Chapter 25

Transfection of Leishmania and Trypanosoma brucei by Electroporation

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1. Introduction

DNA transfection is the introduction of molecular constructs into parasites in a manner permitting the expression of encoded genes. Transfection is an exceedingly powerful technique for analyzing genetic regulatory elements and gene function, and is widely used in molecular biology. Transfection by electroporation has now been accomplished in five trypanosomatid genera. We describe protocols used in our laboratories for transfection of Trypanosoma brucei and Leishmania. There are three basic steps in DNA transfection:

1. Construction of the DNA to be tested using standard recombinant DNA methodology. The design of the DNA constructs is critical and specific to the experimenter's goals (see Note 1).
2. Introduction of the DNA into parasites. This is accomplished by exposing the cells to a brief electrical pulse in the presence of DNA. Electroporation generates transient pores in the cell membrane, permitting a small proportion of the cells to take up DNA. In these cells some proportion of the DNA makes its way to the nucleus and can be expressed.
3. Assay of the parasites for expression of the genes encoded by the DNA constructs. This takes two basic forms.
   a. Transient transfection assays, in which expression of the transfected DNA is monitored one or two days after electroporation. Expression is followed by either direct analysis of RNA or by assay of an encoded
"reporter" enzyme (see Note 2). Reporter enzymes are selected because the parasite lacks endogenous activity, the enzyme is stable, and the assay is convenient. Commonly used reporter genes are those encoding chloramphenicol acetyltransferase (CAT) (1–5), β-galactosidase (βGAL) (6), β-glucuronidase (GUS) (6), or luciferase (unpublished data).

b. Stable transfections, where the transfected DNA is maintained in a functional state for extended periods of time. The transfecting DNA expresses a drug resistance marker, and drug selections are used to identify transfectant cells. Selectable markers currently in use in trypanosomatids are phosphotransferases conferring resistance to the aminoglycosides G418 (NEO) (7–11) or hygromycin B (HYG) (12,13). Resistances to these drugs are independent, allowing them to be used simultaneously or successively (12).

Transient transfections are widely used in eukaryotic molecular biology because they are rapid, convenient, and ideal for dissection of elements mediating gene expression and regulation. Typically, DNA segments suspected to control expression are fused to a DNA fragment encoding the desired reporter enzyme for assay by transfection. If expression is sufficiently high, direct analysis of proteins can be performed (14).

Stable transfections are slower and involve more work, but permit the experimenter to define the structure of the introduced DNA. Because every cell expresses the gene of interest, stable transfectants yield higher levels of encoded RNAs and proteins. DNA molecules can be introduced as extra-chromosomal replicating episomes (7,8,15) or inserted into chromosomes by homologous recombination (9–11,16). Stably transfected lines are well suited for studying biological function, creating lines that either over-express or lack genes of interest. By proper choice of transfecting DNA one can perform “gene targeting” and delete endogenous chromosomal genes (16). Expression vectors can be used to create parasites that produce foreign proteins (15,17). The study of such lines can provide important clues about gene function during the parasite life cycle.

2. Materials

2.1. General

1. Electroporator: Bio-Rad Gene Pulser with capacitance extender, or a BTX (San Diego, CA) Electro Cell Manipulator 600 electroporation system.
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2. Electroporation cuvets, sterile (0.2 cm; Bio-Rad, Richmond, CA; see Note 3).
3. DNA to be transfected, in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4; sterilized by ethanol precipitation for stable transfections (see Note 4).
4. Scintillation counter (CAT assay), Hoefer, (San Francisco, CA) TKO-100 minifluorimeter (βGAL and GUS assays) or luminometer (luciferase assays), as needed.
5. Selective drugs: G418 (Geneticin; BRL, Gaithersburg, MD) or Hygromycin B (Sigma, St. Louis, MO): both 100 mg/mL in culture medium, then filter sterilized and stored frozen.
6. Phosphate buffered saline (sterile). We have used HEPES-saline glucose for trypanosomes (25 mM HEPES, pH 7.4, 0.9% NaCl, 1% glucose) and Hank’s Balanced Saline (Gibco, Gaithersburg, MD) for Leishmania but any standard formulation will do.

