml).

*The colour of the medium is a good guide to growth; trypanosome metabolism produces waste products such as pyruvate, which acidify the medium and change the colour of phenol red from red through orange to yellow. *T. congolense* BSF do not tolerate acidic conditions well, so it is advisable to subculture before the medium becomes yellow.*

4. Before subculture, gently vortex the flask in the horizontal position for a few seconds to release attached cells. Remove spent medium and replace with fresh medium pre-warmed to 34°C.

**T. congolense* IL3000 is well-adapted to culture and grows fast; it needs diluting 1:10 every day, but will tolerate much higher dilutions (1:100 to 1:200), allowing it to be subcultured every few days. Passage frequency can also be decreased by reducing incubator temperature to 30°C.*

4. To bulk up culture to harvest large numbers of cells, increase the volume using T25 then T75 flasks until the desired volume is reached.

5. Cells are harvested by centrifugation at 3000g for 5-10 minutes at 4°C in 15 ml or 50 ml tubes; pour off clear supernatant into a discard pot and recover pellet. For smaller volumes (~1 ml), use a microcentrifuge (5000g for 3 minutes at room temperature); carefully remove supernatant without disturbing pellet using a pipette.

### ***Cryopreserving a BSF culture***

6. Proceed as described for freezing procyclics (Basic Protocol 1, Support protocol 1), substituting HMI-93 medium for CM in the freezing medium (culture medium with 20% v/v glycerol).

### ***Cloning a BSF culture***

7. Proceed as for Basic Protocol 1, Support Protocol 2, substituting BSF culture conditions (HMI-93 medium, incubation at 34°C in a CO<sub>2</sub> incubator) for procyclic conditions.

8. Observe the plate after about a week. Clones will form foci around the founding cell and this helps to verify that there is a single clone in the well. Clones take 7 - 10 days to emerge.

### ***Appearance of bloodstream form cultures (T. congolense IL3000)***

*Location: In a typical BSF culture, ~75% of cells are loosely attached to the bottom of the flask, while the rest are found free-swimming in the medium; attached cells are readily released by shaking or vortexing. The mechanism of attachment to the flask surface is different to that of epimastigotes: BSF do not form rosettes and there is no constant point of attachment; instead a sliding motion is observed (Video 3).*

*Activity/movement: The flexing motion of the BSF cell is more sluggish and serpentine than procyclics (Video 3). Cells adhering by the anterior gently swing the posterior, hinging in the mid-region of the cell (Video 3).*

*Cell division: Cells in the last stage of division remain joined by the posterior (Wheeler et al., 2013).*

*Morphology: The BSF cell is a fairly stout trypomastigote with the kinetoplast posterior to the nucleus and no free flagellum. The cell shape is more tortuous than in procyclics. The kinetoplast is terminal and the nucleus lies in the posterior half of the cell. The mean length of non-dividing cells is 9.7 +/- 0.07 µm, shorter than procyclics.*

## **SUPPORT PROTOCOL 1**

### **DIFFERENTIATION OF BLOODSTREAM FORM TO PROCYCLIC TRYPANOSOMES**

Procyclics may be derived by differentiation of bloodstream forms grown in culture. The efficiency of transformation of cultured BSF (IL3000) to procyclics appears to be less efficient than for trypanosomes taken directly from blood.

### ***Additional materials***

As for culturing procyclics - Basic Protocol 1.



## Protocol steps

1. Take 1 ml ( $\sim 5 \times 10^6$  trypanosomes) from a log phase BSF culture, pellet by centrifugation in a microcentrifuge (3000g, 3 minutes) and resuspend in 500  $\mu$ l of CM.
2. Place the resuspended cells in a T25 flask and add CM to 5 ml. Incubate the flask at 28°C.
3. Inspect growth after about a week. Cells with BSF morphology persist for some time, but most die within 5-6 days. Simultaneously a population of longer, more motile free-swimming cells will emerge, which are the procyclics.
4. Subculture can be attempted once growth of the procyclics is vigorous. Cryopreserve the culture at this stage (Basic protocol 1, Support protocol 1).

