Chapter 18

Using a Hand-Held Gene Gun for Genetic Transformation of *Tetrahymena thermophila*

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Abstract

Biolistic bombardment is widely used as a means of delivering vector-coated microparticles into microorganisms, cultured cells, and tissues. The first particle delivery system contained a helium propulsion unit (the gun) mounted in a vacuum-controlled chamber. In contrast, the hand-held gene gun does not operate within a chamber. It is completely hand-held, easy, and efficient to use, and it requires minimal space on the laboratory bench top. This chapter describes protocols for using a hand-held gene gun to deliver transformation vectors for overexpression of genes or gene replacement into the macronucleus of *Tetrahymena thermophila*. The protocols provide helpful information for preparing *Tetrahymena* for biolistic bombardment, preparation of vector-coated microcarriers, and basic gene gun operating procedures.

Key words *Tetrahymena*, Biolistic bombardment, Gene gun, Overexpression, Gene replacement, Microcarriers

1 Introduction

Tetrahymena thermophila exhibits nuclear dimorphism [1], in which separate nuclei are used for vegetative and sexual processes. A macronucleus (MAC) provides for vegetative functions, while a micronucleus (MIC), which provides for the sexual process of conjugation, is transcriptionally silent during vegetative growth. The MAC contains approximately 225 chromosomes [2] with approximately 45 copies of each gene and divides amitotically at cell division. The MIC contains five chromosomes and divides mitotically. *Tetrahymena* genome databases can be assessed at ciliate.org or at the National Center for Biotechnology Information (NCBI) website.

Biolistic bombardment is the preferred method for inserting overexpression constructs into the macronucleus and for homologous recombination in somatic and germline transformations in *Tetrahymena* [3]. Overexpression transformations target the

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extrachromosomal rDNA at the point in conjugation when the new MAC is under formation. Somatic and germline gene replacements target a respective gene of interest in the MAC or MIC. DNA from a transformation vector engages in homologous recombination with the gene of interest. After many cycles of vegetative growth, a process known as phenotypic assortment [4] ensures that the MAC is homozygous for all alleles, and therefore all copies of the gene of interest have been replaced. However, if the replaced gene is essential, phenotypic assortment may not be completed as the MAC retains a few copies of the endogenous allele, a condition referred to as a knockdown. At conjugation, the MAC is degraded, and a new MAC is formed from a zygotic MIC, effectively destroying the transformations that were achieved with the vector. After conjugation of a somatic transformation, the vegetative cell and its newly formed MAC reflect the genetic constitution of the zygotic MIC. In contrast, germline transformations target the MIC and are expressed in the newly formed MAC after conjugation [5].

Plasmid vectors for transformation of *Tetrahymena* contain a cassette consisting of a gene for antibiotic resistance, transcriptional start and stop sequences, and flanking regions that allow for homologous recombination with the endogenous gene of interest. Some of these vectors have been designed for either disruption or replacement of endogenous alleles [6–9]. The pVGF-1 vector [10] and its pIGF-1 derivatives are frequently used for overexpression of genes in *Tetrahymena*. This vector targets ribosomal genes located in an extrachromosomal segment that replicates autonomously. In current use, the pIGF-1 vector contains genes for antibiotic resistance, a metallothionein promoter that is inducible by ionic cadmium [11], a GFP gene, and a transcriptional stop sequence (Fig. 1). Genes of interest are cloned 3′ to the GFP site in pIGF-1 and therefore are expressed with an N-terminus GFP tag (Fig. 1). Transformation vectors have been used for diverse studies involving overexpression or replacement of *Tetrahymena* genes [12–16]. A list of plasmid vectors is available from the National *Tetrahymena* Stock Center (*see* **Note 1**).