2.2. Trypanosoma brucei
1. Culture medium: Any medium that supports healthy growth may be employed. We have used SDM-79 (18) or MEM-Pros (19).
2. Trypanosoma brucei electroporation buffer (Zimmerman post-fusion medium or ZPFM) (3): 132 mM NaCl, 8 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1.5 mM magnesium acetate, 90 μM calcium diacetate, pH to 7.00 ± 0.05 with NaOH or acetic acid. Filter sterilize.
3. CAT assay reagents:
   a. Lysis buffer, 100 μL/assay: 100 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol.
   b. Chloramphenicol, 200 μL/assay: 1.25 mM chloramphenicol (0.4 mg/mL) in 100 mM Tris-HCl, pH 7.8.
   c. [¹⁴C]-butyryl CoA, 10 μL/assay (NEC-801, New England Nuclear, Boston, MA; 10 μCi/mL).
   d. Econofluor (New England Nuclear; 4 mL/assay).
4. T. brucei luciferase assay:
   a. Lysis buffer (100 μL/assay): 100 mM potassium phosphate buffer, pH 7.8, 2 μg/mL leupeptin.
   b. Triton X-100.
   c. Reaction mix (350 μL/assay): 9 mL of 25 mM glycyglycine, pH 7.8; 1 mL of 20 mM ATP, pH 7.5; 0.1 mL of 1M MgSO₄.
   d. Injection mix (about 100 μL/assay): 4 mL of 25 mM glycyglycine, pH 7.8; 1 mL of 1 mM luciferin (Sigma; dissolved in H₂O).

2.3. Leishmania
1. Electroporation buffer (EPB; 2,8): 21 mM HEPES, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM glucose. Filter sterilize.
2. *Leishmania* lysis buffer: Tris-protease inhibitor-BSA cocktail (Tris/PI/BSA; 80–160 μL/assay): 25 mM Tris-HCl, pH 7.3, 1 mM EDTA, 150 μg/mL benzamidine (stock: 100 mg/mL in ethanol), 20 μg/mL leupeptin (stock: 5 mg/mL in H₂O), 0.2 mg/mL 1,10-phenanthroline (stock: 100 mg/mL in ethanol), 50 μg/mL soybean trypsin inhibitor (stock: 10 mg/mL in H₂O), 50 μg/mL bovine serum albumin (molecular biology grade). Make up this stock the day of the experiment, in advance. Immediately prior to use, add PMSF to 100 μg/mL from a 100 mg/mL stock in DMSO (PMSF is optional as it is quite unstable and rapidly reacts with other buffer components).

3. 10% SDS in water (1 or 2 μL/transient assay).

4. *Leishmania* β-galactosidase and β-glucuronidase assay mixes (320 μL/assay): 23 mM Tris-HCl, pH 7.5, 125 mM NaCl, 2 mM MgCl₂, 12 mM 2-mercaptoethanol. For the β-galactosidase assay include 0.3 mM 4-methylumbelliferyl-β-D-galactoside (0.1 mg/mL; formula weight = 338; Sigma). For β-glucuronidase assay include: 1 mM 4-methylumbelliferyl-β-D-glucuronide (0.34 mg/mL; formula weight = 352; Sigma). The substrates are prepared as a 200X concentrated suspension in ethanol. Prepare the reaction mix at room temperature, add the appropriate volume of 200X substrate, and vortex until dissolved. Use immediately for lowest backgrounds.

5. Glycine-carbonate reagent: 133 mM glycine, 83 mM Na₂CO₃, pH 10.7 (2 mL/assay).

6. M199 medium (Sigma) (8).

7. M199 selective plates. Prepare 2X M199 medium containing 2.4 μg/mL biopoterin, and 2% agar (Difco Bacto-Agar or Noble Agar) in water (sterile). Autoclave or microwave the agar to melt, and place at 55°C to cool. Place the 2X M199 at 37°C. Each 100 mm Petri plate requires about 25 mL. (we generally find it convenient when preparing many plates to pour them in smaller batches to prevent the agar from solidifying). To the warm 2X stock add the selective drug (twice the desired final concentration; see Note 5), add an equal volume of agar, mix gently and pipet onto the plate. Try to avoid air bubbles. After the medium solidifies, the plates need to be dried. This is important, since flagellites can "swim" on wet plates. Drying is done by a brief 10–15 min exposure in a sterile hood (uncovered) or overnight on the bench (covered). Just before use, equilibrate the plate pH by incubation in a 5% CO₂ incubator for 2–4 h. The color of the indicator dye should be monitored.
3. Methods

3.1. Electroporation of T. brucei

All of the protocols below refer only to procyclic trypanosomes; the conditions described kill bloodstream forms. Transfection of bloodstream trypanosomes has been reported (20,21) but the level of expression of a CAT reporter construct was very low.