## BASIC PROTOCOL 4

### TRANSFECTION OF PROCYCLIC FORMS

Transfection is the process by which foreign DNA is introduced and expressed in cells as part of genetic modification. In trypanosomes, the term transfection is preferred to transformation, which refers to the differentiation of one life cycle stage to another. Transfection is an inefficient process, e.g. typical transfection efficiency of 1 in  $10^7$  trypanosomes, and therefore large numbers of cells are required at the outset. As *T. congolense* procyclics grow to higher density than epimastigotes or BSF, it is easier to obtain large numbers of procyclics for transfection and that is the protocol described here. However, other labs have successfully transfected epimastigotes and BSF (Coustou et al., 2010; Sakurai, Tanaka, Kawazu, & Inoue, 2009).

The plasmid DNA constructs developed for transfection of *T. brucei* often also work in *T. congolense* (e.g. (Inoue, Otsu, Ferraro, & Donelson, 2002)), and hence there has been relatively little effort to design constructs specific for *T. congolense*. However, it was demonstrated that some commonly used *T. brucei* promoters, e.g. the procyclin promoter, failed to express reporter genes in *T. congolense*, and to date the only functional promoter element for *T. congolense* is a ribosomal promoter (Downey & Donelson, 1999b). Nevertheless, since trypanosomes use polycistronic transcription units, it is feasible to integrate transgenes downstream of an endogenous promoter such as tubulin (Inoue et al., 2002; Sakurai et al., 2009). The DNA sequences of some protein-coding genes, e.g. alpha and beta tubulin, PFR1, are sufficiently well-conserved between *T. congolense* and *T. brucei* to allow targeted integration into the specific locus (Figure 4); however, the intergenic regions are generally too divergent.

Several different antibiotics have been used for selection of transfectants in *T. congolense*: hygromycin, blasticidin, G418, puromycin and bleomycin. In general, lower concentrations are tolerated by *T. congolense* compared to *T. brucei*.

## Materials

As for culturing procyclics - Basic Protocol 1  
Electroporation equipment, Amaxa or BioRad electroporator  
Electroporation cuvettes, 2 mm gap  
Transfection buffer, Tb-BSF or Downey's – see recipe  
Plasmid construct  
Appropriate antibiotic for selection

### Protocol steps

Figure 5 shows the general scheme.

1. Pellet approximately  $1 \times 10^8$  cells from a late log phase procyclic culture by centrifugation in a 15 ml centrifuge tube at 3000g for 5 minutes at 4°C.
2. Pour off most of the supernatant into a waste pot and resuspend the cells in about 1 ml of ice cold transfection buffer in a microcentrifuge tube.

*Choice of transfection buffer does not seem to be particularly critical in our experience and recipes for two comparably efficient transfection are given.*

3. Pellet cells by centrifugation in a microcentrifuge (3000g, 3 minutes) and remove supernatant as completely as possible with a pipette.

4. Resuspend the cells in 150  $\mu$ l of transfection buffer containing 5-20  $\mu$ g of plasmid DNA and transfer immediately to a sterile 2 mm electroporation cuvette. Keep on ice.

*In our experience the number of transformants is roughly proportional to the amount of DNA in the transfection buffer. In transient transfection, 1  $\mu$ g of DNA yields 0.2-0.4% of transfectants, while 10  $\mu$ g of DNA yields 2-4%; for 100  $\mu$ g DNA yield has been reported between 10-30% (Downey & Donelson, 1999a, 1999b) . For practicality, 10  $\mu$ g is sufficient, an amount conveniently collected from a plasmid miniprep. Transient transfection with two plasmids with different reporters generally results in discrete populations, with only a very small fraction of cells taking up both DNAs. This suggests that transfection is random rather than targeting a subpopulation of super competent cells.*

5. Place cuvette in the electroporator and pulse.

*Optimum electroporation settings are likely to vary between different machine makes. We recommend two pulses on program X-001 for the Amaxa Nucleofector 2b. For Bio-Rad machines (Gene Pulser II) a single or double pulse at 500-1000 V, 25  $\mu$ F and 200  $\Omega$ .*

6. Immediately transfer the electroporated cells from the cuvette to a T25 flask containing 5 ml of CM without glutamine pH 8. Incubate the flask upright overnight at 28°C.