The advent of particle bombardment via the hand-held gene gun led to a more efficient method for transfection of plants, animals, and plant and animal cells in culture and for therapeutic delivery of nucleic acids into cells [17–23]. The basic operation of the gene gun involves adhering microcarriers coated with the biological material of interest (DNA, RNA, or other substances) to the inner surfaces of small plastic tubes. A positively charged organic polymer such as polyethylenimine can be used to neutralize the negative charge on DNA. Polycaprolactone, a biodegradable polyester, is another effective charge neutralizer [24]. High-pressure helium is used to dislodge the microcarriers ("bullets") and force them into the cell. Gold or tungsten microcarriers are widely used for particle bombardment protocols with diverse

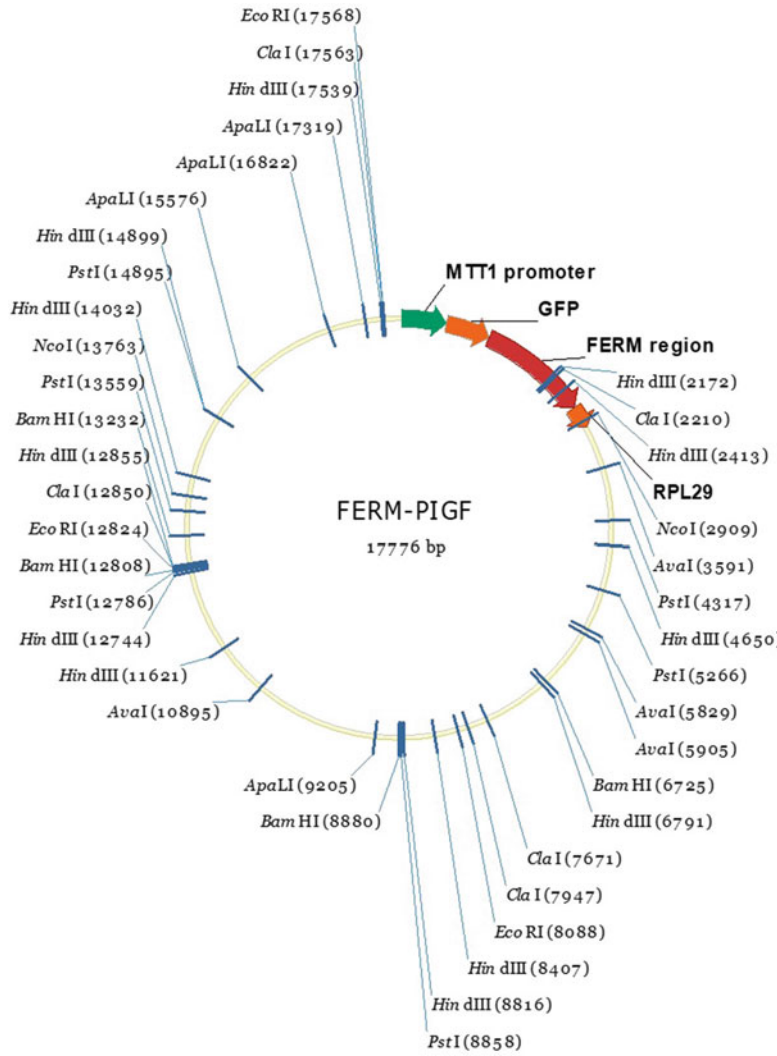

Fig. 1 A pIGF-1 vector was used to prepare a construct for transforming *Tetrahymena thermophila* for overexpression of the FERM domain in a myosin gene [4]. Unique restriction endonuclease cloning sites MTT1 (green) GFP (orange) FERM insert (red) and the transcriptional termination site RPL (darker orange) are pictured from 5′ → 3′

experimental systems [17–23, 25–28]. However, some nanoparticles such as silver can be cytotoxic [29]; therefore, great care must be taken in choosing the correct DNA carrier. Bacterial spores and other degradable compounds have recently been used as microcarriers for both in vitro and in vivo transfection of mice [30] and delivery of therapeutic proteins [31]. Interestingly, *Tetrahymena* is known to ingest microcarriers [32] via phagocytosis. Phagosomes can travel to the nucleus [33], and this raises the possibility that ingested microcarriers could be targeted to the nucleus via phagocytosis.

The Bio-Rad chamber model PDS-1000/He Particle Delivery System (Cat#: 1652257) and the Helios® Gene Gun System (Cat #: 1652431) were recently quoted at a similar price for academic use. However, the chamber requires the purchase of a hepta adaptor (Cat#: 1652225) which increases the overall cost of the chamber by more than 20%. The significant price difference for supplementary components to the chamber model vs hand-held gun model may urge burgeoning *Tetrahymena* researchers to choose the latter model, which is easy to use and requires minimal space on the bench top.