1. In advance grow parasites to late log phase (see Note 6). Each transfection requires about $2 \times 10^7$ cells. Be sure to include appropriate controls (see Note 7).
2. Spin down the trypanosomes at $4^\circ$C, for 10 min at 1000g. Meanwhile prepare flasks (5 mL medium each) to accept the transfected cells. Do not attempt more than 30 cuvets in one experiment; 20 is more manageable. If you need more it is better to divide the experiment.
3. Resuspend the cells in 10–20 mL ice-cold ZPFM, and centrifuge again. Meanwhile place the electroporation cuvets (0.2-cm gap) on ice.
4. Resuspend in ZPFM at a concentration of $4 \times 10^7$ cells/mL (see Note 8). Place on ice for 5 min.
5. Place 0.5 mL of cells into cuvets. Incubate on ice for 10 min.
6. Add DNA to 2 or 4 cuvets (see Notes 9 and 10). Mix very well by pipeting up and down with a Pasteur pipet. Pulse immediately; with the Bio-Rad electroporator, use 800 V, 25 μF (9). With the BTX electroporator, use 1.6 kV with R2 resistance setting. Note the pulse time constant, which should be similar among all samples to ensure consistency. It should be 0.3 for the BTX electroporator.
7. Pipet cells into individual flasks. Rinse out the cuvet with medium, pipeting up and down to remove debris from the sides. Put the rinses into the culture flasks. Put the empty cuvet into a bath of water ready for washing.
8. Continue with steps 5 and 6 until all the DNAs have been processed.
9. Incubate at $27^\circ$C as required: 6 h for measurement of transcription, 7–8 h for preparation of RNA, overnight for enzyme assays or stable transfections.

3.2. Selection for Stable Transfectants of T. brucei

1. After the overnight incubation, add the appropriate selective drug. For G418 selection of neo transfectants use 25–50 μg/mL (9,10). For hygromycin B selection of hyg transfectants use 25 μg/mL, increasing to 50 μg/mL 48 h thereafter (13).
2. After 4–5 d one will begin to see extensive cell death; after about 10 d, outgrowth of resistant cells will be observed. Usually resistant cell populations are not clonal. Until recently, plating methods for single *T. brucei* cells were not available, and clonal populations were obtained by limiting dilution. Unfortunately many procyclic trypanosome cultures die at low density, making cloning by limiting dilution impossible for these lines. Prolonged culture of the new drug-resistant cells may give a clonal population. (See Note 11).

3.3. CAT
*(Chloramphenicol Acetyl Transferase)*

Assays of *T. brucei*

In this two-phase assay CAT mediates the reaction of $[^{14}C]$-butyryl CoA with chloramphenicol to produce $[^{14}C]$-butyryl chloramphenicol. The reaction product partitions into the organic-phase scintillant carefully layered above the aqueous reaction mix. This assay is the most convenient of those we have tried and has the advantage of giving kinetic data (2).

1. After incubation, check the cells under the microscope and measure the cell density in a few of the flasks. If you see more than twofold disparities, count all flasks.
2. To make extracts, place the cells from each flask into separate centrifuge tubes and collect (10 min at 1000g, 4°C). Resuspend the trypanosomes in about 1 mL of ice-cold saline. Transfer to a microcentrifuge and centrifuge for 1 min at 4°C. Remove all the supernatant and proceed immediately; be careful as the cell pellet may be soft.
3. Resuspend the harvested cell pellet in 100 μL CAT lysis buffer. Freeze on dry ice, thaw at 37°C, freeze again and thaw again.
4. Microcentrifuge for 3 min. Take off the supernatant into a new tube.
5. Heat at 65°C for 6 min to inactivate endogenous acetylases or deacetylases. (This step is in most protocols but in our experience is unnecessary for trypanosomes.) The extract can be stored at −20°C until needed. This is a good time to reserve time on the scintillation counter.
6. In a scintillation vial place the transfected cell extract (10–50 μL); if necessary, add 100 mM Tris-HCl, pH 7.8, to give a total final volume of 50 μL.
7. Add 200 μL 1.25 mM chloramphenicol and then 10 μL $[^{14}C]$-butyryl-CoA; mix immediately, as this starts the reaction.
8. Overlay with 4 mL of Econofluor. It is extremely important to wipe the outsides of the vials with a damp cloth to eliminate static.