*Experiments requiring only transient transfection of a non-integrating plasmid construct do not require the next steps. For fluorescent protein expression constructs, fluorescence is observed within 6-8 hours of transfection and increases in intensity over 16 hrs. Fluorescent cells can still be detected several days post transfection.*

7. After 24 hours increase the volume to 50 ml by adding an additional 45 ml of medium to the flask and add selection drug.

*Allowing the flask to stand on end for a few hours is an effective way to remove dead cell debris generated by electroporation. We use the following antibiotic concentrations: Hygromycin 15 - 25 µg/ml; G418 3 - 5 µg/ml; Puromycin 0.25 - 0.5 µg/ml; Blastidicin 2.5 - 5 µg/ml. These antibiotics inhibit protein synthesis and therefore act swiftly to stop the growth of non-selected cells; typically it takes ~10 days to kill ~90% of cells. In contrast, Bleomycin inhibits DNA synthesis and takes time to have effect; this makes its use for selection of *T. congolense* problematic and we avoid using it.*

8. Plate out the culture in 2 ml aliquots in a 24-well plate. Seal the plate with parafilm and incubate at 28°C. Inspect the plate every few days until dividing trypanosomes appear. Subculture in selective medium until a pure culture of actively dividing trypanosomes is obtained. Transfected lines will emerge after 3-4 weeks.

*When selective antibiotic is introduced into the medium, there is a balance between growth of transfectants and the gradual death of non-transfected cells – the majority of the cells. If the selective antibiotic is slow to kill non-transfected cells, then the medium may become exhausted before transfectants have a chance to grow through; in this case, replace 1 ml of medium in each well.*

## REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps.*

### ***Cunningham's Medium (CM)***

This is a modification of the medium originally described by Cunningham (Cunningham, 1977), omitting the redundant amino acid taurine and adding HEPES to improve buffering (M.A. Gray et al., 1987). The solutions can be made in advance and stored frozen at -20 °C.

For 1 litre of base medium, combine the following 5 solutions and add 0.2 x BME vitamin mixture (2 ml of 100x stock) and phenol red (10 mg per litre). Adjust pH to 8.0 using 5M NaOH and then make up to 1 litre with distilled water (NB higher pH than in the original CM recipe, because *T. congolense* is less tolerant of low pH). The base medium can be stored frozen at -20 °C for up to 1 month; longer than this, an insoluble precipitate develops, which may be CaSO<sub>4</sub>; however, the precipitate does not appear to affect the nutritive properties of the medium.

**1. Salts 100 ml**

4.42 mM NaH<sub>2</sub>PO<sub>4</sub>  
14.95 mM MgCl<sub>2</sub>  
15.01 mM MgSO<sub>4</sub>  
40.00 mM KCl

**2. CaCl<sub>2</sub> 50 ml**

1.02 mM CaCl<sub>2</sub>

**3. Sugars 100 ml**

3.89 mM glucose  
2.22 mM fructose  
1.17 mM sucrose

**4. TCA intermediates 50 ml**

5.00 mM L-malic acid  
2.53 mM α-ketoglutaric acid  
0.47 mM fumaric acid  
0.51 mM succinic acid

**5. Amino acids 600 ml**

6.12 mM L-alanine  
22.47 mM beta-alanine  
2.53 mM L-arginine  
1.82 mM L-asparagine  
0.83 mM L-aspartic acid  
0.57 mM L-cysteine  
0.13 mM L-cystine  
1.70 mM L-glutamic acid  
11.23 mM L-glutamine\*  
1.60 mM L-glycine  
1.03 mM L-histidine  
0.69 mM L-isoleucine  
0.69 mM L-leucine  
1.03 mM L-lysine  
1.34 mM L-methionine  
1.21 mM L-phenylalanine  
60.00 mM L-proline  
1.90 mM L-serine  
0.84 mM L-threonine  
0.49 mM L-tryptophan  
1.10 mM L-tyrosine  
1.79 mM L-valine  
20.00 mM HEPES

Thaw and supplement with 10-20% v/v of heat-inactivated foetal calf serum, 0.2x hemin (100x stock: 2.5 mg/ml hemin dissolved in 50 mM NaOH) and antibiotic if required (0.01 mg/ml gentamycin). Filter sterilize using a 0.2 µm filter and store at 4°C for up to 2 weeks. \*Omit from CM for conditions where trypanosomes are at low density, such as cloning and transfection; when adapted to medium without glutamine, trypanosomes grow perfectly well, so glutamine may be left out without detriment.