This chapter describes protocols for using a hand-held gene gun to deliver transformation vectors for overexpression of genes or gene replacement in the macronucleus of *Tetrahymena thermophila*. Protocols provide helpful information for preparing cells for biolistic bombardment, preparation of DNA-coated gold particle "bullets" as adapted from a JoVE video article by Woods and Zito [34], and basic gene gun operating procedures for the Bio-Rad Helios Hand-Held Gene Gun based on the Bio-Rad Handbook for the Helios Gene Gun. We are not aware of any other maker of a hand-held gene gun. However, the protocols described in this chapter should be adaptable to other versions of the gene gun.

2 Materials

2.1 Growth and Starvation Media

1. Modified Neff's growth medium [35]: 0.25% proteose peptone, 0.25% yeast extract, 0.5% glucose, 333 μM $FeCl_3$. Add 500 mL of dH_2O to an autoclavable bottle. Add 2.50 g proteose peptone, 2.50 g yeast extract, 5.0 g glucose, and 1 mL of 1000× 33.3 μM $FeCl_3$. Bring the solution up to 1 L with dH_2O, and mix vigorously. Autoclave and store media at 4 °C after sufficient cooling has taken place.
2. Dryl's starvation medium [36]: 2 mM $C_6H_5Na_3O_7$, 1 mM NaH_2PO_4, 1 mM Na_2HPO_4, and 15 mM $CaCl_2$. Add 50 mL of dH_2O to an autoclavable bottle. Add 590 mg Na citrate•$2H_2O$, 140 mg NaH_2PO_4•H_2O, 140 mg Na_2HPO_4. Bring the solution up to 100 mL with dH_2O, and mix vigorously. Add 50 mL of dH_2O to a separate autoclavable bottle. Add 130 mg $CaCl_2$. Bring the solution up to 100 mL with dH_2O, and mix vigorously (*see* **Note 2**). Autoclave each solution for 20 min, and allow it to cool before using. Mix the two solutions under aseptic conditions by allowing each solution to pass through the same filter sterilization column, and adjust the volume to 1 L with dH_2O.

2.2 DNA Coating of Microcarriers

1. Vector DNA: ~1 μg/μL in dH_2O (*see* **Note 3**).
2. 100% ethanol.

3. Spermidine: Add 1.45 g of spermidine to 100 mL pure ethanol to make a 0.10 M solution.
4. 1.0 M calcium chloride.
5. Polyvinylpyrrolidone (PVP) stock: Dissolve 200 mg PVP into 10 mL of dH_2O to make a 20 mg/mL stock solution. Prepare a working solution of PVP: Add 175 μL of 20 mg/mL PVP stock solution to a sterile screw cap container, and bring the volume up to 3.5 mL with 100% ethanol to yield a concentration of 0.01 mg/mL PVP (*see* **Note 4**).
6. Gold (Au): 0.6–1.0 μm gold particles (*see* **Note 5**).

2.3 Gene Gun

1. Helios Gene Gun System (Bio-Rad 165-2431) includes the helium hose assembly with regulator tubing, prep station, syringe kit, Tefzel tube cutter, and optimization kit.
2. Tank of compressed helium.
3. Tank of compressed nitrogen.

2.4 Reagents for Selecting Transformants

1. 100× stock paromomycin: Dissolve 120 mg of paromomycin in 10 mL of sterile dH_2O.
2. 100× stock cycloheximide: Dissolve 30 mg of cycloheximide in 10 mL of sterile dH_2O.
3. 100× antibiotic/antimycotic mix: Use a commercially available product.
4. 1000× stock cadmium chloride: Dissolve 10 mg of $CdCl_2$ into 10 mL of sterile dH_2O.