9. Count the vials sequentially for 0.2 min on the $^{14}$C channel. Do this at time 0, then at 15 min or hourly intervals depending on the activity.

**3.4. Luciferase Assay of T. brucei**

Firefly luciferase catalyzes a reaction between luciferin, ATP, and oxygen, leading to the production of AMP, oxyluciferin, and light; the light is measured in a dedicated luminometer (23).

1. Harvest the parasites as described in steps 1 and 2 in Section 3.3. Resuspend the harvested cell pellet in 100 µL 100 mM potassium phosphate buffer plus leupeptin and place on ice. Add Triton X-100 to 0.2%. Mix, and spin down the debris (2 min, 4°C in a microcentrifuge). Use the supernatant as soon as possible.

2. Aliquot 350 µL of reaction mix into luminometer tubes. Make two per extract and about four spares. Install the luciferin injection mix into the apparatus according to the manufacturer’s instructions. Before starting, remember to wash out the tubes and inject several times to wash out old injection mix.

3. For each assay, add 10–30 µL of cell extract to 350 µL of reaction mix. Tap well or vortex to mix. Put in the luminometer, inject luciferin according to instructions, and measure for about 30 s.

**3.5. Electroporation of Leishmania**

These protocols have been performed on cultured promastigotes from all four pathogenic complexes of Leishmania, and with Crithidia and Endotrypanum. Transfected infective promastigotes are capable of differentiating into amastigotes in vivo and infecting animals (17). Additionally, cultured Leishmania amastigotes have been transiently transfected with two reporter gene constructs (V. Bajjaj and S. M. Beverley, unpublished data).

1. In advance inoculate sufficient medium with cells so that there will be enough late log phase cells (each transfection requires $4 \times 10^7$ cells; see Note 6). For stable transfections sterility should be maintained throughout. Be sure to plan appropriate controls (see Note 7).

2. Place DNAs (see Notes 9 and 10) and electroporation cuvets on ice.

3. Collect the cells by centrifugation (5 min at 1000g; speed 5 in a Sorval T6000 tabletop centrifuge). Discard the supernatant; resuspend the cells
by pipeting up and down in 5–10 mL EPB until completely dispersed. Repeat the centrifugation.

4. Resuspend the cells at a density of $10^8$/mL in EPB (working stock). Place on ice if they are to be used immediately or store at room temperature. Cells have been stored up to 2 h with no loss in efficiency. When you are ready, aliquot 0.4 mL of the working stock cell suspension into electroporation cuvets, and leave on ice for a few minutes.

5. Turn on the Gene Pulser. Set the voltage at 0.45 kV (with a 0.2 cm cuvet, the delivered pulse will be 2.25 kV/cm; see Note 12). Set the main unit for “capacitance extender” and the capacitance to 500 μF.

6. For each tube of DNA, perform the next steps in the sterile hood, with some rapidity.
   a. Add DNA to the cell suspension in the electroporation cuvet; tap vigorously or use a sterile Pasteur pipet to mix well; then tap the cuvet to ensure that the mixture is completely between the electrodes.
   b. Place the cuvet in the chamber, and electroporate (“zap”) immediately. This is done by pressing both buttons on the Gene Pulser unit simultaneously and holding until the display stops blinking CHG and the beep sounds (be sure to read the manual for the Gene Pulser, since high voltages are involved). Note the pulse time constant, which should be 3.5–4.5 ms for all samples.
   c. Remove the cuvet from the chamber and place on ice.

7. After 10 min, still working in the sterile hood, transfer the cells to 10 mL of M199 medium without selective drugs. Use some of the culture medium to rinse the cells out of the electroporation cuvet. Incubate overnight to allow expression of the introduced genes.

3.6. Harvesting and Plating Leishmania for Stable Transfections

1. In advance prepare selective plates containing the appropriate drug (see Note 5).

2. Transfer the 10 mL of electroporated cells in medium to a 15-mL polystyrene centrifuge tube. Spin for 5 min at 1000g (speed 5 in a T6000 Sorvall tabletop centrifuge).