### ***HMI-93***

Iscove's modified Dulbecco's medium (IMDM) with glutamine, supplemented with 36 mM NaHCO<sub>3</sub>, 0.05 mM bathocuproine disulphonic acid (disodium salt), 1.5 mM L-cysteine, 1 mM hypoxanthine, 1 mM sodium pyruvate, 0.16mM thymidine, 0.2 uM 2-mercaptoethanol, pH 7.5. Store base medium frozen at -20°C. Thaw and supplement with: 10% v/v goat serum and antibiotic if required (0.01 mg/ml gentamycin), though trypanosomes may do better without antibiotic. Store at 4°C for up to 2 weeks. Warm in 37 °C water bath directly before use.

### ***Freezing medium***

Make a 20% v/v solution of glycerol in growth medium and filter sterilize. Store at 4°C for up to 1 month.

### ***Anti-contamination cocktail***

For a 100x stock solution (Maser et al., 2002), dissolve the following in sterile distilled water: 6 mg/ml Penicillin G sodium salt, 10 mg/ml Kanamycin sulphate, 5 mg/ml Flucytosine (5-fluorocytosine), and add 1% v/v Chloramphenicol (100 mg/ml stock in ethanol). Store frozen at -20°C as small aliquots of ~1 ml; keep working stock at 4°C for 1 month.

### ***Tb-BSF buffer (Schumann Burkard, Jutzi, & Roditi, 2011)***

67 mM Na<sub>2</sub>HPO<sub>4</sub>, 23 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM KCl, 0.15 mM CaCl<sub>2</sub>, 50 mM HEPES, pH 7.3. Filter sterilize and store at 4°C for several months.

### ***Downey's transfection buffer (Downey & Donelson, 1999a)***

1.75 mM KH<sub>2</sub>PO<sub>4</sub>, 4 mM MgCl<sub>2</sub>, 68 mM sucrose, 90 mM KCl, 0.113 mM CaCl<sub>2</sub>, 19 mM HEPES, 1.5 mM EDTA, pH 7.6. Filter sterilize and store at 4°C for several months.

## **COMMENTARY**

### **Background Information**

The tsetse-transmitted trypanosomes have been intensively studied in the laboratory for over 100 years, because they are major pathogens of humans and their livestock in subSaharan Africa. African Animal trypanosomiasis, particularly in draught animals such as

cattle and horses, had a profound impact on the development of agriculture in tsetse-infested regions, and the disease continues to be a major economic drain.

In the early days, most laboratory work was carried out using bloodstream form (BSF) trypanosomes grown in experimental rodents and laboratory strains were continuously maintained by serial sub-passage until cryopreservation became a routine tool in the 1960's. Culture media suitable for longterm maintenance of procyclics in suspension culture were developed in the 1970's (Brun & Schonenberger, 1979; Cunningham, 1977). BSF were initially cultured with mammalian cells, e.g. *T. brucei* (Brun et al., 1981), *T. congolense* (Hemphill & Ross, 1995), with the subsequent development of systems without feeder layer cells, e.g. (Hirumi & Hirumi, 1989, 1991). Most effort has focussed on *T. brucei*, because of its importance as a human pathogen, while *T. congolense* has received limited attention.

### **Critical Parameters**

For trypanosome culture, particularly BSF, the type of serum appears to be a critical factor, e.g. animal source - goat serum supported optimal growth of *T. congolense* BSF (Hirumi & Hirumi, 1991); batch effects - batches of serum may differ in their ability to sustain growth.

Trypanosome cell density affects growth, e.g. some procyclic cultures may not recover if diluted beyond 1:5 or 1:10, perhaps because trypanosomes themselves alter the composition and pH of the medium through their metabolic products. Until you are confident about how great a dilution the trypanosomes will tolerate, it is wise to be cautious and dilute growing cultures frequently rather than drastically diluting the cells less often.