3 Methods

3.1 Coating Vector DNA to Gold (AU) Microcarriers

1. Add 50 μg (0.6–1.0 μm) gold particles to a sterile microfuge tube containing 50 μL of ethanol and 50 μL of 0.10 M spermidine. Sonicate for 1–2 min, and put the mixture through a vortex for 2–3 s. Next, add between 50 and 100 μg of 1 μg/μL vector DNA to the solution, and gently sonicate (*see* **Note 6**).
2. Dropwise, add 100 μL of 1.0 M calcium chloride to the previously prepared solution (**step 1**), and sonicate for 2–3 min. Allow the mixture to remain at room temperature, with the microfuge tube cap open, for 10 min.
3. After the 10-min incubation, briefly sonicate, and then pulse-centrifuge the mixture for 15 s. Carefully remove and discard supernatant (*see* **Note 7**).
4. Repeat three washes by adding 1 mL of fresh ethanol, and pulse-centrifuge the mixture for 15 s. Carefully remove and discard supernatant.

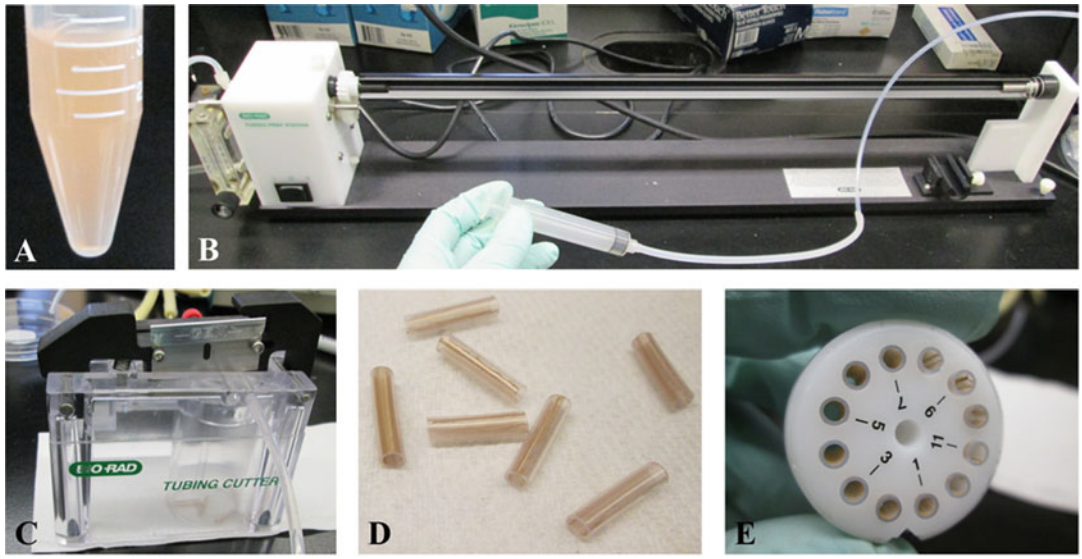

Fig. 2 Preparation of "bullets" for use in the gene gun. (**a**) DNA-gold mixture suspended in solution. (**b**) Loading the mixture in the drying chamber. (**c**): Cutting "bullets" in the tube cutter. (**d**) Cut bullets. (**e**) Loading the bullets into the barrel of the gene gun

5. After the final wash, use 3.5 mL PVP ethanol working solution to transfer the DNA-coated gold particles to a sterile 10 mL screw cap container by repeatedly adding 200 μL of PVP (0.01 mg/mL) aliquots.
6. Complete the transfer of the DNA-coated gold particles, and add the remaining PVP working solution to the container, cap, and invert the container several times to ensure an even suspension (Fig. 2a).

3.2 Coating the Plastic Tubing with Gold Microcarriers (See Note 8)

1. Insert Tefzel tube into prep station rotator (Fig. 2b) until the free end of the tube reaches the "O" ring inside the tube prep station (*see* **Note 9**).
2. Cut the overhanging exposed end of the Tefzel tube ~2 in. (~5 cm) past the external hole of the prep station rotor (right side of Fig. 2b) to form a ~30 in. (~80 cm) Tefzel tube.
3. Dry the inner surface of the tube by adjusting the flow nob (immediately above "B" in Fig. 2b) to allow nitrogen flow between 0.35 and 0.4 L/min for a minimum of 15 min.
4. Draw up the DNA-coated gold particles (prepared in Subheading 3.1, **step 6**) into a 5 mL syringe.
5. Use connector tubing to attach the filled syringe to the dried Tefzel tube (Fig. 2b).
6. Quickly transfer the DNA-coated gold particles from the syringe to the dried tubing situated in the tubing prep station (*see* **Note 10**).