3. Decant or pipet the supernatant medium carefully; the pellet is very loose. Lightly vortex, or flick with a finger to resuspend the cells in the residual M199 in the tube (there should be no more than 100–200 μL).

4. Transfer to a plate with a sterile pipet. Spread gently on the plate with a sterile spreader (bacterial-style triangle). If wet, the plates may need to air dry briefly, although this may change the pH.

5. Wrap the plates in parafilm and put in a 26°C incubator, agar side up.
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We find that wrapping is essential despite the maintenance of appropriate humidity in the incubator. It will take 4–10 d for control colonies (without the drug) to grow, and 6–14 d (or more) for transfectants to emerge.

6. Count the colonies after all have emerged. Visualization is easy using indirect back-lighting (see Note 13).

7. Identify well-isolated colonies and then check under a dissecting microscope for small microcolonies nearby. To pick the colonies use sterile needles, toothpicks, or pipets. Place them in 1 mL of medium containing the selective drug in 24-well plates. Once dense growth is evident in the well, transfer to a 10 mL culture. The efficiency of recovery of colonies is quite high (nearly 100%). To store plates with colonies for at least 1 mo, wrap tightly with parafilm, place in a styrofoam box, seal, and place at 4°C with the agar side up. This allows the plates to cool slowly and prevents condensation (which would disperse the cells). To recover, place the box back at room temperature and wait 6–8 h. Alternatively, store at room temperature for up to several weeks.

3.7. Harvesting Leishmania for β-Galactosidase and β-Glucuronidase Assays

These enzymes are measured using a fluorescence-based assay using either 4-methylumbelliferyl-β-D-galactoside or 4-methylumbelliferyl-β-D-glucuronide. The methylumbelliferone formed by enzymatic hydrolysis can be detected by its fluorescence in a dedicated fluorimeter (excitation at 365 nm, emission at 460 nm). The fluorescence yield is maximal at alkaline pH values.

1. Transfer all of the 10 mL culture of transfected cells to a 15-mL polystyrene centrifuge tube.
2. Centrifuge for 5 min at 1000g. Decant the supernatant (be careful not to pour out the cells), resuspend the cells in 1.2 mL of HBSS, and transfer to a 1.5-mL microcentrifuge tube.
3. Centrifuge in a microcentrifuge for 20 s at 6000–8000 rpm (approx 3000–4000g).
4. Remove the supernatant carefully with a Pasteur pipet. Be careful since the pellet is soft.
5. Resuspend the pellet by vigorous addition of 80 μL Tris/PI/BSA (use 160 μL if both β-galactosidase and β-glucuronidase are to be assayed). Chill on ice.
6. Add SDS to 0.1%; vortex vigorously and incubate for 15 min on ice.
7. Centrifuge for 15 min in a microcentrifuge at 4°C. Remove 80 μL of
clear supernatant to new 1.5-mL microcentrifuge tubes for assays (keep on ice until ready to perform the assay).

8. Add 320 µL of fresh reaction mix (β-galactosidase or β-glucuronidase) to tubes containing 80 µL cell extract and mix gently. This starts the reaction, so note the time.

9. Incubate in a 37°C waterbath for the desired time; 1–2 h is convenient, although the reaction is linear for at least 20 h. Near the end of this time prepare tubes containing 2 mL of glycine-carbonate reagent.

10. Stop the reaction by rapidly transferring the 400 µL assay mix to the tubes containing the glycine-carbonate reagent; mix.

11. Read the fluorescence in a Hoefer TKO 100 minifluorimeter. If off-scale, accurate readings can be obtained by diluting with the glycine-carbonate reagent.

3.8. Use of Hoefer TKO 100 Minifluorimeter

1. Allow the machine to warm up for 30 min. Zero the machine using a blank consisting of 80 µL Tris/PI/BSA plus 320 µL reaction mix plus 2 mL of glycine-carbonate reagent.

2. Read the fluorescence; for optimum accuracy, do not remove the cuvet between samples. Instead, use 3 mL polypropylene transfer pipets to remove samples and flush the cuvet. If desired, fluorescence units can be converted to moles of product (see Note 14).