### **Troubleshooting**

It is wise to cryopreserve a trypanosome culture in multiple aliquots as soon as it is growing well. This provides insurance against loss through contamination, a bad batch of medium or serum, or other disaster. Power failure is usually not a problem, leastways for procyclic and epimastigote cultures, which can survive at room temperature for some days.

### **Time Considerations**

In general cultures need attention every few days for optimal growth, so it is better to cryopreserve cultures that cannot be monitored for a week or more. However, clones will take around 10 days to grow to density, depending on the rate of cell division, and after transfection, at least a week of antibiotic selection is needed before drug-resistant cells grow through.

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## INTERNET RESOURCES (optional)

The website of George Cross's Laboratory of Molecular Parasitology hosted at Rockefeller University provides a compendium of useful information on trypanosome culture and transfection methods: <http://tryps.rockefeller.edu/>

Genome data for *Trypanosoma congolense* and other trypanosomes is available via [www.genedb.org](http://www.genedb.org) and [www.TriTrypDB.org](http://www.TriTrypDB.org).

## FIGURE LEGENDS

**Figure 1:** Diagram illustrating the life cycle of *Trypanosoma congolense* in the tsetse fly. **A.** Developmental stages in the midgut. Bloodstream forms (BSF) enter the midgut when the fly feeds on an infected animal and transform to procyclics, which proliferate and pass through the peritrophic matrix into the ectoperitrophic space. They continue to proliferate and migrate forward to the proventriculus, the valve separating the foregut and midgut. Here they become long proventricular forms and cease division. **B.** Developmental stages in the proboscis. Proventricular forms migrate to the mouthparts via the foregut. They attach to the chitinous lining of the food canal and start to proliferate as epimastigotes before invading the hypopharynx, the duct that carries saliva from the paired salivary glands to the tip of the proboscis. Infective metacyclics are produced in the hypopharynx and pass into the next animal that the fly feeds on. The whole cycle takes about 2 weeks.

**Figure 2:** Procylic culture. **A.** Live procylic trypanosomes from culture. The arrow indicates two trypanosomes in the final stage of cell division, connected by a thin cytoplasmic bridge between the cell posteriors. **B.** Two T25 culture flasks showing the colour change brought about by cell growth. The flask on the left is in the final stage of log growth and the medium has acidified as indicated by the yellow colour of the phenol red in the medium; the flask on the right has just been subcultured with fresh medium at neutral pH.

**Figure 3:** Epimastigote culture. **A.** Epimastigotes attached to a glass coverslip, with several rosettes of dividing trypanosomes. **B.** The bottom of a T25 culture flask showing the visible layer of epimastigote colonies covering the surface of the plastic.

**Figure 4:** Genetically modified *Trypanosoma congolense* with a fluorescent flagellum. Procylics were transfected with a plasmid construct containing the gene for yellow fluorescent protein (YFP) in fusion with the gene for PFR1, a major structural protein of the paraflagellar rod that runs parallel to the axoneme. Although the plasmid construct was originally designed for *T. brucei* (Peacock et al., 2011) and has no *T. congolense*-specific sequences, it is efficiently expressed in *T. congolense*. **A - C.** Phase contrast; yellow fluorescence; merge.

**Figure 5:** Scheme of transfection as described in Basic Protocol 4.

## VIDEOS

- 1.** Procylic culture. *T. congolense* IL3000 procylics. The trypanosomes are very active and occasionally swim rapidly in a straight line; the anterior of the cell shows the greatest movement, while the posterior is relatively stiff. In this mid-log phase culture, some pairs of trypanosomes in the final stage of cell division can be seen, which are joined by their posterior ends.
- 2.** Epimastigote culture. *T. congolense* WG81 epimastigotes attached to the bottom surface of a tissue culture flask. Two large rosettes are visible. Epimastigotes are firmly attached by the anterior and gently pivot about this point; the cell body shows little undulatory movement.
- 3.** Bloodstream form culture. *T. congolense* IL3000 bloodstream forms. The majority of cells loosely adhere to the bottom of the flask as single cells rather than rosettes. Cells adhering by the anterior gently swing the posterior, hinging in the mid-region of the cell. The flexing motion of the BSF cell is more sluggish and serpentine than procylics.