7. Mark off the distal region of the tube where there are no microcarriers. Exclude this region when preparing bullets in a subsequent step.
8. Allow the microcarriers to settle for 3–5 min. Then remove the mixture at a rate of 0.5–1.0 in./s. The tube should be emptied within 30–45 s.
9. Rotate the tube 180° by holding the tubing prep station on/off switch to position II, and allow the gold to coat the inside of the tubing for 3–4 s.
10. Turn the tubing prep station switch to position I for 20–30 s to rotate the tube and smear it with gold.
11. Allow nitrogen to flow through the tube at a rate of 0.35–0.4 L/min for 3–5 min.
12. Turn off the tubing prep station switch to position 0 (off), and close the nitrogen valve.
13. Remove the section of tubing that does not contain DNA-coated gold particles (*see* Subheading 3.1, **step 7**).

3.3 Tube Cutting and Barrel Preparation

1. Transfer the gold smeared tube from the tubing prep station to the tube cutter.
2. Ensure that the end of the tubing contacts the rear end of the prep station. Cut the tubing into sections approximately 1 in. (2.5 cm) in length (Fig. 2c).
3. Load precut tubes (Fig. 2d) into the barrel of the gun (Fig. 2e) or store the tubes at −20 °C until cells have been prepared.

3.4 Preparing Cells for Transformation Using Overexpression Vectors

1. The pIGF-1 vector and its derivatives target developing macronuclei at conjugation. Therefore, two different mating-competent strains are required for this type of transformation. Mating-competent cells can be purchased from the National *Tetrahymena* Stock Center (*see* **Note 11**).
2. Separately grow wild-type *Tetrahymena* cells (e.g., strains 427 and 428) in bottles containing 30 mL of modified Neff's medium at 30 °C overnight, usually 12–18 h dependent upon the concentration of the inoculum.
3. Initiate a starvation regime while cells are still in exponential phase of growth. Gently centrifuge cells at 300 × *g* for 5 min in a sterile glass centrifuge tube. Carefully decant the Neff's medium without disturbing the soft pellet of cells. Resuspend the pellet in Dryl's starvation medium and wash 3× using the gentle centrifugation regime with fresh Dryl's starvation medium for each wash.
4. After the third wash, resuspend each strain in 30 mL of Dryl's starvation medium to yield an optical density of 0.2 as measured at 540 nm. Incubate the cells overnight (approximately 18 h) at 30 °C (*see* **Note 12**).

5. The following day, add ~2.5 mL of each starved *Tetrahymena* strain to a sterile 100 × 15 mm plastic Petri dish, and incubate at 30 °C. Expect conjugal pairing in 2–3 h after the initial cell mixing.

3.5 Preparing Conjugal Pairs for Biolistic Bombardment

1. Concentrate ~30 mL of conjugal *Tetrahymena* pairs (8–10 h after mixing) by gentle centrifugation at 300 × *g* for 5 min in a sterile glass centrifuge tube.
2. Gently decant the Dryl's medium, and resuspend the conjugal pairs in the small volume (approximately 2–3 mL) of Dryl's that remains in the centrifuge tube.
3. Decant the cell suspension onto the surface of sterile 24 cm GF/A Whatman filter paper placed in 100 × 15 mm plastic Petri dishes.

3.6 Using the Helios Hand-Held Gene Gun for Particle Bombardment

1. Load the stored bullets (Fig. 2d) into the barrel of the gun (Fig. 2e); load the barrel into the gun, and reassemble the remaining components (Fig. 3a).
2. Pressurize the gun to 200 psi with helium.