4. Notes

1. The design of experimental constructs is currently at the forefront of trypanosomatid research, so the rules are just becoming known. Because transcription of protein coding genes occurs in a polycistronic manner followed by trans-splicing and polyadenylation to generate mature mRNAs, dissection of regulatory elements has been more complicated than for other organisms (24-26). To obtain expression, it seems that one minimally needs to provide only a functional splice acceptor sequence upstream of the reporter gene (2,3,15). Authentic RNA polymerase II promoters have not been described; for the DHFR-TS gene of *Leishmania* the level of expression directed by short flanking sequences in stable episomal transfectants is comparable to that found in the parental chromosomal gene (8,15; unpublished data). In contrast, in *T. brucei* inclusion of the PARP promoter increases the level of expression at least two orders of magnitude (3,4). Most workers find that the nature of the splice acceptor site, the 3' flanking sequences, the specific bacterial vector, and the orientation within the vector can have effects of varying magnitude. Thus, it is important when testing specific elements
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to keep the overall background of the constructs constant. In at least one case it appears that the bacterial vector sequences serve as splice acceptors at some efficiency (2), so care must be taken to ensure biological relevancy.

2. The choice of reporter enzyme is often a matter of preference and availability of equipment. Luciferase assays are the most sensitive but require a dedicated expensive machine not needed for other work. Luciferase, β-galactosidase, and β-glucuronidase assays are nonradioactive, relatively cheap, and linear over at least three orders of magnitude. Since β-galactosidase and β-glucuronidase assays are performed in the same reaction buffer, one can readily transfec in two molecular constructs, an experimental construct expressing one and a control construct expressing the other enzyme, and thereby control for transfectional variability.

3. The "disposable" electroporation cuvets can be reused at least 20 times for transient assays, although we use new cuvets for stable transformations. For reuse, make sure that cells and DNA do not dry onto them immediately after use, either cap them or drop them into a waterbath. At the end of the experiment, wash them out thoroughly with distilled water. Sterilize by washing them with ethanol and allow to dry in the tissue culture hood overnight under UV illumination.

4. In general DNA samples should be pure. DNA banded twice in CsCl/ethidium bromide density gradients or prepared using commercially available columns, such as Qiagen or Nucleobond, works equally well in both stable and transient assays. The column methods are somewhat more expensive but much quicker and more convenient. DNA prepared by alkaline lysis "miniprep" methods will also work, often with a somewhat reduced expression or transfection efficiency (also, for some reason the colonies appear more slowly). For some purposes this is quite sufficient, however. Miniprep DNA should be treated with RNase, phenol extracted, and ethanol precipitated (with 70% ethanol washes to remove excess salt). DNAs are suspended in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 7.4) at about 0.5–1 mg/mL. If the concentrations of the samples vary greatly the pulse time constant will vary; the most important consideration is that it be constant. We use a Hoefer minifluorimeter to determine DNA concentrations. DNA samples need to be sterile but this is readily accomplished by ethanol precipitation.

5. For stable transfections of many species of Leishmania and the genera Endotrypanum and Crithidia, a drug concentration 2–3 times the EC50 (the concentration required to inhibit growth by 50%) is appropriate for selectively killing untransfected but not transfected cells (8,17). The
EC50 appears to vary in different media and with different species. To establish the proper drug concentration on plates, plate 4 x 10^7 cells (with or without mock electroporation) on solid medium containing varying drug concentrations; use the minimal drug concentration that yields no surviving colonies. Occasionally resistance to G418 by chromosomal mutations may occur, causing background problems. To eliminate this, use sufficient G418 and freshly cloned cell lines. Increased G418 pressure leads to increased copy number of episomal DNAs (8).

6. The cells should be in mid log phase (typically about 3–8 x 10^6 cells/mL). If they do not look healthy or are not growing well, do not start the experiment. In general it appears that any medium that supports healthy growth is suitable, although one will need to check the concentration of selective drug required in stable transfections (see Note 5).

7. Experiments are performed in duplicate or triplicate, and replicates agree quite well. In transient transfections a zero DNA and positive control should be included. One can add a constant control DNA to all experimental samples prior to transfection to serve as an internal standard. One can use β-galactosidase as the reporter gene for the experimental samples, and β-glucuronidase as the control reporter gene (see Note 2 and ref. 6). In stable transfections include the following controls: on drug-free plates examine the plating efficiency before and after electroporation; on drug-containing plates include a mock (no DNA) and some active construct control. For stable transfections of trypanosomes also include a positive and negative control.

8. For T. brucei, the cell density during electroporation can be varied slightly. 0.5 mL of a 2–6 x 10^7/mL suspension is acceptable. In one experiment where the density was greater, the expression decreased. The electroporation buffer has to be of high ionic strength. ZPFM works best, although the Leishmania EPB also works (K. Fung and C. E. Clayton, unpublished). Addition of an energy source (proline or glucose) had no effect. The temperature of the parasites is not vital; good results are obtained by keeping everything at room temperature throughout. However, storage of the parasites on ice in ZPFM for 10 min prior to electroporation doubled the level of CAT expression. Further incubation on ice after electroporation had no effect.

9. For transient expression assays one needs intact circular DNAs. For extrachromosomal transformations in Leishmania circular DNAs are used (8), although linear DNAs with cohesive ends or in high amounts can yield episomal transfectants (7,16). For chromosomal integration linear DNAs are used, preferably with blunt or noncohesive ends (9–11,16); the DNA should be cut in a region of homology to the chro-
mosomal target site, not in the vector. In *Leishmania* the type of integration has been shown to be dramatically affected by DNA amounts: with less than 5 µg DNA, homologous replacements at the DHFR-TS locus were observed, whereas at higher DNA amounts homologous integration of multiple copies of the transfecting DNA were observed (16). This feature is quite reproducible and can be useful. Recombination between extrachromosomal DNAs has also been observed in *Leishmania* (27). The minimum amount of homology thus far is 639 bp in *T. brucei* (9) and 900 bp in *Leishmania* (12). Larger extents of homology work somewhat more efficiently.

10. The amount of DNA needed depends on the desired end result. In *T. brucei* 50 µg of a PARP-CAT construct yields 6 ng of CAT from 3 x 10^7 cells (if 1/10 of the sample is assayed after transfection of 10 µg DNA, 20,000 cpm are obtained in 1 h); 10 µg DNA can yield 100,000 or more luciferase units from 3 x 10^6 cells. Between 5 and 50 µg DNA, expression is linear with increasing DNA (K. Fung and C. E. Clayton, unpublished data; see also ref. 28). To get permanent transfectants 8–50 µg of linear DNA were used (9–11). In *Leishmania*, for both β-galactosidase and β-glucuronidase, 10 µg of active constructs yields more than 10,000 fluorescence units (the background is less than 100), and enzyme activity is linear with the time of assay and up to 80 µg of DNA (6). In stable transfections, colony formation is roughly linear to at least 80 µg DNA (8).

11. Plating of bloodstream and procyclic trypanosomes has been recently reported and should become exceedingly useful in future transfection studies (29).

12. The conditions provided are the best we have found for transfection of *Leishmania* thus far. Under these conditions one can obtain stable transfection at frequencies approaching 10^-4/cell (8,17).

13. Colonies can be directly assayed prior to picking. Colonies can be transferred to filter membranes by gently pressing the membrane to the plate. The filter replica can then be processed for nucleic acid hybridization studies (colony hybridization), for western analysis (colony immunoscreening), or for enzymatic assay (8,15). If sterile membranes are used in making the replica and the colony plate saved, one can identify cells with relevant phenotypes. You may need to experiment with plates of differing “dryness” to obtain the best replicates with different membranes.

14. To quantitate fluorescence units, prepare a 1 mM stock of the product, 4-methylumbelliferone (7-hydroxy-4-methylcoumarin, free acid) in ethanol. The concentration can be established by spectrophotometry
(molar extinction coefficient at 325 nm = 15,850). Dilute varying amounts of this stock into a total volume of 80 µL aqueous buffer, 320 µL of reaction mix, and 2 mL of glycine-carbonate reagent, and read the fluorescence. We reproducibly find 24 fluorescence units = 1 nM.

Acknowledgments

We thank V. Bajjaj, V. Bellofatto, H. Callahan, V. Carruthers, C. Coburn, G. Cross, A. Cruz, K. Fung, H. Ip, J. LeBowitz, M. Lee, G. Kapler, D. Sherman, and L. Van der Ploeg for assistance, discussions, and/or sharing unpublished data. Supported by grants from the NIH to S. M. B. and C. E. C.

References

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