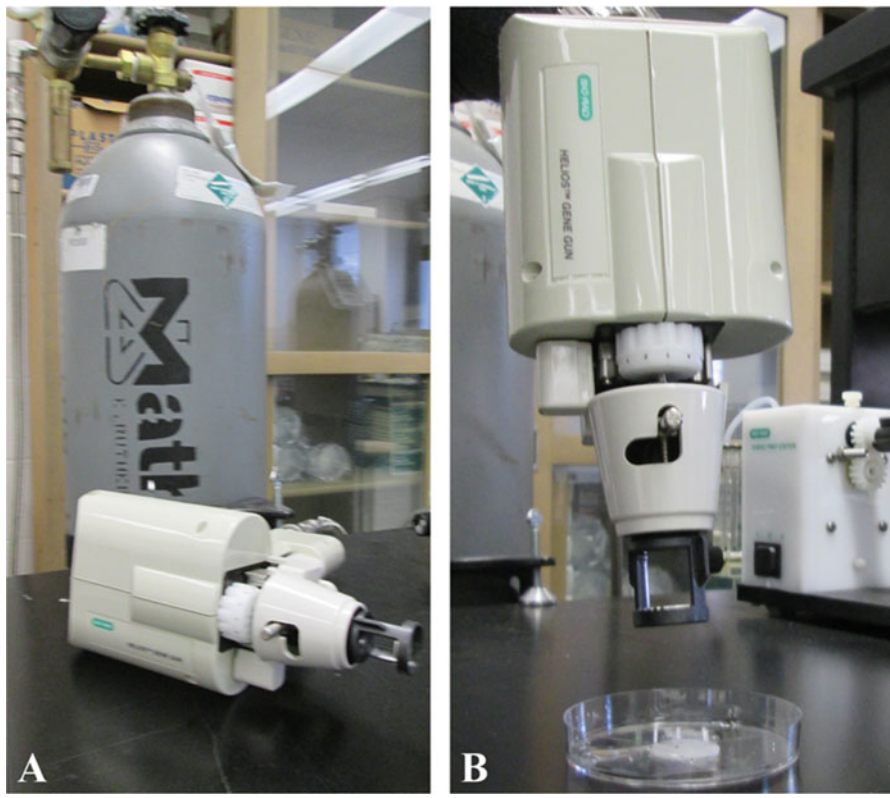

Fig. 3 Assembled gene gun. (**a**) Gene gun attached to a helium tank. (**b**) Firing position of gun

3. Hold the gene gun approximately 20 cm above the Petri dish containing the conjugal pairs (Fig. 3b), and discharge the gene gun with a pressure of 120–180 psi (*see* **Note 13**).

3.7 Screening for Overexpression Transformants

1. Wash the bombarded cells from the Petri dish into Neff's medium containing antibiotic/antimycotic mix, and incubate at 30 °C overnight.
2. Add the appropriate selection antibiotic either paromomycin (120–300 μg/mL) or cycloheximide (30–120 μg/mL) depending on the vector of choice (*see* **Note 14**). Vectors that contain a metallothionein promoter can be activated for expression of antibiotic resistance by addition of 1.0 μg/mL $CdCl_2$ (*see* **Note 15**).
3. Next dispense 250 μL aliquots of cell culture to each well of a 96-well plate, and incubate at 30 °C.
4. Examine each well for antibiotic-resistant clones after 3–5 days. Antibiotic-sensitive cells (non-transformers) will die within 3–5 days. Transformants will display rapid movement, and over time the population density will increase dramatically (*see* **Note 15**).
5. Remove 100 μL of culture from each well containing transformants, and put each aliquot into fresh 3 mL tubes. Add the appropriate concentration of selection marker either 120 μg/mL paromomycin or 30 μg/mL cycloheximide.
6. Repeat **step 4**, except using higher concentration of the appropriate selection antibiotic (either 180–240 μg/mL paromomycin or 45–60 μg/mL cycloheximide) (*see* **Note 15**).

3.8 Somatic Transformations

Somatic transformations are achieved by using plasmid vectors that target genes in the macronucleus of vegetative cells. Therefore, conjugal pairs are not used for this technique.

1. Grow wild-type *Tetrahymena* cells (e.g., strain 427 or 428) in bottles containing 30 mL of modified Neff's medium at 30 °C overnight, usually 12–18 h dependent upon the concentration of the inoculum.
2. Initiate a starvation regime while cells are still in exponential phase of growth. Gently centrifuge cells at 300 × *g* for 5 min in a sterile glass centrifuge tube. Carefully decant the Neff's medium without disturbing the soft pellet of cells. Resuspend the pellet in Dryl's starvation medium and wash 3× using the gentle centrifugation regime with fresh Dryl's starvation medium for each wash.
3. After the third wash, resuspend each strain in 30 mL of Dryl's starvation medium to yield an optical density of 0.2 as measured at 540 nm. Incubate the cells overnight (approximately 18 h) at 30 °C.

4. Transfer concentrated cells onto the surface of sterile 24 cm GF/A Whatman filter paper placed in 100 × 15 mm plastic Petri dishes.
5. Prepare the gene gun and use it as described in Subheading 3.6, **steps 1–3**.

3.9 Screening for Somatic Transformants

Screen for transformants using the protocols described in Subheading 3.7, **steps 1–4**.

4 Notes

1. National *Tetrahymena* Stock Center Plasmid list can be found at (last accessed 10.25.2020) https://tetrahymena.vet.cornell.edu/plasmids.php. The pVGF-1 plasmid can be purchased under Plasmid ID: PID00008.
2. Alternatively Dryl's can be prepared as 10× aliquots.
3. Prepare a suitable transformation vector for either overexpression or replacement of genes in the macronucleus. A list of plasmid vectors is available from the National *Tetrahymena* Stock Center (*see* **Note 1**).
4. Polyvinylpyrrolidone (PVP) serves as an adhesive during the cartridge preparation process. The working concentration of PVP should be between 0.01 and 0.1 mg/mL. We found that 0.01 mg/mL of PVP worked well for us.
5. We have experienced variability in the effectiveness of some commercially available gold particles. However, we achieved good results with gold microcarriers from Seashell Tech: (http://www.seashelltech.com/dnadel.shtml).
6. We used the Branson Model B200 Ultrasonic Cleaner. For gentle sonication, 20–30 s of sonication is sufficient.
7. We used a standard benchtop microcentrifuge for pulse centrifugations. Do not disturb the pellet when decanting the supernatant; it is acceptable to leave a small fraction of the supernatant behind.
8. Tefzel tubing is supplied with the Bio-Rad Gene Gun. It is advisable to begin drying the tube shortly before coating the gold with DNA.
9. Insert the Tefzel tube at the front end of the tubing prep station (right side of Fig. 2b), and gently guide the Tefzel tube until it reaches the internal contact point at the rear end of the tubing prep station (left side of Fig. 2b).
10. Work rapidly and carefully when coating the plastic with gold. A vacuum pump can be used to fill and to empty the Tefzel

tube with the gold-DNA mixture. Alternatively, with a bit of practice, this step can be performed manually with a syringe.

11. *Tetrahymena* mating strains can be purchased from the National *Tetrahymena* Stock Center (last accessed 10.25.2020): https://tetrahymena.vet.cornell.edu/strains.php. We achieved successful transformations with mating pairs CU427.4 (Stock ID: SD00715) and CU428.2 (Stock ID: SD00178).
12. In order to prevent low mating efficiency, the Neff's medium must be completely washed from the culture and replaced with Dryl's starvation medium in order to ensure adequate starvation of cells. Incompletely starved cells will continue to feed and will not mate. Excessive centrifugal force will lyse some of the cells, and the cell debris becomes nutrient material for the cells and consequently, they are not starved. In our hands, optimal starvation time is 18 hours assuming no nutrient medium is present in the culture.
13. *Tetrahymena* cells are very sensitive to biolistic force and will splatter out of the Petri dish when subjected to bombardment at pressures greater than 200 psi. We observed very good results at psi range between 120 and 180. For higher discharge pressures, preparing cartridges with higher PVP concentrations can increase the total number of particles delivered but may increase the number of cells that splatter out of the Petri dish.
14. Begin screening with low concentration of antibiotic (120 μg/mL paromomycin or 30 μg/mL cycloheximide). Increase antibiotic concentration until cells can no longer tolerate a further increase in concentration. It is advisable to replenish the 96-well plates with fresh Neff's medium containing antibiotic/antimycotic mix and the appropriate selection marker after 2–3 days. $CdCl_2$ should not be added to the replenishment media.
15. We observed basal expression of GFP or GFP-tagged epitopes with the pIGF-1 vector in the absence of $CdCl_2$ consistent with another observation [37]. It is possible that the $FeCl_3$ in the Neff's media mildly activates the MTT1 promoter.

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